Chapter 7: Elucidating the Mechanism of CS-E Mediated Neuronal Outgrowth [*†]

Background

Glycosaminoglycans (GAGs) have an inherent capacity to encode functional information that rivals DNA, RNA, and proteins. Specifically, these polysaccharides display diverse patterns of sulfation that are tightly regulated *in vivo*.[1, 2] Over the past several decades, genetic and biochemical studies have established the importance of GAGs in regulating many physiological processes, including morphogenesis and development, viral invasion, cancer metastasis and spinal cord injury.[3 -- 6] However, a key unresolved question is whether GAGs utilize specific sulfation sequences to modulate biological processes.

Chondroitin sulfate (CS) GAGs have been shown to play critical roles in various physiological processes, ranging from cell division to spinal cord injury.[6, 7] The complexity of these polysaccharides has hindered efforts to relate structure to function and to generate defined molecular tools for manipulating CS activity. Comprising 40 – 200 sulfated disaccharide units, CS is thought to contain "blocks" of high and low sulfation, with highly sulfated regions serving as binding sites for growth factors, cytokines, and other proteins.[8 -- 11] For instance, several growth factors, including midkine, pleiotrophin, and FGF-16, exhibit preferential binding to highly sulfated CS

---

[*] 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. The CS-E glycopolymers were synthesized by Dr. Manish Rawat. Computational modeling studies were performed by Peter M. Clark, a graduate student in the Hsieh-Wilson laboratory.

[†] Portions of this chapter were taken from C. I. Gama et al. (2006) *Nat. Chem. Biol.* **2**, 467 – 473 and M. Rawat et al. (2008) *J. Am. Chem. Soc.* **130**, 2959 – 2961.

polysaccharides containing either CS-E or CS-D sulfation patterns.[12, 13] Furthermore, the highly sulfated CS-E polysaccharide has been shown to antagonize the activity of the proinflammatory cytokine TNFα as well as activate phospholipase C signaling in dopaminergic neurons.[14, 15]

Although GAGs contribute to diverse physiological processes, an understanding of their molecular mechanisms has been hampered by the inability to access homogeneous GAG structures. As described in Chapter 6, we assembled well-defined CS oligosaccharides with sulfate groups installed at precise positions along the carbohydrate backbone. Using these defined structures, we demonstrated that distinct sulfation patterns modulate neuronal growth. Specifically, we have shown that the CS-E motif enhances neuronal outgrowth of hippocampal, dorsal root ganglion, and dopaminergic neurons.[9, 16] Here, we extend these studies to examine how specific sulfation motifs function as molecular recognition elements for growth factors and investigate the mechanism by which the CS-E sulfation pattern stimulates neuronal outgrowth.

The CS-E sulfation motif regulates neuronal outgrowth

CS GAGs have been shown to influence neuronal growth as both a stimulatory and inhibitory cue.[6, 17, 18] These conflicting effects are most likely due to the heterogeneity of the polysaccharides employed in these studies. However, it is also possible that inconsistent experimental designs and the examination of differing cell types may contribute to the opposing effects observed. Our initial studies demonstrated that the CS-E motif specifically stimulates neuronal growth across various neuron types

(Chapter 6). In an attempt to resolve the paradoxical nature of CS, we used CS-E-enriched polysaccharides to modulate the growth of hippocampal neurons. First, we grew hippocampal neurons on a substratum of polyornithine in the presence or absence of CS-E polysaccharide. Consistent with previous studies,[19] we saw that neuronal outgrowth was dramatically stimulated by treatment with CS-E polysaccharide (Figure 7.1). Importantly, the enhanced neuronal outgrowth was specific to the polysaccharide, as treatment with chondroitinase ABC, an enzyme that digests CS, abolished the stimulatory effects observed. These results suggest that CS-E polysaccharide present on the surface is presumably interacting with growth factors in the culture media and thus enabling activation of neuronal growth pathways.

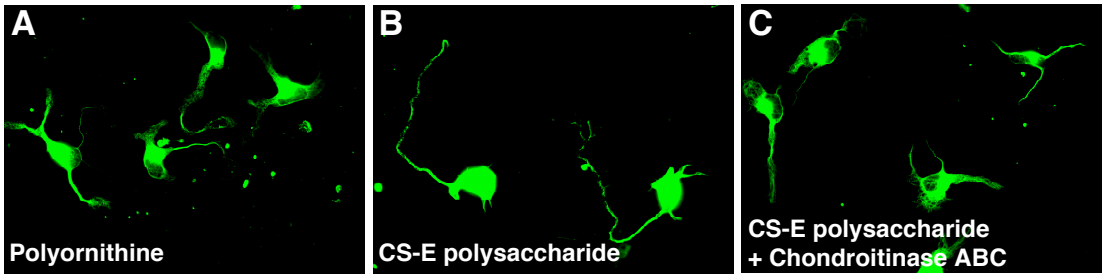

Figure 7.1. A substratum of CS-E polysaccharide stimulates the outgrowth of hippocampal neurons. A) Neurons grown on polyornithine-coated coverslips. B) Neurons grown on coverslips coated with CS-E polysaccharide (16 μg/mL) have longer neurites than control neurons. C) Neurons grown on coverslips coated with CS-E polysaccharide (16 μg/mL) previously digested with chondroitinase ABC (10 mU/mL) do not have longer neurites and are similar to control neurons.

To systematically examine the mechanism of CS-E-mediated neuronal growth, we then presented the polysaccharide to the cells in solution. Interestingly, soluble CS-E polysaccharide caused dramatic inhibition of neurite outgrowth (Figure 7.2). Although these results appear contradictory to the substratum effects of CS-E, they are consistent with the model that CS-E, present on cell-surface proteoglycans or coated on a substratum, recruits growth factors to the cell surface, thereby stimulating downstream

signaling pathways involved in neuronal growth.[9, 15] By adding CS-E in solution to neurons, growth factors are presumably sequestered away from the cell surface, resulting in neurite inhibition.

