Understanding the Chemical Basis of Neuronal Development and Communication:

I. The Role of Fucose α(1-2) Galactose Carbohydrates in Neuronal Growth

II. Structure-Function Analysis of Chondroitin Sulfate in the Brain

Thesis by

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...for my family...

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Abstract

Although carbohydrates are known to participate in many important processes including inflammation, cancer metastasis and pathogenic infection, their functional roles are only beginning to be understood on a molecular level. The challenge is that carbohydrates and glycoproteins are inherently difficult to study. Unlike DNA and proteins, carbohydrate structures are not template-encoded, and the modifications are challenging to detect *in vivo* and manipulate for structure-function analyses. As such, new tools are needed to complement the traditional biochemical and genetic approaches in order to advance our understanding of carbohydrates and their physiological roles. Here, we seek to understand the roles of carbohydrates in regulating the structure and function of proteins in the brain. Our major focus will be on two carbohydrate modifications that play important roles in neuronal communication, development and memory storage: fucosylation (Part I) and chondroitin sulfate glycosaminoglycan modifications (Part II).

In Part I, we describe our progress in elucidating the molecular mechanisms by which fucosyl saccharides regulate neuronal communication in the brain. Information flow in the brain is regulated by synapses, which are specialized sites of contact between neurons. Synaptic connections involve numerous molecular recognition events among proteins, carbohydrates, and small molecules. One of the molecules enriched at the synapse is the sugar L-fucose. Previous studies have suggested that fucoseα(1-2)galactose (Fucα(1-2)Gal) saccharides play essential roles in learning and memory. For instance, preventing formation of Fucα(1-2)Gal linkages has been shown to cause reversible amnesia in animals. Despite these intriguing observations, proteins that

express the Fucα(1-2)Gal epitope (glycoproteins) or proteins that bind this epitope (lectins) have not been identified. Through the use of several chemical probes, we have established that Fucα(1-2)Gal associated proteins participate in a novel carbohydrate-mediated pathway for regulating neuronal growth. Additionally, we have found that Fucα(1-2)Gal glycoproteins are prevalent in the developing brain and that synapsin Ia/Ib are the major Fucα(1-2)Gal glycoproteins in the adult brain. In our attempts to identify Fucα(1-2)Gal lectins, we have established that multivalent polymers enhance our ability to capture and characterize such proteins.

In Part II, we describe our efforts toward understanding the role of chondroitin sulfate glycosaminoglycans in neuronal development. Chondroitin sulfate (CS) glycosaminoglycans are linear, sulfated polysaccharides implicated in cell division, neuronal growth, and spinal cord injury. The structural complexity and heterogeneity of CS has hampered efforts to understand its precise biological roles. Although they exist as a heterogeneous mix in nature, it is thought that CS activity is dictated by a sulfation code where distinct sulfation sequences are spatially and temporally regulated and direct the biological activity of CS glycosaminoglycans. We have developed a chemical approach to evaluate the structure-activity relationship of CS as it effects neuronal growth. We generated the first synthetic library of well-defined CS oligosaccharides containing various sulfation sequences and have demonstrated that the CS-E sulfation sequence is a stimulatory motif that promotes the growth of several neuron types. Moreover, we determined that CS-E mediated stimulation of neurite outgrowth was facilitated by activation of midkine/PTPζ and BDNF/TrkB signaling pathways.

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List of Abbreviations

1D	one-dimensional
2D	two-dimensional
2-dGal	2-deoxy-D-galactose
2-fucosyllactose	L-fucoseα(1-2)galactoseβ(1-4)glucose
3-dGal	3-deoxy-D-galactose
6-dGal	6-deoxy-D-Galactose
AAA	*Anguilla anguilla* agglutinin
Ab	antibody
Ac	acetyl, acetate
aq	aqeous
BCA	bicinchoninic acid
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
°C	degree Celsius
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
CH_3N	acetonitrile
$CHCl_3$	chloroform
CMF-HBSS	Calcium and Magnesium Free Hank's Balanced Salt Solution
CNS	central nervous system
CO_2	carbon dioxide
CRD	carbohydrate recognition domain
CS	chondroitin sulfate
ddH_2O	double distilled water
D-Gal	D-galactose
diazirine	trifluoromethylphenyldiazirine
DIV	days in vitro
DMEM	Dulbecco's Minimal Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DP	degree of polymerization
DRG	dorsal root ganglion
DTT	dithiothreitol
E18	embryonic day 18
ECL	enhance chemiluminescence
EDTA	ethylenediaminetetraacetic acid
endo N	endoneuraminidase
EXT1 and 2	exostoses enzymes 1 and 2
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor, tyrosine kinase receptor
Fuc	L-fucose
Fucα(1-2)Gal	fucose α(1-2) galactose
FX	epimerase-reductase enzyme

g	gram, gravitational force
GAG	glycosaminoglycan
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GDP-fucose	guanosine diphosphatyl-fucose
GlcA	D-glucuronic acid
GlcN	D-glucosamine
GlcNAc	*N*-acetylglucosamine
Gluc	glucose
GluR1	glutamate receptor 1
GMD	GDP-mannose-4,6-dehydratase
GPC	gas phase chromatography
GTP	guanosine triphosphate
h	hour
HIO_4	periodate
HRP	horse-radish peroxidase
HS	heparan sulfate
IC_{50}	inhibition concentration at 50%
IdoA	L-iduronic acid
IgG	immunoglobulin
IP	immunoprecipitated
K^+	potassium ion
K_{assoc}	association constant
KCl	potassium chloride
kDa	kilodalton
L	liter
LTL	*Lotus tetragonolobus* lectin
LTP	long-term potentiation
M	molar
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MAP2	microtubule associated protein 2
MAPK	mitogen-associated protein kinase
MEM	Minimal Eagle's Medium
MeOH	methanol
μg	microgram
$MgCl_2$	magnesium chloride
min	minutes
m	milli or meter
μ	micro
mol	mole
MS	mass spectrometry
n	nano
N	normal
Na^+	sodium ion
NaCl	sodium chloride
NaOH	sodium hydroxide

NCAM	neural cell adhesion molecule
NDST	*N*-deacetylase-*N*-sulfotransferase
NH_4HCO_3	ammonium bicarbonate
NP-40	nonidet P-40 detergent
NSF	*N*-ethylmaleimide sensitive factor
OEt	*O*-ethyl
P2	insoluble fraction 2
PAA	polyacrylamide polymer lacking saccharide
PAGE	polyacrylamide gel electrophoresis
PAO	phenyl arsine oxide
PAPS	3'phosphoadenosine 5'phosphosulfate
PBS	phosphate buffered saline
PI3-K	phosphatidylinositol 3-kinase
PSA	polysialic acid
PSD-95	post synaptic density protein 95
PTPζ	protein tyrosine phosphatase zeta
PVDF	polyvinylidene difluoride
Qeq	charge equilibrium
RNA	ribonucleic acid
rpm	revolutions per minute
rt	room temperature
S2	soluble fraction 2
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Syn KO	synapsin knockout
TBST	tris buffered saline with Tween-20
TNFα	tumor necrosis factor alpha
Tris-Cl	tris chloride
TrkA	tyrosine kinase A receptor
TrkB	tyrosine kinase B receptor
U	unit
UEA I	*Ulex europeaus* agglutinin I
UV	ultraviolet
vol	volume
w/v	weight per volume
WGA	wheat germ agglutinin
WT	wild type

Part I—Chapter 1: Fucosylation in the Brain

Carbohydrates in cellular communication

The cell surface is a fluid, dynamic structure composed of many different biomolecules, including proteins, lipids, and carbohydrates. Most often, carbohydrates are found on the cellular membrane covalently bound to lipids and proteins, where they form glycolipids and glycoproteins, respectively. These membrane-bound carbohydrates serve as markers that distinguish individual cells and are crucial elements in cell-cell recognition and communication. Many glycoproteins are known to be involved in numerous biological processes including cell differentiation, neuronal growth, interneuronal recognition, and signal transduction.[1, 2] In the brain, about 80% of glycoproteins are found in the microsomal fraction and in synaptic membranes.[3]

The reorganization of synaptic membranes (synaptic remodeling) allows for the formation of memory traces and involves changes in the synthesis of glycoproteins as well as posttranslational modifications to existing glycoproteins.[4] This synaptic remodeling is thought to underlie information processing in the brain and leads to memory formation. One of the leading models for how memory traces are mechanistically formed in the brain is long-term potentiation (LTP), a process where high-frequency stimulation of a nerve cell causes an abrupt and sustained increase in the efficiency of synaptic transmission of the signal.[4] LTP has been found to occur in both vertebrates and invertebrates in a variety of neural systems, ranging from the mammalian peripheral nervous system to the arthropod neuromuscular junction to subcortical mammalian nuclei, such as the amygdala.[5]

Changes in neuronal morphology have been directly associated with LTP in various systems. Inducing LTP in cultured rat hippocampal slices caused new spines to appear on post-synaptic dendrites, whereas no significant spine growth was found in regions where LTP was blocked.[6] Similar results were seen in hippocampal slices from spatially trained rats, where a significant increase in dendritic spine density was observed in trained rats compared to nontrained rats.[7] Such synaptic remodeling could involve glycosylation of synaptic proteins, as supported by the presence of protein glycosylation machinery in dendrites.[8] Interestingly, the presence of glycosylation machinery in dendrites suggests that the synthesis of glycoproteins at the synapse may be dynamically regulated. Moreover, protein glycosylation has been shown to be necessary for maintaining hippocampal LTP. Treating hippocampal slices with different protein glycosylation inhibitors (tunicamycin, brefeldin A, swainsonine) during induction of LTP prevented maintenance of LTP and caused postsynaptic potentials to return to baseline levels within 100 minutes after induction of LTP.[9] However, postsynaptic potentials from control slices remained elevated for more than 200 minutes following induction of LTP.[9] These findings provide a direct link between learning, LTP, synaptic remodeling, and protein glycosylation.

Evidence for the importance of fucose α(1-2) galactose glycoproteins in the mammalian brain

The formation of synaptic connections and synaptic remodeling involves the recognition of different molecules present at the membrane, including various proteins, lipids, carbohydrates, and other small molecules. We are particularly interested in the

sugar L-fucose (Figure 1.1), one of the small molecules enriched in the synaptosomal fraction.[10]

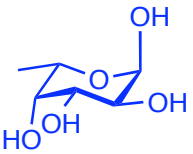

Figure 1.1. The monosaccharide L-fucose

L-Fucose (Fuc) is one of the 9 standard carbohydrate building blocks commonly found in mammals, and fucose-containing oligosaccharides play important roles in many biological processes, including blood transfusion reactions, host-microbe interactions, and cancer pathogenesis.[11] Fucose is appended to various biomolecules by fucosyltransferases, of which thirteen have been identified in the human genome.[11] All currently identified fucosyltransferases utilize guanosine diphosphatyl-fucose (GDP-fucose) as the monosaccharide donor. In mammalian cells, two pathways exist for the synthesis of GDP-fucose, the *de novo* pathway and the salvage pathway (Figure 1.2).

The *de novo* pathway converts GDP-mannose to GDP-fucose via three enzymatic reactions carried out by two enzymes. First, GDP-mannose-4,6-dehydratase (GMD) converts GDP-mannose to GDP-4-keto-6-deoxymannose through oxidation of the hydroxyl group at C-4 of the mannose ring and reduction of the hydroxyl group at C-6. Next, the dual functional epimerase-reductase enzyme known as the FX protein converts GDP-4-keto-6-deoxymannose to GDP-fucose. In the salvage pathway, GDP-fucose is synthesized from free fucose via a two-step process. In the first step, free fucose derived from extracellular or lysosomal sources is transported into the cytosol and converted to fucose-1-phosphate via fucose kinase. Next, GDP-fucose pyrophosphorylase catalyzes the reversible condensation of fucose-1-phosphate with GTP to form GDP-fucose. All

GDP-fucose, synthesized from either pathway, must then enter the Golgi apparatus to be used for fucosylation reactions by the fucosyltranferases.

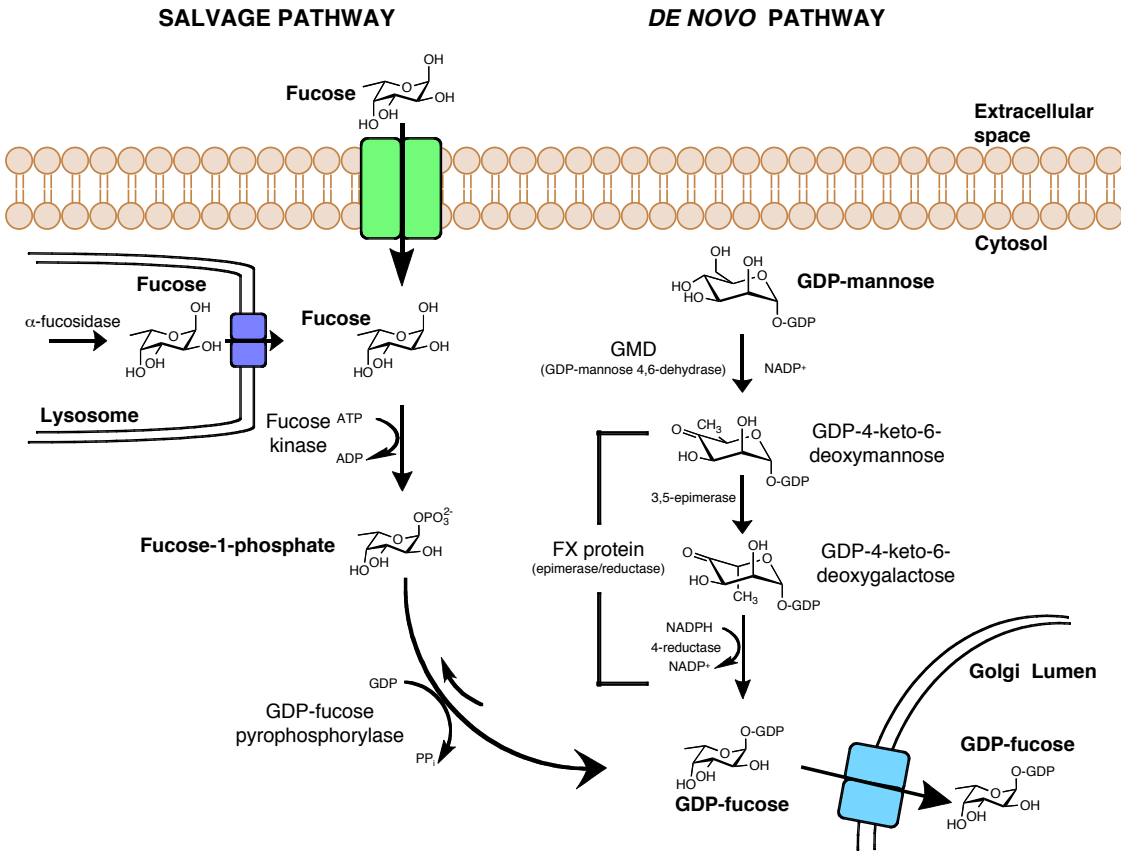

Figure 1.2. In mammalian cells, the biosynthesis of GDP-fucose occurs through two distinct pathways. The *de novo* pathway converts GDP-mannose to GDP-fucose via three enzymatic reactions carried out by two proteins, GMD and the FX protein. The salvage pathway utilizes free fucose found in the cytosol to synthesize GDP-fucose using the enzymes fucose kinase and GDP-fucose pyrophosphorylase. GDP-fucose generated by either pathway is transported into the Golgi lumen to become available to the fucosyltransferases.

In the brain, L-Fuc is usually found as the terminal residue on carbohydrate chains attached to proteins via *N*- or *O*-linkages to asparagine or serine/threonine residues, respectively.[12, 13] Frequently, L-Fuc is attached to the C-3 and C-6 positions of *N*-acetylglucosamine (GlcNAc) or to the C-2 position of galactose (Gal) (Figure 1.3).[12] Several studies support the idea that oligosaccharides containing terminal fucose α(1-2)

galactose (Fucα(1-2)Gal) moieties contribute to information storage and processing in the brain.

The importance of Fucα(1-2)Gal is supported by behavioral and electrophysiological studies using the unnatural sugar analog 2-deoxy-D-galactose (2-dGal). In these studies, the sugar analog 2-dGal competes with Gal for incorporation into carbohydrate chains.[14] Once inserted, 2-dGal inhibits the formation of a specific 1-2 glycosidic linkage with fucose because it lacks the hydroxyl group at the C-2 position (Figure 1.4).

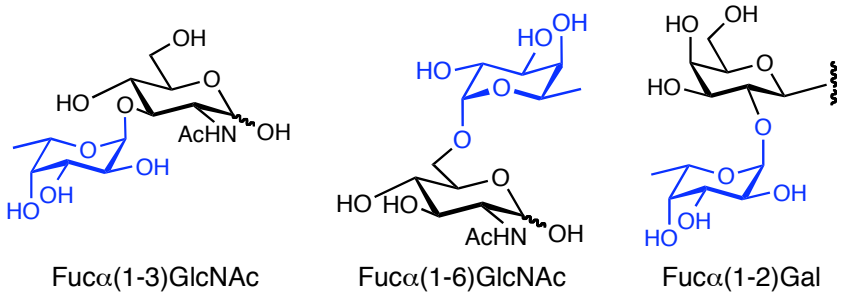

Figure 1.3. Common fucose-galactose linkages found on the terminal ends of carbohydrate chains

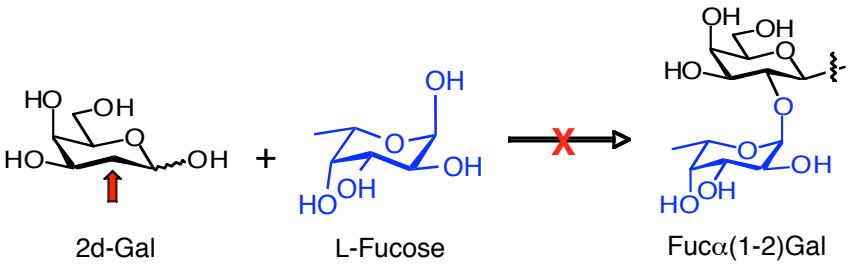

Figure 1.4. Incorporation of 2-deoxy-D-Galactose (2-dGal) inhibits formation of Fucα(1-2)Gal linkages. 2-dGal is missing the hydroxyl group at the C-2 position (red arrow) and thus prevents the incorporation of fucose into carbohydrate chains.

Fucose incorporation into hippocampal glycoproteins was found to be significantly reduced in trained rats injected with 2-dGal compared to saline-treated rats.[15, 16] Furthermore, treating rats with 2-dGal either 30 minutes before or 15 minutes

after retrieval testing caused amnesia in a passive avoidance response.[17] Similar experiments performed in trained chicks also resulted in decreased fucose incorporation and amnesic effects in animals treated with 2-dGal.[18, 19] In electrophysiological studies, hippocampal LTP maintenance was also suppressed upon treatment with 2-dGal.[20] These studies underscore the importance of Fucα(1-2)Gal glycoproteins in long-term memory formation.

Although many studies have looked at the amnesic effects of 2-dGal in the brain, studies have yet to examine the impact of 2-dGal on synaptic remodeling or neuronal structure. However, related small molecules have been shown to elicit varying effects in cell culture. Incorporation of the sugar analog 2-deoxy-2-fluoro-D-galactose in cultured rat hepatocytes inhibited *N*-glycosylation of membrane proteins.[21, 22] However, addition of the analog 2-deoxy-2-fluoro-D-glucose only partially inhibited *N*-glycosylation.[22] Furthermore, neither analog affected *O*-glycosylation.

Additional support for the importance of fucose-expressing glycoproteins in learning and memory can be found in studies examining the levels of fucose incorporation following learning tasks. When incorporation of [^{14}C]-labeled fucose was monitored in trained chicks, a linear increase of fucose could be seen in forebrain slices for up to 3 hours after training.[3] Furthermore, a 26% increase in tritium-labeled fucose 24 hours following learning in trained chicks, as compared to nontrained chicks, was observed in a separate study.[23] Collectively, these findings support the view that glycoproteins expressing the Fucα(1-2)Gal epitope play a critical role in neuronal communication.

It is interesting to note that glycoproteins present at synapses have been previously implicated in cellular recognition and adhesion steps during synaptic development. A well-studied example is the neural cell adhesion molecule (NCAM), a member of the immunoglobulin superfamily that is glycosylated with polysialic acid (PSA) residues. NCAM is crucial in the development and regeneration of the nervous system and is also involved in synaptogenesis.[24, 25] Interestingly, the PSA residues present on NCAM greatly affect these functions. Enzymatic removal of PSA with endoneuraminidase (endo N) inhibited LTP in the hippocampus and disrupted neuron migration and axon outgrowth.[26 -- 29] Polysialylated NCAM also helps maintain membrane fluidity and neural plasticity by increasing intermembrane repulsion and inhibiting cellular adhesion.[30]

Evidence for the existence of Fucα(1-2)Gal lectins in the mammalian brain

In addition to the role of fucose-containing glycoproteins, there is evidence that proteins which specifically recognize fucose may also be involved in regulating neuronal communication. The importance of lectins (proteins that recognize carbohydrates) that specifically recognize the Fucα(1-2)Gal moiety in memory formation has been demonstrated in several behavioral studies using an antibody specific to the Fucα(1-2)Gal epitope.

The monoclonal antibody A46-B/B10, which recognizes the trisaccharide Fucα(1-2)Galβ(1-4)GlcNAc[31] (Figure 1.5), was injected intrahippocampally to rats trained on a brightness discrimination task. Administration of the antibody both before and after training did not interfere with learning but drastically reduced the retention of

the task in relearning sessions, whereas an antibody selective for other trisaccharides had no amnesic effect.[32] Although the potential molecular mechanism(s) by which the antibody causes amnesia has not been elucidated, one potential explanation is that the antibody prevents the interaction between Fucα(1-2)Gal-containing glycoproteins and lectins. These studies support the notion that Fucα(1-2)Gal lectins may be involved in modulating neuronal communication.

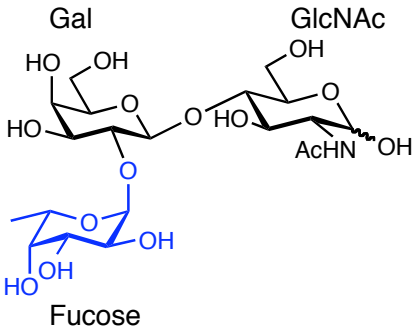

Figure 1.5. The trisaccharide Fucα(1-2)Galβ(1-4)GlcNAc recognized by antibody A46-B/B10

Studies using free fucosyl saccharides provide further evidence for the importance of Fucα(1-2)Gal lectins in the brain. Several reports have shown that free fucosyl saccharides enhance memory retention and LTP. For instance, rats injected with L-Fuc or the trisaccahride L-Fucα(1-2)Galβ(1-4)Glucose (2'-Fucosyllactose) exhibited prolonged, enhanced potentiation following induction of LTP compared to control animals injected with saline or lactose.[33] Higher potentiation following LTP induction was also observed in rat hippocampal slices incubated with L-Fuc and 2'-Fucosyllactose.[34] Importantly, the observed effects were stereo- and regiospecific as neither D-fucose or 3'-Fucosyllactose showed any effect on LTP or memory consolidation.[34] While these effects may also be due in part to fucosylation of

glycoproteins, another potential explanation is that these fucosyl saccharides could be interacting with specific Fucα(1-2)Gal lectins on the cell surface to promote LTP.

The binding of lectins to specific carbohydrate motifs is well precedented in mediating cellular recognition events, including leukocyte adhesion, microbial phagocytosis, and neuronal outgrowth.[35 -- 37] One well-studied example is the galectin family of lectins, which specifically recognize galactose-containing oligosaccharides. Galectins are found on both the cell surface and the extracellular matrix, as well as in the cytoplasm and nucleus, and are involved in cell adhesion, cell growth, and apoptosis.[38] Notably, galectin-3 mediates cell adhesion in a carbohydrate-dependent manner, as treatment of cells with either endo-β-galactosidase (a protein that cleaves the galactose moieties from the protein) or a polyvalent carrier of terminal β-galactosides inhibited adhesion.[39]

Proposed mechanisms of Fucα(1-2)Gal action

As detailed in the above studies, L-Fuc and, in particular, the Fucα(1-2)Gal saccharide appear to be critical components for long-term information storage. Despite the overwhelming evidence implicating the Fucα(1-2)Gal epitope in memory formation, relatively little is known about the proteins that display (glycoproteins) or bind (lectins) this epitope. One research group has reported the identification of several proteins to be Fucα(1-2)Gal glycoproteins.[40] However, the existence of a Fucα(1-2)Gal epitope on these proteins was not rigorously proven and was only shown through antibody binding, which can be somewhat promiscuous. Additionally, another group has reported the fucose-mediated binding of the proteoglycan versican to the adhesion protein tenascin-R.

Importantly, only the C-type lectin domain of versican, expressed as a recombinant protein, was shown to bind to insolubilized fucose.[41] Aside from these two reports, no other Fucα(1-2)Gal glycoproteins or lectins have been identified. Moreover, the functional significance of the Fucα(1-2)Gal epitope has not yet been investigated.

Based on literature findings and our own results, we have developed three working models to explain the role of fucosyl saccharides in modulating neuronal communication. In the first model, we propose that the Fucα(1-2)Gal epitope functions as a "recognition element" to mediate protein-protein interactions at the cell surface (Mechanism A, Figure 1.6). In this model, specific molecular recognition between Fucα(1-2)Gal glycoproteins and lectins would be expected to trigger intracellular signaling cascades that modulate neuronal communication. In the second model, we propose that the Fucα(1-2)Gal epitope may function as a "targeting element" to regulate the folding, function and/or trafficking of Fucα(1-2)Gal glycoproteins to the synapse (Mechanism B, Figure 1.6). By directing glycoproteins to the synapse, the Fucα(1-2)Gal epitope may position the glycoproteins to perform essential functions, as well as help recruit proteins to the synapse during cell communication events. Alternatively, it is possible that fucosyl glycoproteins may act intracellularly to modulate proteins and signaling pathways involved in synaptic plasticity (Mechanism C, Figure 1.6).

Given the complexity of the brain, it is possible that these three mechanisms are operating in parallel to mediate neuronal communication. Regardless of which model is correct, it is clear that identification of Fucα(1-2)Gal lectins and glycoproteins is necessary to understand the potential role of fucosyl saccharides in the formation and strengthening of synaptic connections. Furthermore, the discovery of new lectins and

glycoproteins will enable a molecular-level understanding of the role of fucosyl saccharides on neuronal communication and provide new insights into cell communication and synaptic plasticity in the brain.

