Chapter 6: Investigations into the Sulfation Code of Chondroitin Sulfate Glycosaminoglycans[*†]

Background

Chondroitin sulfate glycosaminoglycans are sulfated polysaccharides implicated in cell division, neuronal development, and spinal cord injury.[1 -- 3] While considerable attention has been focused on heparan sulfate (HS) glycosaminoglycans, much less is known about the chondroitin sulfate (CS) class. As with all glycosaminoglycans (GAGs), the complexity and heterogeneity of CS has hampered efforts to understand its precise biological roles. For instance, CS has been shown to prevent the growth of axons; yet it is also found in developing, growth-permissive regions.[1, 4] Synthetic access to CS molecules of defined length and sulfation pattern, in combination with biological studies, should enable a systematic examination of structure-activity relationships.

Although GAGs exist as a heterogeneous mix in nature, several studies have shown that specific sulfation sequences direct the activity of both HS and CS. In fact, it is now thought that GAG activity is dictated by a sulfation code where distinct sulfation patterns are spatially and temporally regulated and direct the biological activity of HS and CS GAGs. For example, mutational deletion of HS 2-*O*-sulfotransferase activity disrupted development of both the kidney and cerebral cortex,[5 -- 7]

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[*] Synthesis of all of the chondroitin sulfate oligosaccharides was carried out by Dr. Sarah E. Tully, Dr. Sherry Tsai, Dr. Ross Mabon, and Dr. Manish Rawat, former graduate students and postdoctoral scholars in the Hsieh-Wilson laboratory. Biological studies using dopaminergic neurons were carried out by Naoki Sotogaku, a graduate student in Akinori Nishi's laboratory in the Department of Pharmacology at the Kurume University School of Medicine, Kurume, Fukuoka, Japan.

[†] Portions of this chapter were taken from S. E. Tully et al. (2004) *J. Am. Chem. Soc.* **126**, 7736 – 7737 and C. I. Gama et al. (2006) *Nat. Chem. Biol.* **2**, 467 – 473.

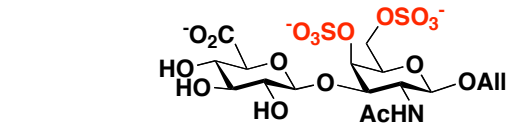
while 6-*O*-sulfotransferase activity was necessary for muscle differentiation and proper tracheal development.[8, 9] Growth factors also display preferential binding to distinct sulfation sequences; for instance, FGF-2 requires 2-*O*-sulfation but not 6-*O*-sulfation of HS and both pleiotrophin and midkine were shown to preferentially bind CS enriched in the disulfated CS-E motif.[10, 11] Distinct sulfation patterns of CS have also been shown to influence neuronal growth, as both a stimulatory and inhibitory cue.[1, 12, 13]

Advancing a molecular-level understanding of GAGs will require new tools for studying their structure-function relationships. Although several strategies have been developed, there are currently no methods to systematically explore the role of specific sulfation sequences. For instance, genetic approaches that target a particular sulfotransferase gene perturb many sulfation patterns throughout the polysaccharide chain and therefore cannot be used to study the impact of a single structural motif.[14, 15] Biochemical methods afford a mixture of heterogeneously sulfated compounds of poorly defined linear sequence,[16] thereby complicating efforts to relate a biological function to a particular sulfation sequence.

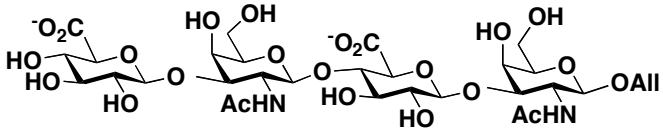
We have developed a chemical approach to evaluate the structure-activity relationship of CS as it effects neuronal growth. Through the synthesis of oligosaccharides of defined length and sulfation pattern, we have demonstrated that the CS-E sulfation sequence is a stimulatory motif that promotes the growth of hippocampal, dorsal root ganglion, and dopaminergic neurons.

