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.

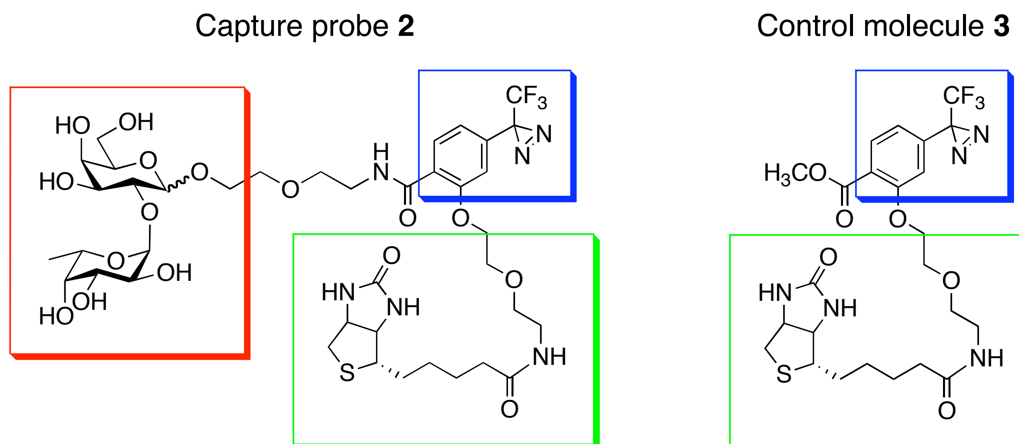

Figure 4.1. Monovalent capture probe **2** and control molecule **3**. The three main features of probe **2** are the Fucα(1-2)Gal disaccharide (red) for protein binding, the photoreactive diazirine moiety (blue) for crosslinking to bound proteins, and the biotin handle (green) for affinity column purification. 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.

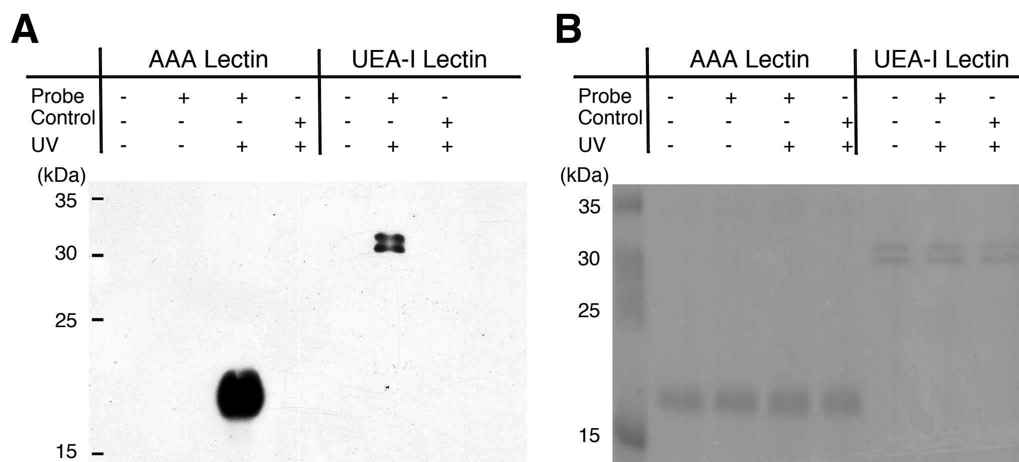

Figure 4.2. Capture probe **2** specifically labels the fucose-binding lectins AAA and UEA-I. A) Purified samples of AAA and UEA-I were treated with probe **2** and control molecule **3** and analyzed for specific labeling by streptavidin-HRP. B) Samples were also analyzed by Coomassie stain for total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

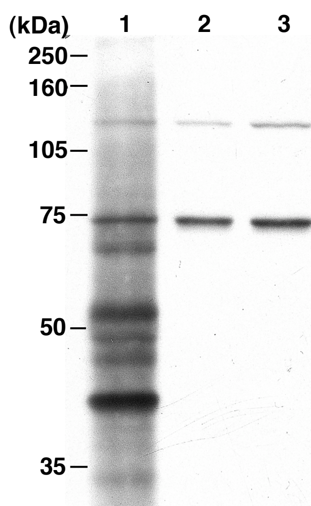

Figure 4.3. Capture probe **2** labels distinct proteins in dissociated neurons. Lane 1: Proteins from dissociated cells captured by probe **2**. Lane 2: Proteins captured by control molecule **3**. Lane 3: Proteins non-specifically detected by streptavidin-HRP in untreated cells

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.