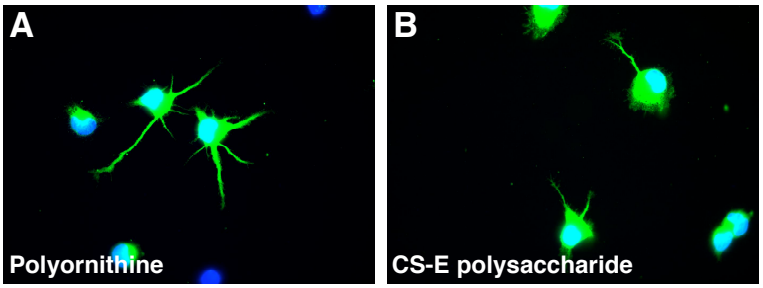

Figure 7.2. CS-E polysaccharide added in solution inhibits the outgrowth of hippocampal neurons. A) Neurons grown on polyornithine-coated coverslips. B) Neurons grown on coverslips coated with polyornithine and treated CS-E polysaccharide (0.1 μg/mL) added in solution have shorter neurites than control neurons.

Although we observed such striking effects on neuronal growth using CS-E-enriched polysaccharides, it is possible that there are other sulfation sequences present in the polysaccaharide that may be contributing to the observed effects. Therefore, we proceeded to develop CS glycopolymers that would retain key properties of CS polysaccharides but would be of a defined, homogeneous sulfation sequence. Dr. Manish Rawat developed a new methodology to generate various glycopolymers made up of CS-E disaccharide, CS-E tetrasaccharide, and unsulfated tetrasaccharide building blocks (Figure 7.3).[20]

We evaluated the biological activity of the glycopolymers by measuring their ability to modulate the outgrowth of hippocampal neurons. Attempts to evaluate the activity of the glycopolymers in a substratum-based system proved to be inconclusive; when the glycopolymers were coated on coverslips neither stimulatory nor inhibitory effects were observed in this system. Therefore, we proceeded to evaluate the

glycopolymers using a solution-based assay where neurons were cultured on polyornithine coated coverslips and each glycopolymer was added in solution. Solution studies were chosen to evaluate the glycopolymers in order to study the importance of multivalency and macromolecular structure of CS in directing its activity. Furthermore, adding the glycopolymers in solution avoids any potential confound multivalency effects naturally imparted by immobilization of the compounds on surfaces.

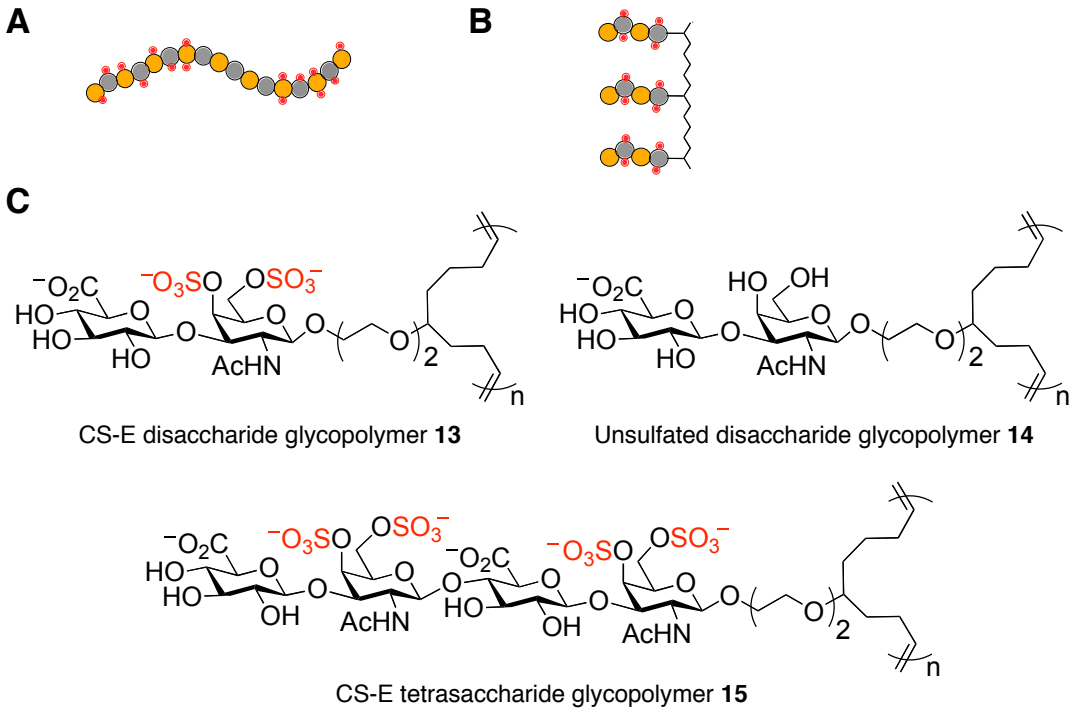

Figure 7.3. Schematic representation of (A) natural CS and (B) CS glycopolymers. Grey and orange circles represent the monosaccharide subunits, red circles represent sulfation. C) Structures of CS glycopolymers

Remarkably, CS-E disaccharide glycopolymer **13** mimicked the activity of the natural polysaccharide (Figure 7.4). While the isolated CS-E disaccharide was insufficient for biological activity, incorporation of the disaccharide into a polymeric framework endowed it with the ability to inhibit neuronal growth. Moreover, the potency of the glycopolymers was valence-dependent: polymers with 25 disaccharide units

exhibited moderate activity (40.9 ± 5.5% inhibition), while those with 80 disaccharide units showed significantly enhanced activity (86.0 ± 5.8% inhibition) at the same glucuronic acid concentration. These findings highlight the importance of multivalency in modulating the activity of CS. In addition, the unsulfated glycopolymer **14** had little effect on neurite outgrowth (5.9 ± 5.6% inhibition), confirming earlier observations that sulfation is a prerequisite for activity[16] and highlighting the ability of these glycopolymers to recapitulate features of natural CS polysaccharides.