CS-E sulfated tetrasaccharide enhances neurite outgrowth

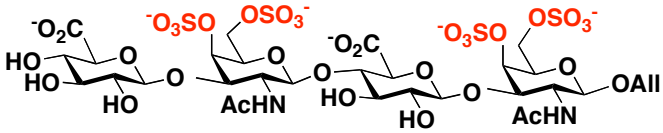
CS polysaccharides have been shown to both stimulate and attenuate the growth of cultured neurons.[17 -- 19] Notably, the molecules used in those studies were ~ 200 saccharides in length, poorly defined, and heterogeneously sulfated, features that might account for the contradictory observations. Therefore, we sought to investigate the biological properties of CS-E and establish the minimal structural determinants needed for activity. Toward this end, di- and tetrasaccharides bearing the CS-E sulfation pattern were first synthesized by Dr. Sarah E. Tully, Dr. Sherry Tsai, Dr. Ross Mabon, and Dr. Manish Rawat (Figure 6.1).[20]



CS-E Disaccharide **6**



Unsulfated Tetrasaccharide **7**



CS-E Tetrasaccharide **8**

Figure 6.1. Structures of the initial synthetic CS oligosaccharides

To explore the ability of compounds **6** – **8** to modulate neuronal growth, we cultured primary hippocampal neurons with or without each compound. After a 48 h treatment, the neurons were fixed, immunostained with anti-tau antibodies, and examined

by fluorescence microscopy. Sulfated tetrasaccharide **8** exhibited striking effects on neuronal morphology and growth (Figure 6.2).[20] The growth of the major neurite extension was dramatically stimulated by 39.3 ± 3.6% relative to the untreated, polyornithine control. In contrast, sulfated disaccharide **6** and unsulfated tetrasaccharide **7** had no significant effect on neuronal outgrowth.

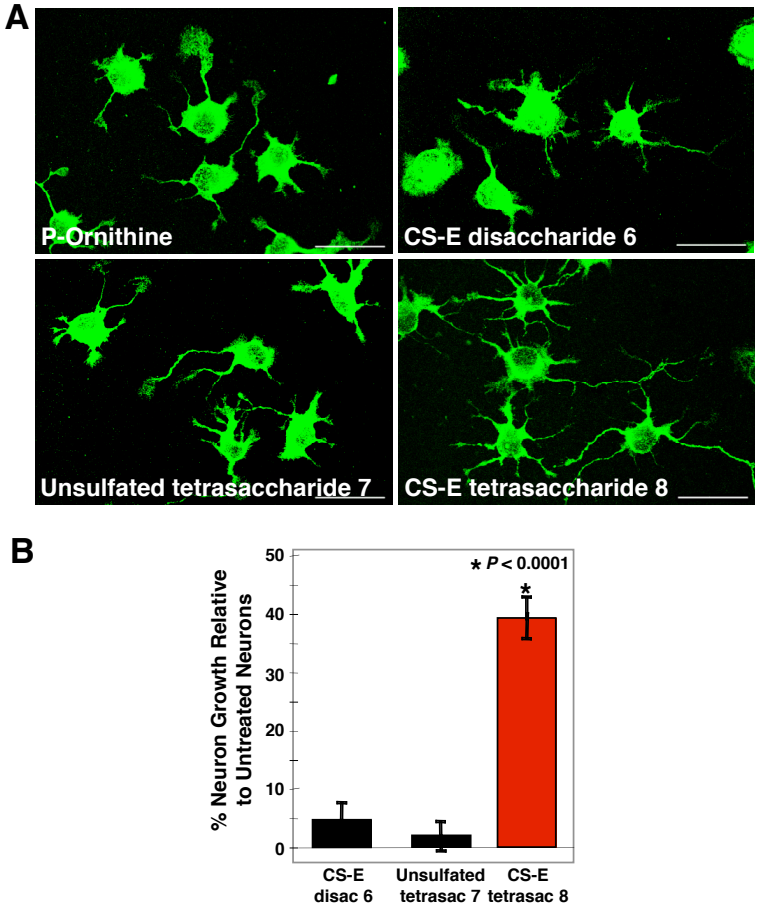


Figure 6.2. CS-E tetrasaccharide **8** stimulates the outgrowth of hippocampal neurons. A) Immunofluorescence images of neurons 48 h after treatment with the indicated compound. B) Statistical analysis of neurite length. *P* value is relative to poly-ornithine control. Scale bars represent 45 μm.