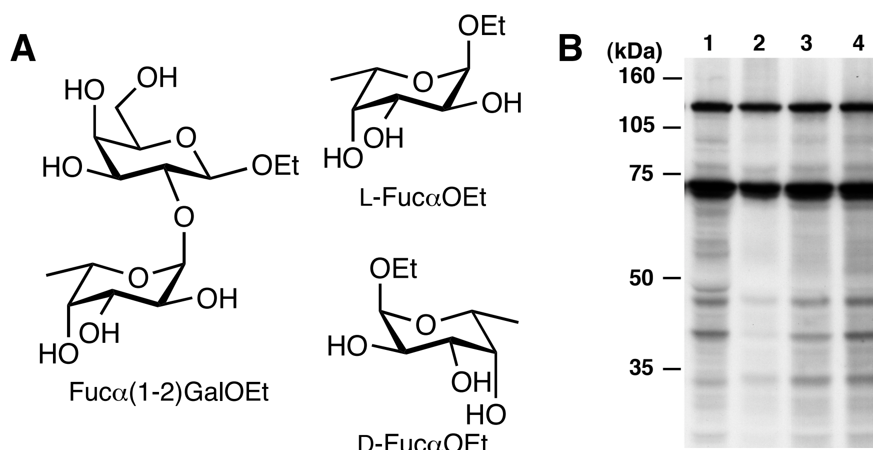

Figure 4.4. Probe **2** specifically labels Fucα(1-2)Gal lectins in dissociated neurons. A) Compounds synthesized for competition experiments with probe **2**. B) Competition binding with specified molecules shows that probe **2** is specifically labeling Fucα(1-2)Gal lectins. Lane 1: Cells treated with 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.

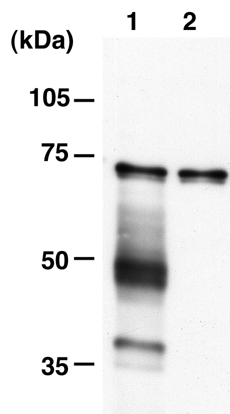

Figure 4.5. Fucα(1-2)Gal lectins were specifically captured by probe **2** and isolated on a streptavidin column. Dissociated cells were incubated with probe **2** (lane 1) or control molecule **3** (lane 2), crosslinked with UV light, and then lysed for protein purification. Eluents from the streptavidin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

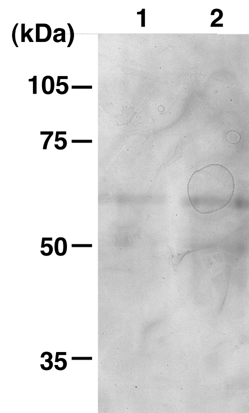

Figure 4.6. Coomassie stain analysis of total protein captured on streptavidin column after probe **2** labeling. Lane 1: Protein labeled with probe **2**; Lane 2: Protein labeled with control molecule **3**

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).