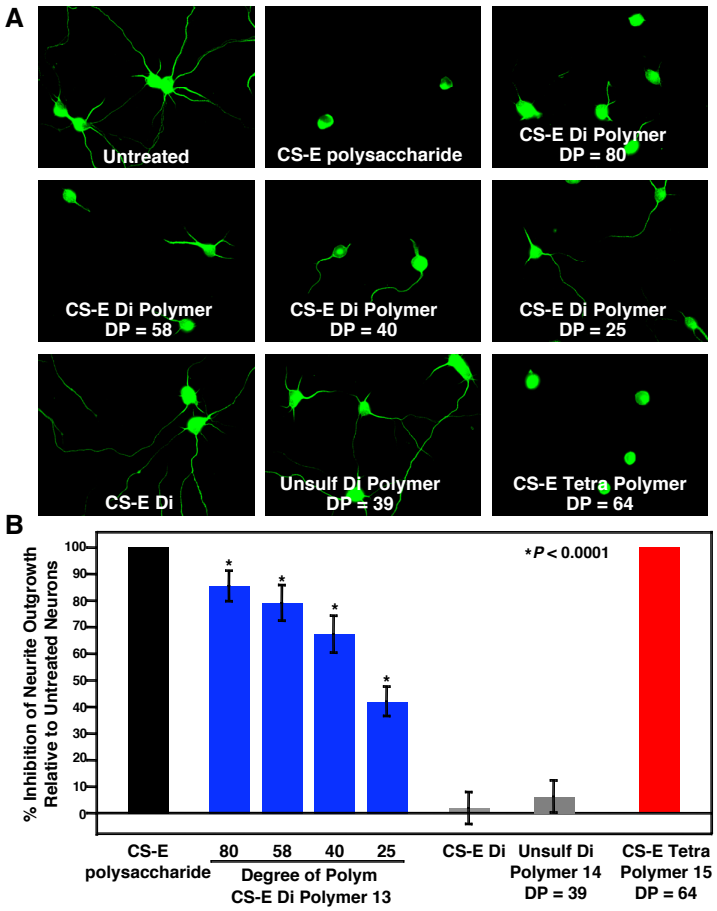

Figure 7.4. CS-E glycopolymers inhibit the outgrowth of hippocampal neurons. A) Immunofluorescence images of neurons 48 h after treatment with the indicated compound. B) Statistical analysis of neurite length. *P* values are relative to the untreated neurons. DP, degree of polymerization, equals the number of repeating subunits.

We have previously shown that a tetrasaccharide represents a minimum functional domain for protein recognition and neuronal growth-promoting activity when adhered to a substratum.[9, 14, 16] In this solution-based assay, a monovalent tetrasaccharide at 0.5 μg/mL glucuronic acid concentration had minimal activity (Figure 7.5). In contrast, CS-E tetrasaccharide glycopolymer **15** at the same glucuronic acid concentration exhibited maximal activity, inducing neurite inhibition to the same extent as the natural polysaccharide (Figure 7.4).

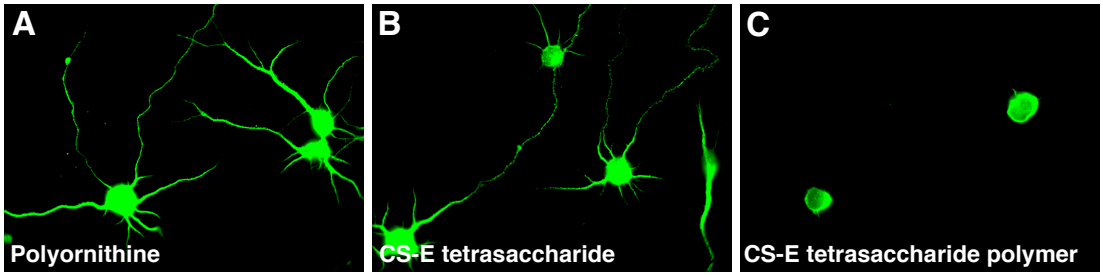

Figure 7.5. CS-E tetrasaccharide glycopolymer inhibits hippocampal neurite outgrowth. A) Untreated neurons. B) Neurons treated with CS-E tetrasaccharide (0.5 μg/mL). C) Neurons treated with CS-E tetrasaccharide polymer **15**. Monovalent CS-E tetrasaccharide displays no inhibitory activity whereas CS-E tetra glycopolymer completely inhibits neurite outgrowth.

To compare the relative potencies of glycopolymer **15** and the natural polysaccharide, we measured their inhibition values at various concentrations (Figure 7.6). We were excited to find that the inhibitory potency of **15** was comparable to that of the natural polysaccharide (IC_{50} values of 1.3 ± 0.1 and 1.2 ± 0.1 nM, respectively), despite considerable changes to the macromolecular structure. Given the challenges inherent in the synthesis of large polysaccharides, our approach greatly simplifies the synthesis of complex glycosaminoglycans, providing synthetically accessible, bioactive structures of programmable sulfation sequence. The ability to control the sulfation pattern within the glycopolymer is significant as it should allow for the generation of CS

type-specific polymers (e.g. CS-E, CS-A, CS-C) with distinct functions. We anticipate that these glycopolymers will be powerful tools for further exploring how the macromolecular structure of CS directs its activity and for manipulating the functions of CS *in vivo*. In all, these studies demonstrate that the CS-E motif is particularly associated with modulating growth in the brain. As such, we proceeded to investigate the mechanism of CS-E stimulated outgrowth and determine which growth factors are specifically involved and interacting with the CS-E motif.