These results indicate that a tetrasaccharide represents a minimum structural motif with biological activity and that sulfation is necessary for function. Moreover, these studies are the first, direct investigations into the structure-activity relationships of CS

using homogeneous, synthetic molecules. The ability of CS small molecules to recapitulate the activity of larger, natural polysaccharides provides a new chemical approach to understand and manipulate neuronal growth and regeneration.

The CS-E sulfation pattern is a stimulatory motif that enhances neurite outgrowth

Once we established that a tetrasaccharide was a minimum structural determinant displaying biological activity, we proceeded to evaluate the sulfation code hypothesis for CS function. Several tetrasaccharides representing three important subclasses of CS found *in vivo* were then synthesized by Dr. Sarah E. Tully, Dr. Ross Mabon, and Dr. Manish Rawat (Figure 6.3).[21] Tetrasulfated molecule **8** contains the CS-E sulfation sequence. Disulfated molecules **9** and **10** represent the most abundant sulfation patterns found *in vivo*, CS-A and CS-C, respectively.[22] For comparison, we also generated two unnatural CS molecules, tetrasulfated molecule **11**, denoted CS-R, and a dimer of CS-E disaccharides (molecule **12**). These two molecules have not yet been isolated from natural sources, however they represent important controls because they possess the same overall negative charge as CS-E tetrasaccharide **8**, unlike molecules **9** and **10**, which are only disulfated, not tetrasulfated. Moreover, these control molecules display the negative charge in a different manner and will further elucidate whether the specific placement of the sulfate groups, and not just the overall charge, is crucial for biological activity of the CS molecules.

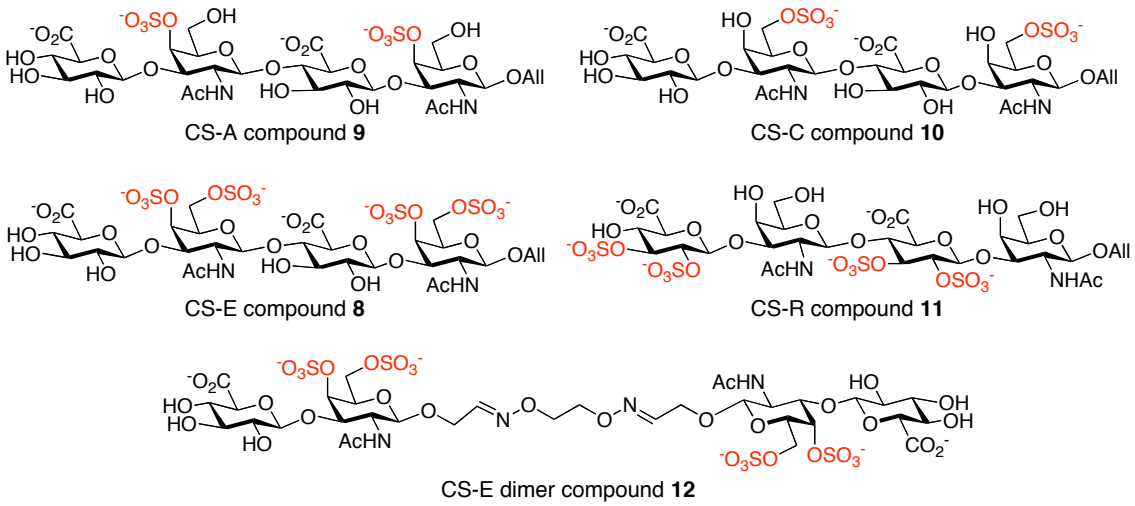


Figure 6.3. Structures of synthetic CS oligosaccharides containing distinct sulfation patterns