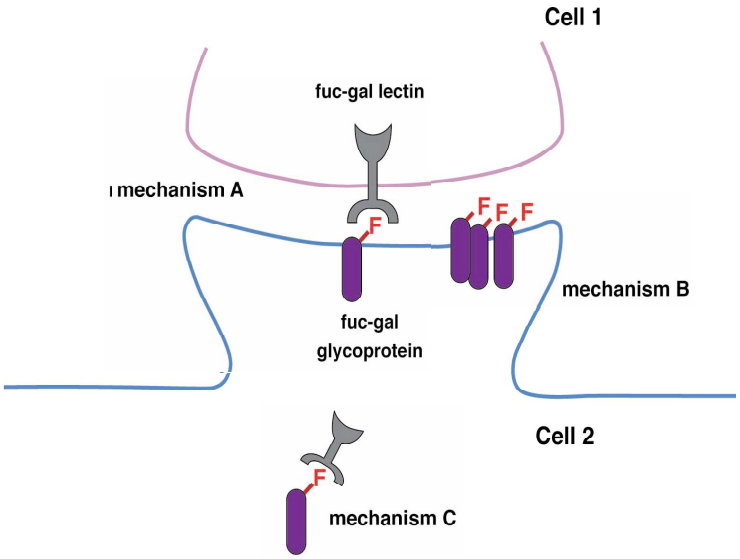

Figure 1.6. Proposed mechanisms by which the Fucα(1-2)Gal epitope acts in neurons. Fucα(1-2)Gal saccharides (**F**) may function as recognition elements to mediate protein-protein interactions between Fucα(1-2)Gal lectins and glycoproteins (mechanism A) or as targeting elements to increase the concentration of fucose at the synapse (mechanism B). Additionally, Fucα(1-2)Gal proteins may operate intracellularly to modulate proteins at the synapse.

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Chapter 2: Fucose α(1-2) Galactose Carbohydrates Regulate Neuronal Growth[*†]

Background

Carbohydrates play important roles in numerous biological processes and are key modulators of molecular and cellular recognition. The diverse chemical structures of carbohydrates encode vast amounts of information and serve as critical determinants of protein folding, trafficking, and stability.[1] Carbohydrates are highly abundant in the brain and are involved in various neural functions including learning and memory, brain development, and spinal cord injury.[2 -- 4] Despite their importance in neurobiology, the precise molecular mechanisms by which carbohydrates influence these processes in the brain are not well understood.

In the brain, information flow from one cell to another is regulated by synapses, which are specialized sites of contact between neurons. Not surprisingly, about 80% of glycoproteins are found in the microsomal fraction and in synaptic membranes.[5] One of the molecules enriched at the synapse is the sugar L-fucose.[6] L-Fucose primarily exists as a terminal residue on *N*- or *O*-linked glycoproteins and is frequently attached to the C-3 and C-6 position of N-acetylglucosamine or the C-2 position of galactose.[7] Interestingly, the fucose α(1-2) galactose (Fucα(1-2)Gal) linkage has been implicated in cognitive processes such as learning and memory.

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[*] Synthesis of fucose α(1-2) galactose probe **1** was done by Dr. Lori W. Lee, a former graduate student in the Hsieh-Wilson laboratory, and Dr. Stacey A. Kalovidouris, a former postdoctoral scholar in the Hsieh-Wilson laboratory. Treatment of neuronal cultures with multivalent polymers and known fucose lectins were done in collaboration with Dr. Kalovidouris.

[†] Portions of this chapter were taken from S.A. Kalovidouris et al. (2005) *J. Am. Chem. Soc.* **127**, 1340 – 1341.

Several lines of evidence suggest that Fucα(1-2)Gal carbohydrates play essential roles in modulating neuronal connections important for learning and memory. For instance, blocking the formation of Fucα(1-2)Gal linkages on glycan chains using 2-deoxy-D-galactose (2-dGal) causes reversible amnesia in animals and interferes with the maintenance of long-term potentiation (LTP).[8 -- 11] Since 2-dGal specifically inhibits the incorporation of [^{14}C]-radiolabeled fucose into glycoproteins at the synapse[8], it is possible that Fucα(1-2)Gal glycoproteins contribute to memory storage. Additionally, injection of a monoclonal antibody selective for Fucα(1-2)Gal also impairs memory formation in animals[12, 13], presumably by blocking the Fucα(1-2)Gal epitope. Furthermore, both task-specific learning and LTP have been shown to increase fucosylation of proteins at the synapse and addition of L-fucose or 2'-fucosyllactose was found to enhance LTP.[5, 14 -- 16]

Despite these intriguing observations, relatively little is known about the proteins that express the Fucα(1-2)Gal epitope (glycoproteins) or those proteins that bind this epitope (lectins). Furthermore, no Fucα(1-2)Gal-associated proteins have been identified from the brain. We therefore sought to establish the existence of Fucα(1-2)Gal lectins and glycoproteins in the brain. Through the use of chemical and biochemical tools, we have demonstrated that Fucα(1-2)Gal and its associated proteins promote the growth of hippocampal neurons and identify a novel, carbohydrate-mediated pathway for regulating neuronal growth and morphology.

Fucα(1-2)Gal lectins exist in neurons

The overall goal of our research project is to understand how fucosyl saccharides are involved in cell-cell recognition in the brain and to determine how these

carbohydrates impact different processes such as learning and memory. Toward this end, we developed a chemical probe for detecting Fucα(1-2)Gal lectins in neurons. The small molecule probe was synthesized by Dr. Lori W. Lee and Dr. Stacey A. Kalovidouris and was made to mimic endogenous glycoproteins containing Fucα(1-2)Gal linkages (Figure 2.1).[17] This probe, **1**, has two key structural elements: (1) the Fucα(1-2)Gal moiety and (2) a biotin moiety.

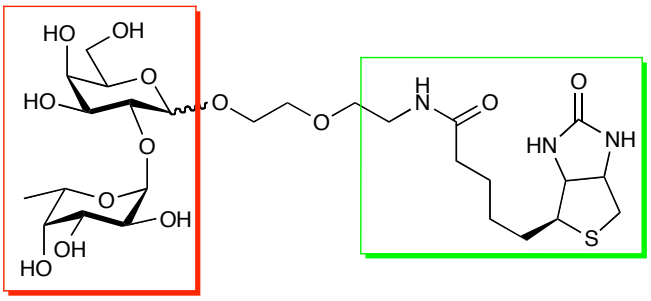

Figure 2.1. Fucα(1-2)Gal-biotin probe **1** was designed to mimic endogenous glycoproteins. The two main structural elements are a Fucα(1-2)Gal moiety (red) for protein recognition and a biotin moiety (green) for fluorescent labeling.

The Fucα(1-2)Gal disaccharide was selected as the recognition element to conclusively demonstrate the importance of the Fucα(1-2)Gal linkage. We decided against using a trisaccharide, as previous research has failed to provide conclusive evidence about the identity or importance of the third sugar. Moreover, L-fucose or 2'-fucosyllactose can stimulate memory formation with approximately equal efficacy, suggesting that Fucα(1-2)Gal may be sufficient for interaction with target lectins. The biotin moiety was included to enable examination of the cellular localization of Fucα(1-2)Gal lectins by fluorescence microscopy. Biotin binds specifically and with high affinity to streptavidin, and a variety of streptavidin-dye conjugates are commercially available for fluorescence staining of cells.

With Fucα(1-2)Gal probe **1** in hand, we tested whether lectins specific for the fucose disaccharide are present in neurons. Using protocols similar to those of Goslin, Asmussen and Banker[18], hippocampal cultures were prepared from embryonic-day 18 (E18) rats and were maintained in culture for at least two weeks. Over the course of two weeks, the cells develop neurites and axons (day 1 – 2), dendrites (day 4 – 5) and, eventually, elaborate networks of neuronal processes and synapses (day > 7). During each stage of development, neurons can be fixed and treated with small molecules or antibodies for specific proteins or carbohydrates. Thus, the expression and subcellular localization of those carbohydrates, lectins, and glycoproteins of interest can be monitored by fluorescence microscopy. This allowed us to study how the expression and distribution of Fucα(1-2)Gal lectins may change with neuronal development or external stimuli.

Fucα(1-2)Gal probe **1** was incubated for 1 hour with neurons that had been cultured for 14 days in vitro (DIV) and then fixed. Following application of probe **1**, the neurons were washed and incubated with streptavidin conjugated to AlexaFluor 488 dye (Molecular Probes) and the Fucα(1-2)Gal probe was detected using confocal fluorescence microscopy. As shown in Figure 2.2, probe **1** binds specifically to neurons and labels the cell body, neuronal processes, and possibly synapses. Several control experiments were conducted to confirm that the observed fluorescence was due to specific recognition of the Fucα(1-2)Gal probe. Upon optimization of the blocking, incubation, and wash steps, we were able to identify conditions where low background staining was obtained, proving that the streptavidin dye conjugate was not simply staining the neurons in the absence of the probe (Figure 2.2B). Second, we confirmed

that the results were not attributable to the biotin portion of the molecule by incubating neurons with D-biotin alone. Once again, we obtained no significant background fluorescence (Figure 2.2C). Thus, our experimental data provide strong evidence that Fucα(1-2)Gal lectins exist in hippocampal neurons.

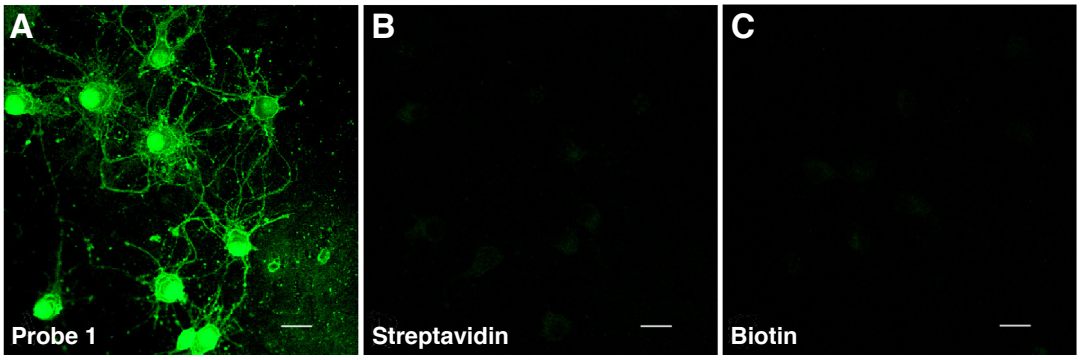

Figure 2.2. Fucα(1-2)Gal probe **1** binds to the cell surface of hippocampal neurons. A) Probe **1** (10 mM) binds selectively to neurons. B) Incubation of neurons with streptavidin dye conjugate produces no background signal. C) Incubation of neurons with biotin (10 mM) also produces no background signal. Scale bars, 20 μm

To prevent intracellular uptake of the compounds, neurons were co-incubated with the endocytosis inhibitor phenyl arsine oxide (PAO).[19] After 23 DIV, hippocampal neurons were treated with 10 μm PAO and either probe **1** or biotin. After 1 hour, neurons were washed, fixed, and stained with streptavidin-dye conjugate and examined by fluorescence microscopy. Again, we saw that probe **1** specifically labels the cell body and neurite processes (Figure 2.3).[17]

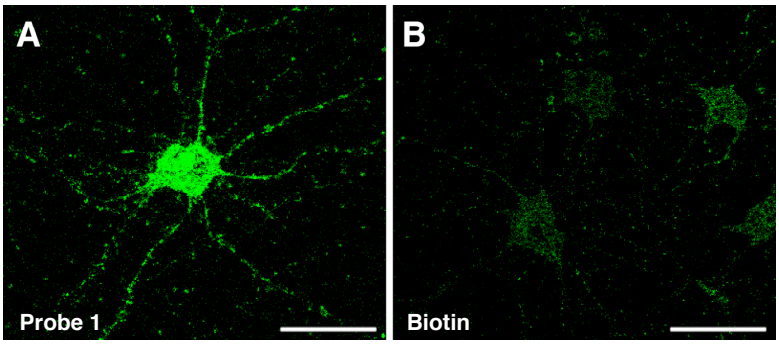

Figure 2.3. Fucα(1-2)Gal probe **1** binds to hippocampal neurons. Neurons were cultured for 23 DIV and then treated with 3 mM of (A) probe **1** or (B) biotin in the presence of 10 μM PAO. Scale bars, 45 μm

To confirm that probe **1** was binding specifically to proteins rather than interacting with the membrane lipids, neurons were delipidated following the protocol of Yavin and Yavin.[20] Briefly, after 23 DIV, cells were rinsed with PBS and exposed to a methanol/chloroform mixture ($MeOH/CHCl_3$; 1/2 by volume) for 15 minutes at –80 °C. This procedure fixes the cells to the glass coverslip and extracts cellular lipids. After removing the $MeOH/CHCl_3$ mixture, neurons were treated with PAO and either probe **1** or biotin. Lipid extraction of cellular membranes prior to treatment with probe **1** did not diminish the labeling (Figure 2.4),[17] consistent with a carbohydrate-protein interaction.

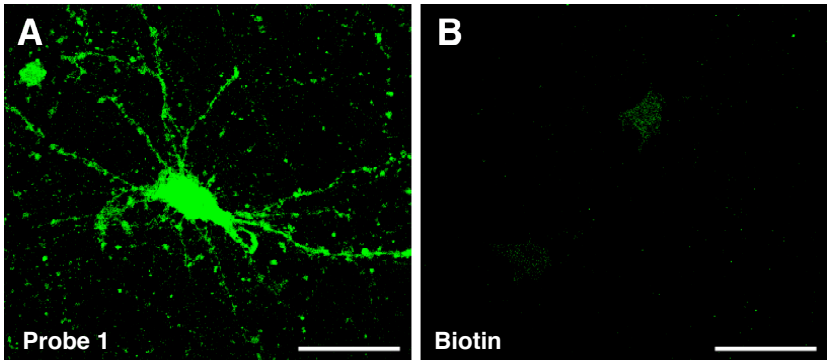

Figure 2.4. Lipid extraction does not alter labeling with Fucα(1-2)Gal probe **1**. Neurons were delipidated with $MeOH/CHCl_3$ prior to labeling with 3 mM of (A) probe **1** or (B) biotin in the presence of 10 μM PAO. Scale bars, 45 μm

After determining that the Fucα(1-2)Gal probe recognized proteins in cultured neurons, we conducted several experiments to determine the subcellular localization of the Fucα(1-2)Gal lectins that were being detected. First, we simultaneously incubated neurons with probe **1** and an antibody to tau protein. Tau is a microtubule-binding protein that is found in cell bodies, axons, and dendrites.[21] After incubating with the streptavidin-dye conjugate and the appropriate secondary antibody for the tau antibody, neurons were visualized with a confocal laser microscope equipped with 488 nm and 546

nm laser lines (Figure 2.5). Again we see that probe **1** (green) binds to the cell surface and along both dendrites and axons and overlaps almost completely with the tau labeling (red).

Second, we concurrently stained neurons with Fucα(1-2)Gal probe **1** and anti-MAP2 antibodies. MAP2 is a selective marker for dendritic processes.[22] In Figure 2.6, we see that probe **1** (green) clearly binds to dendrites labeled with the MAP2 antibody (red). We also see binding of Fucα(1-2)Gal probe **1** to axons and on the cell surface (Figure 2.6A).

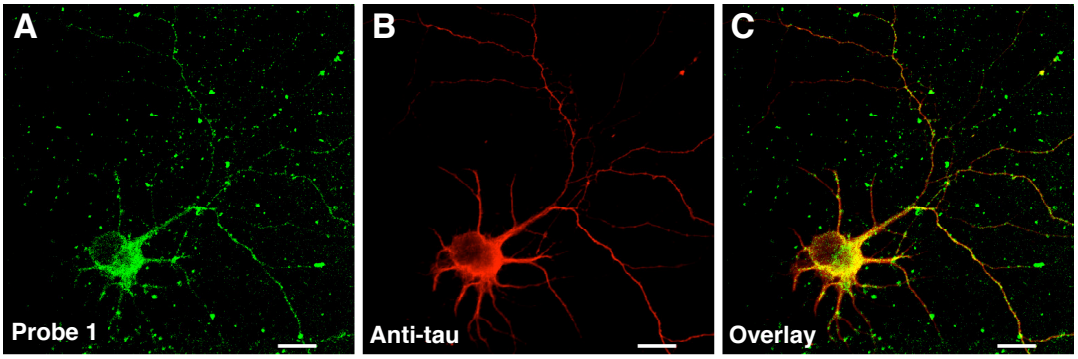

Figure 2.5. Costaining of neurons with Fucα(1-2)Gal probe **1** and an anti-tau antibody. A) Probe **1** staining (green). B) Tau antibody labels axons, dendrites, and cell bodies. C) Overlay of probe **1** and tau labeling (yellow indicates colocalization) shows that Fucα(1-2)Gal staining is distributed on the cell body and along dendrites and axons. Scale bars, 25 μm

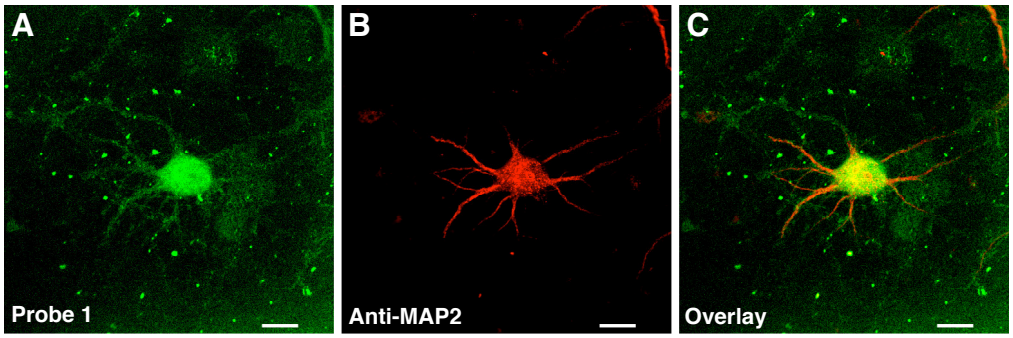

Figure 2.6. Costaining of neurons with Fucα(1-2)Gal probe **1** and a MAP2 antibody. A) Probe **1** staining (green). B) MAP2 labeling (red) shows dendrites and cell body. C) Overlay of probe **1** and MAP2 labeling (yellow indicates colocalization) shows that Fucα(1-2)Gal staining is distributed on the cell body, along dendrites and axons. Scale bars, 25 μm

Next, we sequentially stained neurons first with Fucα(1-2)Gal probe **1**, followed by an antibody specific for synapsin. Synapsin is a marker for synapses and is found in pre-synaptic terminals.[23] As shown in Figure 2.7, Fucα(1-2)Gal binding (green) does not completely overlap with synapsin labeling (red). Interestingly, many of the puncta for the probe and the antibody are adjacent to one another, suggesting a post-synaptic localization for the Fucα(1-2)Gal-binding proteins.

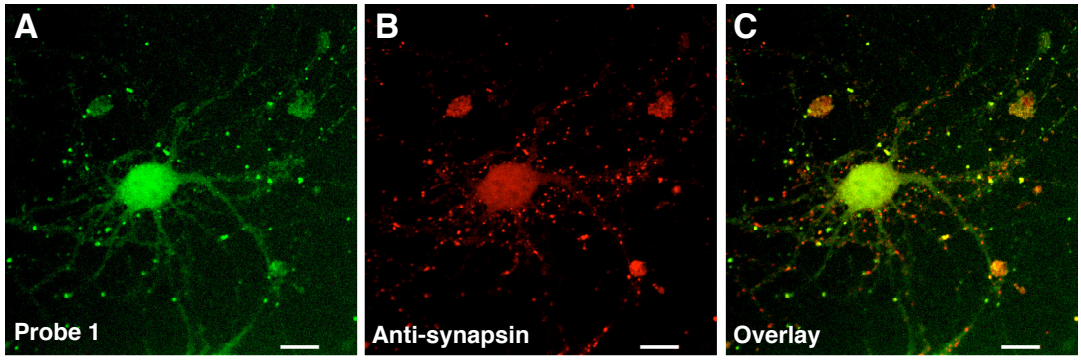

Figure 2.7. Costaining of neurons with Fucα(1-2)Gal probe **1** and an anti-synapsin antibody. A) Probe **1** staining (green). B) Synapsin antibody (red) labels presynaptic terminals. C) Overlay of probe **1** and synapsin labeling (yellow indicates colocalization) shows that Fucα(1-2)Gal staining does not completely overlap with synapsin staining. Scale bars, 25 μm

Fucα(1-2)Gal glycoproteins are present in neurons

The presence of potential lectins specific for Fucα(1-2)Gal implies the existence of glycoproteins covalently modified by the disaccharide epitope. To determine whether such glycoproteins are present in neurons, we treated cells with *Ulex europeaus* agglutinin I lectin (UEA-I) conjugated to fluorescein. UEA-I has been used previously to detect Fucα(1-2)Gal glycoproteins in cells and tissues.[24, 25]

Hippocampal neurons were cultured for 23 DIV before treatment with PAO and fluorescein-conjugated UEA-I. Following fixation and immunostaining with anti-tau antibody, neurons were visualized by fluorescence microscopy. As shown in Figure 2.8,

UEA-I lectin specifically labels neurons on the cell body and along axons and dendrites.[17] Furthermore, UEA-I displays a punctate staining consistent with localization to synapses.

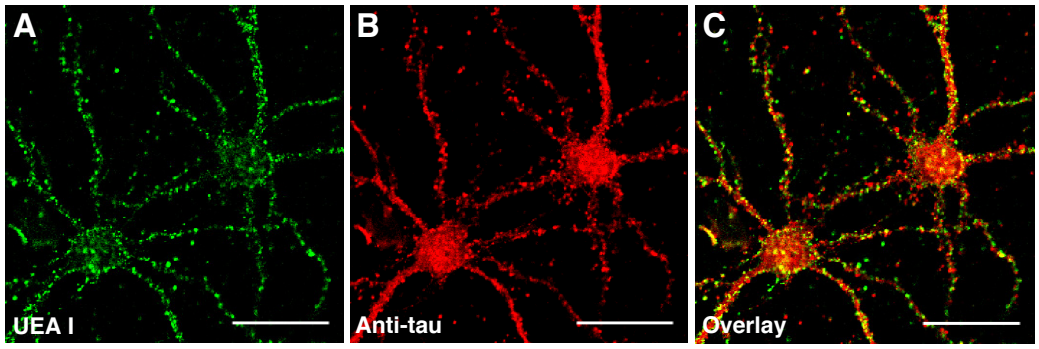

Figure 2.8. Costaining of neurons with UEA-I lectin and an anti-tau antibody. Neurons were stained with (A) fluorescein-conjugated UEA-I lectin and (B) an anti-tau antibody in the presence of 10 μm PAO. C) Overlay of both images (yellow indicates colocalization). UEA-I lectin labels neurons on the cell body and along axons and dendrites. Scale bars, 45 μm

To validate that the UEA-I lectin was labeling glycoproteins rather than simply interacting with the lipid membrane, neurons were delipidated prior to treatment with UEA-I lectin. Specifically, neurons cultured for 23 DIV were treated with a MeOH/$CHCl_3$ mixture followed by incubation with PAO and fluorescein-conjugated UEA-I. In Figure 2.9, we see that lipid extraction prior to treatment with UEA-I lectin does not diminish the labeling of neurons, and we see staining of the cell body and neurite processes.[17]

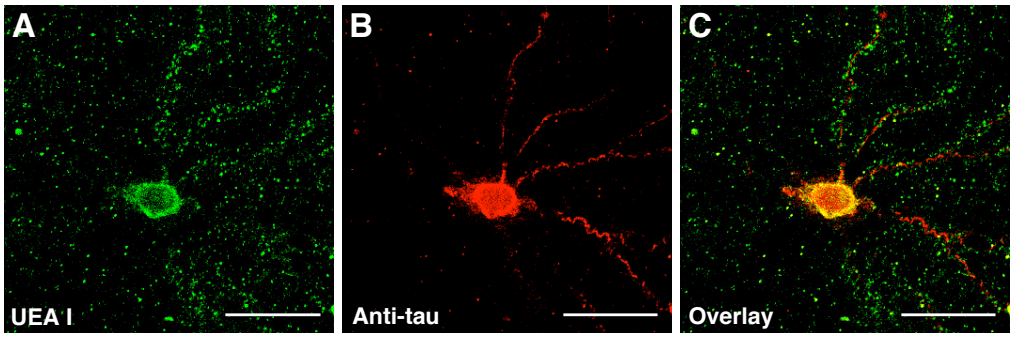

Figure 2.9. Lipid extraction using MeOH/$CHCl_3$ did not diminish UEA-I lectin labeling. Costaining of neurons with (A) UEA-I lectin (green) and (B) anti-tau antibody (red) in the presence of 10 μM PAO after lipid extraction. C) Overlay of both images (yellow indicates colocalization). Scale bars, 45 μm

Fucα(1-2)Gal carbohydrates modulate neuronal outgrowth

Once we established the presence of Fucα(1-2)Gal lectins and glycoproteins in neurons, we sought to investigate the impact of Fucα(1-2)Gal carbohydrates on neuronal function. First, we examined whether the association of Fucα(1-2)Gal with potential lectins would elicit a neuronal response. As carbohydrates have weak binding affinities for lectins (K_{assoc} =10^3 – 10^6 M)[26], we used polyacrylamide polymers bearing multiple Fucα(1-2)Gal epitopes (FucGal-PAA) to stimulate endogenous lectins and enhance the interactions. Treatment of hippocampal neurons with the multivalent polymers was carried out by Dr. Kalovidouris and revealed a striking impact on neuronal morphology.[17] Hippocampal neurons were cultured for 20 hours before treatment with the polyacrylamide polymers in solution for an additional 24 hours. Neurons were then immunostained with anti-tau antibodies and quantified for neurite outgrowth. Remarkably, the multivalent polymers stimulated neurite outgrowth by 50 ± 6% relative to the untreated control (Figure 2.10). Furthermore, the growth-inducing activity was specific to the Fucα(1-2)Gal disaccharide, as polymers lacking the disaccharide (PAA) had no significant effect. Polymers containing *N*-acetylglucosamine (GlcNAc-PAA) or D-galactose (Gal-PAA) failed to promote neuronal outgrowth. Interestingly, other L-Fuc-bearing polymers, such as L-Fuc PAA (Fuc-PAA) and Fucα(1-3)GlcNAc (FucGlcNAc-PAA), displayed neuronal processes similar to those of untreated cells, suggesting that the observed neuritogenic activity is specific for Fucα(1-2)Gal.

