CHAPTER 2

Identifying the Fucose Proteome from Rat Cortical Neurons

Introduction

Detailed studies of glycans and their functional roles have been hindered by a lack of tools. However, the recent development of methods such as metabolic labeling with non-natural sugar analogs has made it possible to more readily perturb and profile glycans. The strategy of metabolic labeling with non-natural sugar analogs, originally pioneered by Bertozzi and co-workers (1), relies on the uptake of non-natural monosaccharide analogs into endogenous biosynthetic pathways and their subsequent incorporation into glycoconjugates. As the non-natural analog contains a reactive bioorthogonal group (e.g., azide, alkyne, ketone), glycoconjugates containing the monosaccharide can be covalently tagged with a variety of chemical reporters, including enzymatic, fluorescent, and affinity labels (2-4).

In order to specifically tag a metabolically labeled glycan, we can take advantage of a bioorthogonal ligation strategy, in which a detection tag is chemoselectively reacted with the metabolically labeled glycan. Bioorthogonal ligation strategies include oxime chemistry, in which ketone/aldehyde is reacted with an amine nucleophiles (5, 6); the Staudinger ligation, in which a triarylphosphine reacts with an azide to form an amine (7, 8); the Cu(I)-catalyzed [3+2] azide-alkyne cycloaddition (CuAAC), in which an azide reacts with an alkyne to form a triazole (9); or strain-promoted azide-alkyne cycloaddition (SPAAC), in which an azide reacts with a strained alkyne (Figure 2.1) (10). In each of these reactions, two bioorthogonal reactive groups, which are not natively found within the cellular environment, are reacted rapidly at physiological conditions to form a covalent bond between the structure of interest and a tag that is conducive to the desired mode of detection.

Metabolic labeling can be a powerful approach to enrich glycoproteins for proteomic analysis (2-4). Once a non-natural sugar analog is incorporated within cellular glycans, the glycan of interest

can be tagged using a bioorthogonal ligation strategy with an affinity probe. Subsequent enrichment and purification of the target glycans allows for proteomic identification. Non-natural sugar analogs that have been utilized for proteomic analyses include azido-*N*-acetylgalactosamine (GalNAz) to label O-linked mucin type glycoproteins (11), alkynyl- or azido-*N*-acetylmannosamine (alkynyl ManNAc or ManNAz) to label sialylated glycoproteins (3), and alkynyl- or azido-*N*-acetylglucosamine (GlcNAz

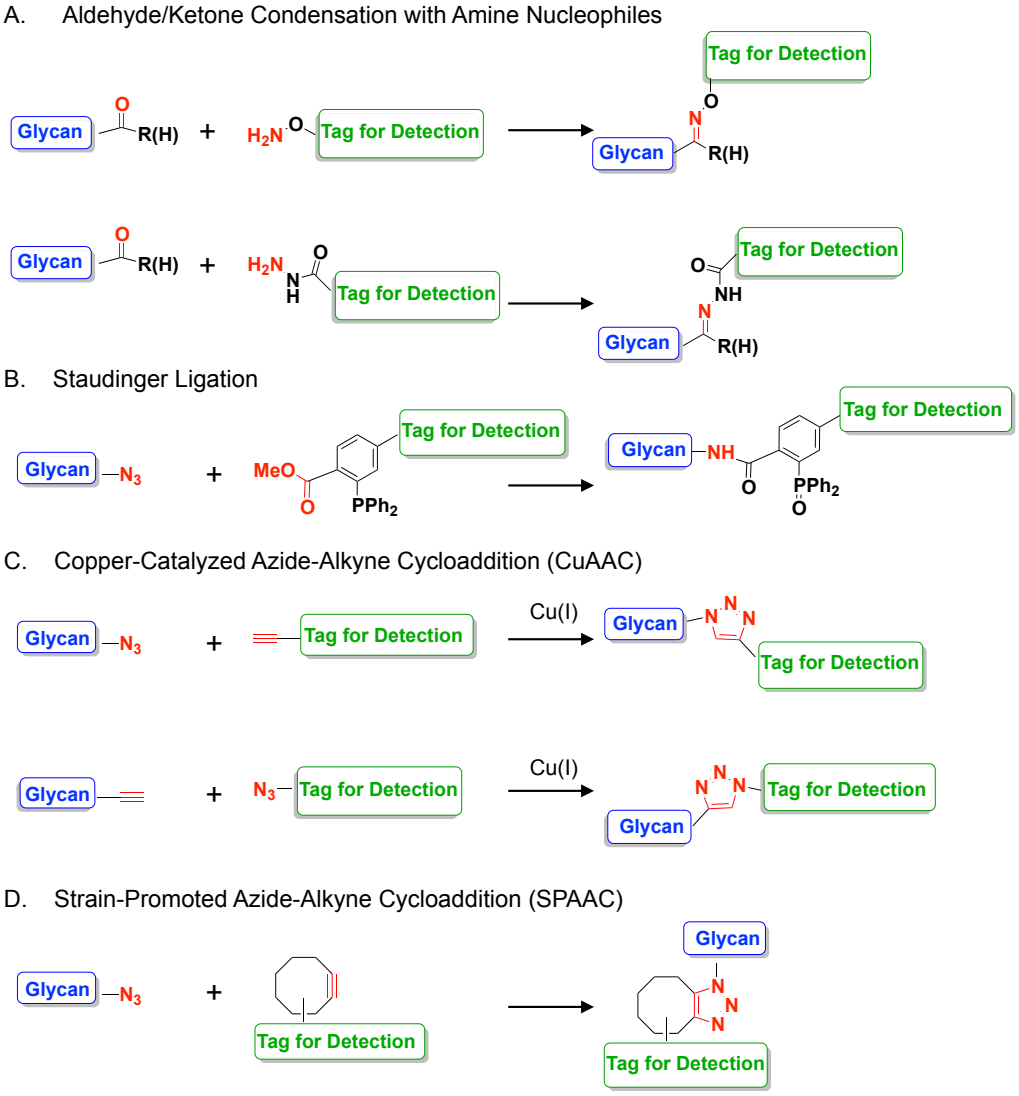

Figure 2.1 Bioorthogonal ligation strategies. (A) Aldehyde/ketone condensation with amine nucleophiles (B) the Staudinger ligation (C) CuAAC chemistry (B) SPAAC chemistry

or GlcNAlk) to label *O*-glcnacylated proteins (12). For example, Chi-Huey Wong and coworkers have used the alkynyl ManNAc analog for the enrichment and proteomic identification of sialylated glycoprotein in a strategy termed GIDmap (glycoprotein identification and glycan mapping) (3). In this strategy, sialylated N-linked glycoproteins in PC3 cells were metabolically labeled with alkynyl ManNAc, bioorthogonally tagged with an affinity probe, and isolated before analysis by multidimensional nano-LC-MS^2. Similarly, O-linked mucin type glycoproteins have been metabolically labeled with GalNAz and enriched for proteomic identification in PC3 cells (11). While metabolic labeling with non-natural fucose analogs has been used to detect fucosylated glycans in multiple biological systems (2, 13), the technique has not yet to date been applied to proteomic analyses in the mammalian neuronal system.