To compare the neuritogenic activity of the differentially sulfated tetrasaccharides, we cultured hippocampal neurons on coverslips coated with polyornithine and each compound. After 48 h, the neurons were fixed, immunostained with antibodies to tubulin, and then examined by fluorescence microscopy. A specific sulfation pattern was required for the growth-promoting activity of CS. As shown in Figure 6.4, the CS-E tetrasaccharide was the only molecule that stimulated neurite outgrowth.[21] Following quantitation of neuronal growth and comparison to untreated controls, we found that the CS-E tetrasaccharide stimulated neurite outgrowth by 48.6 ± 2.3%, while tetrasaccharides representing CS-A and CS-C motifs had no appreciable activity (Figure 6.4). Importantly, CS-R and the dimer had no effect on neurite outgrowth, despite having the same overall negative charge as CS-E. These results are consistent with previous studies reporting that CS polysaccharides enriched in the CS-E sulfation pattern posses neuritogenic activity.[16] However, it is important to note that in

those studies the CS polysaccharides used were heterogeneous and contain variations in size and, significantly, in the sulfation patterns present. It is therefore possible that other sulfation patterns present could have contributed to the reported effects. Our results further extend those findings and establish conclusively for the first time that it is the precise placement and orientation of the sulfate groups that determines the biological activity of CS. Moreover, our studies indicate that the CS-E sulfation motif is responsible for the growth-promoting effects observed.

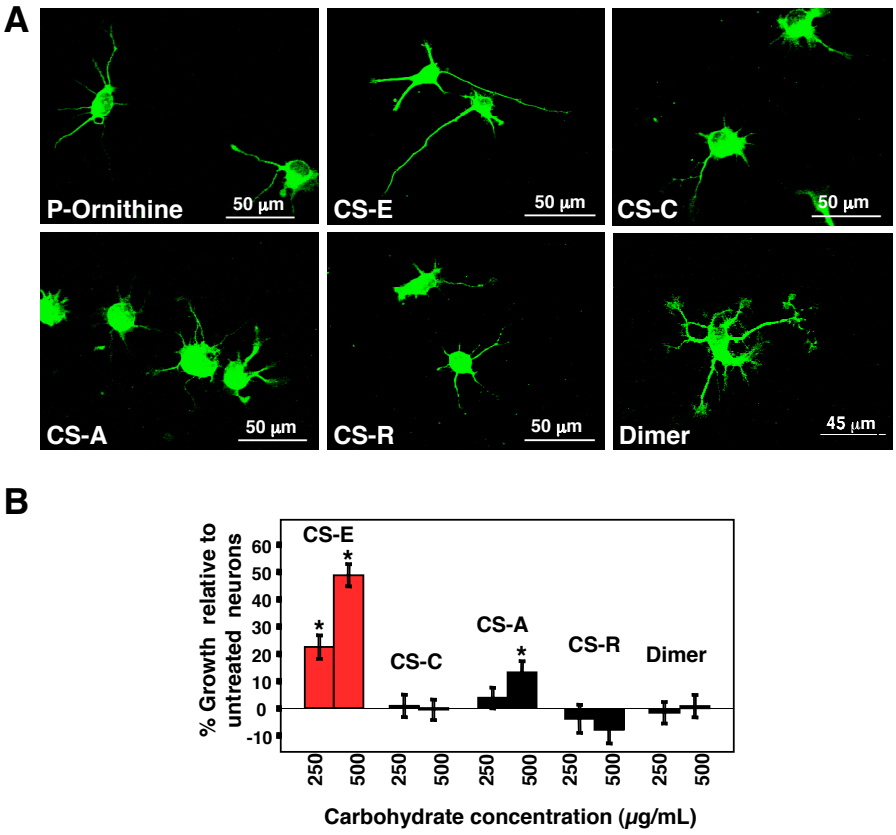


Figure 6.4. The sulfation pattern directs the neuritogenic activity of CS. A) Representative immunofluorescence images of neurons cultured on a substratum of polyornithine and the synthetic molecules. B) Quantification of neurite length, expressed as percentage growth relative to polyornithine control. **P* < 0.0001, relative to polyornithine control.