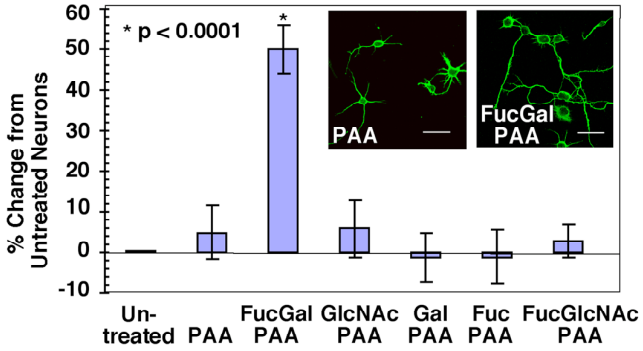

Figure 2.10. Fucα(1-2)Gal promotes neuronal growth. Neurite outgrowth was quantified by measuring the longest neurite per cell after treatment with 130 μM of the indicated compounds. Error bars represent SEM from 100 total neurons in two separate experiments.

We next examined whether Fucα(1-2)Gal glycoproteins are associated with neuronal growth pathways. Since previous studies have shown that lectins activate and promote the clustering of glycoproteins at the cell surface,[27] we used exogenous lectins to stimulate the Fucα(1-2)Gal glycoproteins found in neurons. Dr. Kalovidouris treated hippocampal neurons cultured for 20 hours with the Fucα(1-2)Gal specific lectins UEA-I or *Lotus tetragonolobus* lectin (LTL)[24] and found that neurite outgrowth was stimulated by 21 ± 6% and 20 ± 6%, respectively, relative to the untreated control (Figure 2.11)[17]. Competition experiments with 400-fold excess probe **1** abolished the stimulatory activity of UEA-I and LTL. Additionally, lectins selective for other carbohydrates such as glucosamine (wheat germ agglutinin, WGA) or Fucα(1-3)Gal (*Anguilla anguilla* agglutinin, AAA)[24] did not enhance neurite outgrowth. Together, these results suggest that the growth-promoting activity is specific for Fucα(1-2)Gal carbohydrates.

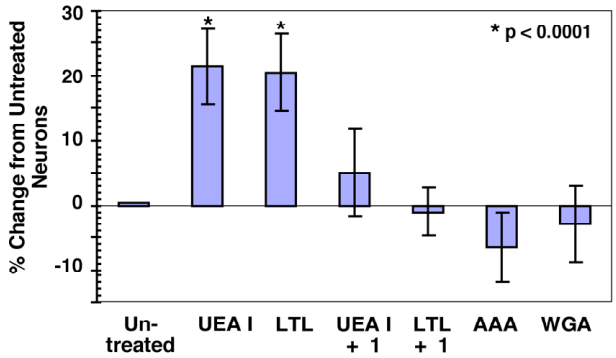

Figure 2.11. Only the Fucα(1-2)Gal-selective lectins UEA I and LTL stimulate neuronal growth. Neurite outgrowth was quantified after treatment with 3.7 μM of the indicated lectins. Error bars represent SEM from 100 total neurons in two separate experiments.

These intriguing observartions suggest that Fucα(1-2)Gal saccharides may play important roles in neuronal growth. To further investigate the effect of Fucα(1-2)Gal saccharides on neuronal morphology and development, we treated neuronal cultures with the unnatural sugar analog 2-dGal. As described earlier, disruption of Fucα(1-2)Gal linkages using 2-dGal caused amnesia in animals and prevented the maintenance of LTP.[9, 11, 28] We first examined the effect of 2-dGal on the expression of the Fucα(1-2)Gal epitope. Hippocampal neurons were grown for 1 day and then treated with or without 30 mM 2-dGal. After 4 days, cells were harvested and cell lysates were probed by Western blotting using the anti-Fucα(1-2)Gal antibody A46-B/B10. Consistent with earlier studies,[8, 29] treatment of neurons with 2-dGal disrupted synthesis of the Fucα(1-2)Gal epitope on glycoproteins (Figure 2.12).[17] Specifically, a significant decrease of the Fucα(1-2)Gal signal on the two major glycoproteins detected in untreated neurons was observed.

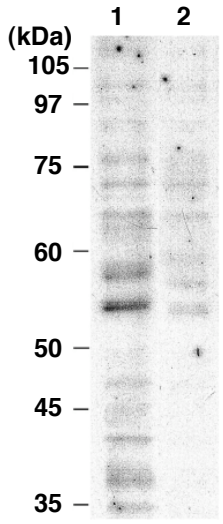

Figure 2.12. Treatment with 2-dGal diminishes the expression of the Fucα(1-2)Gal epitope on glycoproteins. Neurons were treated for 4 days with or without 30 mM 2-dGal. Protein lysates were then analyzed by Western blotting using antibody A46-B/B10. Lane 1: Untreated neurons. Lane 2: Neurons treated with 30 mM 2-dGal. Each lane contains 75 μg total protein.

Once we confirmed that treatment with 2-dGal was indeed disrupting the synthesis of Fucα(1-2)Gal linkages on neuronal glycoproteins, we examined the effects of 2-dGal on neuronal morphology. A dose-response experiment was initially performed to determine the minimum concentration of 2-dGal needed to elicit an effect. Neurons were treated with varying concentrations of 2-dGal for 2 days before immunostaining with anti-tau antibodies. As shown in Figure 2.13, treatment with increasing concentrations of 2-dGal caused neurite retraction.[17] Importantly, no cellular toxicity was observed at concentrations up to 30 mM 2-dGal, as demonstrated by trypan blue staining, adherence of the cells to the coverslip, and healthy cellular morphology. A concentration of 15 mM was used in subsequent experiments, as it was the minimal concentration that produced a strong effect on neurite outgrowth.

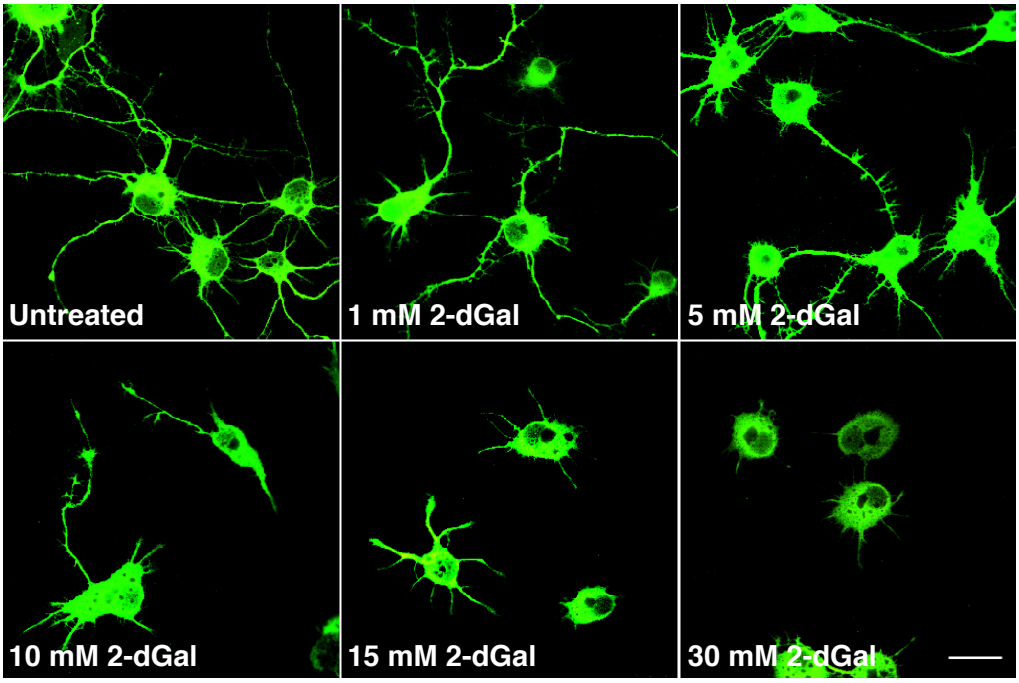

Figure 2.13. Hippocampal neurons treated with varying concentrations of 2-dGal exhibit increasing defects in neuronal growth. After 1 day in culture, neurons were treated with the specified concentrations of 2-dGal for 2 days, followed by immunostaining with anti-tau antibodies.

To fully establish the effects of 2-dGal on neuronal morphology, hippocampal neurons were incubated with 2-dGal and compared to those incubated with 3-deoxy-D-galactose (3-dGal), D-galactose (D-Gal), and untreated neurons. Neurons were treated under four different conditions: (1) incubation with 15 mM 2-dGal for 2 days, (2) incubation with 15 mM 3-dGal for 2 days, (3) incubation with 15 mM 2-dGal for 2 days followed by incubation with 75 mM D-Gal for 2 days, or (4) no treatment for 2 days. Following treatment with the various molecules, cells were fixed and immunostained with anti-tau antibodies. As shown in Figure 2.14, 2-dGal causes severe morphological defects in cultured neurons.[17] Compared to untreated cells that have many neuronal processes, cells treated with 2-dGal exhibited severely stunted neurites and failed to form synapses. Interestingly, the effects were fully reversible: subsequent addition of D-Gal led to regeneration of neuronal processes. In contrast, addition of 3-dGal had no impact

on neurite outgrowth. These results are consistent with a stimulatory role for Fucα(1-2)Gal glycoproteins and demonstrate the striking influence of Fucα(1-2)Gal carbohydrates in neuronal growth.

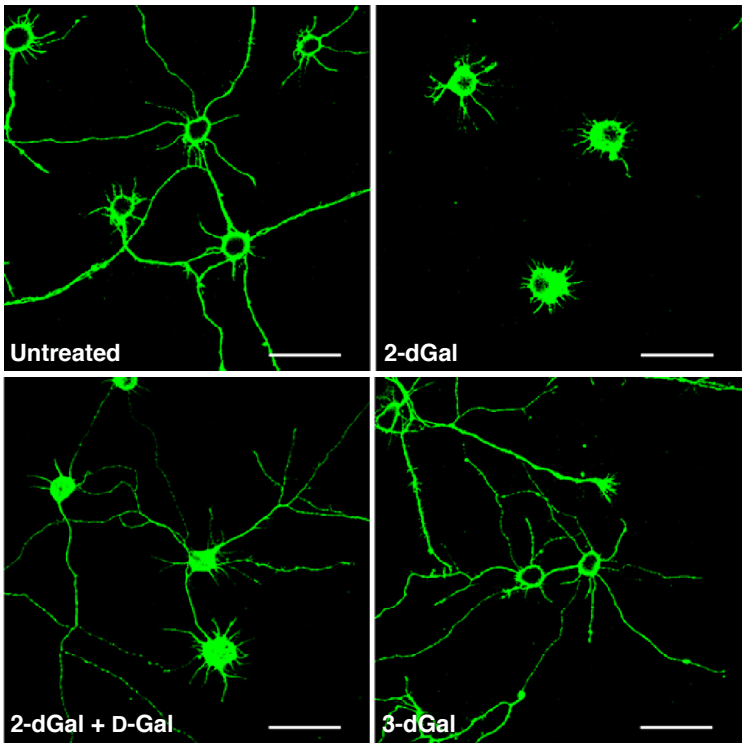

Figure 2.14. Treatment of hippocampal neurons with 2-dGal (15 mM), but not 3-dGal (15 mM), for 2 days inhibits neuronal growth. The effects of 2-dGal can be reversed by subsequent treatment with D-Gal (75 mM) for an additional 2 days. Neurons were immunostained with anti-tau antibodies.

Discussion

Our studies demonstrate that Fucα(1-2)Gal carbohydrates are capable of modulating neuronal outgrowth and morphology. We provide strong evidence for the presence of Fucα(1-2)Gal lectin receptors and glycoproteins in hippocampal neurons. Specifically, we have determined that proteins binding the Fucα(1-2)Gal disaccharide and proteins expressing this epitope are found on the cell surface, along axons and

dendrites, and at synapses. Furthermore, we show that preventing formation of Fucα(1-2)Gal linkages by treatment with 2-dGal induces dramatic morphological changes and severely stunts neurite outgrowth. Consistent with a role for Fucα(1-2)Gal carbohydrates in neuronal growth, stimulation of either Fucα(1-2)Gal lectins or glycoproteins with exogenous Fucα(1-2)Gal polymers or lectins promotes neurite outgrowth. Together, these findings identify a novel, carbohydrate-mediated pathway for modulating neuronal growth and development.

Manipulation of Fucα(1-2)Gal–associated proteins using small molecule and lectin probes elicited striking effects on neuronal morphology, suggesting that Fucα(1-2)Gal may be important for maintaining structural plasticity. This prospect may shed light on behavioral and electrophysiological studies implicating Fucα(1-2)Gal in long-term memory formation. Alterations in neuronal morphology, such as dynamic changes in dendritic spine number and shape, occur during memory consolidation and LTP.[30, 31] Additionally, protein glycosylation has been shown to be necessary for maintaining LTP.[32] Furthermore, fucose incorporation levels increase following learning tasks and have been shown to enhance memory retention and LTP.[5, 14 -- 16] One possibility is that Fucα(1-2)Gal and its associated proteins are involved in structural remodeling events that contribute to synaptic plasticity and are thereby impacting learning and memory processes in the brain.

With the establishment of Fucα(1-2)Gal glycoproteins and lectins in neurons, identification of these proteins is necessary to enable a detailed study of Fucα(1-2)Gal saccharides and their impact on neuronal communication. Using affinity-based and genomics tools, we will first identify and study Fucα(1-2)Gal glycoproteins in the

hippocampus. With the demonstration of the use of small molecules in culture and the development of a chemical tool in probe **1,** we will also detect and seek to identify the first Fucα(1-2)Gal lectins in neurons.

Experimental Procedures for Chapter 2

Buffers and Reagents:

Chemicals and molecular biology reagents were purchased from Fisher (Fairlawn, NJ) unless stated otherwise. Protease inhibitors were purchased from Aldrich Chemicals (St. Louis, MO) and Alexis Biochemicals (San Diego, CA). Cell culture media was purchased from Gibco BRL (Grand Island, NY). German glass coverslips were purchased from Carolina Biologicals (Burlington, NC).

Embryonic Hippocampal Dissection:

Timed-pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Kingston, Mass) and housed at the Caltech laboratory animal facilities. Timed-pregnant rats at embryonic day 18 (E18) were euthanized by carbon dioxide inhalation. A quick C-section was performed and the uterus placed in a 100 x 15 mm petri dish containing ice-cold Calcium and Magnesium Free Hank's Balanced Salt Solution (CMF-HBSS) and transferred to the tissue culture dissecting hood. The embryos were decapitated and the heads placed onto the lid of a petri dish on ice. The skin and skull were cut to expose the brain and the brain was removed by "scooping" it out from the olfactory bulbs to the cerebellum and placed in a new dish on ice containing CMF-HBSS. Under a dissecting microscope, the cerebral hemispheres were separated from the midbrain and the cerebellum and meninges were removed. The hippocampus was cut out with a scalpel and placed in a separate dish with CMF-HBSS and kept on ice until all hippocampi were removed.

Hippocampal Neuronal Cultures:

Hippocampal neuronal cultures were prepared using a modified version of the Goslin, Asmussen, and Banker[18] protocol. Embryos at the E18 stage were obtained from timed-pregnant Sprague-Dawley rats. The hippocampus from each embryo was dissected as described above. All the hippocampi from one prep were transferred to a 15 mL conical containing 4.5 mL of ice-cold CMF-HBSS. Trypsin (2.5%, no EDTA) was added to 5 mL and the tissue was digested for 15 min at 37 °C. The trypsin solution was removed and the tissue rinsed with 5 mL of CMF-HBSS three times. Cells were then dissociated from the tissue in 1 mL of CMF-HBSS by passing through a P1000 pipet tip 15 to 20 times. The cells were counted with a hemacytometer, diluted into Minimal Eagle's Medium (MEM) plus 10% fetal bovine serum, and seeded on poly-DL-ornithine (15 μg/mL; Sigma)-coated 15 mm glass coverslips at a density of 75 cells/mm^2 (100 μL/coverslip) for 30 min. After this time, 500 μL of supplemented neurobasal medium (neurobasal media without L-glutamine, 2 mM L-glutamine, 250 μg/mL penicillin / 250 μg/mL streptomycin, 1X antibiotic-antimycotic, 1X B-27 supplement, 50 mM kynurenic acid in 1 N NaOH) was added to each coverslip. The cultures were maintained in 5% CO_2 at 37 °C until specified.

Immunocytochemistry of Hippocampal Neuronal Cultures:

After specified days in culture, hippocampal neurons on coverslips were used for immunostaining. Cells were rinsed one time with PBS (120 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer pH 7.4), fixed in 4% paraformaldehyde for 20 min at rt, washed twice with PBS, permeabilized in 0.3% Triton X-100 for 5 min at rt, and washed twice

with PBS. Non-specific binding was blocked by incubating with 3% BSA for 1 h at rt and then rinsing once with PBS. Cells were then incubated with anti-tau antibodies (rabbit polyclonal, 1:600; Sigma) in 3% BSA for 2 h at rt. Excess antibody was rinsed away 5 times with PBS. The secondary antibody, anti-rabbit IgG AlexaFluor 488 (1:600; Molecular Probes), was added for 1 h at 37 °C in 3% BSA. Excess secondary antibody was washed off 5 times with PBS. The coverslips were mounted onto glass slides using Vectashield mounting medium (Vector Labs) and sealed with clear nail polish. Cells were then subjected to confocal laser microscopy.

*Staining of Hippocampal Neurons with Probe **1** and Fluorescein-Conjugated UEA-I Lectin:*

Hippocampal neuronal cultures were prepared as described above and maintained at 37 °C, 5% CO_2 in supplemented neurobasal medium. After specified days in culture, the medium was replaced, and neurons were treated with the endocytosis inhibitor phenylarsine oxide[19] (PAO; 4 μL in DMSO, final concentration 10 μM) and either probe **1** (24 μL in PBS, final concentration 3 mM), biotin (24 μL in PBS, final concentration 3 mM), or fluorescein-conjugated UEA I lectin (4 μL, 1:100 final dilution) in supplemented neurobasal medium (400 μL final volume) for 1 h at 37 °C, 5% CO_2. After 1 h, neurons were rinsed twice with PBS, fixed in 4% paraformaldehyde for 20 min at rt, washed twice with PBS, permeabilized in 0.3% Triton X-100 for 5 min at rt, and washed another 2 times with PBS. Non-specific binding was blocked with 3% BSA for 1 h at rt and then rinsed once with PBS. Anti-tau antibody (rabbit polyclonal, 1:400; Sigma), anti-MAP2 antibody (mouse monoclonal, 1:400; Sigma), or anti-synapsin I antibody

(rabbit polyclonal, 1:250; Sigma) was added in 3% BSA for 2 h at rt and the excess antibody rinsed off 5 times with PBS. Probe **1** was detected with streptavidin conjugated to AlexaFluor 488 (1:200; Molecular Probes) while anti-tau, anti-MAP2, or anti-synapsin I antibodies were detected with secondary antibodies conjugated to AlexaFluor 568 (1:600; Molecular Probes). Both dye-conjugated streptavidin and secondary antibodies were added in 3% BSA for 1 h at 37 °C and the excess reagent washed off 5 times with PBS. Coverslips were then mounted onto slides with Vectashield, sealed, and imaged using confocal laser microscopy.

*De-lipidation of Neurons with MeOH/$CHCl_3$ Prior to Treatment with Probe **1** and Fluorescein-Conjugated UEA I Lectin:*

To confirm that probe **1** was binding specifically to proteins rather than interacting with the membrane lipids, neurons were delipidated following the protocol of Yavin and Yavin.[20] Briefly, after specified days in culture, cells were rinsed once with PBS then exposed to MeOH/$CHCl_3$ (1/2 by vol) for 15 min at –80 °C. After removing the MeOH/$CHCl_3$ mixture, coverslips were dried at rt and neurons were then stained as described above.

Treatment of Neuronal Cultures with 2-Deoxy-D-Galactose, 3-Deoxy-D-Galactose, and D-Galactose:

Hippocampal neurons were plated on poly-DL-ornithine-coated glass coverslips as described above. After one day in culture, the medium was replaced with fresh medium, and the small molecules added. A dose-response experiment was initially performed to

determine the minimum concentration of 2-dGal needed to elicit an effect. Neurons were treated with varying concentrations of 2-dGal (1, 5, 10, 15, or 30 mM in 25 μL PBS with 475 μL of supplemented neurobasal medium) for 2 days before immunostaining with anti-tau antibodies as described above. A concentration of 15 mM was used in subsequent experiments, as it produced a strong effect on neurite outgrowth. Cells were treated as above under 4 different conditions: (1) incubation with 15 mM 2-dGal for 2 days, (2) incubation with 15 mM 3-deoxy-D-galactose for 2 days, (3) incubation with 15 mM 2-dGal for 2 days followed by incubation with 75 mM D-galactose for 2 days, or (4) no treatment for 2 days. After adding the small molecules, cultures were incubated at 37 °C, 5% CO_2, then washed once with PBS, and immunostained with the anti-tau antibody as described above.

Analysis of the Fucα(1-2)Gal Epitope on Neuronal Proteins Following Treatment with 2-Deoxy-D-Galactose:

In addition to cells plated on coverslips, hippocampal neurons were grown in 30 mm dishes and treated with or without 30 mM 2-dGal (25 μL in PBS with 475 μL supplemented neurobasal medium). After 4 days, cells were harvested with 2.5% trypsin, lysed with 1 % boiling SDS with protease inhibitors, and cell lysates probed by Western blotting using the anti-Fucα(1-2)Gal antibody A46-B/B10[13]. Protein concentrations of the neuronal lysates were determined using the BCA Protein Assay (Pierce). Equal amounts of total protein were resolved by 10% SDS-PAGE, and proteins were transferred to PVDF membrane (Millipore) in 20 mM Tris-Cl pH 8.6/ 120 mM glycine/ 20% methanol. Western blots were blocked for 1 h with 3% periodated BSA[33] and rinsed with

TBST (50 mM Tris-Cl pH 7.4/ 150 mM NaCl/ 0.1% Tween-20). Blots were incubated with anti-Fucα(1-2)Gal antibody A46-B/B10 (0.5 μg/mL) in TBST overnight at 4 °C with constant rocking, then rinsed and washed twice for 10 min with TBST. Immunoreactivity was visualized by incubation with a horseradish peroxidase conjugated goat anti-mouse antibody (1:2500; Pierce) in TBST for 1 h followed by a rinse and four washes of 20 min with TBST. Blots were visualized by chemiluminescence using ECL reagents (Amersham) on X-Omat R film (Kodak).

Confocal Laser Microscopy:

All cells were imaged on a Zeiss Axiovert 100M inverted confocal laser microscope in the Biological Imaging Center in the Beckman Institute. The images were captured with LSM Pascal software using a 40X plan-neofluar air objective or a 63X plan-neofluar oil objective. All cells were excited with 488 nm and 568 nm light. The scan speed, collection mode, and zoom were changed slightly, as were the gain and black levels, for optimization of the images. All images were then copied into and analyzed by Adobe Photoshop.

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Chapter 3: Fucose α(1-2) Galactose-Containing Glycoproteins are Prevalent in the Brain and Regulate Neuronal Morphology*†

Background

Fucose α(1-2) galactose (Fucα(1-2)Gal) carbohydrates have been implicated in modulating neuronal communication that underlies learning and memory. For instance, preventing formation of Fucα(1-2)Gal linkages by incorporation of 2-deoxy-D-galactose (2-dGal) into glycan chains has been reported to cause reversible amnesia in animals.[1, 2] The sugar analog 2-dGal precludes the formation of Fucα(1-2)Gal linkages because it lacks the hydroxyl group at the C-2 position and inhibits the formation of a specific 1-2 glycosidic linkage with fucose.[1] 2-dGal has also been shown to interfere with the maintenance of long-term potentiation (LTP), a leading model for memory formation in the brain.[3, 4] Additionally, injection of a monoclonal antibody selective for Fucα(1-2)Gal also impairs memory formation in animals,[5, 6] presumably by blocking the Fucα(1-2)Gal epitope. These intriguing results suggest important roles for Fucα(1-2)Gal-associated proteins in regulating neuronal communication.

Interestingly, evidence suggests that protein fucosylation is regulated in response to neuronal activity. Both task-specific learning and LTP have been shown to induce the fucosylation of proteins at the synapse, with linear incorporation of [^{14}C]-labeled fucose

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* Identification of synapsin I was carried out by Wen I. Luo, a former graduate student in the Hsieh-Wilson laboratory, and Heather E. Murrey, a graduate student in the Hsieh-Wilson laboratory. Analysis of the Fucα(1-2)Gal epitope on synapsin function was performed by Heather E. Murrey

† Portions of this chapter were taken from H.E. Murrey et al. (2006) *Proc. Natl. Acad. Sci. USA* **103**, 21 – 26.

up to 3 hours after training and a 26% increase in tritium-labeled fucose levels 24 hours after the learning task.[7, 8] Addition of exogenous L-fucose or 2'-fucosyllactose (but not D-fucose, D-lactose, or 3-fucosyllactose) was also found to enhance LTP in hippocampal slices.[9] Furthermore, the activity of fucosyltransferases, enzymes involved in the transfer of fucose to glycoproteins, has also been demonstrated to increase substantially during synaptogenesis and passive avoidance training.[10, 11] Together, these studies suggest that protein fucosylation is likely a highly regulated process that contributes to synaptic plasticity.

Although there is significant evidence implicating Fucα(1-2)Gal carbohydrates in learning and memory processes, little is known about the location of this epitope or the molecular mechanisms by which it impacts neuronal communication. Notably, no Fucα(1-2)Gal glycoproteins have been characterized from the brain. As our initial data demonstrated the presence of Fucα(1-2)Gal glycoproteins in neurons (Chapter 2), we sought to further characterize the Fucα(1-2)Gal glycoproteins present in the hippocampus. We have established that Fucα(1-2)Gal carbohydrates are expressed on several glycoproteins during neuronal development and demonstrate that synapsin Ia and Ib are the predominant Fucα(1-2)Gal glycoproteins in the adult rat brain.