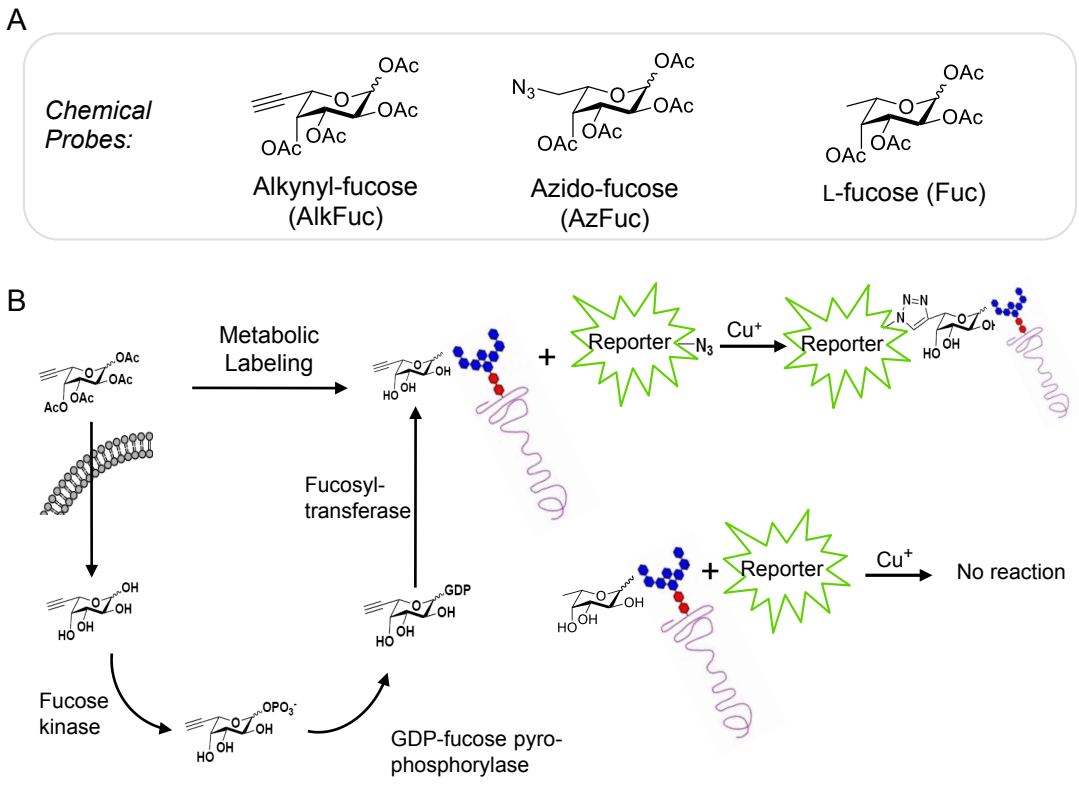

Figure 2.2 Metabolic labeling with non-natural fucose analogs. (A) Non-natural fucose analogs AlkFuc, AzFuc and the natural control analog, Fuc (B) AlkFuc, AzFuc, or Fuc diffuse through the cell membrane and are incorporated into cellular glycans via the salvage pathway. Once incorporated, the fucose analogs can be detected using bioorthogonal ligation strategies with the appropriate reporters.

In the following chapter, we explore the potential of metabolic labeling using non-natural fucose analogs to identify fucosylated glycoproteins in primary neurons. We use the non-natural fucose analogs alkynyl fucose (AlkFuc), azido fucose (AzFuc), and as a control, natural fucose (Fuc; Figure 2.2A). In all cases, the sugar analogs are peracetylated to allow diffusion across the cellular membrane. These molecules are incubated in the cellular growth media where they diffuse into the cells and are available for incorporation into cellular glycans via the fucose salvage pathway, one of the two cellular pathways by which fucose is incorporated into glycans (Figure 2.2B). Once the non-natural analogs are incorporated, the azide or alkyne chemical handle facilitates the chemical detection of fucosylated glycans. Cellular lysates that have been metabolically labeled with AlkFuc or AzFuc can be tagged with the corresponding reactive probe to allow enrichment and identification. Cellular lysates that have been metabolically labeled with the control analog Fuc will not undergo any reaction. We demonstrate that an AlkFuc analog can be successfully incorporated into neuronal proteins, both in culture and *in vivo*. Additionally, we identify the first fucose proteome from cortical neurons, identifying proteins involved in cell adhesion, neuronal signaling, and synaptic transmission.

Results and Discussion

Non-natural Fucose Analogs are Incorporated into Neuronal Glycoproteins

We first examined if the non-natural fucose analogs AlkFuc and AzFuc could be incorporated into neuronal glycoproteins. We expected that labeling of neuronal glycoproteins might be challenging due to the facts that neuronal cells are post-mitotic and that fucose is expressed in fairly low abundance (14). Indeed, when labeling with AzFuc was compared to parallel labeling experiments with azido-*N*-acetylmannosamine (ManNAz) and azido-*N*-acetylgalactosamine (GalNAz), two sugar analogs commonly used to probe N- and O- glycoproteins and glycolipids, the extent of AzFuc labeling was significantly lower than that of ManNAz or GalNAz (Figure 2.2). Cortical neurons at 4 DIV were treated with either 50 μM AzFuc, ManNAz, GalNAz, or an equivalent volume of the vehicle DMSO. Subsequent to 3 d of treatment, cell were lysed and the lysates were reacted with the

corresponding alkyne-biotin tag via the [3+2] copper catalyzed azide-alkyne cycloaddition (CuAAC). Analysis by SDS-PAGE followed by Western blot and detection with streptavidin conjugated to Alexa Fluor 680 revealed AzFuc, ManNAz, and GalNAz labeled proteins. Though each sampled contained an equivalent amount of protein as determined by tubulin blot, the extent of labeling by ManNAz and GalNAz was significantly higher. Besides the low abundance of fucosylated glycans in general, it is also possible that the relevant biosynthetic enzymes are less tolerant of the non-natural fucose analogs than other sugar analogs, contributing to the low-level of metabolic incorporation (15).

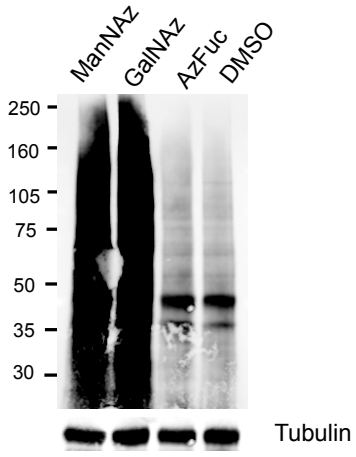

Figure 2.3 Incorporation of ManNAz, GalNAz, and AzFuc into neuronal glycoproteins. Cortical neurons at 4 DIV were incubated with 50 μM ManNAz, GalNAz, AzFuc, or an equivalent volume of vehicle, for 3 d. Cells were lysed, "click" labeled, and probed for labeling after Western blot.