We next investigated whether the effects of the CS-E motif are unique to specific cell types. Paradoxically, CS has been reported both to stimulate and inhibit neuronal

growth, depending on the cellular context. For example, CS proteoglycans can repel migrating neurons or extending axons during brain development or after injury.[1, 13] However, CS staining was also found to coincide with developing axon pathways, and tissues expressing CS do not always exclude axon entry.[23] To examine whether sulfation is important for the growth of other neuron types, we cultured dorsal root ganglion (DRG) neurons from rat embryos on a substratum of each tetrasaccharide. DRG neurons are a good model for investigations into the effects of CS on spinal cord neuron growth. We found that the CS-E tetrasaccharide had a similar activity toward both DRG and hippocampal neurons, where the outgrowth of DRG neurons was stimulated by 32.5 ± 2.9% (Figure 6.5A).[21] In contrast, the CS-C, CS-A, CS-R, and dimer motifs showed no appreciable activity. In a similar manner, our collaborator Naoki Sotogaku found that dopaminergic neurons were stimulated by the CS-E tetrasaccharide but not the other sulfation patterns (Figure 6.5B).[21] Dopaminergic neurons are derived from the mesencephalon and are the neurons affected in Parkinson's disease, schizophrenia and attention deficit/hyperactivity disorder.[24] The ability of the CS-E sulfation motif to elicit a response in various cell types suggests that protein receptors, which can be shared by many cell types, are likely present to engage the carbohydrate. These results indicate that the molecular structure of CS GAGs is critical for the function of CS, largely independent of neuron type.

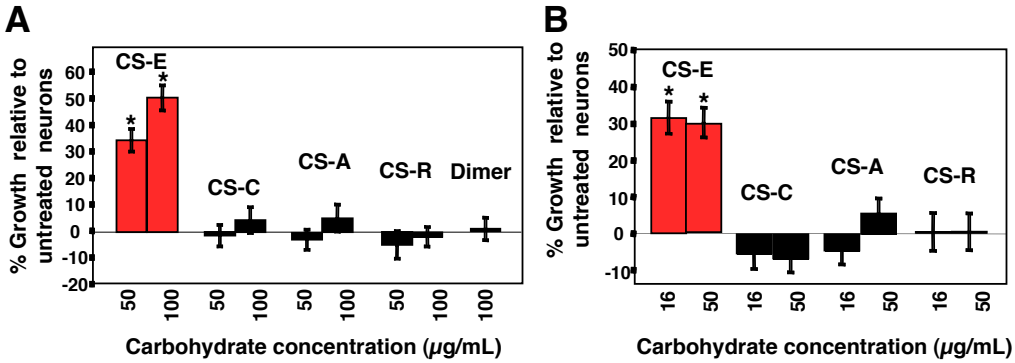


Figure 6.5. The CS-E sulfation motif stimulates the outgrowth of DRG (A) and dopaminergic neurons (B). Neurons were cultured for 2 – 5 days on coverslips coated with polyornithine and the tetrasaccharides at the indicated concentrations. Neurite length is expressed as percentage of growth relative to polyornithine control. *$P < 0.0001$, relative to polyornithine control.

Discussion

Together, our studies provide compelling support for the existence of a sulfation code, whereby the precise position of sulfate groups along the carbohydrate backbone permits GAGs to encode information in a sequence-specific manner. Using well-defined oligosaccharides, we have directly shown that distinct CS sulfation sequences stimulate neuronal outgrowth. Moreover, the activity of CS-E relative to other CS subclasses, CS-R and the dimer, as well as the preservation of activity across different cell types, suggests the importance of specific molecular interactions rather than nonspecific, electrostatic effects. HS has also been proposed to operate through a sulfation code,[15] and the concept of a sulfation code finds precedent in the sequence-specific manner in which other biopolymers (DNA, RNA and proteins) interact with their molecular targets.