Fucα(1-2)Gal glycoproteins are enriched at synapses

We previously demonstrated the presence of potential glycoproteins specific for the Fucα(1-2)Gal disaccharide using the *Ulex europeaus* agglutinin I (UEA-I) lectin.[12] To allow for further characterization and identification of Fucα(1-2)Gal glycoproteins, we obtained an antibody selective for Fucα(1-2)Gal epitopes (Figure 3.1),[6] the mouse

monoclonal antibody A46-B/B10, which was a generous gift from Dr. Uwe Karsten. Importantly, antibody A46-B/B10 has been shown to induce amnesia in animals[5], suggesting that it recognizes one or more physiologically relevant epitopes.

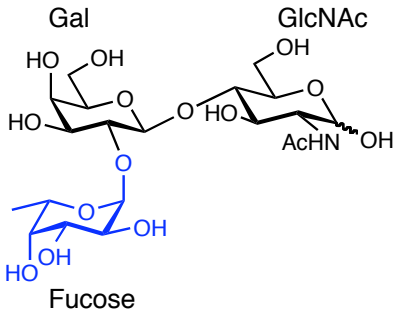

Figure 3.1. Trisaccharide Fucα(1-2)Galβ(1-4)GlcNAc recognized by antibody A46-B/B10

Fucose-containing glycoproteins are known to exist in the brain, but they have not been conclusively established to be present at synapses. Therefore, we sought to establish the location of Fucα(1-2)Gal glycoproteins in neurons. Hippocampal neurons were cultured for 14 DIV to allow for synapse formation and were subsequently fixed, permeabilized, and coimmunostained with antibody A46-B/B10 and an antibody against the neuronal marker tubulin. The neurons were visualized by confocal laser microscopy following incubation with appropriate dye-conjugated secondary antibodies. Using antibody A46-B/B10, we found that Fucα(1-2)Gal glycoproteins are located on the surface of neurons and are highly enriched at synapses.[13] In Figure 3.2, we see that Fucα(1-2)Gal glycoproteins (green) have remarkable punctate staining along axons and dendrites (red) as well as some staining on the cell body surface. As controls in every experiment, the primary and secondary antibodies were tested individually and in various combinations to ensure that the observed fluorescence was due to the desired antibody. In addition, several rounds of optimization of blocking conditions, wash conditions,

antibody concentrations, incubation times and temperatures were necessary to optimize signal and minimize the non-specific background.

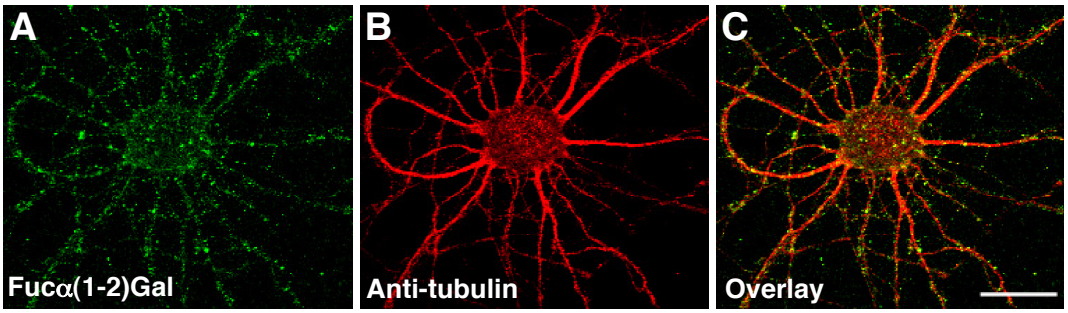

Figure 3.2. Costaining of neurons with Fucα(1-2)Gal antibody A46-B/B10 and an anti-tubulin antibody. A) A46-B/B10 antibody staining (green). B) Anti-tubulin labeling shows axons, dendrites, and cell bodies. C) Overlay of A46-B/B10 antibody and anti-tubulin labeling (yellow indicates colocalization) shows that Fucα(1-2)Gal staining is distributed on the cell body and along dendrites and axons. Scale bar, 25 μm

To confirm that the Fucα(1-2)Gal antibody was labeling glycoproteins rather than simply interacting with the lipid membrane, neurons were delipidated[14] prior to treatment with antibody A46-B/B10. Specifically, neurons cultured for 20 days were treated with a methanol/chloroform ($MeOH/CHCl_3$) mixture followed by coimmunostaining with antibody A46-B/B10 and an anti-tau antibody. Indeed, lipid extraction prior to immunostaining does not diminish the labeling of neurons, and we see staining of the cell body and neurite processes (Figure 3.3),[13] confirming the labeling of glycoproteins rather than glycolipids.

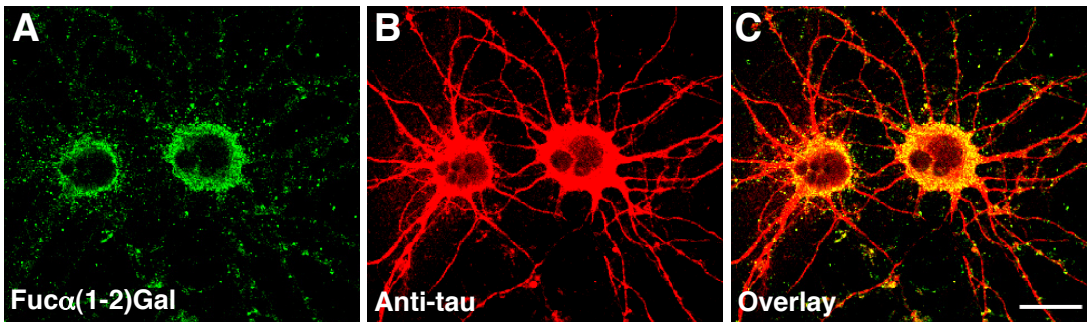

Figure 3.3. Lipid extraction of cellular membranes does not abolish staining with antibody A46-B/B10. Neurons were delipidated with $MeOH/CHCl_3$ before staining with (A) antibody A46-B/B10 (green) and (B) an anti-tau antibody (red). C) Overlay of images (yellow indicates colocalization). Scale bar, 25 μm

The punctate staining pattern observed suggested that Fucα(1-2)Gal glycoproteins were present at synapses. To validate this result, we compared the subcellular localization of the Fucα(1-2)Gal glycoproteins to that of synapsin I and spinophilin. Synapsin I is a conventional marker for presynaptic terminals while spinophilin is found at postsynaptic terminals.[15, 16] We performed co-localization studies of antibody A46-B/B10 with synapsin I antibodies (Figure 3.4A).[13] The results confirmed the presence of Fucα(1-2)Gal at pre-synapses, as antibody A46-B/B10 labeling (green) overlapped with 58 ± 2% of synapses labeled with anti-synapsin I (red). Colocalization studies of antibody A46-B/B10 with spinophilin antibodies (Figure 3.4B) revealed mostly apposition of Fucα(1-2)Gal labeling (green) with spinophilin labeling (red).[13] These findings demonstrate that Fucα(1-2)Gal sugars are enriched on glycoproteins present at presynaptic terminals.

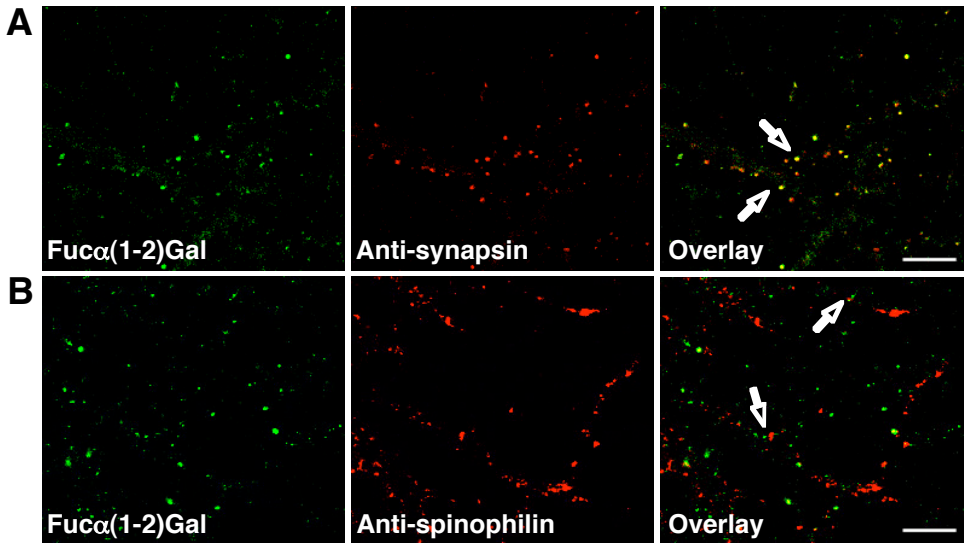

Figure 3.4. Costaining of neurons cultured for 14 DIV with Fucα(1-2)Gal antibody A46-B/B10 and either an anti-synapsin antibody or an anti-spinophilin antibody. A) A46-B/B10 antibody staining (green) colocalizes with anti-synapsin staining (red), suggesting that Fucα(1-2)Gal glycoproteins are enriched at presynaptic terminals. B) A46-B/B10 antibody staining (green) does not colocalize with anti-spinophilin staining (red), suggesting that Fucα(1-2)Gal glycoproteins are mostly apposed to post-synaptic terminals. Arrows indicate colocalization in (A) and apposition in (B). Scale bars, 10 μm

Expression of Fucα(1-2)Gal on glycoproteins is developmentally regulated

In order to better understand the precise function(s) of the Fucα(1-2)Gal motif on glycoproteins, it is essential to identify specific glycoproteins displaying the disaccharide at the synapse. Toward this end, we evaluated the expression of the Fucα(1-2)Gal epitope on glycoproteins in the hippocampus. Cellular lysates from adult rat hippocampus, E18 hippocampus, and cultured embryonic hippocampal neurons were analyzed by Western blotting with antibody A46-B/B10. We found that the Fucα(1-2)Gal epitope is expressed on distinct proteins during neuronal development (Figure 3.5).[13]

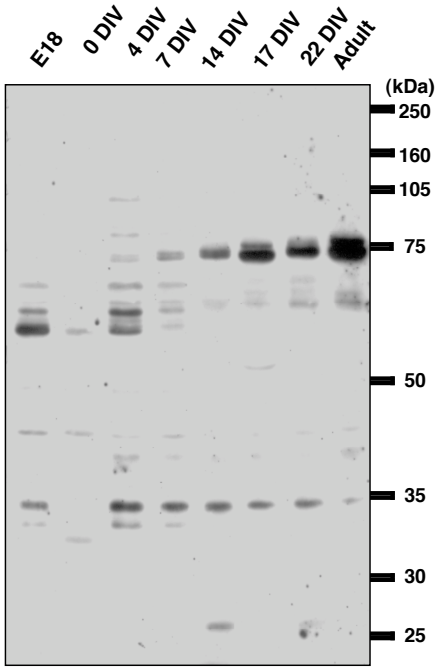

Figure 3.5. Fucα(1-2)Gal is expressed on several glycoproteins in the hippocampus and is developmentally regulated. Comparison of the Fucα(1-2)Gal glycoproteins present in E18 rat hippocampus, embryonic hippocampal neurons cultured for the indicated times, and adult rat hippocampus. Cellular lysates were resolved by SDS-PAGE and probed by Western blotting with antibody A46-B/B10.

In E18 hippocampal tissue, three major glycoproteins of approximately 35, 60, and 65 kDa are prominently observed. Expression of Fucα(1-2)Gal on these

glycoproteins is drastically reduced in the adult hippocampus. Significantly, the major Fucα(1-2)Gal glycoproteins of 73 and 75 kDa found in mature cultured neurons and adult brain tissue are distinct from those in embryonic tissue. Interestingly, expression of Fucα(1-2)Gal is observed on multiple glycoproteins in developing neurons cultured for 4 and 7 DIV, periods when axons, dendrites, and functional synapses are being formed. These results indicate that Fucα(1-2)Gal saccharides are synthesized on distinct proteins and the expression levels of Fucα(1-2)Gal and/or the associated glycoproteins vary dramatically with age and development.

Synapsin Ia and Ib are the major Fucα(1-2)Gal glycoproteins in the hippocampus

We next sought to identify the major Fucα(1-2)Gal glycoproteins found in the brain. Attempts to purify Fucα(1-2)Gal glycoproteins from extracts using antibody A46-B/B10 were unsuccessful due to the relatively weak binding affinity of the antibody for the carbohydrate epitope. Therefore, potential glycoproteins were identified using a combination of subcellular fractionation, gel electrophoresis, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).[13] Heather E. Murrey took adult rat hippocampal lysates enriched in synaptic proteins and resolved the proteins using 1D or 2D gel electrophoresis. Proteins were then analyzed by Western blotting with antibody A46-B/B10 or stained with Coomassie brilliant blue. As observed previously, two major glycoproteins of approximately 73 and 75 kDa were recognized by antibody A46-B/B10. The proteins of interest were identified by immunoblotting and excised from the corresponding Coomassie-stained gel, digested with trypsin, and identified by MALDI-TOF MS. The predominant proteins in adult rat brain were

identified as synapsin Ia and Ib, synaptic-vesicle-associated proteins involved in neurotransmitter release.

To confirm the fucosylation of synapsin Ia and Ib, the proteins were immunoprecipitated and examined by Western blotting with antibody A46-B/B10.[13] Indeed, Heather E. Murrey found that the immunoprecipitated proteins were specifically recognized by the antibody (Figure 3.6). Furthermore, she established that only synapsins Ia and Ib, but not the synapsin II or III isoforms, are covalently modified by the critical Fucα(1-2)Gal epitope and that fucosylation protects synapsin from degradation by the calcium-activated protease calpain.

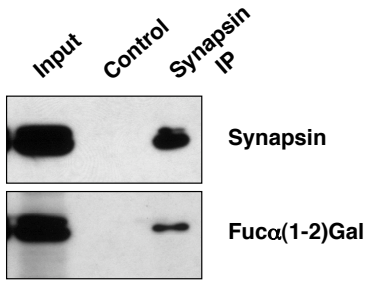

Figure 3.6. Synapsins Ia and Ib are Fucα(1-2)Gal glycoproteins. Immunoprecipitated synapsin I is detected by antibody A46-B/B10. Input, lysate used for immunoprecipitation; Control, immunoprecipitation in the absence of antibody; Synapsin IP, immunoprecipitated synapsin. *Upper panel* was immunoblotted with an anti-synapsin antibody, and *Lower panel* was probed with antibody A46-B/B10. Synapsin Ia appeared in darker exposures of the blot.

Fucosylation regulates synapsin expression in neurons and neurite outgrowth

As our results indicate a critical role for fucosylation on synapsin function, we wanted to further investigate this phenomenon in neurons. Toward this end, the effect of 2-dGal on synapsin fucosylation in neurons was examined. After culturing neurons for 7 DIV to allow for adequate synapsin expression, Heather E. Murrey treated the cultures with either 2-dGal or 6-deoxy-D-Galactose (6-dGal). Expression of synapsin was dramatically reduced by treatment with 2-dGal, but not 6-dGal (Figure 3.7).[13] Moreover,

the effects of 2-dGal were specific to synapsin, as the expression of other synaptic proteins was unchanged by the 2-dGal treatment.

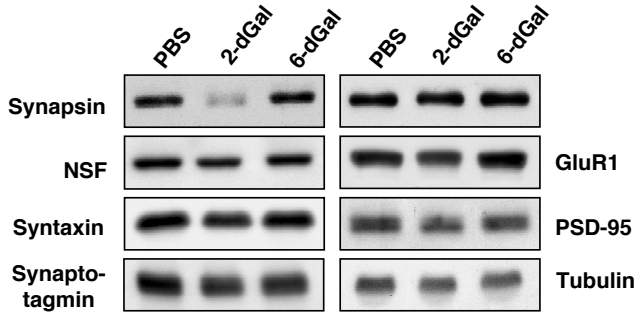

Figure 3.7. Expression of synapsin I was reduced by treatment with 2-dGal but not 6-dGal. Cortical neurons were treated with 2- or 6-dGal (15 mM) for 3 days. Protein lysates were analyzed by Western blotting for the indicated proteins. A significant reduction in the expression of synapsin I was observed, whereas other synaptic proteins were unaffected by the 2-dGal treatment.

Because the synapsins play important roles in neuronal development and synaptogenesis, we investigated how 2-dGal might influence neuronal growth and morphology. Previously, we saw that treatment with 2-dGal severely stunted neurite outgrowth of young, 2 DIV neurons (Chapter 2). With the identification of synapsins Ia/Ib as the major Fucα(1-2)Gal glycoproteins in mature cultures and adult hippocampus, we treated older cultures with 2-dGal and also found dramatic effects on neuronal morphology. Hippocampal neurons were cultured for 7 DIV as above to establish synapses and subsequently incubated for 3-5 days with 2-dGal at various concentrations (Figure 3.8). Treatment with 2-dGal induced retraction of neurites and collapse of synapses, whereas 6-dGal had no effect.[13] Interestingly, the inhibitory effects of 2-dGal could be rescued by subsequent treatment with D-galactose (D-Gal). In fact, treatment with D-Gal caused a 2.06 ± 0.14-fold rescue of neurite length, presumably by reestablishing the Fucα(1-2)Gal linkage. Collectively, we have found that treatment of cultured neurons at different developmental stages with 2-dGal impairs neurite outgrowth

and disrupts neuronal connections, suggesting that Fucα(1-2)Gal is important for maintaining neuronal plasticity.

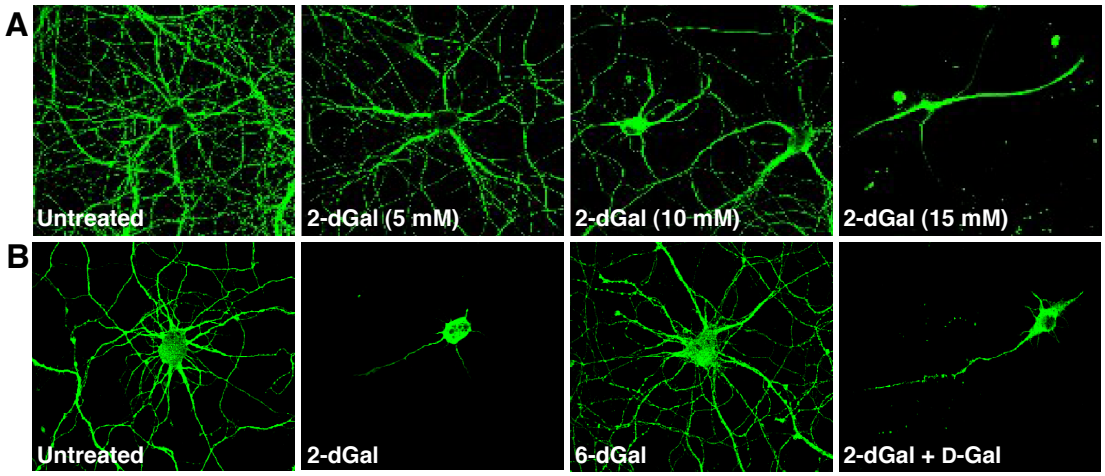

Figure 3.8. The morphology of hippocampal neurons is modulated by 2-dGal in a concentration-dependent manner. A) C57BL/6 mice neurons were cultured for 7 DIV and treated with 2-dGal for 5 days at the concentrations indicated. Neurite retraction becomes more pronounced with increasing concentration of 2-dGal. B) Rat neurons were cultured for 7 DIV and treated with either 2- or 6-dGal (15 mM) for 3 days. The effects of 2-dGal were partially reversed by treatment with D-Gal.

One potential mechanism by which 2-dGal might influence neuronal morphology is by regulating the function and/or expression of synapsin in presynaptic terminals. To examine the relative contribution of synapsin I to the effects elicited by 2-dGal, neurons from synapsin I-deficient or wild-type mice were cultured for 2 days, treated with or without 2-dGal for 3 days, and then examined by fluorescence microscopy (Figure 3.9).[13] We found that neurons from wild-type mice treated with 2-dGal had shorter neurites than their wild-type counterparts (compare Fig 3.9C and A). Interestingly, the effects of defucosylation with 2-dGal were more pronounced than the elimination of the synapsin I gene (compare Figure 3.9C and B). Furthermore, treatment with 2-dGal induced more neurite retraction in wild-type relative to synapsin-deficient neurons (compare Figure 3.9C and D). Although the length and extensive overlap of neuronal processes for

untreated wild-type neurons precluded a quantitative analysis of neurite length, 2-dGal treatment led to neurite retraction and enabled quantification. We found that synapsin-deficient neurons displayed 1.17-fold longer neurites than wild-type neurons upon treatment with 2-dGal (Figure 3.9E).

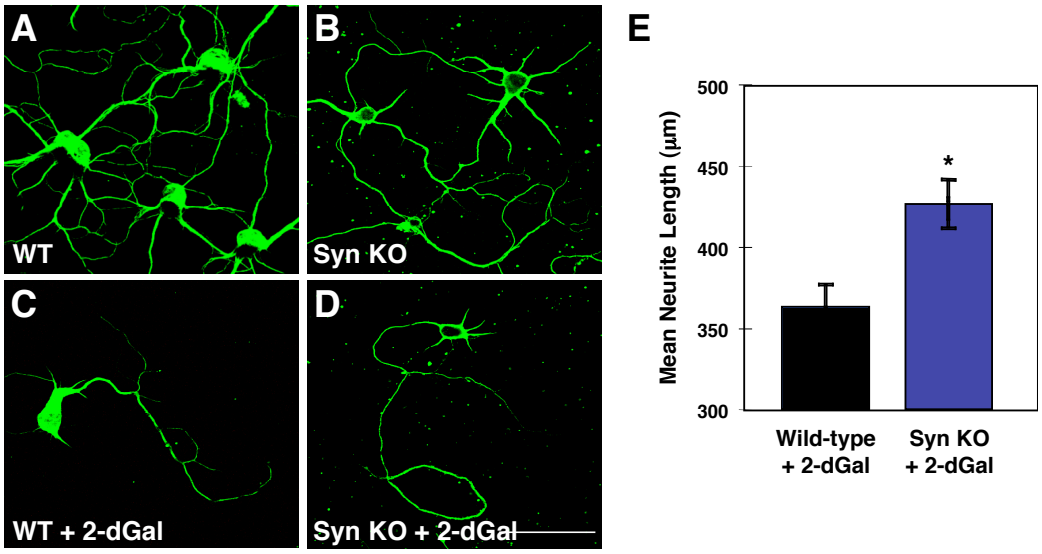

Figure 3.9. Synapsin-deficient neurons display reduced neurite retraction relative to wild-type neurons upon treatment with 2-dGal. Neurons from synapsin I-deficient (Syn KO) or wild-type (WT) mice were cultured for 2 days, treated in the presence (C, D) or absence (A, B) of 2-dGal (15 mM) for 3 days, and examined by confocal fluorescence microscopy. (E) Neurons treated with 2-dGal were analyzed for neurite length and the mean neurite length was compared by the ANOVA test. Error bars represent the SEM from 50 total neurons in three separate experiments (*, $P < 0.03$). Scale bar, 75 μm

Discussion

Increasing evidence has linked synaptic activity with changes in the levels of protein fucosylation in the brain. Both task-specific learning and LTP have been shown to enhance protein fucosylation.[7, 8] Additionally, the activity of fucosyltransferases substantially increases during synaptogenesis and upon passive avoidance training in animals.[10, 11] These studies suggest that protein fucosylation may be dynamically regulated at the synapse. Our findings that synapsins Ia and Ib are the major Fucα(1-

2)Gal glycoproteins in maturing neuronal cultures and the adult rat hippocampus are the first studies identifying synaptic proteins modified by the Fucα(1-2)Gal epitope.

The synapsins are a family of highly conserved neuron-specific proteins that are associated with synaptic vesicles.[17] The synapsins have been shown to modulate neurotransmitter release by regulating the supply of releasable vesicles during periods of high activity.[17, 18] In addition, synapsin I has recently been found to control synaptic vesicle dynamics in developing neurons via a cAMP-dependent pathway.[19] Accordingly, synapsin-deficient mice exhibit reduced numbers of synaptic vesicles within nerve terminals and display significant alterations in neuronal transmitter release and synaptic depression.[17, 20] Our findings indicate that fucosylation of synapsin critically impacts its expression and turnover in presynaptic terminals. Moreover, fucosylation of synapsin increases its half-life and protects against degradation by the calcium-activated protease calpain.

Furthermore, our results demonstrate that synapsin fucosylation has a significant impact on neuronal growth and morphology. We used the small molecule 2-dGal, an inhibitor of Fucα(1-2)Gal linkages, as a tool for defucosylating synapsin and for investigating the role of the carbohydrate on synapsin function. Treatment of neurons with 2-dGal led to stunted neurite outgrowth and delayed synapse formation. Moreover, significant differences were observed between wild-type and synapsin-deficient neurons upon treatment with 2-dGal. The extent of neurite retraction in synapsin-deficient mice is less pronounced, most likely because the primary target of 2-dGal, synapsin I, is missing. Indeed, the bar graph in Figure 3.10 likely represents a lower estimate of the contribution of synapsin, because neurites from synapsin-deficient neurons are shorter than those from

wild-type neurons before treatment with 2-dGal. From these results, we propose that defucosylation may disrupt synapsin function, leading to its degradation and neurite retraction. Although further studies are needed to resolve whether synapsin fucosylation stimulates or inhibits neurite outgrowth, our results strongly support the notion that synapsin fucosylation plays a role in modulating neuronal growth and morphology.

Our findings also implicate other Fucα(1-2)Gal glycoproteins in regulating neuronal morphology. We have shown that Fucα(1-2)Gal carbohydrates are not limited to synapsin but are found on additional proteins in developing neurons (Figure 3.6). Expression of the sugar and/or these glycoproteins changes dramatically during neuronal development. We found that defucosylation of synapsin did not fully account for the striking neurite retraction induced by 2-dGal, suggesting that 2-dGal may disrupt the fucosylation of other Fucα(1-2)Gal glycoproteins that influence neuronal morphology. Moreover, 2-dGal was still capable of inducing neurite retraction in synapsin-deficient neurons and in young cultured neurons where synapsin expression is low.[12] Thus, Fucα(1-2)Gal sugars appear to regulate the functions of multiple proteins involved in neuronal morphology and exert their effects via distinct molecular mechanisms.