However, both the AlkFuc and AzFuc analogs could be incorporated into neuronal proteins. Cortical neurons at 4 DIV were similarly treated with 50 - 200 μM AzFuc, AlkFuc, or Fuc in neuronal growth media for 3 d. Subsequent to treatment, cells were lysed and the lysates were reacted with the alkyne-biotin tag via CuAAC chemistry. The lysates were resolved by SDS-PAGE followed by Western blot and detection with streptavidin conjugated to Alexa Fluor 680, revealing metabolically labeled proteins in cells treated with AzFuc and AlkFuc, but not Fuc (Figure 2.3). However, treatment with AzFuc followed by CuAAC labeling with alkyne-biotin gave significantly higher background than

treatment with AlkFuc followed by CuAAC with azido-biotin (Figure 2.3). Treatment of neurons with any concentration of AzFuc also resulted in significant cytotoxicity, consistent with previous reports (2). Though previous studies have utilized concentrations of 200 μM AlkFuc or AzFuc (2), concentrations above 100 μM were toxic to cultured neurons even in the case of AlkFuc, and thus limited the concentrations of metabolic labeling. Due to the cytotoxicity and increased background resulting from AzFuc treatment and alkyne-biotin labeling, we proceeded with our studies utilizing only AlkFuc. To determine the specificity of the AlkFuc treatment, cells were co-treated with tunicamycin, an N-glycosylation inhibitor. As previous reports suggest that the majority of neuronal fucosylation exists in complex N-linked glycans (16), we expected treatment with tunicamycin to largely abolish AlkFuc labeling.

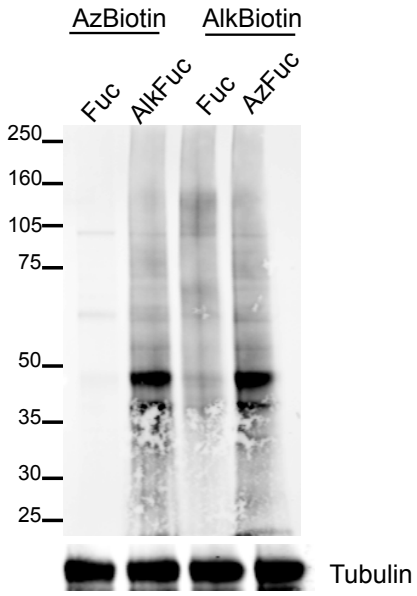

Figure 2.4 AlkFuc labeling results in higher signal. Cortical neurons at 4 DIV were incubated with either 100 μM AlkFuc, AzFuc, or Fuc. Lysates were labeled with either azido-biotin or alkyne-biotin and probed with streptavidin conjugated IR Dye 800. Labeling with alkyne-biotin resulted in increased background when compared with labeling with azido-biotin.

Cortical neurons at 4 DIV were treated with 100 μM AlkFuc or Fuc for 3 d, and co-treated during the last day of metabolic labeling with either 25 μM tunicamycin in ethanol or vehicle (0.01% final concentration), followed by lysis and CuAAC labeling with azido-biotin. Analysis by Western blot indicated that AlkFuc labeling had been abolished in cells treated with tunicamycin (Figure 2.4), suggesting that AlkFuc was specifically incorporated into N-linked glycoproteins. Thus, the AlkFuc analog is incorporated into neuronal glycoproteins, and specifically into N-linked glycoproteins.

Given our goal of undertaking proteomic studies with the AlkFuc analog, it was necessary to maximize the labeling of fucosylated glycoproteins in neurons. In order to optimize labeling, we more closely investigated CuAAC reaction conditions. Previously reported conditions included a labeling solution of 50 mM $CuSO_4$ as the source of the copper catalyst, 2 mM sodium ascorbate as the Cu(II) reducing agent, 0.1 mM tristriazoleamine ligand (triazole), and 0.1 mM azido-biotin; the suggested reaction time was a period of 1 h at rt (17).

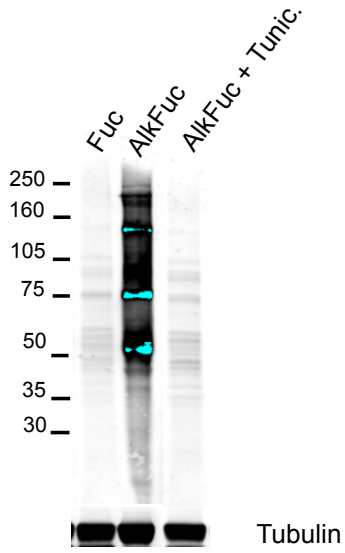

Figure 2.5 AlkFuc is incorporated into N-linked neuronal glycoproteins. Cortical neurons at 4 DIV were metabolically labeled with 100 μM Fuc or AlkFuc for 3 d before the cells were lysed and the lysates labeled with azido-biotin. SDS-PAGE and Western blotting with a streptavidin conjugated IR Dye 800 reveals specifically labeled glycoproteins in cultures metabolically labeled with AlkFuc, but not Fuc. Co-treatment with 25 μM tunicamycin abolished the signal, indicating that AlkFuc is incorporated into N-linked glycoproteins.

These reaction conditions often resulted in indistinct labeling and caused significant protein degradation of AlkFuc treated neuronal glycoproteins. Upon investigation of the specific parameters, we noted that $CuSO_4$ seemed to cause protein degradation, as opposed to CuBr (Figure 2.5). Freshly prepared 50 mM CuBr was subsequently used as the source of the copper catalyst for lysate labeling conditions, and the concentration of the sodium ascorbate was maintained. The addition of sodium ascorbate ensured that the copper catalyst remained as the active Cu (I) species. Additionally, decreased protein degradation was observed when the reaction was allowed to proceed for 3 h at 4 °C, rather than 1 h at rt.

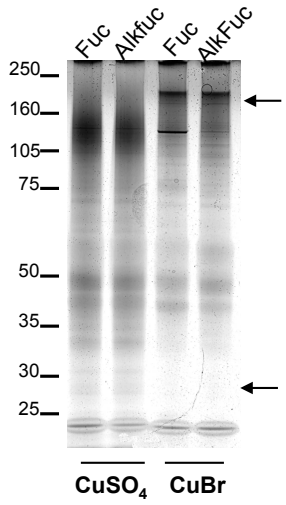

Figure 2.6 $CuSO_4$ causes protein degradation during CuAAC chemistry. Cell lysates, labeled with Fuc and AlkFuc, were labeled via CuAAc using two different sources of copper and visualized by silver stain. Arrows indicate high molecular weight proteins that appear with CuBr, but not with $CuSO_4$, and low molecular proteins that appear with $CuSO_4$, but not with CuBr.