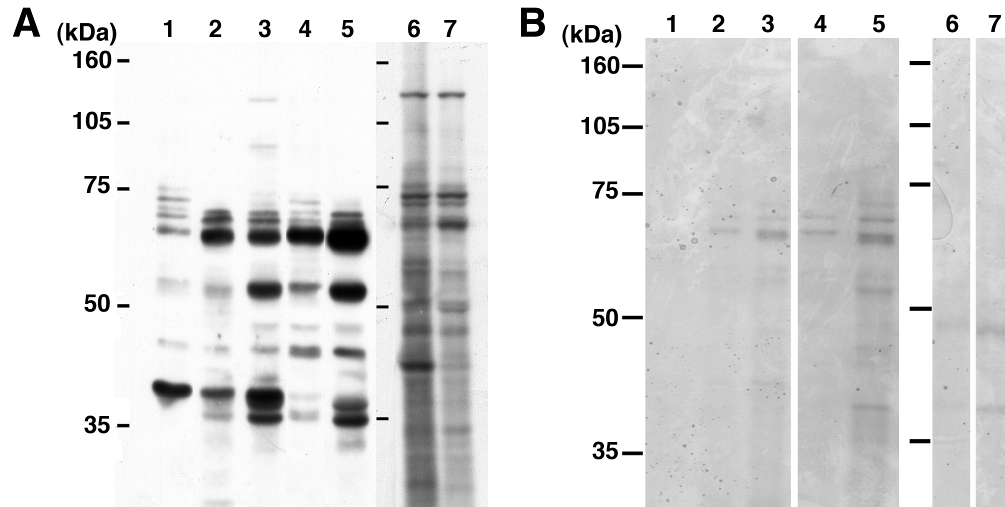

Figure 4.7. Subcellular fractionation of protein lysates labeled with probe **2** and control molecule **3**. A) Streptavidin-HRP Western blot of subcellular fractions further separated by anion exchange chromatography. Lanes 1 – 3: S2 fractions labeled with probe **2**. Lanes 4 – 5: S2 fractions labeled 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).

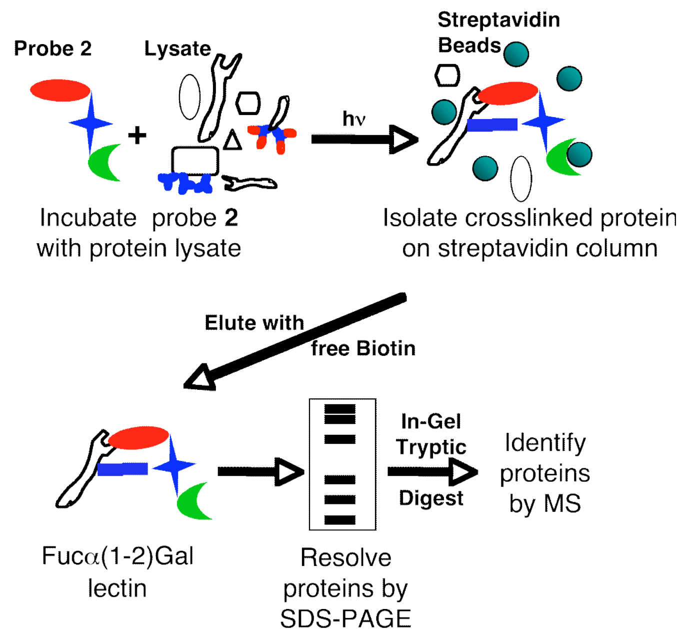

Figure 4.8. Scheme depicting the strategy by which probe **2** is used to label and identify Fucα(1-2)Gal lectins from neuronal protein lysate

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]

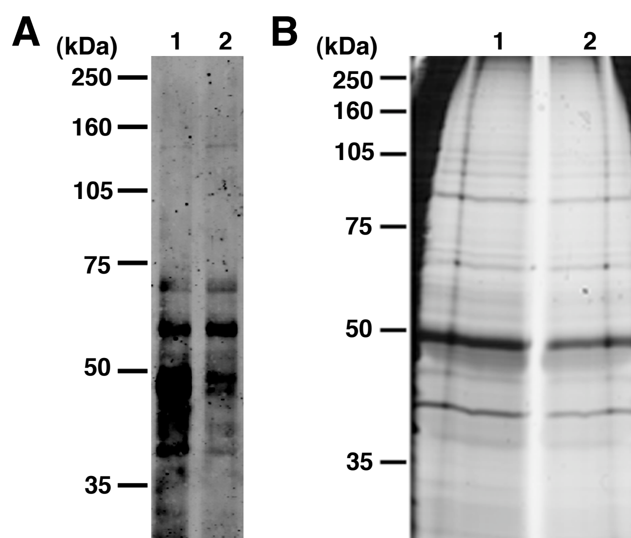

Figure 4.9. Distinct proteins from rat pup lysate were captured and isolated by probe **2**. A) Western blot analysis of rat pup lysate labeled with probe **2** (lane 1) and control molecule **3** (lane 2). B) Silver stain analysis of proteins labeled with either probe **2** (lane 1) or control molecule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