Collectively, our studies provide new molecular-level insights into the role of Fucα(1-2)Gal in mediating the communication between neurons. The finding that synapsin Ia and Ib are fucose-containing glycoproteins is significant because it suggests that fucosyl saccharides may be involved in regulating neurotransmitter release and/or synaptogenesis. The modification of synapsin with Fucα(1-2)Gal fits accordingly with our model of fucosyl saccharides serving as a targeting element for proteins. Although further experiments are needed to show that the Fucα(1-2)Gal epitope directs the

targeting of synapsin to the synapse, it is clear that fucosylation directly affects synapsin stability and, thus, its functions at the synapse.

Experimental Procedures for Chapter 3

Buffers and Reagents:

Chemicals and molecular biology reagents were purchased from Fisher (Fairlawn, NJ) unless stated otherwise. Protease inhibitors were purchased from Aldrich Chemicals (St. Louis, MO) and Alexis Biochemicals (San Diego, CA). Cell culture media was purchased from Gibco BRL (Grand Island, NY). German glass coverslips were purchased from Carolina Biologicals (Burlington, NC).

Embryonic Hippocampal Dissection:

Embryonic tissue was dissected as described in Chapter 2.

Neuronal Cultures and Immunocytochemistry:

Hippocampal neurons were cultured and immunostained as described in Chapter 2. Synapsin I knockout mice[21] were generously provided by H. T. Kao and P. Greengard (The Rockefeller University, New York). Antibody A46-B/B10[6] was a generous gift from U. Karsten (Max-Delbrick Centre for Molecular Medicine, Berlin-Buch, Germany) and was incubated in 3% BSA (2.5 μg/mL) overnight at 4 °C. The anti-tubulin (1:500; Sigma), anti-synapsin (1:5,000; Molecular Probes), and anti-spinophilin [1:10,000 (14)] antibodies were added in 3% BSA for 2 h at 37 °C. Goat anti-mouse IgM AlexaFluor 488 or goat anti-rabbit IgG AlexaFluor 568 (1:250; Molecular Probes) were added for 1 h at 37 °C in 3% BSA.

De-lipidation of Neurons with MeOH/$CHCl_3$ Prior to Immunostaining with Antibody A46-B/B10:

Delipidation of neurons with MeOH/$CHCl_3$ was performed as described in Chapter 2. Antibody A46-B/B10 (2.5 μg/mL) was added in 3% BSA overnight at 4 °C. Anti-tau antibodies (1:500; Sigma) were added in 3% BSA for 2 h at 37 °C. Goat anti-mouse IgM AlexaFluor 488 or goat anti-rabbit IgG AlexaFluor 568 (1:250; Molecular Probes) were added for 1 h at 37 °C in 3% BSA.

Adult Hippocampal Dissection and Lysis:

100 g male Sprague-Dawley rats were purchased from Charles River Laboratories (Kingston, Mass) and housed at the Caltech laboratory animal facilities. Rats were anesthetized with carbon dioxide for 2 min and immediately euthanized by decapitation with a guillotine (Kent Scientific Co.). The brain was promptly removed and placed on ice. The hippocampus was quickly dissected and homogenized in 50 mM Tris-HCl pH 8.0/ 150 mM NaCl/ 0.2% sodium deoxycholate/ 1% Nonidet P-40 supplemented with protease inhibitors with a glass Dounce homogenizer and sonicated briefly. Supernatants were clarified by centrifugation at 12,000 x g for 10 min, and protein concentrations were determined by using the BCA protein assay (Pierce).

Western Blotting:

Protein concentration of hippocampal lysates was determined using the BCA protein assay (Pierce). Lysates were resolved on 10% acrylamide-SDS gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore) for at least 12 h in

20 mM Tris-Cl pH 8.6/ 120 mM glycine/ 20% methanol. Western blots were blocked for 1 h with HIO_4-BSA and rinsed with TBST (50 mM Tris-Cl pH 7.4/ 150 mM NaCl/ 0.1% Tween-20). Blots were incubated with 0.5 mg/mL antibody A46-B/B10 in TBST overnight at 4 °C with constant rocking, then rinsed and washed twice for 10 min with TBST. Immunoreactivity was visualized by incubation with a horse-radish peroxidase conjugated goat anti-mouse antibody (1:2500; Pierce) in TBST for 1 h followed by a rinse and four washes of 20 min in TBST. Blots were visualized by chemiluminescence using Pico Chemiluminescent Substrate (Pierce).

Treatment of Cells with Deoxy-Galactose Analogues:

Rat neuronal cultures were treated after 7 days in culture as described in Chapter 2. Neurons from C57BL/6 and synapsin I knockout postnatal day 0 mice were cultured for 2 days and then treated for 3 days with 15 mM 2-dGal.

Morphometric Analysis:

For quantitative analysis of neurite length, 50 cells were analyzed per experimental condition for three separate experiments. Only cells with neurites longer than one cell body diameter were measured. The length of the longest neurite was measured using NIH Image 1.62 software, and mean neurite lengths were compared by the ANOVA test using the statistical analysis program Statview 4.0.

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Chapter 4: Discovery of Fucα(1-2)Gal-Specific Lectins in the Developing Brain*

Background

L-Fucose is a monosaccharide enriched at synapses that exists primarily as a terminal modification to glycan chains. A majority of the fucose-containing carbohydrates are complex *N*-linked sugars containing two or more peripheral branches or, less often, hybrid structures.[1, 2] Fucose is frequently linked to the C-3 and C-6 positions of *N*-acetylglucosamine or to the C-2 position of galactose.[3] We are particularly interested in the fucoseα(1-2)galactose (Fucα(1-2)Gal) disaccharide, as several studies have implicated this disaccharide in learning and memory processes. Preventing the formation of Fucα(1-2)Gal linkages on glycan chains using 2-deoxy-D-galactose (2-dGal) causes reversible amnesia in animals.[4 -- 6] As 2-dGal specifically inhibits the incorporation of fucose into glycoproteins,[4, 7, 8] it is likely that Fucα(1-2)Gal glycoproteins contribute to memory storage. Moreover, protein fucosylation has been shown to increase in response to neuronal activity, with fucose incorporation and fucosyltransferase activity increasing in direct response to learning and long-term potentiation (LTP).[9 -- 11] These remarkable results suggest critical roles for Fucα(1-2)Gal glycoproteins in regulating the neuronal communication underlying learning and memory.

With such considerable evidence supporting a significant role for Fucα(1-2)Gal glycoproteins in cell-cell communication, it is likely that Fucα(1-2)Gal lectins are also

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* Synthesis of capture probe **2** and control molecule **3** was carried out by Dr. Lori W. Lee, a former graduate student in the Hsieh-Wilson laboratory, and Dr. Stacey A. Kalovidouris, a former postdoctoral scholar in the Hsieh-Wilson laboratory. Synthesis of polymer **5** is being carried out by Arif Wibowo, a graduate student in the Hsieh-Wilson laboratory.

involved in regulating neuronal communication. Indeed, several studies have demonstrated the importance of Fucα(1-2)Gal lectins in information processing and memory formation. Injection of a monoclonal antibody specific for the Fucα(1-2)Gal epitope drastically reduced retention of a learned task and had an amnesic effect in both chicks and rats.[12, 13] Presumably, the amnesic effect is a result of the antibody preventing the interaction between fucosylated glycoproteins and lectins. Additionally, treatment with exogenous fucosyl saccharides in both *in vivo* and *in vitro* models was found to enhance LTP.[14, 15] Together, these studies provide considerable evidence supporting a role for Fucα(1-2)Gal lectins in modulating neuronal communication.

The binding of lectins to carbohydrate motifs is an important phenomenon crucial to many cellular functions, including pathogen recognition, cellular adhesion, and lymphocyte trafficking.[16 -- 18] Although no Fucα(1-2)Gal-specific lectins have been characterized from animals, the total number of animal lectins identified is ever increasing. Most animal lectins identified can be classified into five major groups: C-type or Ca^{2+}-dependent lectins, galactose-binding galectins, P-type phospohorylated mannose receptors, I-type immunoglobulin-like sugar-binding proteins, and L-type lectins related to leguminous plant lectins.[19] Despite the enormous diversity among lectins, the sugar-binding activity can be attributed to the carbohydrate-recognition domain (CRD), typically a globular region of less than 200 amino acids.[18] The CRD among the individual groups of lectins are related to each other in amino acid sequence, thus enabling classification into the separate groups.

While there are increasing numbers of animal lectins being identified each year, there have been no Fucα(1-2)Gal-specific lectins reported to date. As such, we had to

first determine whether Fucα(1-2)Gal lectins exist in the brain. Indeed, we have demonstrated that Fucα(1-2)Gal lectins are present in the mammalian brain and are found on the cell body and neurites of hippocampal neurons (Chapter 2). Furthermore, we found that stimulation of the lectins with Fucα(1-2)Gal dramatically promotes neurite outgrowth. Our studies are the first report of Fucα(1-2)Gal lectins in the brain and identify a novel carbohydrate-mediated pathway for neuronal growth. Through the use of various chemical probes, we now seek to identify Fucα(1-2)Gal lectins from the brain in order to gain a molecular-level understanding of the impact of fucosyl saccharides on neuronal function.

Design of Fucα(1-2)Gal capture probe 2

Recent studies in our laboratory have established that Fucα(1-2)Gal lectins exist in the mammalian brain and are involved in a novel pathway that promotes neuronal growth. To facilitate isolation and identification of these lectins from the brain, our laboratory has synthesized chemical probe **2** (Figure 4.1). Chemical probe **2** was synthesized by Dr. Lori W. Lee and Dr. Stacey A. Kalovidouris and contains the Fucα(1-2)Gal moiety as the critical molecular recognition element as well as the biotin moiety for detection, as was found in probe **1** (Chapter 2). Importantly, probe **2** also contains a trifluoromethylphenyldiazirine (diazirine) moiety, which enables the capture of target lectins via photoactivated crosslinking. By forming a covalent linkage to the proteins of interest, the protein-probe complex can withstand rigorous washing in the purification process. Additionally, control molecule **3** was synthesized to test the specificity of probe

2, as it lacks the Fucα(1-2)Gal disaccharide while still carrying the diazirine and biotin moieties.

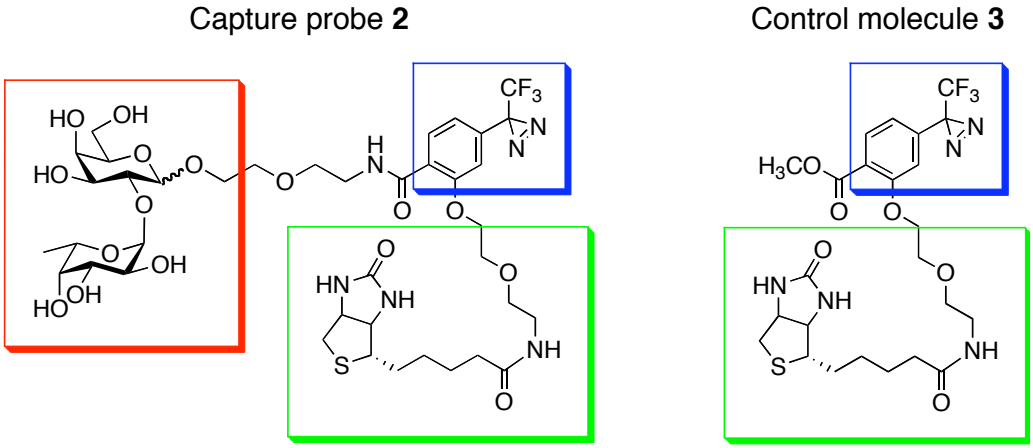

Figure 4.1. Monovalent capture probe **2** and control molecule **3**. The three main features of probe **2** are the Fucα(1-2)Gal disaccharide (red) for protein binding, the photoreactive diazirine moiety (blue) for crosslinking to bound proteins, and the biotin handle (green) for affinity column purification. Control molecule **3** acts as a specificity marker because it lacks the Fucα(1-2)Gal disaccharide while still bearing the diazirine moiety (blue) and biotin handle (green).

Photoaffinity labeling is a well-established technique to elucidate ligand-biomolecule interactions. This technique has been used to successfully label enzymes, protein structures, and RNA/DNA structures.[20 -- 23] Typically, the substrate is modified to bear the photoreactive element and radiolabels are incorporated to allow for the identification of the binding site. The covalently labeled protein can also be visualized with a variety of other techniques, such as spectroscopic analysis and fluorophore methods.[22]

Combining the power of photoaffinity labeling with the advantages of biotinylating substrates creates a chemical probe that allows for a covalent bond to be formed between the protein of interest and the probe and provides a sturdy handle to isolate the complex with the use of immobilized avidin. The biotin moiety also provides the advantage of sensitive, non-radioactive detection of labeled protein using

streptavidin-conjugated-horseradish peroxidase (HRP). The photoreactive, covalent crosslinking element is thought to overcome the weak binding affinities often observed of lectins for their carbohydrate ligands.[24] A variety of biotinylated photoreactive probes have been synthesized and successfully used to study and isolate protein-substrate complexes, ranging from γ-secretase inhibitors to glucose transporter ligands.[25, 26]

Fucα(1-2)Gal capture probe 2 labels known fucose-binding lectins AAA and UEA-I

With probe **2** in hand, we first validated the design of the capture probe and tried to label known fucose-binding lectins. Purified samples of the known fucose-binding lectins AAA from *Anguilla anguilla* and UEA-I from *Ulex europaeus*[27, 28] were incubated with or without probe **2** and control molecule **3,** irradiated with UV light, and analyzed by Western blotting using streptavidin-HRP. Notably, AAA and UEA-I lectins were detected by streptavidin-HRP only after treatment with probe **2** and UV irradiation (Figure 4.2A). No signal was observed when either lectin was incubated with control molecule **3** or when UV irradiation was omitted. The lectins were also analyzed by Coomassie stain (Figure 4.2B) to confirm that equivalent amounts of protein were present in each sample. From these results, we were confident that probe **2** was capable of specifically labeling fucose-binding lectins and proceeded to label Fucα(1-2)Gal lectins in embryonic neurons.

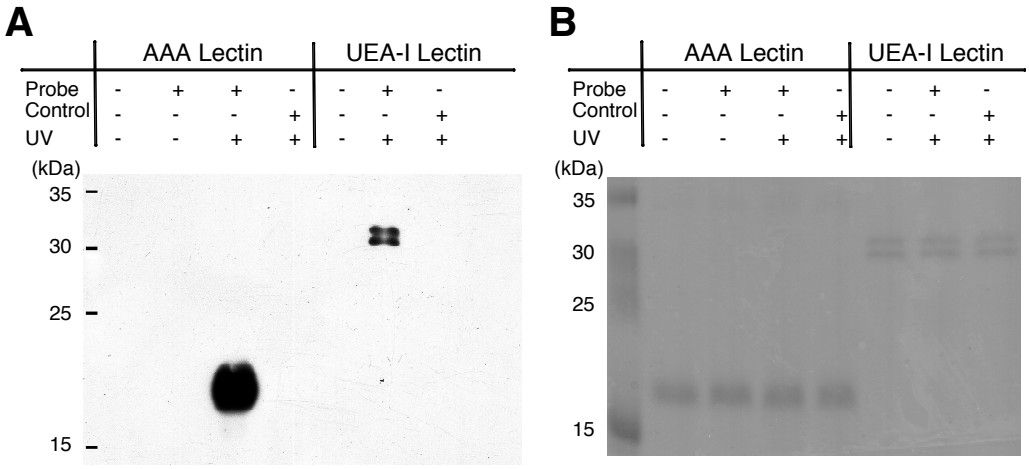

Figure 4.2. Capture probe **2** specifically labels the fucose-binding lectins AAA and UEA-I. A) Purified samples of AAA and UEA-I were treated with probe **2** and control molecule **3** and analyzed for specific labeling by streptavidin-HRP. B) Samples were also analyzed by Coomassie stain for total protein content.

Capture of Fucα(1-2)Gal lectins from dissociated neurons

Once we established the labeling of known fucose-binding lectins, we proceeded to use probe **2** for the capture of Fucα(1-2)Gal lectins. Dissociated neurons prepared from E18 rats were incubated with probe **2** in neurobasal medium. We initially chose to label dissociated neurons because cellular staining with probe **1** (Chapter 1) gave significant signal and thus we reasoned that Fucα(1-2)Gal lectins would be amenable to labeling with probe **2** on the cell surface, as the probe probably does not cross the cell membranes. Following incubation, the cells were irradiated on ice with UV light (365 nm). The optimal time for crosslinking proteins to the probe without damaging the cells was determined to be 2 h. Cells were then lysed with boiling 1% SDS to solubilize all proteins. The proteins were resolved by SDS-PAGE and probed by Western blotting using streptavidin-HRP. As shown in Figure 4.3, probe **2** indeed captured proteins from embryonic neurons. At least two major proteins at approximately 55 and 40 kDa, and several minor proteins, were captured by probe **2** (Figure 4.3, lane 1 vs. 2 and 3).

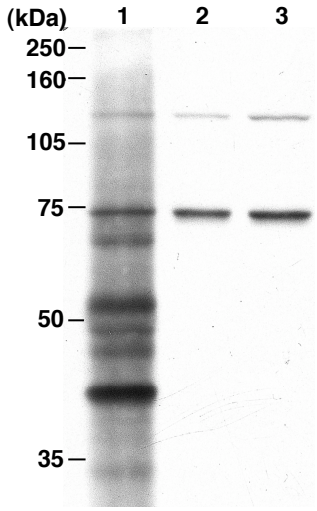

Figure 4.3. Capture probe **2** labels distinct proteins in dissociated neurons. Lane 1: Proteins from dissociated cells captured by probe **2**. Lane 2: Proteins captured by control molecule **3**. Lane 3: Proteins non-specifically detected by streptavidin-HRP in untreated cells

Investigations into the specificity of the target lectins

To confirm that probe **2** is binding selectively through the Fucα(1-2)Gal disaccharide, we performed competition experiments with several sugars. Several alternative sugars were synthesized by Dr. Kalovidouris that vary in the configuration of the fucose saccharide in the model Fucα(1-2)Gal disaccharide (Figure 4.4A). Dissociated forebrain cells were first incubated with Fucα(1-2)Gal-OEt disaccharide, L-FucαOEt, or D-FucαOEt (each at 150 mM) for 2 h and then with probe **2** (0.3 mM) for an additional 2 h. Cells were irradiated and lysed as described above and the proteins were resolved and detected as above by Western blotting (Figure 4.4B). The proteins captured by probe **2** (lane 1) are no longer detected upon treatment with the Fucα(1-2)Gal-OEt competitor (lane 2). Treatment with L-FucαOEt or D-FucαOEt reduced the concentrations of proteins captured, however the reduction was incomplete (lanes 3 and 4). These studies demonstrate that the lectins are recognizing the probe specifically via the sugar moiety. Moreover, comparison of the L-FucαOEt or D-FucαOEt

monosaccharides with the disaccharide competitor suggests that recognition of the galactose moiety by the target lectins is an important factor.

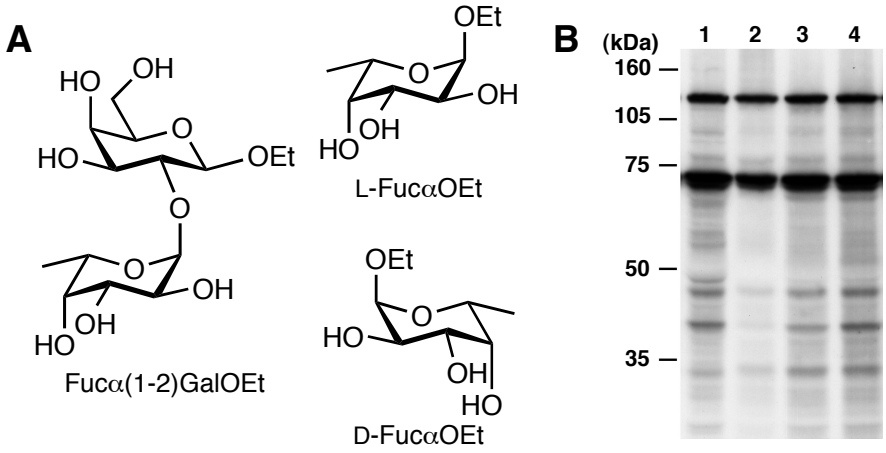

Figure 4.4. Probe **2** specifically labels Fucα(1-2)Gal lectins in dissociated neurons. A) Compounds synthesized for competition experiments with probe **2**. B) Competition binding with specified molecules shows that probe **2** is specifically labeling Fucα(1-2)Gal lectins. Lane 1: Cells treated with probe **2** alone (0.3 mM). Lane 2: Cells treated first with Fucα(1-2)GalOEt (150 mM) followed by probe **2** (0.3 mM). Lane 3: Cells treated with L-FucαOEt (150 mM) followed by probe **2** (0.3 mM). Lane 4: Cells treated with D-FucαOEt (150 mM) followed by treatment with probe 2 (0.3 mM)

Purification of Fucα(1-2)Gal lectins from dissociated neurons

To identify the target proteins, probe **2** was used to isolate Fucα(1-2)Gal lectins from embryonic brain. Dissociated cells were incubated with probe **2**, irradiated, and lysed as described above. After lysis, the proteins were first pre-cleared with agarose beads and then captured with streptavidin-agarose beads. Eluted proteins were resolved by SDS-PAGE and probed by Western blotting using streptavidin-HRP. As shown in Figure 4.5, the major Fucα(1-2)Gal lectins were the same as those observed in the capture experiment in Figure 4.3. Furthermore, control molecule **3** failed to capture any Fucα(1-2)Gal lectins.

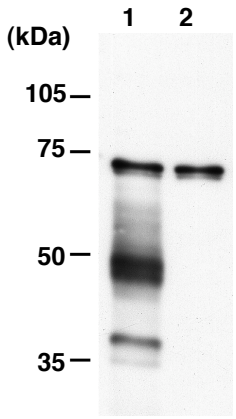

Figure 4.5. Fucα(1-2)Gal lectins were specifically captured by probe **2** and isolated on a streptavidin column. Dissociated cells were incubated with probe **2** (lane 1) or control molecule **3** (lane 2), crosslinked with UV light, and then lysed for protein purification. Eluents from the streptavidin columns were resolved and analyzed by Western blotting with streptavidin-HRP.

With such promising capture of Fucα(1-2)Gal-specific lectins by probe **2**, we thought identification of these proteins would follow accordingly. Dissociated neurons were labeled with probe **2** and captured proteins were isolated on streptavidin beads as described above. Eluted proteins were resolved by SDS-PAGE and visualized by Coomassie staining to allow for subsequent identification by mass spectrometry (MS). As shown in Figure 4.6, very little protein was detected by Coomassie staining. More importantly, there were no differences observed between samples labeled with probe **2** or control molecule **3**. Furthermore, none of the proteins observed by Coomassie staining corresponded to proteins detected by streptavidin-HRP, which visualizes lectins crosslinked to probe **2**. Subsequently, we performed these experiments on a larger scale to try to enhance the total amount of proteins isolated. However, efforts to isolate the lectins in sufficient quantity to be visualized by Coomassie staining and for subsequent MS analysis were unsuccessful.

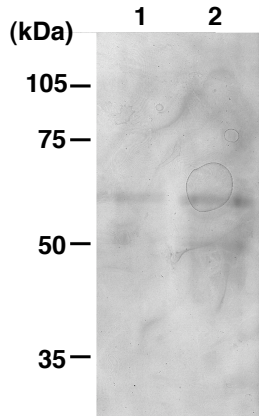

Figure 4.6. Coomassie stain analysis of total protein captured on streptavidin column after probe **2** labeling. Lane 1: Protein labeled with probe **2**; Lane 2: Protein labeled with control molecule **3**

In an effort to enrich the target lectins before MS analysis, we performed subcellular fractionation experiments with labeled neurons before isolating over streptavidin beads. Dissociated neurons were labeled with probe **2** as described above and lysed in 0.32 M sucrose by homogenization. Cell lysates were separated into soluble S2 and membrane P2 fractions. The S2 fractions were further separated by anion exchange chromatography on a Q-sepharose column. The fractions with the highest absorbance at 280 nm were resolved by SDS-PAGE and analyzed by Western blotting with streptavidin-HRP. As shown in Figure 4.7A, several proteins labeled with probe **2** were divided into different fractions and were separated from proteins labeled with control molecule **3**. The separation of labeled proteins by anion exchange was promising for the enrichment of target lectins. However, when the fractions were analyzed by Coomassie staining, very little to no protein was detected (Figure 4.7B).

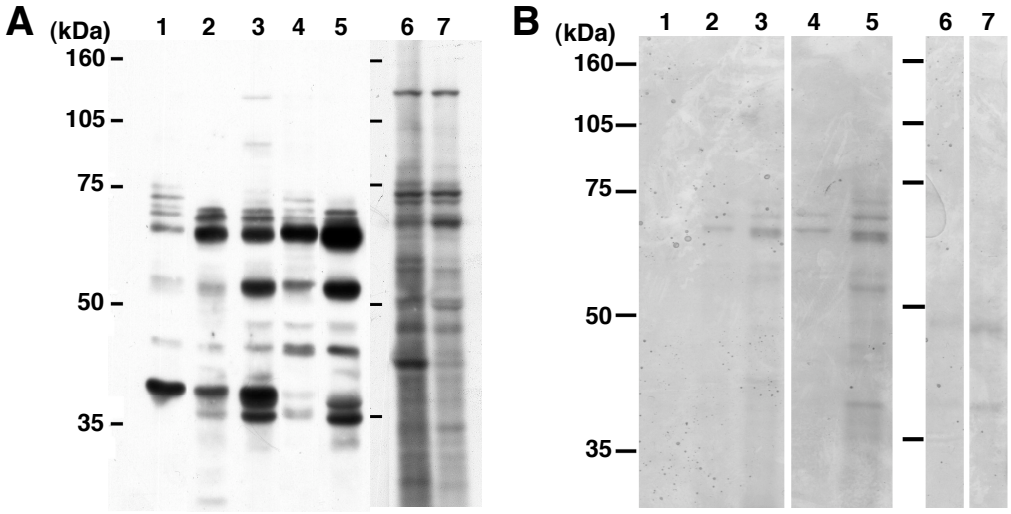

Figure 4.7. Subcellular fractionation of protein lysates labeled with probe **2** and control molecule **3**. A) Streptavidin-HRP Western blot of subcellular fractions further separated by anion exchange chromatography. Lanes 1 – 3: S2 fractions labeled with probe **2**. Lanes 4 – 5: S2 fractions labeled with control molecule **3**. Lane 6: P2 fraction labeled with probe **2**. Lane 7: P2 fraction labeled with control molecule **3**. B) Coomassie stain analysis of the same subcellular fractions as in A)

Our studies indicate quantitative capture of the lectins using streptavidin beads. However, the crosslinking step seems to be inefficient and we estimate that less than 1% of the total protein in the cells is being crosslinked to the probe. These results suggest that the probe has weak binding affinity for the lectins and/or the lectins are present in very low cellular abundance. These challenging obstacles have made it quite difficult to capture sufficient quantities of the Fucα(1-2)Gal lectins for MS analysis from embryonic tissue. As a result, we proceeded to isolate target lectins from protein lysates generated from rat pup brain, as older animals will presumably contain larger quantities of protein.