Capture of Fucosylated Glycoproteins by Streptavidin-Affinity Chromatography

Subsequent to optimizing the CuAAC parameters, we sought to develop a protocol for the affinity purification and proteomic analysis of neuronal fucosylated glycoproteins (Figure 2.7). Briefly, our strategy would entail metabolically labeling fucosylated glycoproteins in primary cortical neurons, tagging labeled proteins with an affinity probe via CuAAC chemistry, and affinity purification of tagged proteins. Isolated proteins would then be resolved by SDS-PAGE and

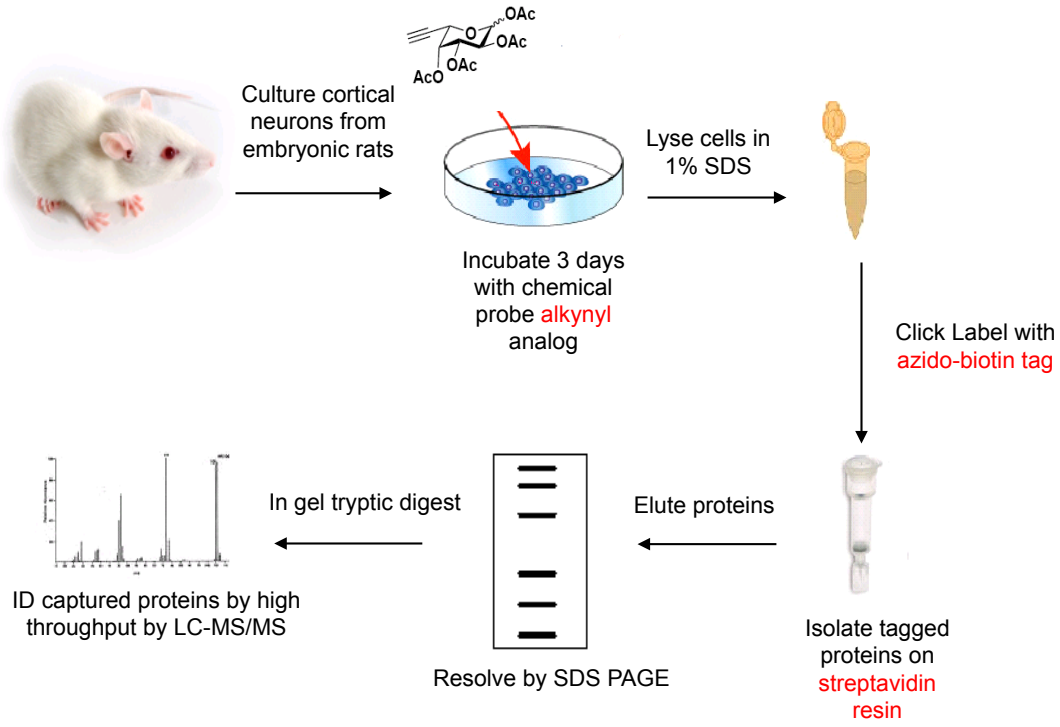

Figure 2.7 Strategy for the identification of the fucose proteome in rat cortical neurons via metabolic labeling, CuAAC chemistry, and affinity chromatography.

identified by LC-MS^2 after in-gel digestion. As we were metabolically labeling relatively low amounts of protein due to the apparent low abundance of fucosylated glycoproteins and the sub-quantitative incorporation of the metabolic labeling analogs, it was critical to minimize any non-specific interactions of highly abundant proteins, such as tubulin or actin, with the enrichment resin**.** Because of these limitations, we decided to utilize streptavidin affinity chromatography to capture biotin-labeled fucosylated glycoproteins due to the strong streptavidin-biotin interaction. Due to the strength of the streptavidin-biotin interaction, it was possible to take advantage of very stringent wash conditions to minimize any non-specific interactions. However, due to the strength of the streptavidin-biotin interaction, we also expected that it would be difficult to elute the captured proteins. As such, we spent a significant amount of time optimizing capture conditions to develop a protocol for the capture and proteomic identification of AlkFuc labeled glycoproteins. Initial efforts

were complicated by low capture efficiency. We briefly attempted other capture and elution methods such as avidin affinity chromatography, or utilizing a cleavable disulfide biotin tag for more efficient elution (data not shown), but we determined that streptavidin resin yielded the most efficient capture. Metabolically labeled neurons were biotinylated via CuAAC and enriched over streptavidin resin. Based on previous reports, we initially limited our washes to 40 CV of PBS (3). However, these washes were insufficient to minimize non-specific background, as there was no significant enrichment apparent in eluates after PBS-only washes (Figure 2.7A). To minimize non-specific interactions, we developed a wash protocol that included (i) a low salt, high detergent wash, (ii) a high salt, low detergent wash (18), (iii) a 4 M Urea, 1% SDS wash, followed by (iv) a 50 mM Tris, pH 7.5 wash. All washes were carried out at 4 °C, except for the urea-SDS wash, which was preformed at rt. Following extensive washing, the proteins were eluted in boiling elution buffer consisting of 6 M urea, 2 M thiourea, 30 mM biotin, and 2% SDS at pH 12 (19). Any milder conditions, such as a standard sample buffer elution, resulted in sub-quantitative elution (Figure 2.8B).

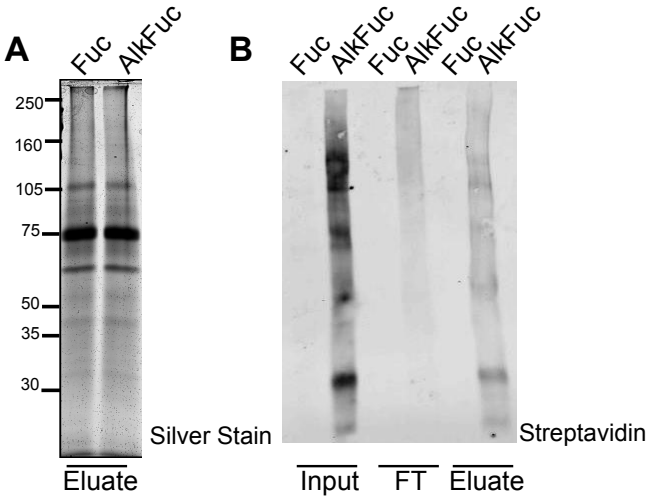

Figure 2.8 Optimization of Streptavidin Capture. (A) Silver stain of streptavidin-enriched proteins after of metabolic labeling and CuAAC. Elution after mild washes shows high non-specific binding. (B) Elution with 2X sample buffer does not yield quantitative elution. Elution shows no enrichment when the input (labeled lysate), flow through (FT), and elution are blotted with streptavidin to detect biotinylated proteins.

With an optimized capture strategy in hand, we proceeded to carry out the enrichment of neuronal fucosylated glycoproteins for LC-MS/MS analysis. Cortical neurons 8 DIV in culture were metabolically labeled and tagged with biotin as described above, and the biotinylated glycoproteins were isolated by streptavidin affinity chromatography. Eluates were concentrated and subsequently resolved by SDS-PAGE and silver stained to identify specific proteins present in the AlkFuc treated lysates, demonstrating a clean capture of neuronal fucosylated glycoproteins (Figure 2.8A). While some background was evident in the Fuc treated lysates, we observed significantly more proteins enriched from the AlkFuc treated lysates. Specifically, we observed fucosylated glycoproteins at 120, 110, 105, 90, 76, 75, 65, 60, 55, 45, 43, 40, 37, and 36 kDa. Seventeen bands from each lane (Figure 2.9B) were analyzed by LC-MS/MS in collaboration with Dr. Eric Peters at the Genomics Institute of the Novartis Research Foundation. The identified peptides were searched in Mascot against the SwissProt Database 57.15 and analyzed in Scaffold (Table 1). Proteins were considered putative fucosylated glycoproteins when at least three unique peptides were observed, resulting in a 99% probability that the protein is accurately identified (additional details in Experimental Methods).