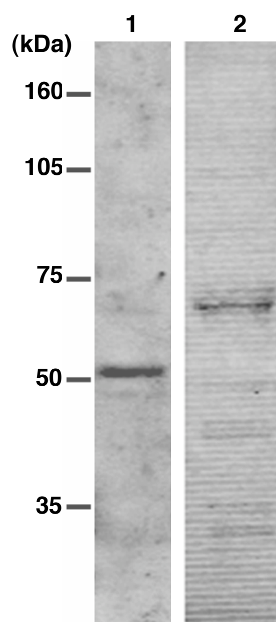

Figure 4.10. Protein lysate labeled with probe **2** did not confirm identification of potential Fucα(1-2)Gal lectins. Lane 1: Labeled lysate probed with Na^+/K^+ ATPase antibody (predicted molecular weight is 112 kDa); Lane 2: Labeled lysate probed with importin β antibody (predicted molecular weight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

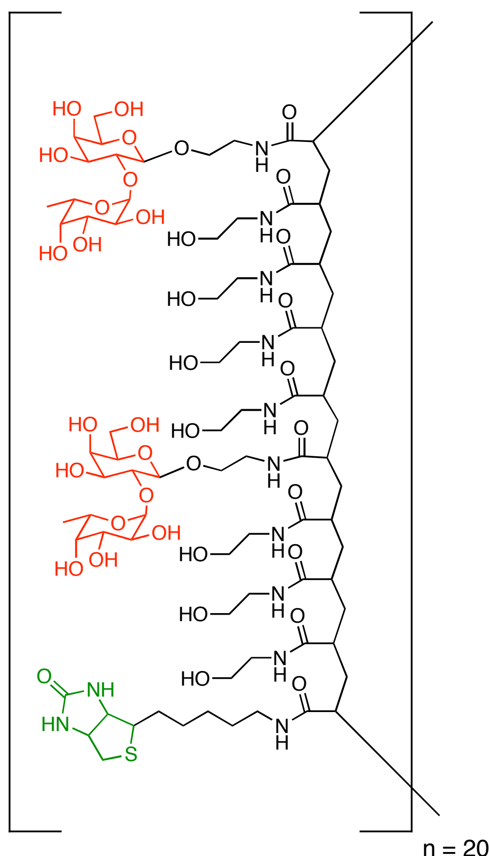

Figure 4.11. Multivalent polymer **4** displays multiple Fucα(1-2)Gal epitopes (red) and multiple biotin moieties (green). Specifically, polymer **4** has a polyacrylamide backbone and is 20% loaded with Fucα(1-2)Gal (approximately 40 units) and 5% loaded with biotin (approximately 20 units).

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.

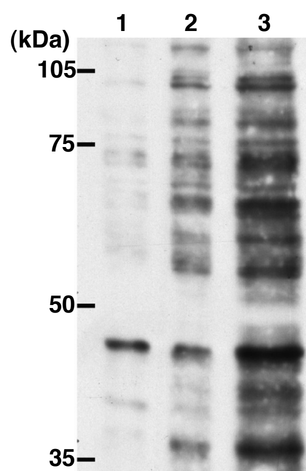

Figure 4.12. Labeling of proteins from dissociated embryonic neurons using the multivalent or monovalent probe. Proteins from the S2 (lane 1) or P2 (lane 2) fractions were labeled on blot with multivalent polymer **4**. Lane 3: Intact cells were labeled with the monovalent probe **2** and lysed.

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).

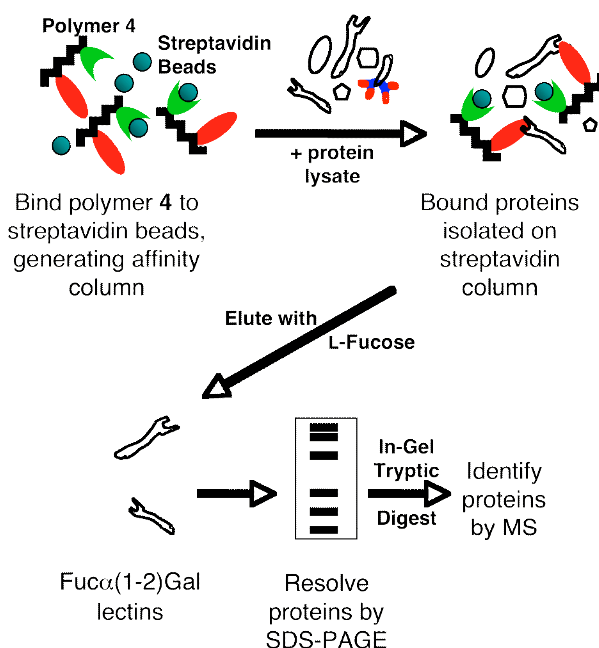

Figure 4.13. Strategy for identification of Fucα(1-2)Gal lectins from neuronal protein lysate using multivalent polymer **4**

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).