Identification of Fucα(1-2)Gal lectins from rat brain lysate

In trying to identify Fucα(1-2)Gal lectins from embryonic tissue, we encountered several complications. Mainly, we were unable to isolate sufficient quantities of target

lectins for MS analysis. Therefore, we decided to move towards using rat pup protein lysate for identifying Fucα(1-2)Gal lectins (Figure 4.8).

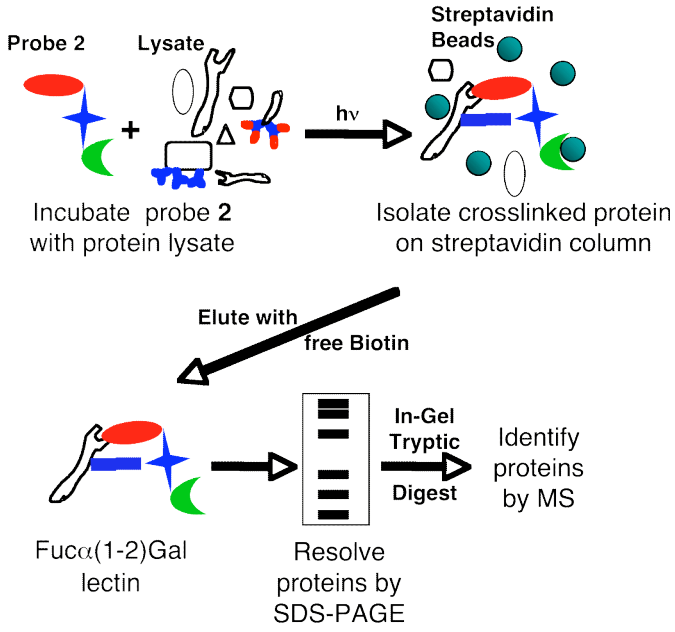

Figure 4.8. Scheme depicting the strategy by which probe **2** is used to label and identify Fucα(1-2)Gal lectins from neuronal protein lysate

Whole cortices from rat pups were lysed by sonication before incubating over agarose beads to eliminate any endogenous, agarose-binding proteins. Lysates were then labeled with probe **2** or control molecule **3** and then captured over streptavidin-agarose beads. Target lectins were eluted off the streptavidin column, resolved by SDS-PAGE, and analyzed by Western blotting and silver staining. As shown in Figure 4.9A, several proteins were enriched with probe **2** labeling as compared to control molecule **3** labeling. Importantly, the silver stain analysis of these samples also showed enrichment of proteins labeled with probe **2**, indicating capture of potential Fucα(1-2)Gal lectins (Figure 4.9B).

Although weak binding was observed, protein bands from both lanes of the silver stain were excised, digested with trypsin, and compared by MS analysis. From this

analysis, we identified the first potential Fucα(1-2)Gal lectins: Na^+/K^+ ATPase and importin β. Despite low signal from the MS data, we were able to duplicate the identification of these two proteins from two separate labeling experiments. These putative protein hits were quite exciting, as both of these proteins have crucial roles in cellular function. The Na^+/K^+ ATPase is essential in generating the electrochemical gradient necessary to maintain cellular potential.[29] Importin β is a major transport receptor crucial to the import of proteins into the nucleus.[30]

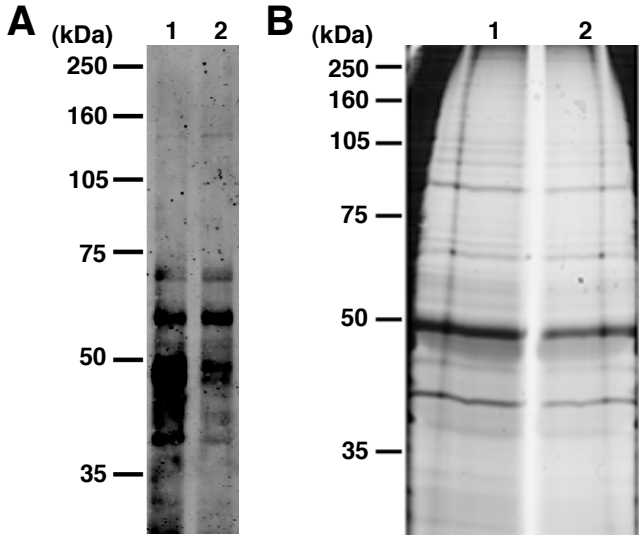

Figure 4.9. Distinct proteins from rat pup lysate were captured and isolated by probe **2**. A) Western blot analysis of rat pup lysate labeled with probe **2** (lane 1) and control molecule **3** (lane 2). B) Silver stain analysis of proteins labeled with either probe **2** (lane 1) or control molecule **3** (lane 2)

To confirm that these proteins are Fucα(1-2)Gal lectins, lysates labeled with probe **2** were resolved by SDS-PAGE and then analyzed by Western blotting using either Na^+/K^+ ATPase or importin β antibodies. Unfortunately, we did not detect any signal at the predicted molecular weights of either protein (Figure 4.10). Moreover, further attempts at confirming these results with more labeling experiments using probe **2** were not possible. Limited quantities of probe **2** were generated in the laboratory and after

many rounds of optimization and experimental trials, we had exhausted all stocks synthesized.

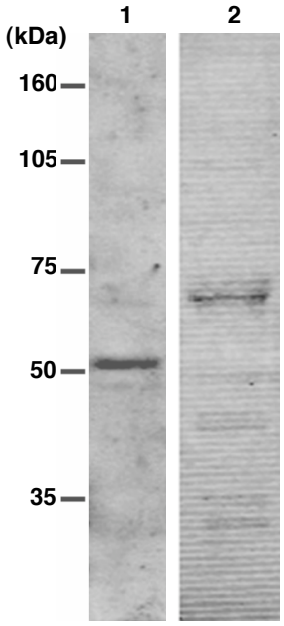

Figure 4.10. Protein lysate labeled with probe **2** did not confirm identification of potential Fucα(1-2)Gal lectins. Lane 1: Labeled lysate probed with Na^+/K^+ ATPase antibody (predicted molecular weight is 112 kDa); Lane 2: Labeled lysate probed with importin β antibody (predicted molecular weight is 97 kDa)

Capture of Fucα(1-2)Gal lectins using multivalent polymers

Although we have used probe **2** to label several potential Fucα(1-2)Gal lectins in embryonic brain, efforts to capture and isolate sufficient quantities of target lectins for MS analysis were only mildly successful. Therefore, we proceeded to use multivalent probes to enhance our ability to capture larger quantities of lectins. As carbohydrates have weak binding affinities for lectins ($K_{assoc} = 10^3 – 10^6$ M), multivalent probes greatly enhance the binding affinity and cluster of lectins to their targets.[24, 31] Remarkably, we found that polyacrylamide polymers displaying multiple Fucα(1-2)Gal epitopes stimulated neurite outgrowth by 50 ± 6% (Chapter 2). To establish whether the polymers would function to isolate target lectins, we first determined if these polyacrylamide

polymers displaying multiple Fucα(1-2)Gal epitopes (polymer **4**, Glycotech Corporation, Figure 4.11) could label target lectins.

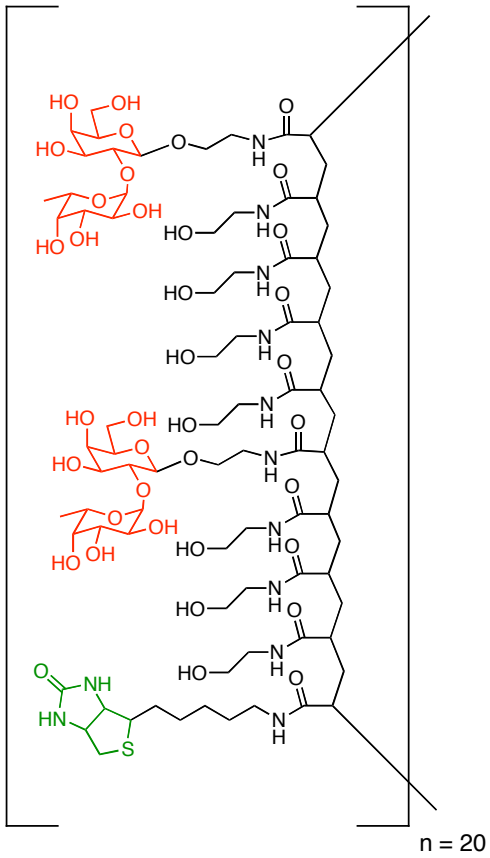

Figure 4.11. Multivalent polymer **4** displays multiple Fucα(1-2)Gal epitopes (red) and multiple biotin moieties (green). Specifically, polymer **4** has a polyacrylamide backbone and is 20% loaded with Fucα(1-2)Gal (approximately 40 units) and 5% loaded with biotin (approximately 20 units).

Dissociated neurons from embryonic rat brains were lysed, and proteins were fractionated into S2 and P2 fractions, resolved by SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membranes. The lectins were visualized by Far-Western analysis where the blot was probed with polymer **4**, followed by incubation with streptavidin-HRP. Importantly, we found that polymer **4** labeled similar lectins to those labeled with monovalent probe **2** (Figure 4.12). Comparing lane 2 versus lane 3, we see

many of the same protein bands detected with multivalent polymer **4** as were labeled with monovalent probe **2**, respectively.

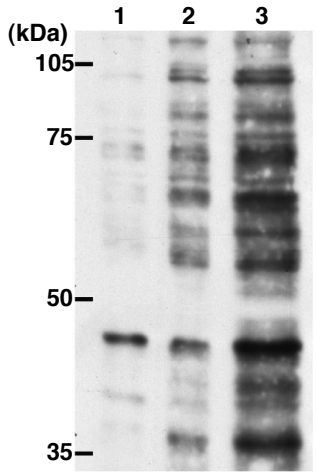

Figure 4.12. Labeling of proteins from dissociated embryonic neurons using the multivalent or monovalent probe. Proteins from the S2 (lane 1) or P2 (lane 2) fractions were labeled on blot with multivalent polymer **4**. Lane 3: Intact cells were labeled with the monovalent probe **2** and lysed.

As the polyacrylamide polymers successfully labeled target lectins, we proceeded to capture the lectins using polymer **4**. In combination with streptavidin beads, polymer **4** was used to generate a Fucα(1-2)Gal affinity column for the isolation of desired lectins (Figure 4.13).

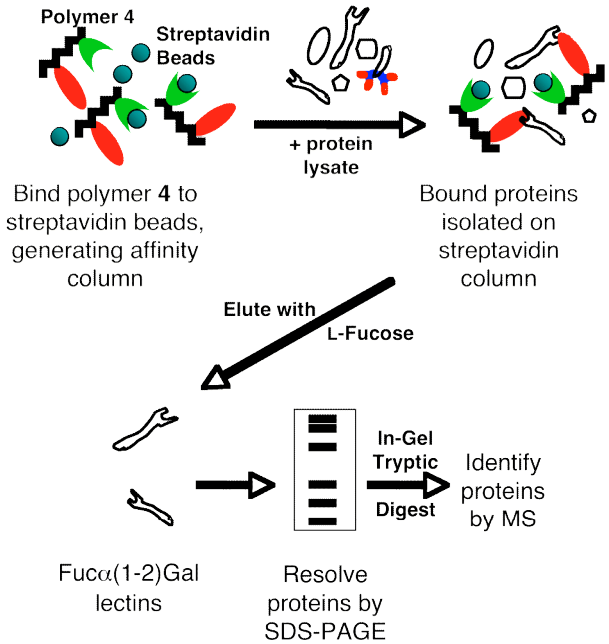

Figure 4.13. Strategy for identification of Fucα(1-2)Gal lectins from neuronal protein lysate using multivalent polymer **4**

First, we used the known fucose-binding lectin UEA-I to optimize binding and capture conditions. The Fucα(1-2)Gal affinity column was generated by incubating polymer **4** with streptavidin beads. After unbound polymer was removed, UEA-I lectin was bound on the column and then eluted with binding buffer containing 200 mM L-fucose. This procedure enabled specific capture of the UEA-I lectin by the Fucα(1-2)Gal affinity column compared to the control streptavidin column (Figure 4.14).

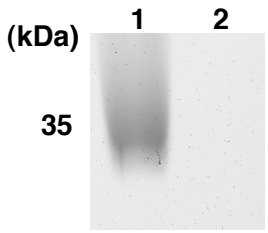

Figure 4.14. Capture of purified UEA-I lectin using the multivalent Fucα(1-2)Gal polymer **4**. Equal amounts of UEA-I lectin (10 μg) were bound on the Fucα(1-2)Gal affinity column and streptavidin control column. Lane 1: Eluent from Fucα(1-2)Gal affinity column; Lane 2: Eluent from streptavidin column

With successful isolation of the UEA-I lectin by the Fucα(1-2)Gal affinity column, we proceeded to capture the fucose-specific lectins from rat pup protein lysate. Rat pup cortical tissue was lysed by sonication with ice-cold binding buffer before incubating with the Fucα(1-2)Gal affinity column. As a positive control, we added UEA-I lectin to the lysate mixture before incubating with the affinity column. Again, we were able to specifically capture the UEA-I lectin (Figure 4.15). However, the efficiency of lectin capture from the lysate was not very high and resulted in smeared protein bands visualized in the silver staining.

Figure 4.15. Capture of UEA-I and Fucα(1-2)Gal lectins from rat pup lysate. UEA-I lectin was specifically captured by the affinity column (Lane 1). Other Fucα(1-2)Gal lectins were also captured, albeit to a lesser extent, exhibited by the smear of protein bands in Lane 1. Lane 1: Eluent from Fucα(1-2)Gal affinity column; Lane 2: Eluent from streptavidin column

To enhance the efficiency of the Fucα(1-2)Gal affinity column, we optimized the lysis and binding conditions to facilitate the capture of Fucα(1-2)Gal lectins. Rat pup cortical tissue was lysed in ice-cold binding buffer that contained no detergent, and non-specific proteins were reduced by pre-clearing the lysate over a streptavidin column for 1 h at room temperature prior to incubation with the Fucα(1-2)Gal affinity column for 4 h at room temperature. Again, we added UEA-I to the lysate prior to incubating with either affinity column as a positive control. Furthermore, we added a competition affinity column where L-fucose (1000-fold excess) was added to the lysate during incubation with the Fucα(1-2)Gal affinity column. The competition column would better indicate which captured proteins are fucose-specific lectins and will allow us to eliminate false positives that might arise from non-specific crosslinking to proteins or non-specific

binding of proteins to the polyacrylamide backbone or streptavidin beads. As shown in Figure 4.16, several proteins were enriched on the Fucα(1-2)Gal affinity column relative to the control column where lectin binding was competitively inhibited by L-fucose (arrows). Moreover, the Fucα(1-2)Gal affinity column captured the positive control lectin UEA-I (arrowhead). The bands from the affinity and control columns were excised, digested with trypsin, and compared by MS analysis. Although specific proteins were visualized by silver staining, the total amount of protein isolated from each band was too low and MS signal strength was weak and inconclusive.

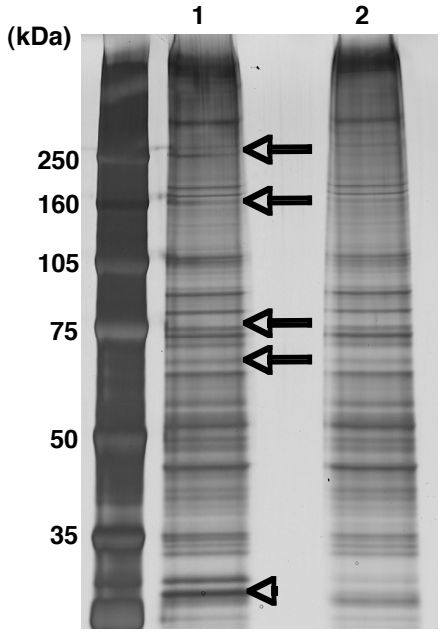

Figure 4.16. Capture of Fucα(1-2)Gal lectins from rat pup lysate using the Fucα(-2)Gal affinity column. Proteins at ~ 65, 80, 140, and 300 kDa (arrows) were selectively captured by the Fucα(1-2)Gal affinity column (Lane 1). UEA-I lectin was also specifically captured (arrowhead, Lane 1). Lane 1: Eluent from Fucα(1-2)Gal affinity column; Lane 2: Eluent from L-fucose competition column

Design of new multivalent Fucα(1-2)Gal probes for isolating neuronal lectins

As described in Chapter 2 and above, our studies demonstrate that Fucα(1-2)Gal lectins exist in the mammalian brain and are involved in a novel pathway for neuronal growth. Using polyacrylamide polymers displaying multiple disaccharide and biotin

moieties, we created an affinity column to capture the target lectins. While this approach has enhanced the binding of desired lectins to the Fucα(1-2)Gal affinity column, we were still unable to isolate sufficient quantities of proteins for MS analysis. Therefore, we feel that we can significantly improve our ability to capture the lectins by generating a multivalent, biotinylated glycopolymer containing photoreactive crosslinking groups. Arif Wibowo will synthesize polymer **5**, which has Fucα(1-2)Gal recognition elements, photoreactive phenyl azide groups, and is end-labeled with biotin (Figure 14.17).

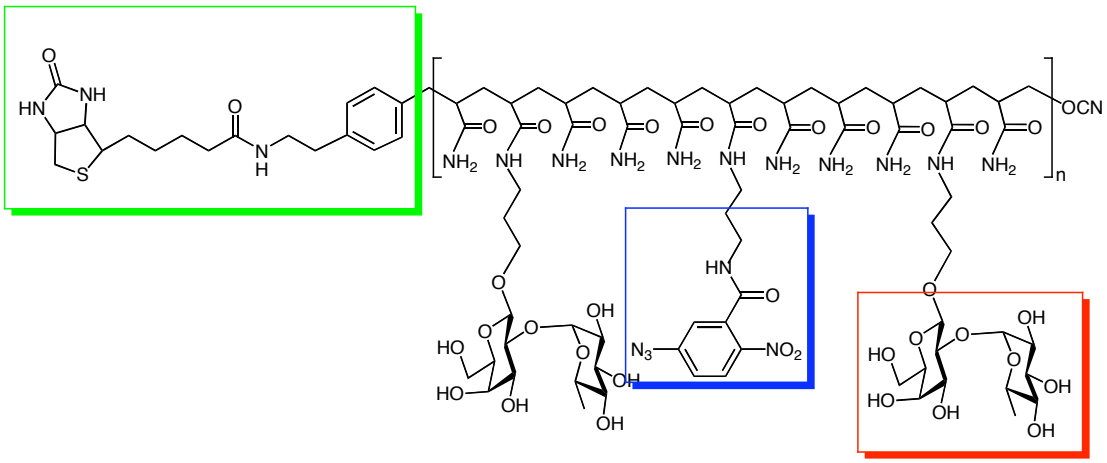

Figure 4.17. Design of multivalent capture polymer **5**. Polymer **5** will have a polyacrylamide backbone, multiple Fucα(1-2)Gal epitopes (red), photoreactive phenyl azide groups (blue), and will be end-labled with biotin (green).

The presence of multiple Fucα(1-2)Gal sugars on a polyacrylamide backbone ensures the binding of polymer **5** to the target lectins and the biotin group provides a convenient handle for capturing the lectins by affinity chromatography. The photoreactive phenyl azide moieties should allow more efficient lectin capture by forming a covalent linkage to the target proteins. Importantly, the addition of a covalent linkage between the polymer and protein of interest allows for vigorous washing to reduce non-specifically bound proteins. Phenyl azides were chosen as a starting point

because they are well precedented and can be readily synthesized from commercially available starting materials. Other photoactivatable groups such as trifluorometylphenyldiazirines (as in probe **2**) and benzophenone are also possible.

Discussion

Our overall goal is to understand the molecular mechanisms by which fucose-containing carbohydrates regulate neuronal communication and function. As fucose-associated lectins and glycoproteins from the brain have not been identified previously, we developed new methods to identify and characterize these proteins. Our studies have revealed that such proteins do indeed exist in the mammalian brain and play a key role in the regulation of synaptic proteins and neuronal morphology.

We have established that Fucα(1-2)Gal-associated proteins participate in a novel carbohydrate-mediated pathway for regulating neuronal growth (Chapter 2).[32] Specifically, we developed a chemical probe for detecting Fucα(1-2)Gal lectins in neurons and found that the probe bound to the cell body and neurites of hippocampal neurons. Furthermore, we discovered that association of the fucose disaccharide with these lectins dramatically promotes neurite outgrowth. Additionally, we have found that Fucα(1-2)Gal glycoproteins are prevalent in the developing brain and that synapsin Ia and Ib are the major Fucα(1-2)Gal glycoproteins in the adult rat brain (Chapter 3).[33] Notably, fucosylation protects the synapsins from rapid degradation, while inhibition of fucosylation drastically impairs the outgrowth of neurons and delays synapse formation. Together, our studies suggest that Fucα(1-2)Gal carbohydrates play a significant role in regulating neuronal growth and communication.

Once we established the existence of Fucα(1-2)Gal lectins in neurons, we sought to identify and characterize these proteins. As no Fucα(1-2)Gal lectins have been previously reported and no methods existed to isolate such proteins, we designed a chemical probe (probe **2**) and developed a strategy for the capture and identification of the target lectins from the brain. The probe includes 3 key features: 1) a Fucα(1-2)Gal recognition element for binding to lectins, 2) a photoactivatable group to covalently crosslink the probe to target lectins, and 3) a biotin handle for affinity capture. Efforts to isolate lectins from embryonic tissue in sufficient quantity for MS analysis were unsuccessful. Attempts to enrich the captured lectins using subcellular fractionation were also unsuccessful. Based on our experimental results, we estimated that less than 1% of the total protein in the cells is being crosslinked to the probe. This suggests that the probe has a weak binding affinity for the target lectins and/or that the target lectins are of very low abundance in the embryonic cell.

Unable to characterize Fucα(1-2)Gal lectins from embryonic neurons, we proceeded to isolate target lectins from juvenile rats in an attempt to obtain sufficient quantities of protein for MS analysis. This strategy was a move in the right direction and provided the identity of the first potential Fucα(1-2)Gal lectins, the Na^+/K^+ ATPase and importin β. Despite such promising results, we were unable to confirm the identity of these putative lectins by Western blotting and further attempts to isolate more proteins using probe **2** were not possible. As such, our efforts then focused on using multivalent probes to capture the target lectins.

Carbohydrates typically have weak binding affinities for lectins,[24] making it quite difficult to study and identify specific lectin targets. Multivalent probes can help

overcome these challenges by enhancing the binding affinity between the carbohydrate and lectin of interest.[31] We used a biotinylated polyacrylamide polymer containing multiple Fucα(1-2)Gal epitopes and generated a lectin affinity column using streptavidin beads. Although weak binding was observed, we found that we could successfully capture Fucα(1-2)Gal-specific lectins from neuronal lysates. Importantly, the affinity column captured the protein UEA-I, a Fucα(1-2)Gal-specific plant lectin[27, 28] which was added to the lysates as a positive control. After many rounds of optimization of experimental conditions, several proteins were enriched relative to a control column in which lectin binding was competitively inhibited with L-fucose (Figure 4.16). Despite such distinct protein staining, the efficiency of capture was not enhanced relative to that of the first probe (probe **2**), which was monovalent but contained a photoactivatable crosslinking group. Based on these results, we have reasoned that adding photo-crosslinking groups to multivalent polymers should solve these technical issues, combining enhanced lectin binding with efficient covalent capture. Efforts in the lab have now focused on generating multivalent polymers that will enable control over carbohydrate density, type, and number of photo-crosslinking groups, and allow for the addition of biotin or other moieties for affinity chromatography.

Although we were unable to conclusively identify any Fucα(1-2)Gal lectins from neurons, the possibility that the Na^+/K^+ ATPase and importin β are fucose-specific lectins leads to significant implications. First, the Na^+/K^+ ATPase is an essential protein crucial to cellular function. It creates an electrochemical gradient across the cell membrane by exchanging cytoplasmic Na^+ for extracellular K^+ in a 3:2 ratio.[29] This gradient plays a role in maintaining cell volume and pH, in keeping the cell resting membrane potential,

and in providing the energy necessary for the secondary transport of other ions, solutes and water across the cell membrane. The Na^+/K^+ ATPase is made up of two main subunits, α and β, both of which exist in different isoforms. Interestingly, the β subunit is known to be glycosylated, and the glycoproteins have been shown to contain fucose carbohydrates.[29, 34, 35] Although glycosylation is not essential to formation, cell trafficking, or activity of the Na^+/K^+ ATPase, glycosylation aids in the structural stability of β subunits and also specifies the assembly of distinct α and β subunits.[29] As such, one possibility is that the α subunit may serve as a lectin receptor, binding to the carbohydrate expressed on the β subunit. Upon binding, correct αβ heterodimer assembly can occur and thus allow delivery of an active Na^+/K^+ ATPase to the cell membrane. In this instance, fucosylation of the Na^+/K^+ ATPase would be acting as an intracellular signal modulating the assembly of different αβ heterodimers, generating separate Na^+/K^+ ATPase isozymes with distinct functions.

The possibility that importin β is also a fucose-specific lectin has significant implications for nuclear transport and neuronal communication. Importin β is a key protein essential to nuclear transport in most mammalian cells and can bind macromolecular cargo indirectly via the adaptor protein importin α.[30] Importin α must first bind importin β before binding to any cargo in the cytoplasm via a nuclear localization sequence. Although the classical mode of nuclear import incorporates the binding of importin β to importin α, it is also possible for importin β to bind directly to cargo.[30, 36] As fucose levels have been shown to increase during learning and memory processes, it is possible that importin β may bind to and translocate newly fucosylated glycoproteins into the nucleus to initiate the cellular response needed for learning. In this

manner, fucosylation would act as a recognition element enabling the binding of fucosylated glycoproteins to importin β, which in turn would transport these proteins into the nucleus. Moreover, the transport of newly fucosylated proteins would provide direct communication of synaptic activity to the nucleus.