We identified eleven proteins, 4 of which had not yet been previously identified (starred). The putative fucosylated glycoproteins could be broadly categorized into four classes, including cell adhesion molecules, selectin antigens, cell signaling proteins, and those proteins involved in neuronal growth and morphology (Table 1). We did observe significant protein degradation in the sample, as many of the proteins identified ran lower than their true molecular weights, possibly due to harsh processing conditions. However, we successfully identified several fucosylated glycoproteins, including NCAM and lysosomal-associated membrane glycoproteins 1 and 2, which have been previously reported to be fucosylated (20-22), validating this approach for the identification of fucosylated glycoproteins. Additionally, we identified proteins involved in neuronal growth and modulation, such as neuromodulin/growth-associated protein 43 (GAP-43) and the myristoylated alanine-rich protein kinase C susbtrate (MARCKS) (23-29). Notably, both MARCKS and GAP-43

are known to be membrane-associated, cytosolic proteins GAP-43 does have several potential N-glycosylation sites

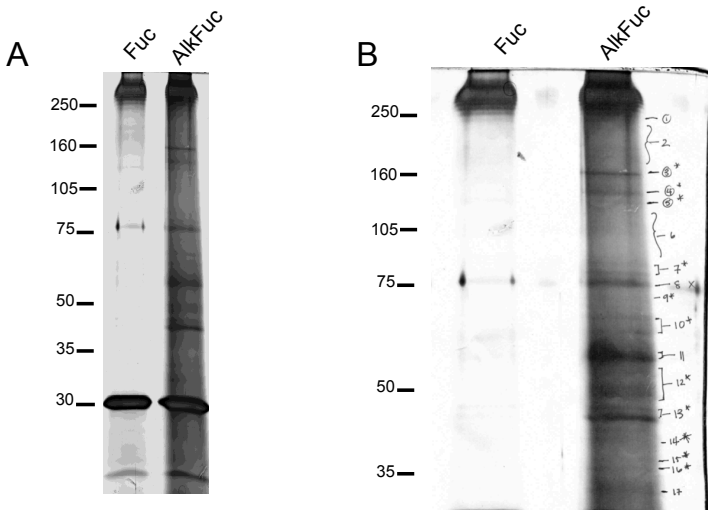

Figure 2.9 (A) Silver stain of proteins isolated from Fuc and AlkFuc labeled cortical neurons after CuAAC with azido-biotin and streptavidin affinity chromatography. (B) Bands excised for proteomic analysis.

Table 1 Fucosylated proteins identified from rat cortical neurons at 8 DIV

Identified Proteins	Function	Accession No.	MW (kDa)	Peptide No.	Seq. Coverage
	Cell Adhesion Molecules				
Neural Cell Adhesion Molecule	cell adhesion	P13596.1	95	12	22
Cell Adhesion molecule 2	cell adhesion	Q1WIM2.2	48	4	13
Phospholipase	Lipid metabolism	Q5FVH2.1	54	3	6
Prolow-density lipoprotein receptor-related protein	Regulates lipid metabolism and neural development	Q91ZX7.1	505	4	1
	Cell Growth/Morphology				
Myristoylated alanine-rich C-kinase substrate	Maintain Dendritic Spine Morphology	P30009.2	30	4	21
MARCKS-related protein	Maintain Dendritic Spine Morphology	Q3KRE8.1	20	3	31
Neuromodulin	Axonal Growth Development and Plasticity	P07936.1	24	4	22
	Cell Signaling				
14-3-3 protein gamma	Regulation of signaling cascades	P61983.2	28	3	20
14-3-3 protein theta	Regulation of signaling cascades	P63102.1	28	4	19
	Selectin Antigens				
Lysosome-associated membrane glycoprotein 1	Cellular Protein Degradation	P14562.1	44	4	11
Lysosome-associated membrane glycoprotein 2	Cellular Protein Degradation	P17046.2	45	3	9

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based on consensus sequence, and has been suggested to be glycosylated (30). The MARCKS protein was recently suggested to bind the polysialic acid structures of NCAM (PSA-NCAM) (31), raising the possibility that MARCKS, also reported to be fucosylated by lectin affinity chromatography (32), was purified non-specifically along with NCAMWe also undertook the identification of the fucose proteome in 14 DIV neurons with the goal of identifying proteins that may be more highly expressed in mature neurons. We relied on the biotin-streptavidin enrichment strategy (Figure 2.9A and 2.9B); proteins identified are summarized in Table 2. We identified 51 putative fucosylated glycoproteins, of which 23 have not yet been reported (starred). Proteins identified have a wide range of biological roles and can be broadly categorized into four major functional classes: cell adhesion molecules, ion channels and solute carriers/transporters, selectin antigens, and synaptic proteins. Once again, several of the putative fucosylated glycoproteins identified, including NCAM, have been previously

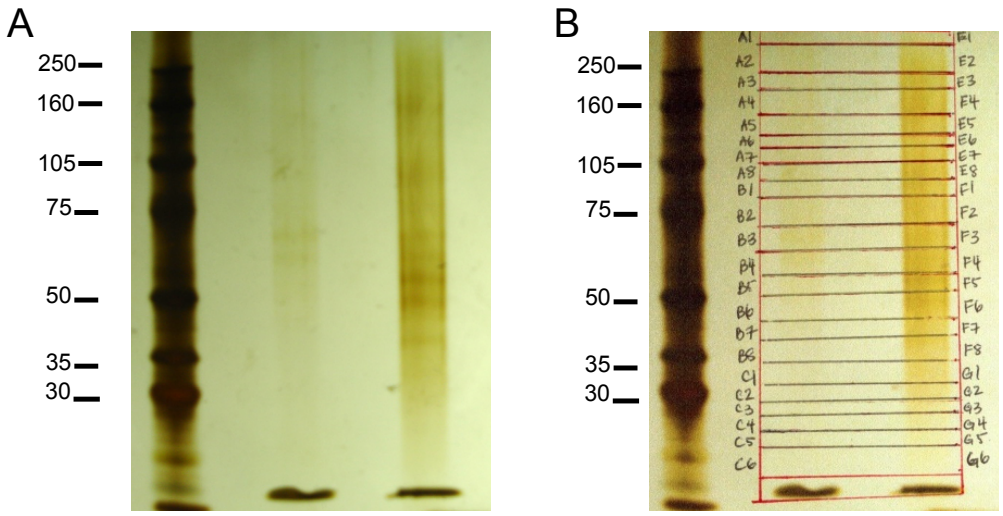

Figure 2.10 Streptavidin enrichment of metabolically labeled fucosylated glycoproteins in 14 DIV neurons. (A) Silver stain of captured fucosylated proteins (B) Bands excised for proteomic analysis.

characterized as fucosylated glycoproteins (21). In addition to NCAM1, we identify L1 CAM and NCAM2, cell adhesion molecules involved in cell adhesion, neuronal migration, axonal fasciculation, and synaptogenesis (33, 34). We also identified the ion channels, voltage-gated calcium channel alpha2/delta subunit and the sodium/potassium transporter ATPase subunits α3 and β1 as putative