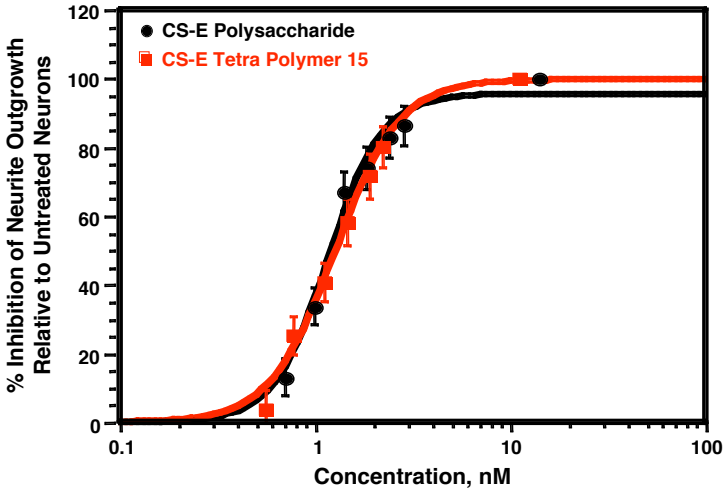

Figure 7.6. Comparison of the inhibitory potencies of CS glycopolymer **15** and the natural polysaccharide at various concentrations. IC_{50} values are based on the molar concentration of compound required to inhibit neurite outgrowth by 50% relative to untreated neurons.

Molecular dynamic simulations of CS oligosaccharides reveal distinct structures for each sulfation sequence

With the ability to access well-defined CS sequences, we embarked on systematic investigations into the role of sulfation. HS and CS GAGs play critical roles in cell growth and development by regulating various growth factors, including FGFs, Hedgehog, Wingless, and semaphorins.[3, 18, 21] Peter M. Clark first investigated computationally whether subtle variations in the sulfation pattern would favor distinct

structural conformations of glycosaminoglycans. He used the Dreiding force field[22] (modified slightly using quantum mechanics) with charges from the charge equilibrium[23] (QEq) method and carried out Boltzmann jump simulations[24] on tetrasaccharides **8** – **11** to obtain the lowest-energy CS conformations. These conformations were then used to perform molecular dynamics simulations[24] in explicit water to predict the optimum conformation in solution. Interestingly, we found that each CS tetrasaccharide favors a distinct set of torsion angles and presents a unique electrostatic and van der Waals surface for interaction with proteins (Figure 7.7).[9] Whereas the negatively charged sulfate and carboxylate groups on CS-C point toward either the top or bottom of the face of the molecule, as oriented in Figure 7.7, the same charges on CS-A point in several different directions. Similarly, although CS-E and CS-R have the same number of sulfate groups, the relative orientation of these groups along the carbohydrate backbone leads to distinctly different predicted solution structures. Whereas the CS-R tetrasaccharide has the sulfate groups distributed along several faces of the molecule, the CS-E tetrasccharide presents all four sulfate groups along a single face. This architecture may position the sulfate groups to interact with basic amino acid residues characteristic of GAG binding sites found on proteins.[4]

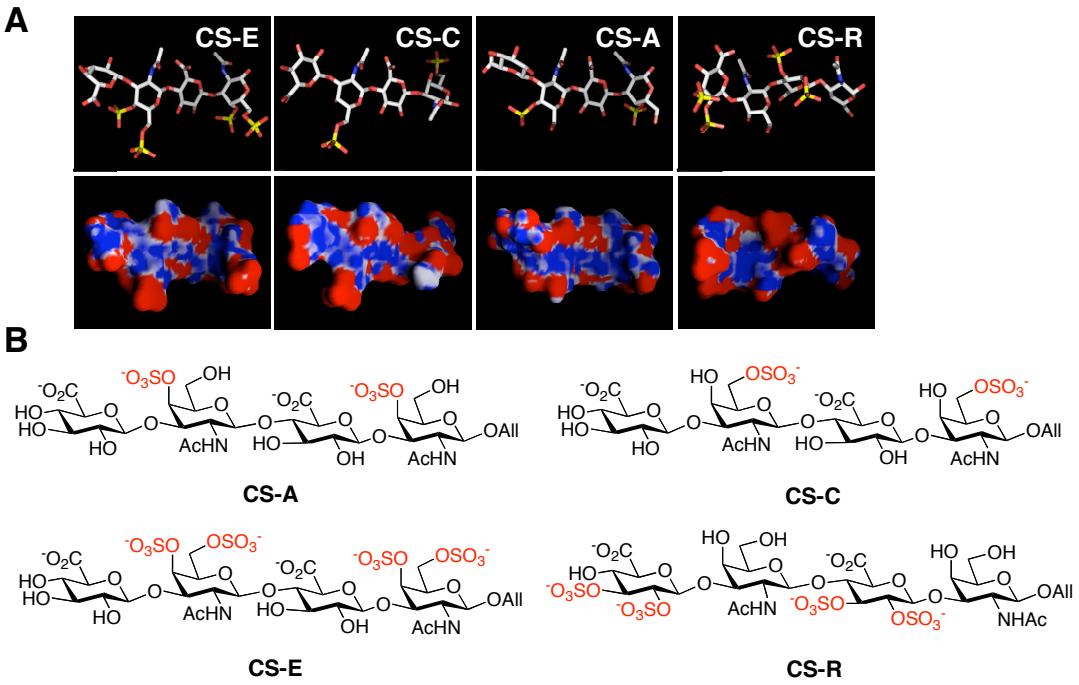

Figure 7.7. Each sulfation pattern exhibits a distinct structural conformation. A) Average structures from molecular dynamics simulations of the CS tetrasaccharides in water. B) Chemical structures of the CS tetrasaccharides. The sulfation pattern influences the structure of CS, allowing it to present distinct electrosatic and van der Waals surfaces to proteins. The CS ball-and-stick figures were created in PyMol and the electrostatic maps were created using GRASP.