In all, our studies provide new molecular-level insights into the function of L-fucose in the mammalian brain. We have shown that fucose-specific lectins are found in hippocampal neurons and have made progress in identifying these specific proteins. In addition, we have determined that synapsin Ia and Ib are fucose-containing glycoproteins. Taken together, our studies provide compelling evidence for an important physiological role for fucosyl sugars in the brain. By understanding the molecular underpinnings of communication in the brain, we hope to ultimately provide new targets for therapeutic intervention when learning and memory become impaired.

Experimental Procedures for Chapter 4

Buffers and Reagents:

Chemicals and molecular biology reagents were purchased from Fisher (Fairlawn, NJ) unless stated otherwise. Protease inhibitors were purchased from Aldrich Chemicals (St. Louis, MO) and Alexis Biochemicals (San Diego, CA). Cell media was purchased from Gibco BRL (Grand Island, NY).

*Labeling of Known Fucose-Binding Lectins AAA and UEA I with Probe **2**:*

Purified lectins were purchased from EY Laboratories (San Mateo, CA) and used to make 1 mg/mL stocks in water. 10 μg of each lectin was incubated with or without probe **2** (1 mM) or control molecule **3** (1 mM) for 1 h at 37 °C with gentle mixing in neurobasal media in the dark. Samples were then irradiated on ice with UV light (365 nm) for 1 h before resolving proteins by SDS-PAGE and analyzing by streptavidin-HRP Western blotting and Coomassie staining.

Capture of Fucα(1-2)Gal Lectins from Dissociated Embryonic Neurons:

Embryonic brain was dissected and neurons were dissociated as described in Chapter 2. Dissociated neurons were spun briefly (1,000 rpm, 5 min), pelleted, resuspended in neurobasal media and incubated with probe **2** (1 mM) or molecule **3** (1 mM) for 4 h at 37 °C with gentle mixing in the dark. Neurons were pelleted (3,000 rpm, 5 min) and washed twice with PBS in the dark to remove any unbound probe. Cells were resuspended in PBS and irradiated on ice with UV light (365 nm) for 2 h. After a brief spin (3,000 rpm,

5 min), cells were lysed with boiling 1% SDS-containing protease inhibitors to solubilize all proteins. Proteins were resolved by SDS-PAGE and analyzed by streptavidin-HRP Western blotting.

*Competition Experiments Investigating Specificity of Probe **2** Labeling:*

Dissociated embryonic neurons were first incubated with the competitor molecules Fucα(1-2)Gal-OEt disaccharide, L-FucαOEt, or D-FucαOEt (each at 150 mM) for 2 h at 37 °C with gentle mixing in neurobasal media. Probe **2** (0.3 mM) was then added and cells were incubated for an additional 2 h in the dark at 37 °C with gentle mixing. After washing away unbound probe, cells were irradiated and lysed as above. Proteins were then resolved and analyzed by streptavidin-HRP Western blotting.

Purification of Labeled Proteins from Dissociated Embryonic Neurons:

Dissociated embryonic neurons were incubated with probe **2**, irradiated and lysed as described above. After lysis, the proteins were pre-cleared with agarose beads (Sigma) for 1 h at room temperature to deplete agarose-binding proteins. After a brief spin to pellet the beads, the protein lysates were removed and incubated for 2 h at room temperature with streptavidin-agarose beads (Pierce) in 0.2% SDS/PBS. The streptavidin beads were washed three times with 0.2% SDS/PBS, once with PBS, and bound protein was eluted with 2X SDS-PAGE loading buffer containing 1000-fold excess free biotin. Eluted proteins were resolved and probed by Western blotting as above.

*Subcellular Fractionation and Separation by Anion Exchange Chromatography of Dissociated Neurons Labeled with Probe **2**:*

Dissociated neurons were labeled with probe **2** as described above. Cells were lysed in 5 volumes of 0.32 M sucrose/ 5 mM Tris pH 8.0 by passing through a 22-gauge needle 5 times. The sample was diluted to 1.5 mL with sucrose buffer and passed through the needle again another 5 times. The cell lysate was spun at 800 x g for 10 min, supernatant was transferred to a new tube and spun again at 16,000 x g for 15 min. The supernatant was saved as S2 and the pellet was saved as P2 and later lysed with boiling 1% SDS before analysis. The S2 fractions were measured for protein concentration using the BCA protein assay (Pierce) and then desalted on a PD-10 column (Amersham). The PD-10 column was first washed with 20 mL of 50 mM Tris pH 8.0/ 0.1% Triton X-100 before adding S2 sample onto the column in a total volume of 2.5 mL. The void volume was discarded and protein was eluted in 3.5 mL of loading buffer. Protein concentration was measured again before loading desalted S2 sample onto a Q-sepharose anion exchange column (1 mL HiTrap Q HP column; Amersham). The Q-sepharose column was run at a flow rate of 0.5 mL/min and was washed first with 5 column volumes of 50 mM Tris pH 8.0, then with 5 column volumes of 50 mM Tris pH 8.0/ 1 M NaCl, and then equilibrated with 10 column volumes of 50 mM Tris pH 8.0. The S2 sample was loaded onto the column, washed with 5 column volumes of 50 mM Tris pH 8.0, and then proteins were eluted with a gradient of 0 – 1 M NaCl for 20 column volumes. Samples were combined based on absorbance at 280 nm, concentrated and dialyzed with Centricon filters (Millipore), and then analyzed by Western blotting and Coomassie

staining. Separate columns were used for cells labeled with control molecule **3** and treated as above.

Purification of Fucα(1-2)Gal Lectins from Rat Pup Brain:

Whole cortices from rat pups were dissected as described in Chapter 3 and lysed by sonication in ice cold binding buffer I (100 mM Tris pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 % NP-40, 0.2% sodium deoxycholate) plus protease inhibitors and pre-cleared over agarose beads for 1 h. Lysates were then incubated with probe **2** or control molecule **3** for 4 h in the dark at room temperature, followed by irradiation with UV light for 2 h at 4 °C. After a couple washes in binding buffer I, lysates were incubated with streptavidin beads equilibrated in binding buffer for 2 h at room temperature. The bead-lysate mixture was packed into a 1 mL spin column and washed as follows: 3 column volumes of binding buffer I, 3 column volumes of high salt binding buffer I (300 mM NaCl), and 3 column volumes of binding buffer I without detergent. Target lectins were eluted with 3 column volumes of 2X SDS-PAGE loading buffer containing 1000-fold excess free biotin, concentrated with Microcon filters (Millipore), then resolved by SDS-PAGE and analyzed by Western blotting and silver stain.

Trypsin Digestion and MS Analysis:

Bands were excised from Coomassie-stained gels and treated essentially as described by Shevchenko et al.[37] Briefly, excised bands were destained overnight in 50% methanol/ 5% acetic acid. Destained bands were dehydrated in acetonitrile (CH_3N), dried by vacuum, and rehydrated in 10 mM DTT. After 30 min reduction at room temperature,

excess DTT was removed, and proteins were alkylated in 50 mM iodoacetamide for 30 min at room temperature in the dark. After alkylation, excess iodoacetamide was removed and protein bands were washed in 100 mM ammonium bicarbonate (NH_4HCO_3; pH 8.0) for 10 min, followed by two successive dehydrations in CH_3N. Wash and dehydration steps were repeated once more, and excess CH_3N was removed under vacuum. Protein bands were rehydrated in 15 ng/μL trypsin (Promega) in 50 mM NH_4HCO_3. Excess trypsin solution was removed after rehydration, and 20 – 30 μL of 50 mM NH_4HCO_3 was then added to cover the gel slices. Proteins were digested overnight at 37 °C. Following digestion, peptides were extracted with successive washes of water followed by 50% CH_3N/ 5% formic acid in water, and dried by vacuum centrifugation. Eluted peptides were sent to our collaboraters at the Genomics Institute of the Novartis Research Foundation (San Diego, CA) and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

*Capture of UEA-I Using Multivalent Polymer **4** Affinity Column:*

The known fucose-binding lectin UEA-I was used to optimize binding and capture conditions. The Fucα(1-2)Gal affinity column was generated by incubating polymer **4** (3 mg; Glycotech Corporation) with streptavidin-agarose beads (1 mL) for 30 min at room temperature in binding buffer I. Unbound polymer was removed by washing with 20 column volumes of binding buffer I. UEA-I (10 μg) was bound on the affinity column for 4 h at room temperature followed by several wash steps (3 column volumes of binding buffer I; 3 column volumes of high salt binding buffer I, 300 mM NaCl; and 3 column volumes of binding buffer I without detergent) and then eluting with binding

buffer I containing 200 mM L-fucose. Eluted samples were analyzed by SDS-PAGE and silver staining. UEA-I was also bound over a streptavidin column, generated as above without polymer **4**, as a control.

*Capture and Purification of Fucα(1-2)Gal Lectins Using Multivalent Polymer **4**:*

The Fucα(1-2)Gal affinity column was generated as described above. After incubating the samples on the column for 4 h at room temperature, the column was washed with 3 column volumes of binding buffer I, 3 column volumes of high salt binding buffer I (300 mM NaCl), and 3 column volumes of binding buffer I without detergent. Proteins were eluted with 3 column volumes of binding buffer I containing 200 mM L-fucose, concentrated with Microcon filters (Millipore), then separated on Tris-acetate gels and visualized by silver staining. For the competition affinity column, L-fucose (1000-fold excess) was added at the same time the sample was added to the Fucα(1-2)Gal affinity column.

Western Blotting:

Protein concentration of samples was determined using the BCA protein assay (Pierce). Lysates were resolved on 10% acrylamide-SDS gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore) for at least 12 h in 20 mM Tris-Cl pH 8.6/ 120 mM glycine/ 20% methanol. Western blots were blocked for 1 h with 3% BSA and rinsed with TBST (50 mM Tris-Cl pH 7.4/ 150 mM NaCl/ 0.1% Tween-20). Blots were incubated with streptavidin-HRP (1:500; Pierce) for 1 h at room temperature with constant rocking, then rinsed and washed 4 times for 20 min with TBST.

Immunoreactivity visualized by chemiluminescence using Pico Chemiluminescent Substrate (Pierce). For blots probed with Na/K ATPase and importin β antibodies, blots were blocked in 3% BSA followed by incubation with either Na/K ATPase antibody (1:500; Abcam) or importin β antibody (1:500; Abcam) for 2 h at room temperature. Blots were rinsed and washed twice with TBST, followed by incubation with IRDYE680 goat anti-mouse antibody (1:5000; Rockland Immunochemicals) in TBST/0.2% SDS for 1 h at room temperature in the dark. After 4 washes of 20 min in TBST, immunoreactivity was visualized using the Odyssey infrared imaging system (LICOR).

Coomassie Staining:

After the proteins were resolved by SDS-PAGE, the gel was immersed in 0.1% Coomassie/ 50% methanol/ 10% acetic acid for 30 min to 1 h at room temperature. The gel was then de-stained with 50% methanol/ 10% acetic acid at room temperature until the desired contrast was obtained.

Silver Staining:

Resolved gels were fixed in 50% methanol/ 10% acetic acid for 10 min, fixed in 5% methanol/ 1% acetic acid an additional 15 min, rinsed briefly in 50% methanol, then washed in double distilled water (ddH_2O) 3 times for 10 min each or overnight. The gels were then sensitized in freshly prepared sodium thiosulfite (0.02% in ddH_2O) for 90 seconds exactly and rinsed in ddH_2O 3 times for 30 sec each, before silver staining in freshly prepared 0.2% silver nitrate for 30 min at room temperature or overnight at 4 °C.

After rinsing 3 times for 60 sec each in ddH_2O, the stain was developed in freshly made 6% sodium carbonate/ 0.018% formaldehyde/ 0.0004% sodium thiosulfite for up to 10 min at room temperature with constant shaking until the desired contrast was attained. Developing was stopped in 6% acetic acid for 10 min and the gel was stored in ddH_2O.

Far-Western Analysis:

Proteins were resolved by SDS-PAGE and transferred to PVDF membrane. The blot was blocked for 1 h at room temperature with 3% BSA in TBST. After a few washes in TBST, the blot was incubated with polymer **4** (13 μM in TBST) overnight at 4 °C with gentle shaking. After two washes of 10 min each in TBST, streptavidin-HRP (1:5000) was added in TBST for 1 h at room temperature. Following 4 washes of 10 min each in TBST, immunoreactivity was visualized using Pico Chemiluminescent substrate.

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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

* Portions of this chapter were taken from C. I. Gama and L. C. Hsieh-Wilson (2005) *Curr. Opin. Chem. Biol.* **9**, 609 – 619.

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.

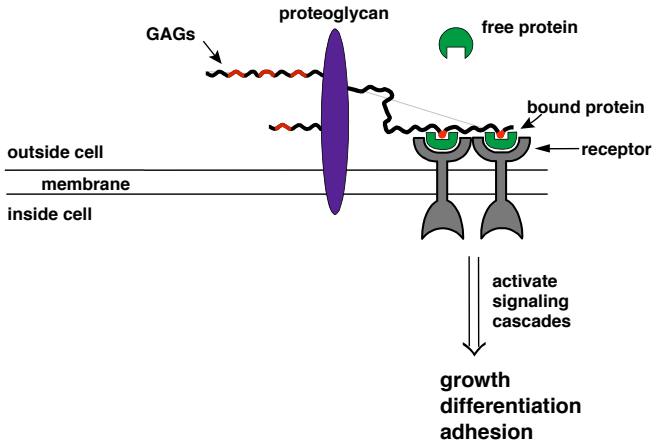

Figure 5.1. One proposed mechanism of GAG function at the cell

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.

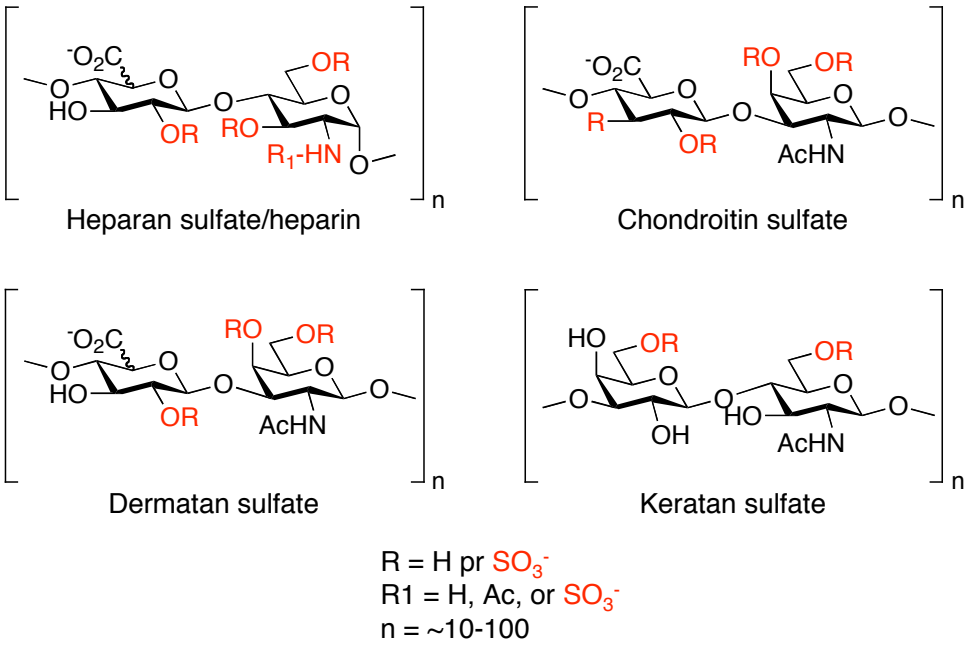

Figure 5.2. Structures of representative classes of GAGs

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,

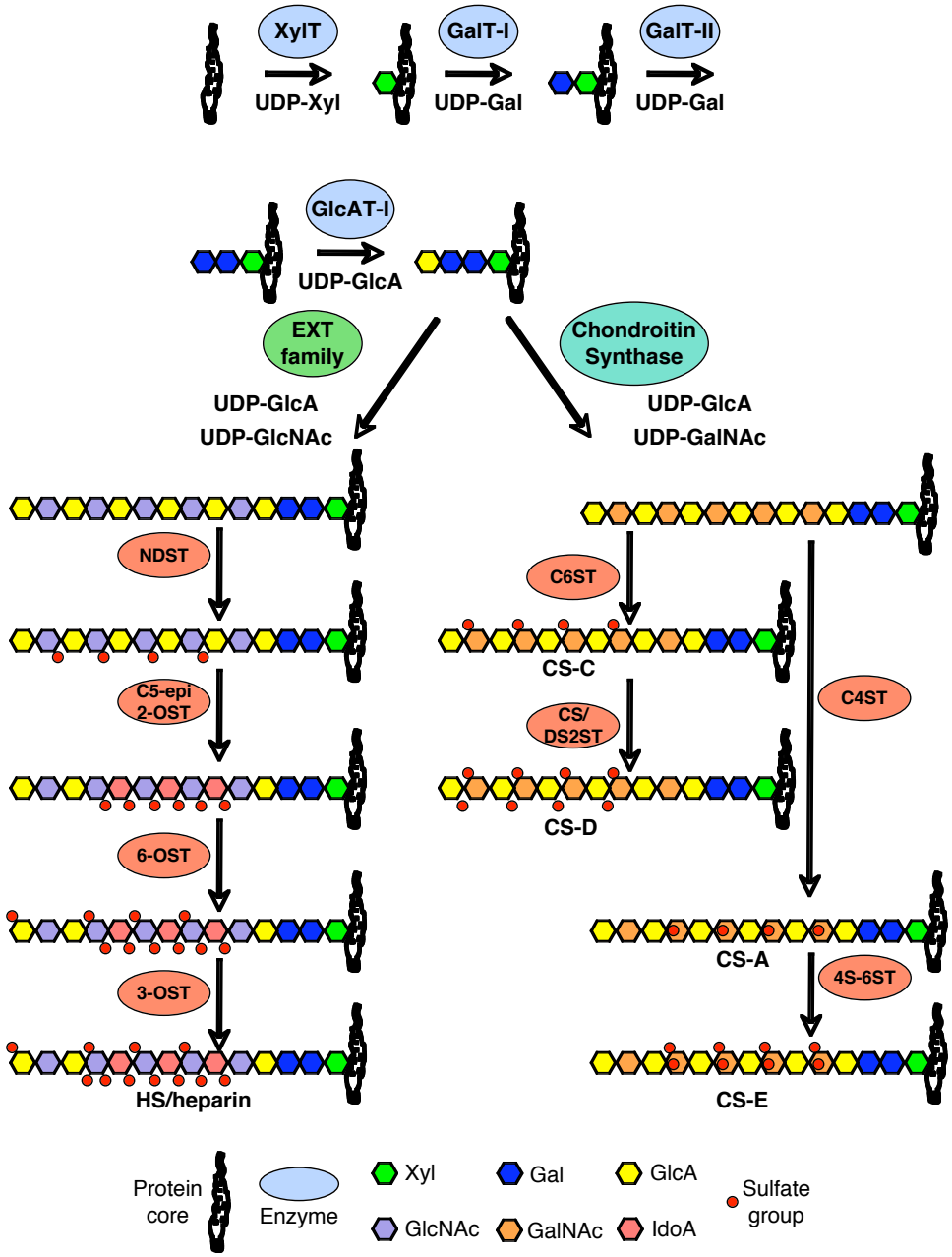

Figure 5.3. Biosynthesis of HS/heparin and CS GAGs takes place in the Golgi apparatus.

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).

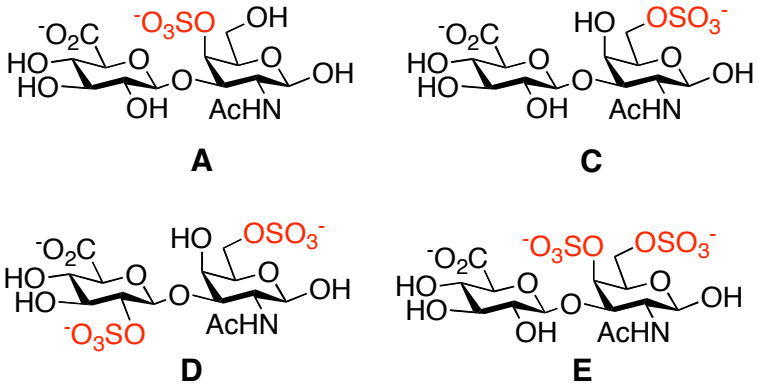

Figure 5.4. Structures of the most common CS sulfation motifs found *in vivo*

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.

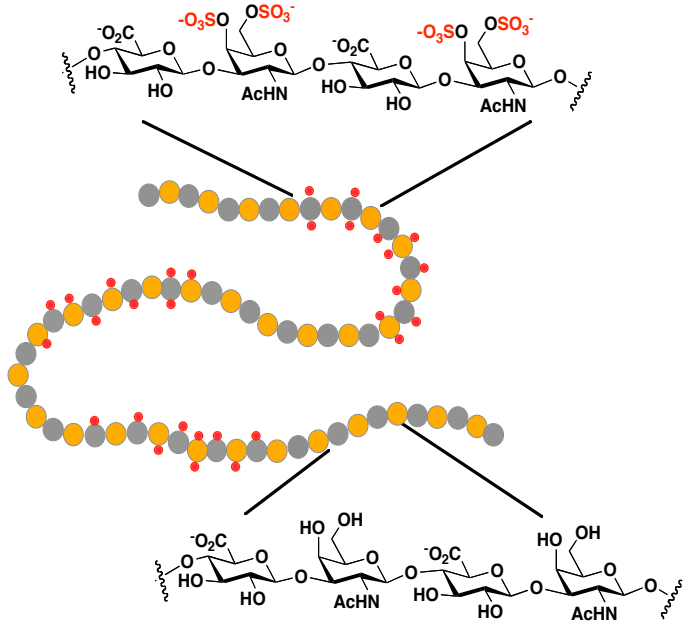

Figure 5.5. Heterogeneous GAG polysaccharides exhibit areas of low and high sulfation.

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.

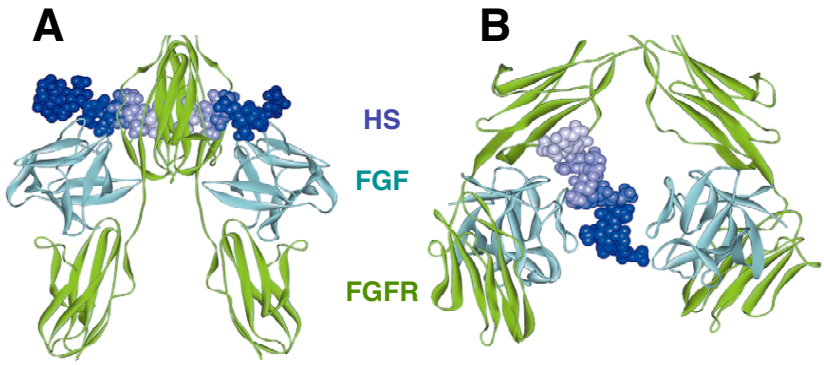

Figure 5.6. Crystallographic models of the FGF-FGFR-HS complex. A) The crystal structure of an FGF2-FGFR1-heparin complex has a 2:2:2 stoichiometry with two chains enabling complex formation. B) The crystal structure of an FGF1-FGFR2-heparin complex has a 2:2:1 stoichiometry with a single chain initiating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

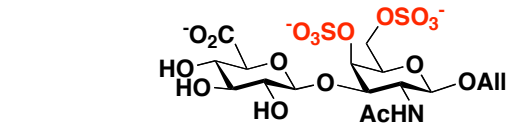
while 6-*O*-sulfotransferase activity was necessary for muscle differentiation and proper tracheal development.[8, 9] Growth factors also display preferential binding to distinct sulfation sequences; for instance, FGF-2 requires 2-*O*-sulfation but not 6-*O*-sulfation of HS and both pleiotrophin and 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.

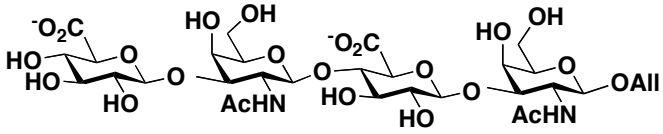
We have developed a chemical approach to evaluate the structure-activity relationship of CS as it effects neuronal growth. Through the synthesis of oligosaccharides of defined length and sulfation pattern, we have demonstrated that the CS-E sulfation sequence is a stimulatory motif that promotes the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

CS-E sulfated tetrasaccharide enhances neurite outgrowth

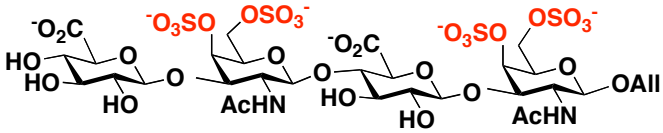
CS polysaccharides have been shown to both stimulate and attenuate the growth of cultured neurons.[17 -- 19] Notably, the molecules used in those studies were ~ 200 saccharides in length, poorly defined, and heterogeneously sulfated, features that might account for the contradictory observations. Therefore, 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.

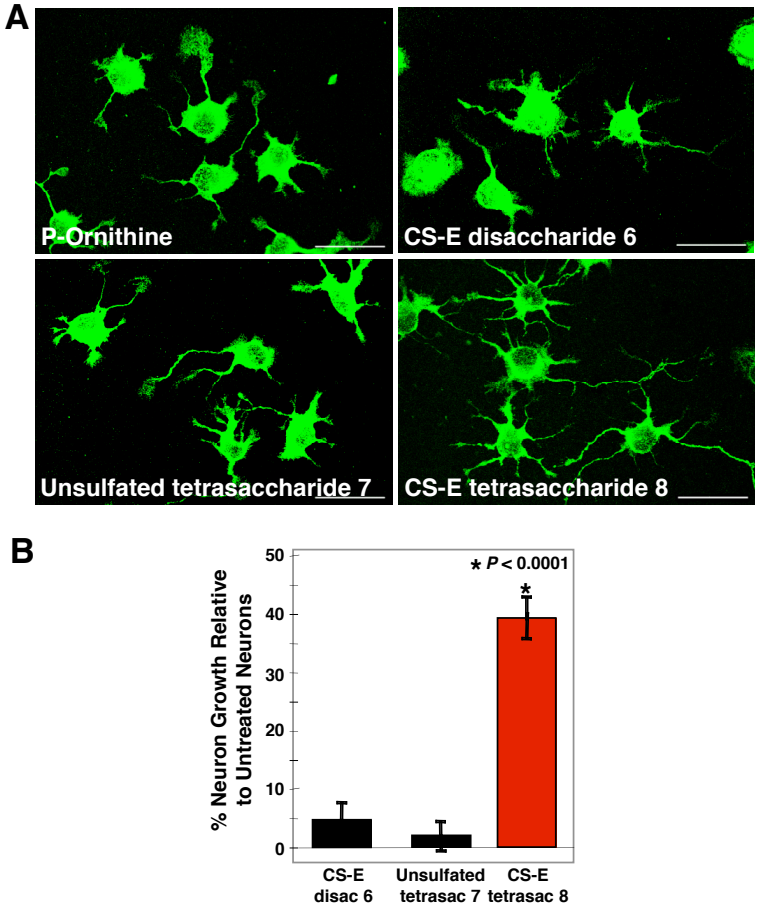


Figure 6.2. CS-E tetrasaccharide **8** stimulates the outgrowth of hippocampal neurons. A) Immunofluorescence images of neurons 48 h after treatment with the indicated compound. B) Statistical analysis of neurite length. *P* value is relative to poly-ornithine control. Scale bars represent 45 μm.