Table 2. Fucosylated proteins identified from rat cortical neurons at 14 DIV

Proteins	Function	Accession No.	MW (kDa)	Peptide No.	Seq. Coverage
	Cell Adhesion Molecules				
Contactin-1	Axonal guidance	Q63198.2	113	23	30
Neural cell adhesion molecule 1	neurite outgrowth	P13596.1	95	16	29
Limbic system-associated membrane protein	neurite outgrowth	Q62813.1	38	8	29
Neural cell adhesion molecule L1	neuronal migration and differentiation	Q05695.3	141	4	5
*Neuronal growth regulator 1	axon sproutin	Q9Z0J8.1	38	10	34
Neural cell adhesion molecule 2	neurite outgrowth	O35136.1	93	5	7
*Cadherin-13	axon guidance	P55290.1	78	4	9
Intercellular adhesion molecule 5	leukocyte migration	Q60625.2	97	4	4
Neuronal cell adhesion molecule	neurite outgrowth	P97686.2	134	7	7
Neurofascin	axon targeting, synapse formation	P97685.2	138	4	3
Opioid-binding protein/cell adhesion molecule	synapse formation	P32736.2	38	6	21
*Tyrosine-protein phosphatase non-receptor type substrate 1	neurite outgrowth, synapse formation	P97710.1	56	4	12
Neurotrimin	neurite outgrowth	Q99PJ0.2	38	4	21
*Coxsackievirus and adenovirus receptor homolog	cell adhesion	Q9R066.2	40	6	16
*Protocadherin-10	synapse formation	Q9P2E7.2	113	6	8
Cell adhesion molecule 2	neurite outgrowth	Q00657.2	48	4	9
CD166 antigen	neurite outgrowth	O35112.1	65	5	10
*Neogenin (Fragment)	axon guidance	P61983.2	151	4	4
*Cell adhesion molecule 4	neurite outgrowth	Q1WIM1.1	43	3	12
*Contactin-5	neurite outgrowth, development	P97527.1	121	3	4
*Receptor-type tyrosine-protein phosphatase delta	dendrite morphology	P23468.2	215	3	2
*Protocadherin-9	cell adhesion	Q9HC56.2	136	3	3
*Receptor-type tyrosine-protein phosphatase S	cell adhesion	Q64605.2	212	3	2
IgLON family member 5	cell adhesion	Q8HW98.2	37	5	20
	Selectin Antigens				
Lysosome membrane protein 2	lysosomal transport	P27615.2	54	3	4
Lysosome-associated membrane glycoprotein 1	lysosomal transport	P14562.1	44	7	17
Lysosome-associated membrane glycoprotein 2	lysosomal transport	P17046.2	45	5	13
Golgi apparatus protein 1	binds to E - selectin	Q61543.1	132	7	7
	Ion Channels/Transporters				
4F2 cell-surface antigen heavy chain	solute carrier	Q794F9.1	58	9	24
Sodium/potassium-transporting ATPase subunit alpha-3	ion channel/atp hydrolysis	P06687.2	112	8	10
Sodium/potassium-transporting ATPase subunit beta-1	ion channel/atp hydrolysis	P07340.1	35	5	17
Voltage-dependent calcium channel subunit alpha-2/delta-1	ion channel	O08532.1	125	9	9
*V-type proton ATPase 116 subunit a isoform 1	proton pump	Q9Z1G4.3	96	3	4
*Voltage-dependent anion-selective channel protein 2	ion channel	P81155.2	32	5	21
*ADP/ATP translocase 1	transporter		33	3	
ATP synthase subunit beta, mitochondrial	ion transporter	P19023.1	59	4	11
Basigin	blood brain barrier	P26453.2	42	3	7
*Neuronal membrane glycoprotein M6-b	maintenance of actin cytoskeleton	P35803.2	36	3	6
	Neurotransmission and Synaptic Vesicle Proteins				
*Neuronal pentraxin receptor	Ca^{2+} binding glycoproteins	O35764.1	52	3	6
*Metabotropic glutamate receptor 7	neurotransmission	P35400.1	102	3	4
*Gamma-aminobutyric acid receptor subunit alpha-5	neurotransmitter receptor	P19969.1	52	3	8
Excitatory amino acid transporter 1	glutamate transporter	P24942.2	60	3	9
Large neutral amino acids transporter small subunit 1	glutamate transporter	Q63016.2	56	5	10
*Metabotropic glutamate receptor 5	glutamate receptor	P31424.2	132	4	4
*Glutamate receptor 1	neurotransmission	P23818.1	102	3	4
Prolow-density lipoprotein receptor-related protein 1	neurotransmission, endocytosis, intracellular signaling	Q91ZX7.1	505	15	4
*Synaptic vesicle glycoprotein 2A	maintains RRP of secretory vesicles	Q02563.2	83	3	5
Synaptotagmin-1	SV cycling	P21707.3	48	8	21
*Vesicle-associated membrane protein 2	Vesicle fusion	Q9N0Y0.3	13	3	34
ADP-ribosylation factor 1	vesicle regulation, recruit effectors to the GA, G protein	P84079.2	21	4	24
Major prion protein	membrane, GA, role in neuronal development and synaptic plasticity	P13852.2	28	3	13

fucosylated glycoproteins, which have functions in ion transport and neurotransmission (35), suggesting roles for fucosylation in ion transport, membrane excitability, ATP metabolism.

Notably, we identify several synaptic-vesicle associated proteins, such as synaptic vesicle glycoprotein 2A, synaptotagmin 1, and vesicle-associated membrane proteins 2 (Table 2), which have important roles in the docking and regulation of synaptic vesicle fusion (36). Synaptotagmin 1 has been shown to be an N- and O-linked glycoprotein, with glycosylation being critical to the targeting of synaptotagmin 1 to synaptic vesicles (37, 38). The detection of several synaptic proteins is consistent with a role for fucosylated glycans in synaptic signaling (32, 39). We also identify the glutamate receptor 1, metabotropic glutamate receptor 1, and GABA receptor subunit α5, with roles in neurotransmission and hippocampal-dependent learning (40). Taken together, the protein identifications suggest roles for fucose in cell adhesion and morphology, neuronal communication and synaptic signaling.

Cloning and Validation

In order to confirm the fucosylation of the putative fucosylated glycoproteins identified, we undertook a biochemical validation of selected proteins. To probe the fucosylation of endogenous neuronal proteins, neuronal cultures were metabolically labeled with the AlkFuc or Fuc molecules for 3 d, followed by CuAAC labeling with azido-biotin and enrichment of labeled proteins over streptavidin resin. The enriched fraction was probed for NCAM and L1 CAM. The presence of NCAM and L1 CAM in the lysates labeled with AlkFuc, but not in the lysates labeled with Fuc, confirmed the fucosylation of NCAM and L1CAM. As a negative control, we blotted for p44 MAPK, a kinase not known to be glycosylated. P44 MAPK was not enriched in the labeled lysates, indicating the specific labeling and enrichment of fucosylated glycoproteins (Figure 2.10A).

In addition to endogenous proteins, we biochemically validated the fucosylation of proteins over-expressed in HeLA cells. Specifically, the proteins MARCKS, voltage-gated calcium channel alpha2/delta subunit (Cacna3d1), and synaptotagmin 1 (Syt1) were cloned into a pCMV expression vector with a C-terminal 3X FLAG tag to construct FLAG fusion proteins. It should be noted that a

truncated SytI, with the known glycosylation sites intact, but with both lipid domains deleted, was ultimately used for the following validation assay (Syt1_1-140) (37); the presence of the lipid binding domains seemed to lead to non-specific interactions with the CuAAC reagents. The FLAG constructs were over expressed into HeLA cells that were metabolically labeled with AzFuc. The metabolically labeled lysates were then lysed and tagged with alkyne-biotin, followed by FLAG-affinity purification of the protein of interest. The eluates were analyzed by SDS-PAGE and Western blot to probe for the fucosylation of the protein of interest (Figure 2.10B). The detection of the fucosylated protein by streptavidin conjugated to Alexa Fluor 680 in the AzFuc treated lysates, but in the Fuc treated lysates, indicated the specific fucosylation of the protein. As a positive control, FLAG-Synapsin I was similarly expressed in metabolically labeled cells, and affinity purified subsequent to CuAAC labeling. Detection of the enrichment of glycosylated synapsin I in the AzFuc treated lysates is apparent, confirming the validation assay.