According to the sulfation code hypothesis, chemical modifications to the polysaccharide backbone may be introduced in a time- or region-specific manner, such as during development or in response to injury. Precise modifications to GAGs could facilitate or inhibit ligand-receptor interactions in a highly localized fashion, providing an

exquisite means for regulatory control. Specific sulfation motifs could control the diffusion and efficient signaling of growth factors, establishing concentration gradients and boundaries. Indeed, support for this view can be seen in *Drosophila melanogaster* mutants where HS biosynthesis was shown to be essential for Hedgehog signaling during embryonic patterning.[25] Furthermore, specific CS sulfation motifs are upregulated during neuronal development and are enriched along axon growth tracts.[26]

The ability of the CS-E tetrasaccharide to stimulate neuronal outgrowth of various cell types suggests that CS GAGs are involved in directing neuronal growth during nervous system development. Indeed, our findings support previous studies implicating the CS-E motif in the growth and development of neurons. For instance, CS-E is found on the protein appican, an isoform of the amyloid precursor protein that exhibits neurotrophic activity.[27] Moreover, the CS-E motif has been shown to be enriched in the developing brain and is crucial for axonal guidance of growing neurons.[13, 22, 23, 28] With the finding that distinct sulfation patterns display biological activity, it is now necessary to examine how these motifs elicit such responses in neurons. As such, we proceeded to investigate the mechanism by which the CS-E motif induces neuronal outgrowth and to examine which proteins are being recruited and activated by the carbohydrate.

Experimental Procedures for Chapter 6

Buffers and Reagents:

Chemicals and molecular biology reagents were purchased from Fisher (Fairlawn, NJ) unless stated otherwise. Cell culture media was purchased from Gibco BRL (Grand Island, NY). German glass coverslips were purchased from Carolina Biologicals (Burlington, NC).

Neuronal Cultures:

Hippocampal neuronal cultures were prepared from embryonic rats as described in Chapter 2. DRG cultures were prepared from the spinal cord of E18 embryos of Sprague-Dawley rats. We dissected ganglia in Calcium- and Magnesium-Free Hank's Balanced Salt Solution (CMF-HBSS; Gibco), digested them with 0.25% trypsin (Gibco) for 20 min at 37 °C and dissociated the resulting fragments in culture medium consisting of DMEM-F12, 10% horse serum, N2 supplement, and nerve growth factor (50 ng/mL). We plated DRG neurons at 100 cells/mm^2 on coverslips coated with polyornithine and the tetrasaccharides.

Preparation of Coverslips:

German glass coverslips (15 mm) were first sterilized by successive washes in ethanol and water. Specifically, coverslips were dropped one at a time into 95% ethanol, swirled around, then washed twice with double-distilled H_2O. A final rinse with 100% ethanol was added before allowing the coverslips to dry in the sterile tissue culture hood. Coverslips were then coated as described by Clement et al.[12] Briefly: coverslips were

precoated with 0.015 mg/mL poly-DL-ornithine (in 10 mM Borate buffer pH 8.1; Sigma) for 1 h at 37 °C/ 5% CO_2, washed three times with double-distilled H_2O, and coated with 0.05 to 0.5 mg/mL of compounds **6 – 11** in PBS (100 μL) overnight at 37 °C/ 5% CO_2. The coverslips were then washed three times with PBS and flooded with culture media until neurons were ready to be plated. Notably, the use of adhered compounds to glass coverslips has been reported to simulate the extracellular matrix, and the procedure by Clement et al. was used previously to implicate heterogeneous polysaccharides containing the CS-E motif in neuronal growth.