Carbohydrate microarrays reveal distinctive binding of CS sulfation motifs to neuronal growth factors

To explore the functional consequences of sulfation on growth factor binding, we generated carbohydrate microarrays using the CS tetrasaccharides. Carbohydrate microarrays have proven to be powerful tools for investigating the interactions of various glycans with proteins, viruses, and bacteria.[25 -- 27] However they have not been extensively exploited for detailed structure-function analyses of GAGs, which pose the unique challenge of presenting carbohydrate structures closely related in stereochemistry and sulfation sequence. Dr. Sarah E. Tully developed and validated the microarray methodology and proceeded to investigate the effects of sulfation on the binding of CS to the growth factor midkine (Figure 7.8).[9] Midkine participates in the development and

repair of neural and other tissues and binds with nanomolar affinity to heterogeneous polysaccharides enriched in the CS-E motif.[28, 29] We observed selective binding of midkine to the CS-E tetrasaccharide at CS concentrations within the physiological range.[30] Notably, the midkine interaction was highly sensitive to the position of the sulfate groups along the carbohydrate backbone (Figure 7.8B). The interaction of midkine with CS-A and CS-C was significantly weaker than that with CS-E. Midkine did not interact as strongly with CS-R as with CS-E, indicating that the midkine-CS association requires a specific arrangement of sulfate groups and is not dictated by nonspecific, electrostatic interactions.

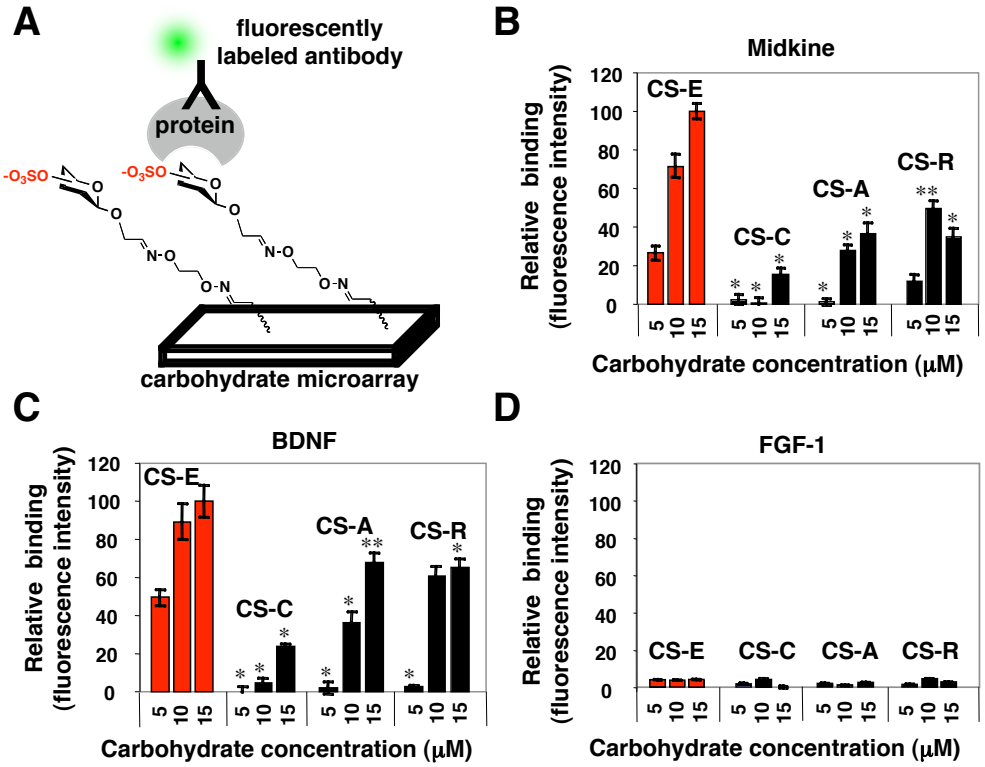

Figure 7.8. A specific sulfation pattern promotes the interaction of CS with neuronal growth factors. A) Overall scheme to detect CS-protein interactions using carbohydrate microarrays. The CS-E tetrasaccharide interacts with the growth factors midkine B) and BDNF C). D) None of the CS tetrasaccharides interact with FGF-1. $*P < 0.0001$, relative to CS-E tetrasaccharide for a given concentration. $**P \leq 0.001$, relative to CS-E tetrasaccharide for a given concentration.

Access to defined sulfation sequences coupled with microarray technologies provides a powerful, rapid means to identify novel GAG-protein interactions and to gain insight into the functions of specific sulfation sequences. In addition to midkine, we discovered that brain-derived neurotrophic factor (BDNF) selectively binds to the CS-E sulfation sequence.[9] The neurotrophin BDNF controls many aspects of mammalian nervous system development and contributes to synaptic plasticity, neurotransmission and neurodegenerative disease.[31] We found that BDNF exhibited a 20-fold preference for the CS-E motif relative to CS-C, CS-A, and CS-R at 5 μM CS concentration, which approximates the estimated concentration of CS-E present in physiological samples (Figure 7.8C).[29, 30, 32] As a control, we demonstrated that none of the tetrasaccharides interacted strongly with FGF-1 (Figure 7.8D), consistent with studies indicating that FGF-1 is regulated by HS but not CS GAGs.[19, 21]

Midkine and BDNF signaling pathways are activated by CS-E interaction

The ability of the CS-E sulfation sequence to interact with growth factors and modulate neuronal growth suggests that CS may recruit specific growth factors to the cell surface, thereby activating downstream signaling pathways. To investigate this potential mechanism, we cultured hippocampal neurons on a CS-E tetrasaccharide or polyornithine substratum in the presence or absence of antibodies selective for midkine or BDNF.[33, 34] The antibodies were expected to block the interaction of the endogenous growth factors with the CS-E substratum and thereby abolish the neuritogenic effects. The effective concentration of each antibody was first determined by treating neurons grown on polyornithine with varying antibody concentrations from 0.5 μg/mL to 20 μg/mL. Only

those concentrations of antibodies that did not affect neuronal growth were then used in the presence of the CS-E tetrasaccharide. The effective concentration of each antibody was determined to be the highest concentration of antibody that elicited the greatest effect in the presence of the tetrasaccharide without eliciting any effect in untreated neurons. Antibodies to midkine or BDNF had no effect on neurite outgrowth in the absence of the tetrasaccharide (Figure 7.9A and 7.10).[9] Importantly, addition of either antibody blocked the neurite outgrowth induced by CS-E. In contrast, neither a control antibody selective for FGF-1 nor class-matched control antibodies were able to abolish the growth-promoting effects of CS-E (Figure 7.9A and 7.10).