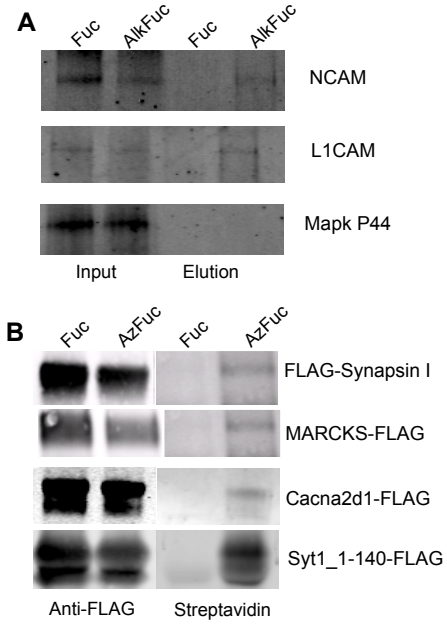

Figure 2.11 Biochemical validation of putative fucosylated glycoproteins. (A) NCAM and L1 CAM, but not the negative control p44 MAPK, are enriched from neurons metabolically labeled with AlkFuc and tagged with azido-biotin before streptavidin affinity purification. (B) FLAG-Synapsin I, MARCKS-FLAG, Cacna2d1-FLAG, and Syt1_1-140 FLAG are labeled with AzFuc when overexpressed in metabolically labeled HeLa cells.

Thus, the specific fucosylation of the proteins NCAM, L1 CAM, MARCKS, Cacna2d1, Syt1_1-140, and Syn I was confirmed using the non-natural fucose analogs, validating their use to probe specific neuronal proteins. Moreover, the fucosylation of two novel proteins, MARCKS and Cacna2d1, was validated, demonstrating the utility of the AlkFuc analog for the proteomic identification of fucosylated glycoproteins.

***In Vivo* Labeling of Fucosylated Glycoproteins.**

Having demonstrated the ability to chemoselectively label fucosylated proteins in dissociated neuronal cultures, we explored the potential to label fucosylated glycans *in vivo* in the brain. Based on previous reports that the expression of fucosylated glycans may be higher in postnatal pups (39), we elected to first attempt *in vivo* labeling in the brains of P1-8 mouse pups. Following protocols approved by the Institutional Animal Care and Use Committee (IACUC), C57/Bl6 pups were cryo-anesthetized and monitored until unresponsive by tail pinch. Using a stereotaxic device modified for mouse pups, 2 µL of 50 mM AlkFuc or Fuc was injected into the hippocampi utilizing a microinjector, using coordinates provided in the Allen mouse brain atlas. Subsequent to the injection, pups were placed on a heating pad and monitored until responsive. After 1 d of survival, pups were humanely euthanized and their brains dissected for analysis by CuAAC. The dissected brain tissue was homogenized and lysed in SDS, and tagged with azido-biotin as previously described. SDS-PAGE and Western blot detection with streptavidin conjugated to Alexa Fluor 680 indicated that some labeling was apparent, but it did not appear that a significant amount of AlkFuc was incorporated (Figure 2.12). To probe the subcellular localization of AlkFuc incorporation *in vivo* in more detail, the brains were removed, immersion fixed in paraformaldehyde solution, and sliced for immunohistochemistry. Briefly, slices

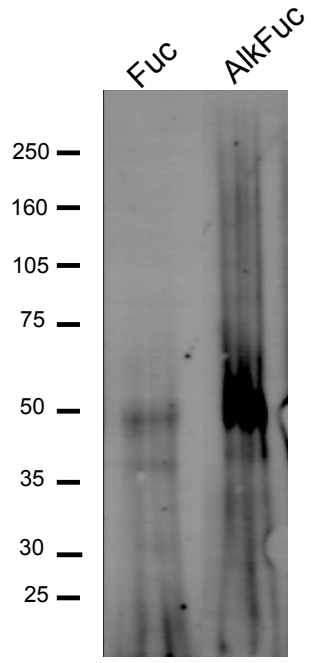

Figure 2.12 *In vivo* labeling of fucosylated glycoproteins in postnatal mice.

prepared on the Lyca Cryostat were blocked in goat serum and labeled via CuAAC overnight. To carry out CuAAC labeling, the CuAAC reaction mixture was prepared in PBS with 25 mM $CuSO_4$, 1 mM sodium ascorbate, .05 mM triazole ligand, and 0.05 mM azido-biotin, and incubated over the slices on the coverslip overnight at 4 °C. Subsequent to CuAAC labeling, the slices were washed in PBS and stained with DAPI or streptavidin conjugated to Alexa Fluor 546 to visualize AlkFuc labeling. When compared to the nuclear stain DAPI, it was not apparent that AlkFuc labeling was specifically localized to any subcellular structures (Figure **2.13).** The staining also seemed localized in close proximity to the injection site, suggesting that the AlkFuc did not permeate the tissue.

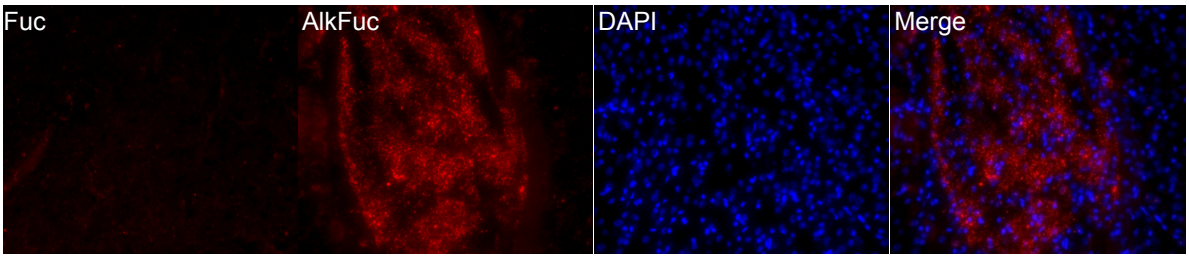

Figure 2.13 *In vivo* labeling of fucosylated glycoproteins in postnatal mice. Fuc or AlkFuc was injected into the hippocampi of WT postnatal mouse pups, after which the pups were humanely euthanized and the brains were dissected and lysed, labeled via CuAAC, and analyzed by histology.

In order to increase AlkFuc incorporation, we reasoned that it might be necessary to administer multiple doses of AlkFuc. As attempting survival surgery in P3 pups to such an extent was impractical, we decided to attempt injections into adult mice, in which we could readily insert cannulas for multiple injections. Importantly, we were able to utilize $Fx^{-/-}$ mice, which are deficient in the FX enzyme, a necessary enzyme for the biosynthesis of fucose (41). Consequently, the transgenic mice must be supplemented with exogenous fucose for survival. The deficiency in the fucose biosynthetic pathways implied to us that incorporation of the non-natural fucose analog could potentially be greater than in mice with intact biosynthetic machinery.