Calibration of CS Molecules:

The relative concentrations of the CS oligosaccharides were calibrated to one another using the carbazole assay for uronic acid residues.[29] Briefly, the acid borate reagent (1.5 mL of a solution of 0.80 g sodium tetraborate, 16.6 mL H_2O, and 83.3 mL sulfuric acid) was added to glass vials. The oligosaccharides were added (50 μL of a 10 mg/mL stock in H_2O) and the solution placed in a boiling H_2O bath for 10 min. Following addition of the carbazole reagent (50 μL of 0.1% w/v carbazole in 100% ethanol), the solution was boiled for 15 min. The absorbance was read at 530 nm and compared to a D-glucuronolactone standard in H_2O.

Immunocytochemistry of Neuronal Cultures:

After 48 h in culture, neurons on coverslips were fixed and treated for immunostaining as described in Chapter 2. Neurons were immunostained with anti-tau antibodies (rabbit

polyclonal, 1:600; Sigma) or anti-β-tubulin III antibodies (mouse monoclonal, 1:500; Sigma) and were examined by confocal fluorescence microscopy.

Confocal Fluorescence Microscopy:

All cells were imaged on a Zeiss Axiovert 100M inverted confocal laser microscope in the Biological Imaging Center in the Beckman Institute at Caltech. The images were captured with LSM Pascal software using a 40X plan-neofluar oil objective. Cells were excited with 488 nm light.

Morphometric analysis:

For quantitative analysis, 50 cells were analyzed per coverslip and each treatment was performed in triplicate. The neurite length is expressed as the total length of the neurite from the perikarya, and only cells with neurites longer than one cell body diameter were counted, as per standard protocol. The length of the longest neurite was measured using NIH Image 1.62 software. The mean neurite lengths were compared among the different substrate conditions with the ANOVA test followed by the Scheffe test using the statistical analysis program StatView (SAS Institute Inc.) and Kaleidograph (Synergy Software).

References

1. Bradbury, E.J., et al. Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* **416**, 636 – 640 (2002).

2. Mizuguchi, S., et al. Chondroitin proteoglycans are involved in cell division of Caenorhabditis elegans. *Nature* **423**, 443 – 448 (2003).

3. Sugahara, K., et al. Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate. *Curr. Opin. Struct. Biol.* **13**, 612 – 620 (2003).

4. Emerling, D.E. & Lander, A.D. Inhibitors and promoters of thalamic neuron adhesion and outgrowth in embryonic neocortex: functional association with chondroitin sulfate. *Neuron* **17**, 1089 – 1100 (1996).

5. McLaughlin, D., et al. Specific modification of heparan sulphate is required for normal cerebral cortical development. *Mech. Dev.* **120**, 1481 – 1488 (2003).

6. Merry, C.L., et al. The molecular phenotype of heparan sulfate in the Hs2st-/- mutant mouse. *J. Biol. Chem.* **276**, 35429 – 354434 (2001).

7. Merry, C.L. & Wilson, V.A. Role of heparan sulfate-2-O-sulfotransferase in the mouse. *Biochem. Biophys. Acta* **1573**, 319 – 327 (2002).

8. Bink, R.J., et al. Heparan sulfate 6-O-sulfotransferase is essential for muscle development in zebrafish. *J. Biol. Chem.* **278**, 31118 – 31127 (2003).

9. Kamimura, K., et al. Drosophila heparan sulfate 6-O-sulfotransferase (dHS6ST) gene. Structure, expression, and function in the formation of the tracheal system. *J. Biol. Chem.* **276**, 17014 – 17021 (2001).

10. Ashikari-Hada, S., et al. Characterization of growth-factor binding structures in heparin/heparan sulfate using an octasaccharide library. *J. Biol. Chem.* **279**, 12346 – 12354 (2004).

11. Deepa, S.S., Umehara, Y., Higashiyama, S., Itoh, N. & Sugahara, K. Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. Implications as a physiological binding partner in the brain and other tissues. *J. Biol. Chem.* **277**, 43707 – 43716 (2002).

12. Clement, A.M., et al. The DSD-1 carbohydrate epitope depends on sulfation, correlates with chondroitin sulfate D motifs, and is sufficient to promote neurite outgrowth. *J. Biol. Chem.* **273**, 28444 – 28453 (1998).