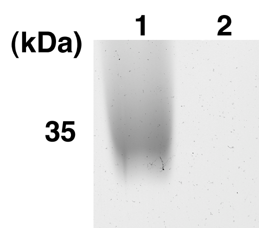

Figure 4.14. Capture of purified UEA-I lectin using the multivalent Fucα(1-2)Gal polymer **4**. Equal amounts of UEA-I lectin (10 μg) were bound on the Fucα(1-2)Gal affinity column and streptavidin control column. Lane 1: Eluent from Fucα(1-2)Gal affinity column; Lane 2: Eluent from streptavidin column

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.

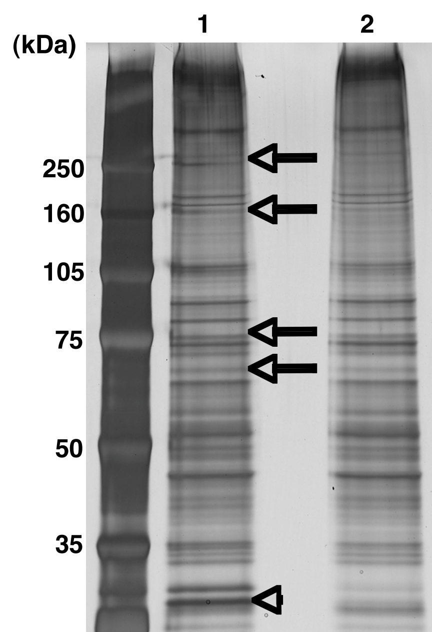

Figure 4.16. Capture of Fucα(1-2)Gal lectins from rat pup lysate using the Fucα(-2)Gal affinity column. Proteins at ~ 65, 80, 140, and 300 kDa (arrows) were selectively captured by the Fucα(1-2)Gal affinity column (Lane 1). UEA-I lectin was also specifically captured (arrowhead, Lane 1). Lane 1: Eluent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

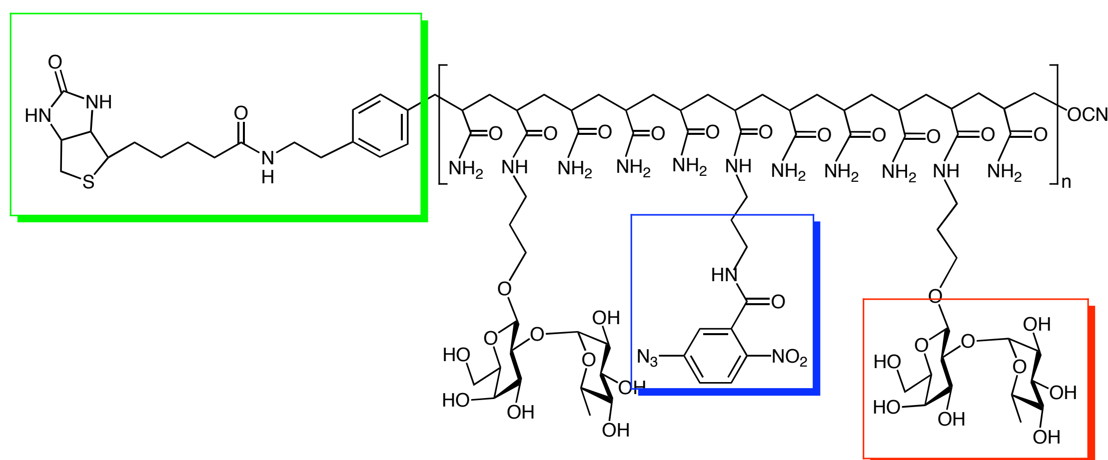

Figure 4.17. Design of multivalent capture polymer **5**. Polymer **5** will have a polyacrylamide backbone, multiple Fucα(1-2)Gal epitopes (red), photoreactive phenyl azide groups (blue), and will be end-labled with biotin (green).

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 $\alpha\beta$ 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 $\alpha\beta$ 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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