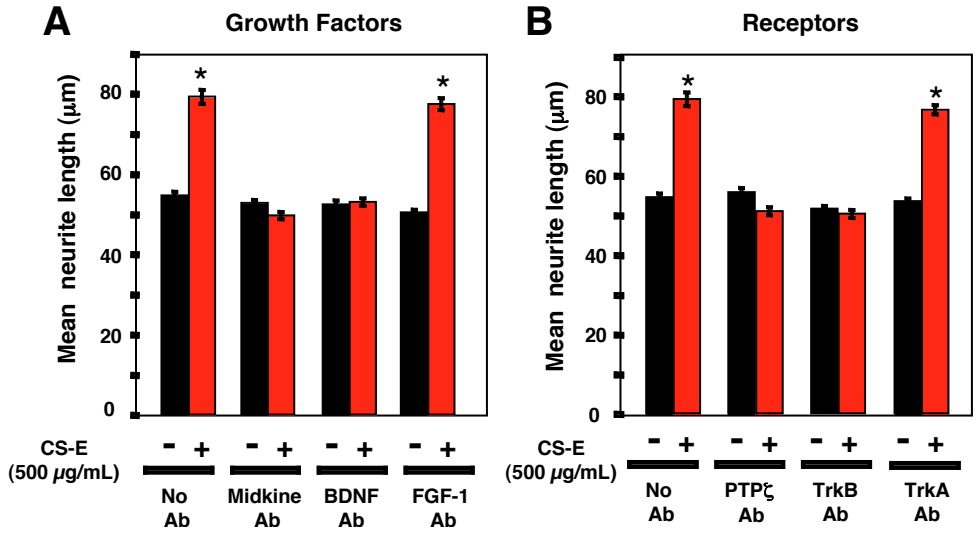

Figure 7.9. The CS-E sulfation motif stimulates neuronal growth through activation of the midkine-PTPζ and BDNF-TrkB signaling pathways. A) Antibodies (Ab) selective for midkine (4 μg/mL) or BDNF (1 μg/mL), but not FGF-1 (4 μg/mL), block the neurite outgrowth induced by CS-E. B) Antibodies against the receptors PTPζ (2 μg/mL) or TrkB (1 μg/mL), but not TrkA (4 μg/mL), abolish the growth-promoting effects of CS-E. $*P < 0.0001$, relative to the no CS-E, no antibody control.

To further confirm the activation of midkine and BDNF signaling pathways by CS-E, we used antibodies that recognize the extracellular domains of the cell surface receptors protein tyrosine phosphatase ζ (PTPζ) and tyrosine kinase B receptor (TrkB).

Binding of midkine and BDNF to PTPζ and TrkB, respectively, has been shown to promote neuronal outgrowth and survival in various systems by activating intracellular pathways such as the mitogen-associated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K) pathways.[28, 31] Notably, antibodies against either PTPζ or TrkB blocked the neuritogenic activity of CS-E (Figure 7.9B and 7.10).[9] In contrast, neither antibody alone had an effect on neurite outgrowth in the absence of CS-E. To demonstrate the specificity of the effects, we showed that function-blocking TrkA and class-matched control antibodies do not influence CS-E mediated neurite outgrowth (Figure 7.9B and 7.10). These results indicate that the CS-E sulfation motif stimulates neuronal growth *in vitro* through selective activation of midkine-PTPζ and BDNF-TrkB signaling pathways.

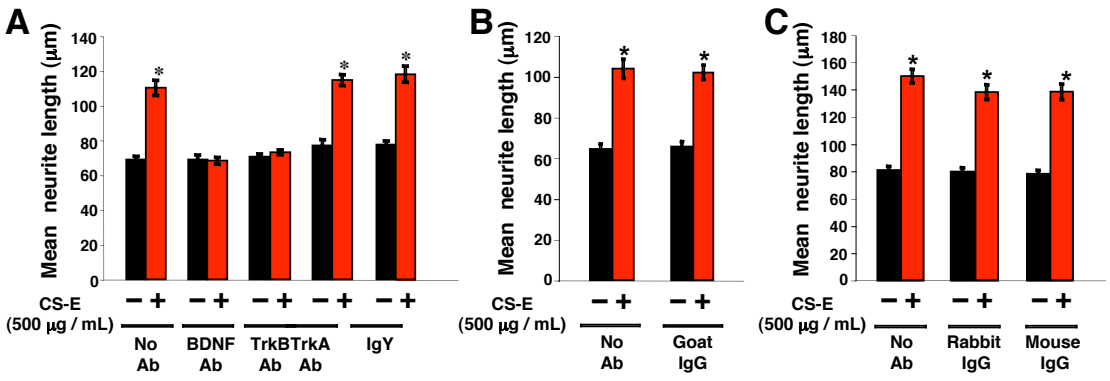

Figure 7.10. Function-blocking antibodies against BDNF and TrkB, but not class-matched control antibodies, disrupt the neuritogenic activity of CS-E. A) The BDNF (10 μg/mL), TrkB (0.5 μg/mL), and TrkA (0.5 μg/mL) antibodies have been shown previously to block endogenous neurotrophin and receptor function. Chicken IgY (10 μg/mL) was the control for the BDNF antibody. B) Goat IgG (4 μg/mL) was the control for the midkine, TrkB, and TrkA antibodies. C) Rabbit IgG (2 μg/mL) was the control for the BDNF, PTPζ, and function-blocking TrkA antibodies; and mouse IgG (4 μg/mL) was the control for the FGF-1 and function-blocking TrkB antibodies. *$P < 0.0001$, relative to the no CS-E, no antibody control

Discussion

The structural diversity of GAGs *in vivo* has led to the hypothesis that specific sulfated structures may modulate the binding and activity of growth factors. However, the complexity and heterogeneity of GAGs has hindered efforts to establish whether growth factors recognize unique sulfation sequences. The overall goal of our research project was to develop a systematic method to investigate the structure-activity relationships of CS carbohydrates. Toward this end, we devised a chemical approach where CS oligosaccharides of defined length and sulfation pattern were synthesized to evaluate the biological activities associated with distinct sulfation sequences.[9, 16] Evaluation of the synthesized molecules revealed that the CS-E sulfation motif was uniquely capable of interacting with neuron growth factors and stimulated the outgrowth of various neuron types. Moreover, we determined that CS-E-mediated stimulation of neurite outgrowth was facilitated by activation of midkine/PTPζ and BDNF/TrkB pathways.