13. Kantor, D.B., et al. Semaphorin 5A is a bifunctional axon guidance cue regulated by heparan and chondroitin sulfate proteoglycans. *Neuron* **44**, 961 – 975 (2004).

14. Häcker, U., Nybakken, K. & Perrimon, N. Heparan sulphate proteoglycans: the sweet side of development. *Nat. Rev. Mol. Cell Biol.* **6**, 530 – 541 (2005).

15. Holt, C.E. & Dickson, B.J. Sugar codes for axons? *Neuron* **46**, 169 – 172 (2005).

16. Nandini, C.D., et al. Structural and functional characterization of oversulfated chondroitin sulfate/dermatan sulfate hybrid chains from the notochord of hagfish. Neuritogenic and binding activities for growth factors and neurotrophic factors. *J. Biol. Chem.* **279**, 50799 – 50809 (2004).

17. Brittis, P.A., Canning, D.R. & Silver, J. Chondroitin sulfate as a regulator of neuronal patterning in the retina. *Science* **255**, 733 – 736 (1992).

18. Dou, C.L. & Levine, J.M. Differential effects of glycosaminoglycans on neurite growth on laminin and L1 substrates. *J. Neurosci.* **15**, 8053 – 8066 (1995).

19. Nadanaka, S., Clement, A., Masayama, K., Faissner, A. & Sugahara, K. Characteristic hexasaccharide sequences in octasaccharides derived from shark cartilage chondroitin sulfate D with a neurite outgrowth promoting activity. *J. Biol. Chem.* **273**, 3296 – 3307 (1998).

20. Tully, S.E., et al. A chondroitin sulfate small molecule that stimulates neuronal growth. *J. Am. Chem. Soc.* **126**, 7736 – 7737 (2004).

21. Gama, C.I., et al. Sulfation patterns of glycosaminoglycans encode molecular recognition and activity. *Nat. Chem. Biol.* **2**, 467 – 473 (2006).

22. Ueoka, C., et al. Neuronal cell adhesion, mediated by the heparin-binding neuroregulatory factor midkine, is specifically inhibited by chondroitin sulfate E. Structural and functional implications of the over-sulfated chondroitin sulfate. *J. Biol. Chem.* **275**, 37407 – 37413 (2000).

23. Bicknese, A.R., Sheppard, A.M., O'Leary, D.D. & Pearlman, A.L. Thalamocortical axons extend along a chondroitin sulfate proteoglycan-enriched pathway coincident with the neocortical subplate and distinct from the efferent path. *J. Neurosci.* **14**, 3500 – 3510 (1994).

24. Nestler, E.J., Hyman, S.E. & Malenka, R.C. Molecular pharmacology: a foundation for clinical neuroscience, 2nd Ed. (McGraw-Hill Professional, New York, 2001).

25. Bellaiche, Y., The, I. & Perrimon, N. Tout-velu is a Drosophila homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion. *Nature* **394**, 85 – 88 (1998).

26. Fernaud-Espinosa, I., Nieto-Sampedro, M. & Bovolenta, P. Developmental distribution of glycosaminoglycans in embryonic rat brain: relationship to axonal tract formation. *J. Neurobiol.* **30**, 410 – 424 (1996).

27. Tsuchida, K., et al. Appican, the proteoglycan form of the amyloid precursor protein, contains chondroitin sulfate E in the repeating disaccharide region and 4-*O*-sulfated galactose in the linkage region. *J. Biol. Chem.* **276**, 37155 – 37160 (2001).

28. Shuo, T., et al. Developmental changes in the biochemical and immunological characters of the carbohydrate moiety of neuroglycan C, a brain-specific chondroitin sulfate proteoglycan. *Glycoconj. J.* **20**, 267 – 278 (2004).

29. Taylor, K.A. & Buchanan-Smith, J.G. A colorimetric method for the quantitation of uronic acids and a specific assay for galacturonic acid. *Anal. Biochem.* **201**, 190 – 196 (1992).