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.

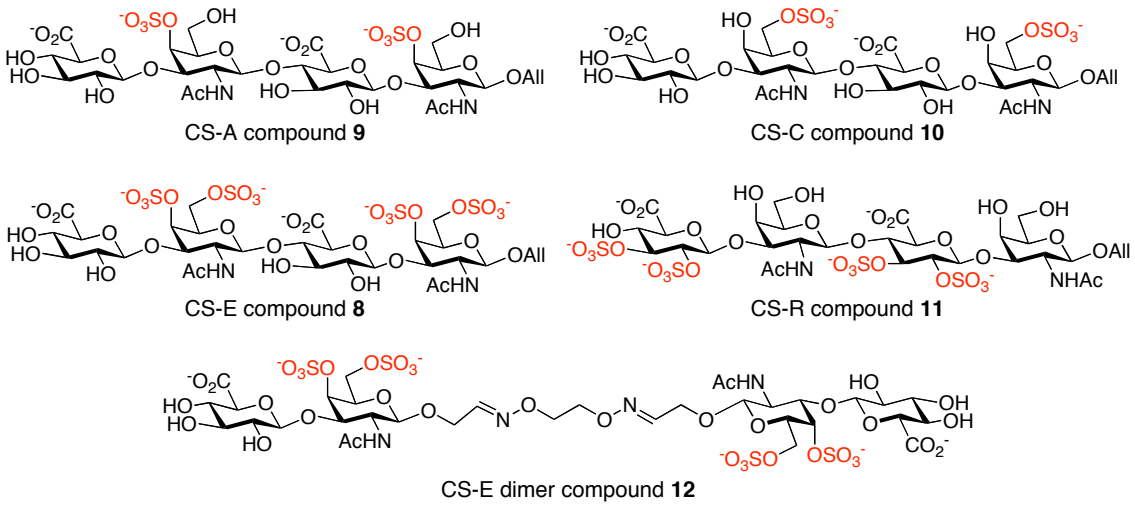


Figure 6.3. Structures of synthetic CS oligosaccharides containing distinct sulfation patterns

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.

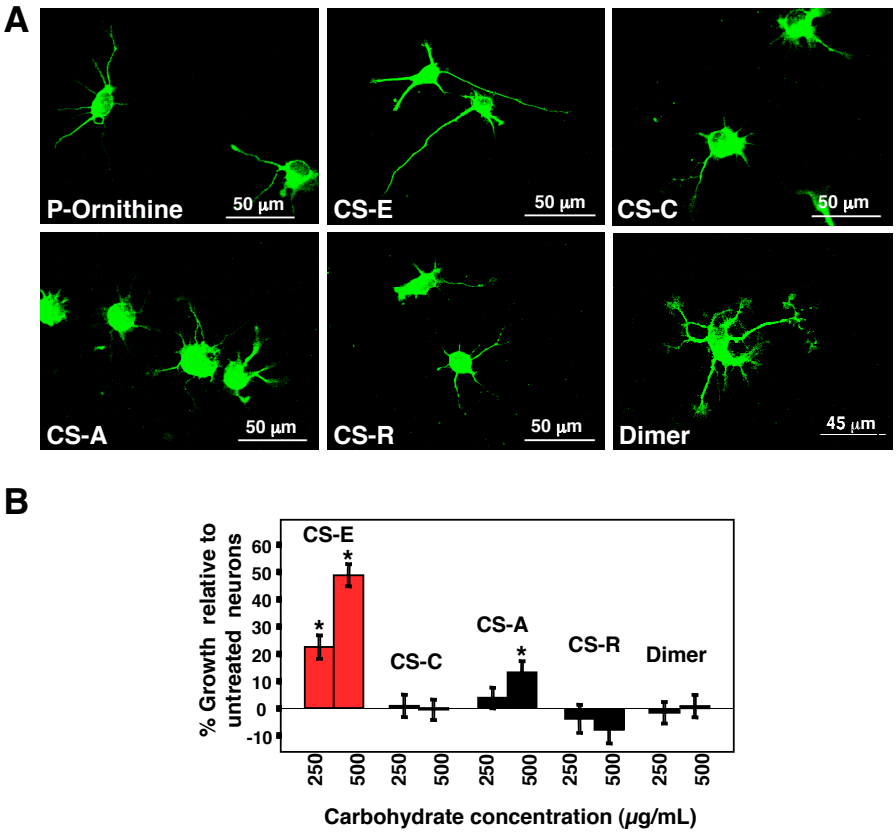


Figure 6.4. The sulfation pattern directs the neuritogenic activity of CS. A) Representative immunofluorescence images of neurons cultured on a substratum of polyornithine and the synthetic molecules. B) Quantification of neurite length, expressed as percentage growth relative to polyornithine control.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

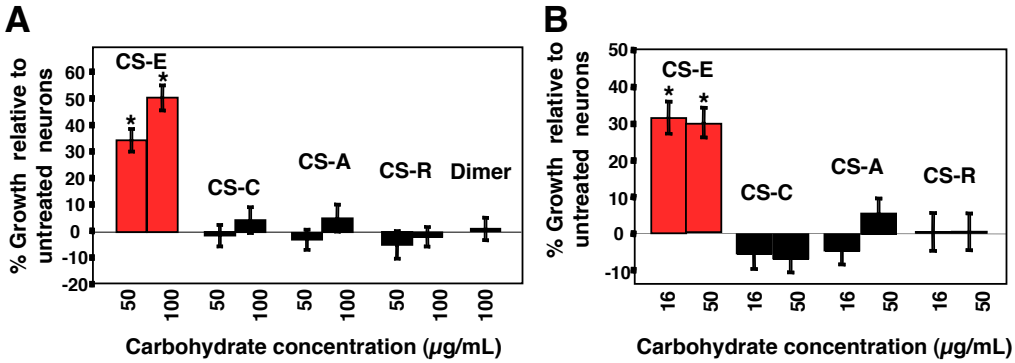


Figure 6.5. The CS-E sulfation motif stimulates the outgrowth of DRG (A) and dopaminergic neurons (B). Neurons were cultured for 2 – 5 days on coverslips coated with polyornithine and the tetrasaccharides at the indicated concentrations. Neurite length is expressed as percentage of growth relative to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

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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

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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. 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.

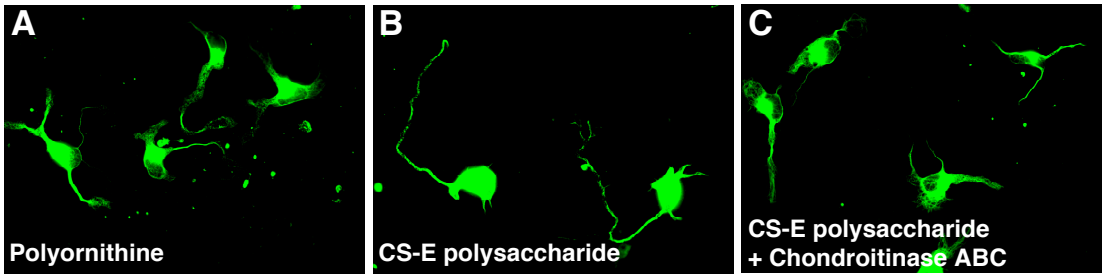

Figure 7.1. A substratum of CS-E polysaccharide stimulates the outgrowth of hippocampal neurons. A) Neurons grown on polyornithine-coated coverslips. B) Neurons grown on coverslips coated with CS-E polysaccharide (16 μg/mL) have longer neurites than control neurons. C) Neurons grown on coverslips coated 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.

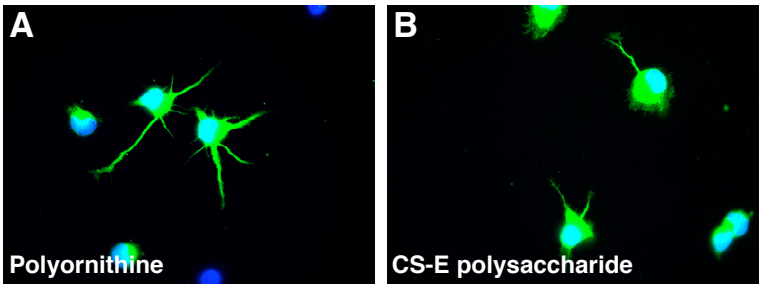

Figure 7.2. CS-E polysaccharide added in solution inhibits the outgrowth of hippocampal neurons. A) Neurons grown on polyornithine-coated coverslips. B) Neurons grown on coverslips coated with polyornithine and treated CS-E polysaccharide (0.1 μg/mL) added in solution have shorter neurites than control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

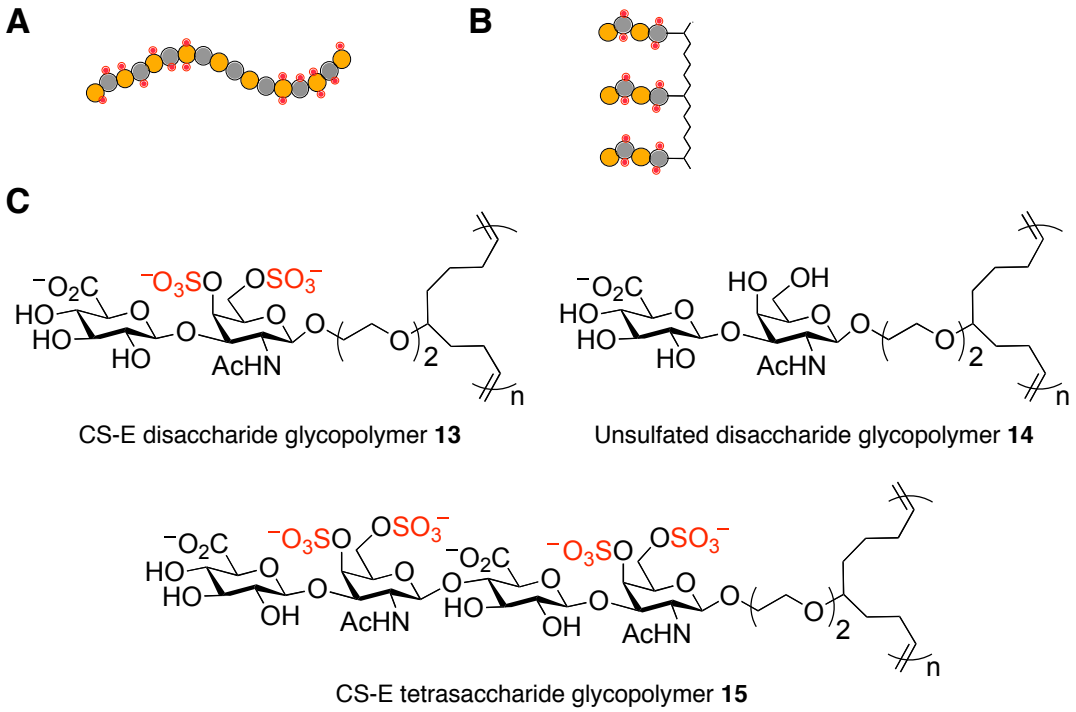

Figure 7.3. Schematic representation of (A) natural CS and (B) CS glycopolymers. Grey and orange circles represent the monosaccharide subunits, red circles represent sulfation. C) Structures of CS glycopolymers

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.

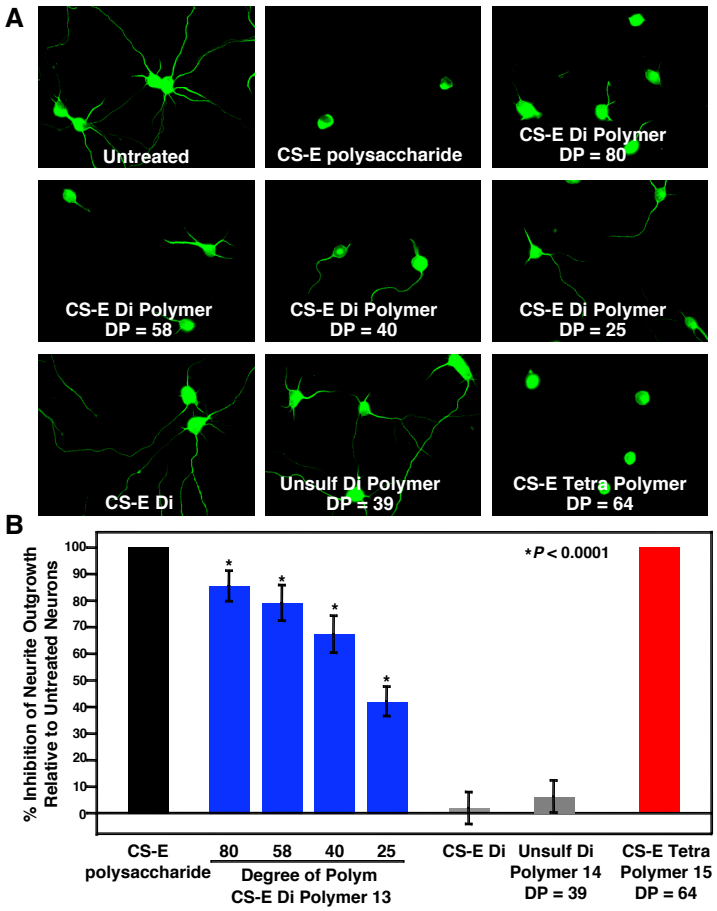

Figure 7.4. CS-E glycopolymers inhibit the outgrowth of hippocampal neurons. A) Immunofluorescence images of neurons 48 h after treatment with the indicated compound. B) Statistical analysis of neurite length. *P* values are relative to the untreated neurons. DP, degree of polymerization, equals the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

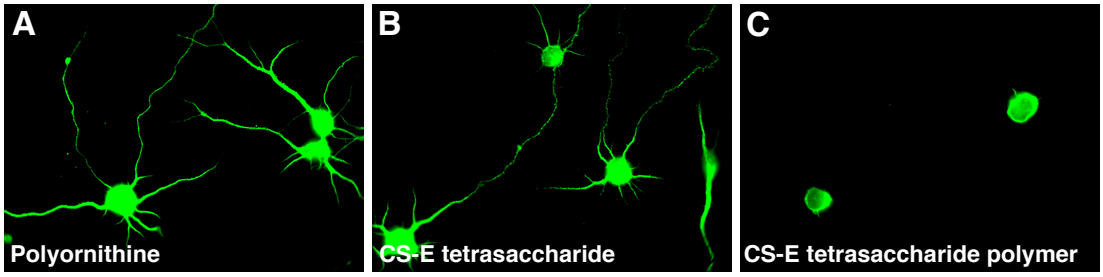

Figure 7.5. CS-E tetrasaccharide glycopolymer inhibits hippocampal neurite outgrowth. A) Untreated neurons. B) Neurons treated with CS-E tetrasaccharide (0.5 μg/mL). C) Neurons treated with CS-E tetrasaccharide polymer **15**. Monovalent CS-E tetrasaccharide displays no inhibitory activity whereas CS-E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

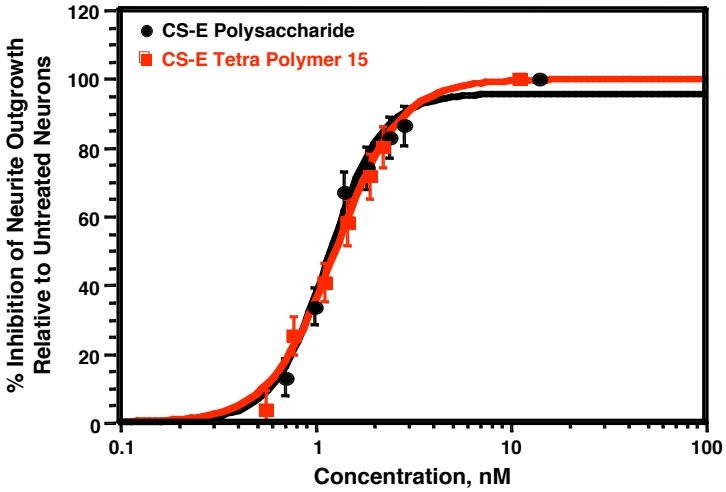

Figure 7.6. Comparison of the inhibitory potencies of CS glycopolymer **15** and the natural polysaccharide at various concentrations. IC_{50} values are based on the molar concentration of compound required to inhibit neurite outgrowth by 50% relative to untreated neurons.

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]

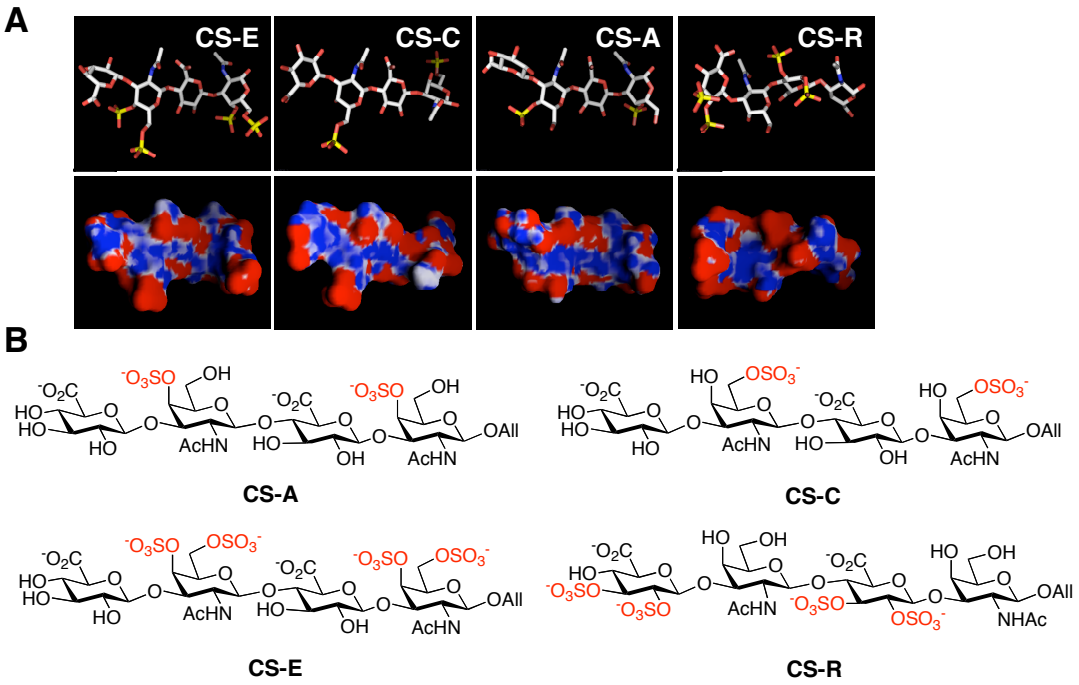

Figure 7.7. Each sulfation pattern exhibits a distinct structural conformation. A) Average structures from molecular dynamics simulations of the CS tetrasaccharides in water. B) Chemical structures of the CS tetrasaccharides. The sulfation pattern influences the structure of CS, allowing it to present 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.

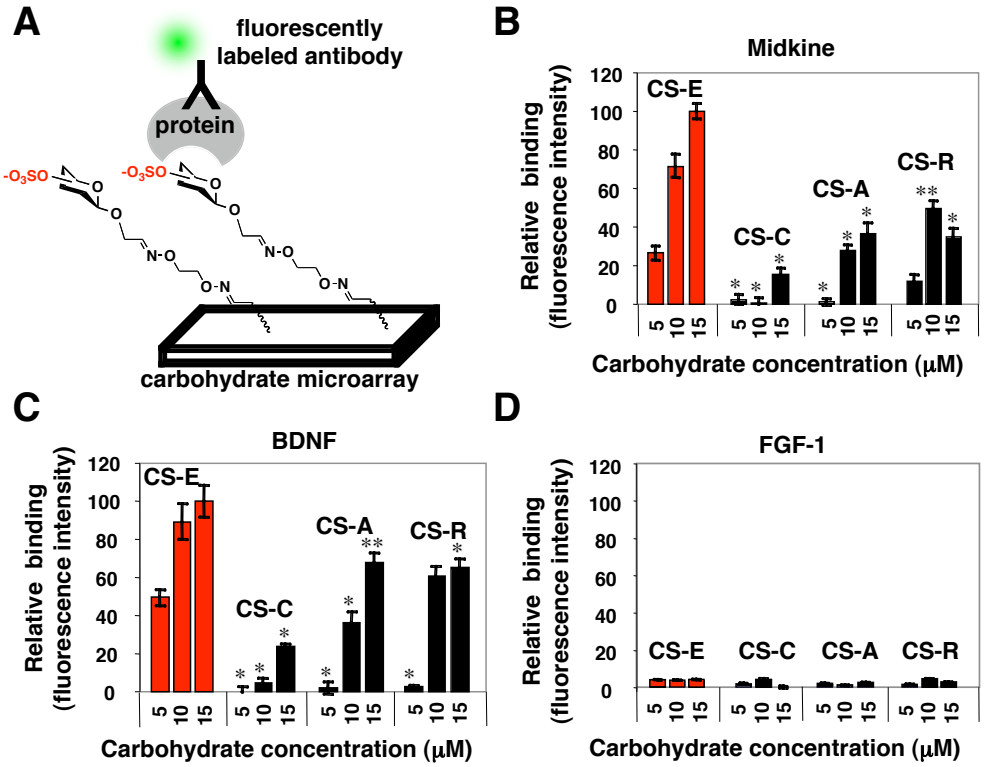

Figure 7.8. A specific sulfation pattern promotes the interaction of CS with neuronal growth factors. A) Overall scheme to detect CS-protein interactions using carbohydrate microarrays. The CS-E tetrasaccharide interacts with the growth factors midkine B) and BDNF C). D) None of the CS tetrasaccharides 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).

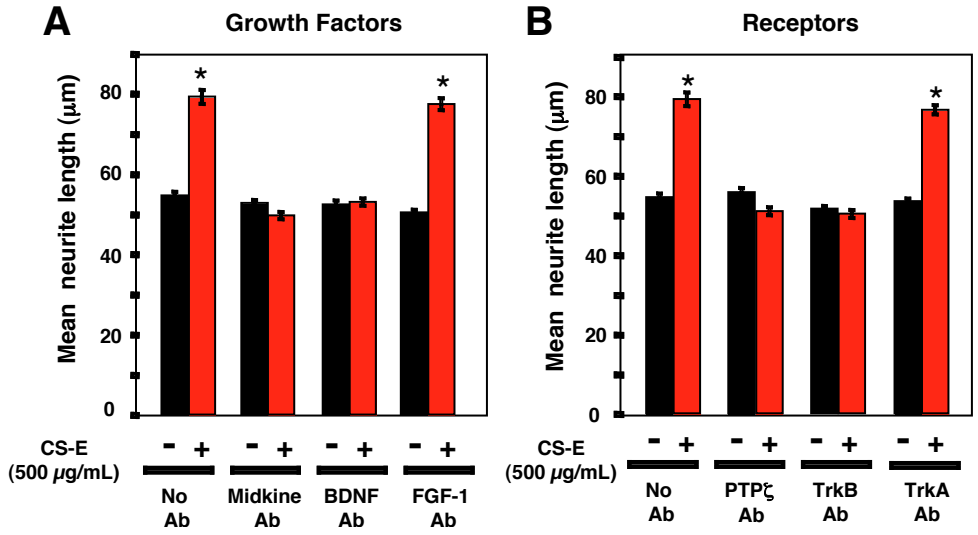

Figure 7.9. The CS-E sulfation motif stimulates neuronal growth through activation of the midkine-PTPζ and BDNF-TrkB signaling pathways. A) Antibodies (Ab) selective for midkine (4 μg/mL) or BDNF (1 μg/mL), but not FGF-1 (4 μg/mL), block the neurite outgrowth induced by CS-E. B) Antibodies against the 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.

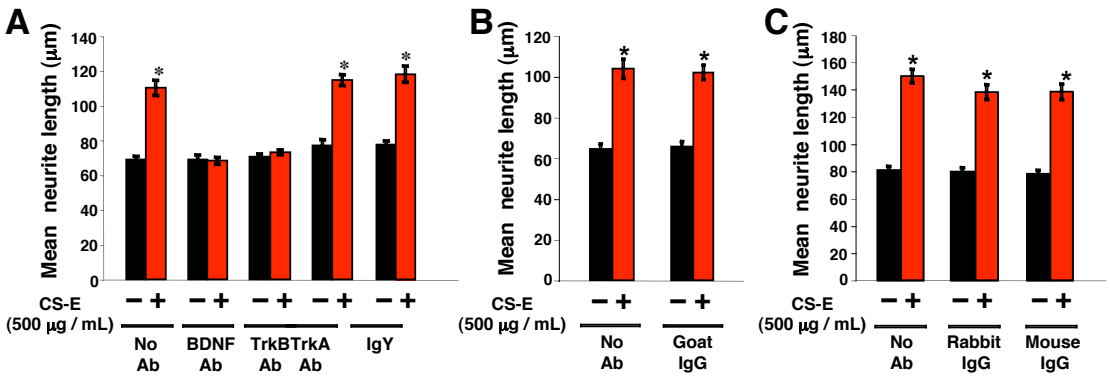

Figure 7.10. Function-blocking antibodies against BDNF and TrkB, but not class-matched control antibodies, disrupt the neuritogenic activity of CS-E. A) The BDNF (10 μg/mL), TrkB (0.5 μg/mL), and TrkA (0.5 μg/mL) antibodies have been shown previously to block endogenous neurotrophin and receptor function. 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.

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