Understanding the many roles of GAGs will require new approaches and reagents to probe and manipulate their structures. We have shown that synthetic chemical approaches are particularly valuable in this regard, enabling the identification of biologically active sulfation motifs, systematic structure-function studies, and the analysis of glycosaminoglycan-protein interactions. While GAGs cannot yet be assembled with the same ease as nucleic acids or proteins, rapid advances in their synthesis and characterization are enabling the first molecular-level investigations of this important class of biopolymers. We anticipate that our approach to systematically explore the role of sulfation sequences will open numerous opportunities for structural

and biophysical studies, as well as facilitate exploration of the roles of GAGs across various proteins and biological contexts.

Experimental Procedures for Chapter 7

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.

Preparation of Coverslips:

Glass coverslips were coated as described in Chapter 6.

Calibration of CS Molecules:

The relative concentrations of the CS-E polysaccharide and CS-E oligosaccharides were calibrated to one another using the carbazole assay as described in Chapter 6.

Treatment of Hippocampal Neurons with the CS-E Polysaccharide and CS Glycopolymers:

Hippocampal neuronal cultures were incubated in 5% CO_2 at 37 °C for 24 h. The media was then removed, and a 1.25 μL solution of CS-E polysaccharide (Seikagaku; ~ 60% of the polysaccharide is estimated to contain the CS-E motif) or glycopolymer in 3.5 M aq.

NaCl was added to supplemented Neurobasal medium (498.75 μL) on each coverslip. A fixed uronic acid concentration of 0.5 μg/mL was used in each case to compare the effects of multivalency. This concentration corresponded to molar concentrations of 14.3 nM for the natural CS-E polysaccharide and 53 nM, 33.2 nM, 2.9 nM, and 16.6 nM for glycopolymer **13** with degree of polymerization (DP) values of 25, 40, 58, and 80, respectively. The molar concentrations of glycopolymers **14** and **15** were 43.2 nM and 12.1 nM, respectively. Importantly, no cellular toxicity was observed at the concentrations used for each compound, as demonstrated by adherence of the cells to the coverslip and healthy cellular morphology. The cultures were incubated for an additional 24 h in 5% CO_2 at 37 °C and analyzed by immunocytochemistry.

*Determining the Relative Potencies of the Natural Polysaccharide and Glycopolymer **15**:* Hippocampal neurons were grown for 24 h before medium was replaced with fresh supplemented Neurobasal medium. Compounds were added at various uronic acid concentrations ranging from 0.01 to 0.5 μg/mL. Neurons were incubated with the compounds for 24 h and then analyzed for neuronal outgrowth as described. To determine the IC_{50} values, the concentrations of the CS-E polysaccharide were calculated based on an average molecular weight of 70,000 g/mol, as provided by the manufacturer. For glycopolymer **15**, a molecular weight of 84,096 g/mol, as determined by GPC, was used. The concentration values were plotted against the % inhibition of neurite outgrowth relative to untreated neurons, and the IC_{50} values represent molar concentrations of compound needed for 50% inhibition of neurite outgrowth. IC_{50} values of 1.2 ± 0.1 nM and 1.3 ± 0.1 nM were determined for the natural polysaccharide and

glycopolymer **15**, respectively (1.2 ± 0.1 nM and 1.6 ± 0.1 nM if calculated based on the saccharide content of each molecule).

Antibody Treatment of Neuronal Cultures:

For the antibody treatments, we cultured hippocampal neurons on a substratum of poly-DL-ornithine in the presence or absence of CS-E tetrasaccharide (100 μL of a 500 μg/mL solution). After 24 h, we added to the medium (final volume of 500 μL) antibodies selective for midkine (Santa Cruz; goat IgG raised against the C terminus of the protein; final concentration of 4 μg/mL), BDNF (Santa Cruz; rabbit IgG raised against residues 130 – 247; final concentration of 1 μg/mL; and Promega; chicken IgY raised against human recombinant protein; final concentration of 10 μg/mL), FGF-1 (R & D Systems; mouse IgG raised against recombinant human protein; final concentration of 4 μg/mL), PTPζ (Santa Cruz; rabbit IgG raised against extracellular domain residues 141 – 440; final concentration of 2 μg/mL), TrkB (Santa Cruz; goat IgG raised against the extracellular domain; final concentration of 1 μg/mL; and BD Transduction Laboratories; mouse IgG raised against extracellular residues 156 – 322; final concentration of 0.5 μg/mL), or TrkA (Santa Cruz; goat IgG raised against the extracellular domain; final concentration of 4 μg/mL; and Abcam; rabbit IgG raised against extracellular residues 1 – 416; final concentration of 0.5 μg/mL). As controls for specificity, we used the corresponding class-matched antibodies (goat IgG, Pierce; rabbit IgG, Pierce; mouse IgG, Pierce; or chicken IgY, Promega) for comparison. The class-matched controls for the midkine, TrkB, and TrkA antibodies were the goat IgG (4 μg/mL), mouse IgG (4 μg/mL), and rabbit IgG (2 μg/mL). The class-matched controls for the BDNF and PTPζ

antibodies were the rabbit IgG and chicken IgY, used at 2 μg/mL and 10 μg/mL, respectively. The class-matched control for the FGF-1 antibody was the mouse IgG, used at 4 μg/mL. We cultured neurons for an additional 24 h before immunostaining them with an antibody to tubulin (Sigma; 1:500) and analyzing them by microscopy.

Immunocytochemistry of Neuronal Cultures:

After 48 h in culture, neurons were treated for immunostaining as described in Chapter 6.

Confocal Laser Microscopy and Morphometric Analysis:

Cells were evaluated by confocal microscopy and quantified for neurite length as described in Chapter 6. Additionally, cells were imaged on a Nikon Eclipse TE2000-S inverted microscope. The images were captured with MetaMorph 6.1 software using a 40x plan fluor oil objective.

References

1. Kitagawa, H., Tsutsumi, K., Tone, Y. & Sugahara, K. Developmental regulation of the sulfation profile of chondroitin sulfate chains in the chicken embryo brain. *J. Biol. Chem.* **272**, 31377 – 31381 (1997).

2. Plaas, A.H.K., West, L.A., Wong-Palms, S. & Nelson, F.R.T. Glycosaminoglycan sulfation in human osteoarthritis. Disease-related alterations at the non-reducing termini of chondroitin and dermatan sulfate. *J. Biol. Chem.* **273**, 12642 – 12649 (1998).

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

4. Capila, I. & Linhardt, R.J. Heparin – protein interactions. *Angew. Chem. Int. Ed. Engl.* **41**, 391 – 412 (2002).

5. Sasisekharan, R., Shriver, Z., Venkataraman, G. & Narayanasami, U. Roles of heparan-sulphate glycosaminoglycans in cancer. *Nat. Rev. Cancer* **2**, 521 – 528 (2002).

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

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

8. Desaire, H., Sirich, T.L. & Leary, J.A. Evidence of block and randomly sequenced chondroitin polysaccharides: Sequential enzymatic digestion and quantification using ion trap tandem mass spectrometry. *Anal. Chem.* **73**, 3513 – 3520 (2001).

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

10. Li, F., Shetty, A.K. & Sugahara, K. Neuritogenic activity of chondroitin/dermatan sulfate hybrid chains of embryonic pig brain and their mimicry from shark liver. Involvement of the pleiotrophin and hepatocyte growth factor signaling pathways. *J. Biol. Chem.* **282**, 2956 – 2966 (2007).

11. Shipp, E.L. & Hsieh-Wilson, L.C. Profiling the sulfation specificities of glycosaminoglycan interactions with growth factors and chemotactic proteins using microarrays. *Chem. Biol.* **14**, 195 – 208 (2007).

12. 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).

13. Maeda, N., Fukazawa, N. & Hata, T. The binding of chondroitin sulfate to pleiotrophin/heparin-binding growth-associated molecule is regulated by chain length and oversulfated structures. *J. Biol. Chem.* **281**, 4894 – 4902 (2006).

14. Tully, S.E., Rawat, M. & Hsieh-Wilson, L.C. Discovery of a TNF-alpha antagonist using chondroitin sulfate microarrays. *J. Am. Chem. Soc.* **128**, 7740 – 7741 (2006).

15. Sotogaku, N., et al. Activation of phospholipase C pathways by a synthetic chondroitin sulfate-E tetrasaccharide promotes neurite outgrowth of dopaminergic neurons. *J. Neurochem.* **103**, 749 – 760 (2007).

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

17. 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).

18. 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).

19. 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).

20. Rawat, M., Gama, C.I., Madson, J.B. & Hsieh-Wilson, L.C. Neuroactive chondroitin sulfate glycomimetics. *J. Am. Chem. Soc.* **130**, 2959 – 2961 (2008).

21. Brickman, Y.G., et al. Structural modification of fibroblast growth factor-binding heparan sulfate at a determinative stage of neural development. *J. Biol. Chem.* **273**, 4350 – 4359 (1998).

22. Mayo, S.L., Olafson, B.D. & Goddard III, W.A. DREIDING: A generic force field for molecular simulations. *J. Phys. Chem.* **94**, 8897 – 8909 (1990).

23. Rappe, A.K. & Goddard III, W.A. Charge equilibration for molecular dynamics simulations. *J. Phys. Chem.* **95**, 3358 – 3363 (1991).

24. Lim, K.-T., et al. Molecular dynamics for very large systems on massively parallel computers: The MPSim program. *J. Comp. Chem.* **18**, 501 – 521 (1997).

25. Feizi, T., Fazio, F., Chai, W.C. & Wong, C.H. Carbohydrate microarrays—a new set of technologies at the frontiers of glycomics. *Curr. Opin. Struct. Biol.* **13**, 637 – 645 (2003).

26. de Paz, J.L., Noti, C. & Seeberger, P.H. Microarrays of synthetic heparin oligosaccharides. *J. Am. Chem. Soc.* **128**, 2766 – 2767 (2006).

27. Park, S., Lee, M.R., Pyo, S.J. & Shin, I. Carbohydrate chips for studying high-throughput carbohydrate-protein interactions. *J. Am. Chem. Soc.* **126**, 4812 – 4819 (2004).

28. Muramatsu, T. Midkine and pleiotrophin: two related proteins involved in development, survival, inflammation and tumorigenesis. *J. Biochem.* **132**, 359 – 371 (2002).

29. 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).

30. Herndon, M.E., Stipp, C.S. & Lander, A.D. Interactions of neural glycosaminoglycans and proteoglycans with protein ligands: assessment of selectivity, heterogeneity and the participation of core proteins in binding. *Glycobiology* **9**, 143 – 155 (1999).

31. Huang, E.J. & Reichardt, L.F. Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* **24**, 677 – 736 (2001).

32. 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).

33. Jiang, B., Akaneya, Y., Hata, Y. & Tsumoto, T. Long-term depression is not induced by low-frequency stimulation in rat visual cortex *in vivo*: A possible preventing role of endogenous brain-derived neurotrophic factor. *J. Neurosci.* **23**, 3761 – 3770 (2003).

34. Seil, F.J. & Drake-Baumann, R. TrkB receptor ligands promote activity-dependent inhibitory synaptogenesis. *J. Neurosci.* **20**, 5367 – 5373 (2000).