Using stereotaxic device, adult transgenic $Fx^{-/-}$ mice that had been fucose-starved (i.e. fed with normal rodent chow, rather than fucose-supplemented chow) for one week and then implanted

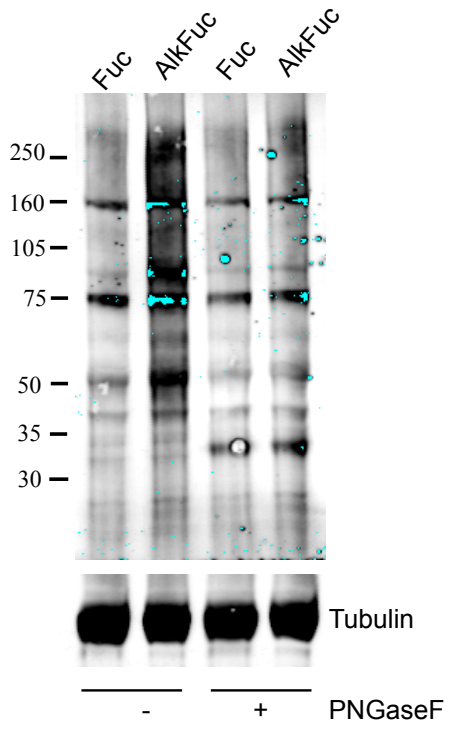

Figure 2.14 *In vivo* labeling of fucosylated glycoproteins in adult mice. Fuc or AlkFuc was injected via microcannula into the hippocampi of $Fx^{-/-}$ mice, after which the mice were humanely euthanized and the tissue was dissected, labeled via CuAAC, and analyzed by Western blot. Treatment with PNGase F significantly abolished signal from the AlkFuc labeled tissue, suggesting that AlkFuc was specifically incorporated into N-linked glycoproteins.

with microcannulae with access to the hippocampus, following approved survival surgery protocols. AlkFuc and Fuc were injected into the hippocampus through the cannula every 48 h for one week, for a total of three injections. One day subsequent to the final injection, the mice were humanely euthanized, and the hippocampal tissue was removed, homogenized, and labeled via CuAAC with azido-biotin. Lysates were resolved by SDS-PAGE and visualized by blotting with IRDye 800 conjugated to streptavidin (Figure 2.14). We observed a number of fucosylated glycoproteins between 50 and 250 kDa in the lysate from the AlkFuc treated animals, whereas there was very little labeling in control fucose injected animals. Notably, AlkFuc labeling was significantly decreased when the lysate was treated with PNGase F, a glycosidase that cleaves N-linked glycans, suggesting that the AlkFuc analog was specifically incorporated into N-linked glycans *in vivo*.

In order to probe the subcellular localization of AlkFuc incorporation *in vivo* in more detail, euthanized mice were perfusion fixed in paraformaldehyde solution. Following dissection, cryopreservation and slicing, brain slices were subjected to immunohistochemistry. However, though signal was apparent by Western blot, no specific signal was apparent in brain slices and the subcellular localization of AlkFuc could not be clarified. Whether incorporation of AlkFuc was too low or whether CuAAC conditions tested were not amenable to histological detection has not yet been determined.

However, the ability to label fucose *in vivo* does open new avenues where we might monitor fucosylation in living animals, particularly to elucidate the proteins involved in learning and memory consolidation. While CuAAC chemsitry is not always conducive to *in vivo* labeling (42), there have been recent developments in reagents that are capable of labeling azide and alkynye reporters *in vivo*, including strained promoted azide alkyne cycloaddition and chelating ligands that reduce the toxicity of copper (10, 43). Notably, these reagents have been used to label glycoconjugates in mice and zebrafish *in vivo*, suggesting their potential use in monitoring fucosylation dynamics in living mice (44, 45). Such studies will reveal molecular insights into learning and memory that have been unattainable by conventional biochemical approaches.

In summary, we exploited the non-natural fucose analog AlkFuc to probe the fucose proteome in cultured neurons and the brain *in vivo.* We show that AlkFuc is incorporated into N-linked glycoproteins in rat cortical neurons and identify the fucose proteome. We identified cell adhesion molecules, selectin antigens, ion channels and ATPases, and synaptic proteins, suggesting roles for fucose in cell adhesion and morphology, neuronal signaling, and synaptic vesicle cycling and neurotransmission.

Experimental Procedures

Copper-catalyzed azide-alkyne cycloaddition (CuAAC) reagents. Peracetylated natural Fucose (Fuc), peracetylated alkynyl-fucose (AlkFuc), peracetylated azido-fucose (AzFuc), azido-biotin, and triazole ligand were synthesized by Sarah Hanson in the laboratory of Dr. Chi-Huey Wong as previously described (17). All molecules were stored at -20 °C in DSMO at the following concentrations: 400 mM Fuc, 400 mM AlkFuc, 400 mM AzFuc, 50 mM azido-biotin, and 50 mM triazole ligand.

Rat Embryonic Cortical and Hippocampal Neuronal Culture. Timed-pregnant Sprague-Dawley rats at embryonic day 18 (E18) were purchased from Charles River Laboratories (Kingston, Mass)

and euthanized in accordance with IACUC protocols. Hippocampal neurons were cultured using a procedure modified from Goslin and Banker (46). Briefly, the hippocampi and cortices of E18 rat embryos were dissected and transferred to 4.5 mL of ice-cold Calcium and Magnesium Free-Hank's Balanced Salt Solution (CMF-HBSS). Trypsin (2.5%, EDTA free) was added to 5 mL, and the tissue was incubated for 15 min at 37 °C. The trypsin solution was then removed and the tissue sample washed three times with 5 mL of CMF-HBSS. Cells were then dissociated from the tissue in 1 mL of CMF-HBSS by passing through a P1000 pipet tip 10-20 times. The cells were counted, diluted into Minimal Eagle's Medium (MEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and penicillin/streptomycin (10,000 U/mL; Life Technologies). Cortical neurons were plated on 10-cm tissue culture plates (BD Falcon) at a density of 20 million cells per plate. Hippocampal neurons were seeded on poly-DL-ornithine-coated glass coverslips (Carolina Biological) at a density of 75 cells/mm^2 (100 µL/coverslip) in a 12-well plate (BD Falcon). After 30 min, 500 µL of supplemented Neurobasal medium (47.5 mL Neurobasal medium (Life Technologies); 0.5 mL L-glutaMAX (200 mM; Life Technologies); 0.5 mL penicillin/streptomycin (10,000 U/mL; Life Technologies); 1.0 mL B-27 serum-free supplement (50X stock; Life Technologies); 50 µL of 0.5 M kynurenic acid in 1 N NaOH) was added to each well. The cultures were maintained in 5% CO_2 at 37 °C, and half the media was changed once a week.

Mouse Embryonic Fibroblast Isolation and Culture. Timed pregnant $Fx^{-/-}$ mice at E15 were euthanized and the embryos removed in accordance with IACUC protocols. Mouse embryonic fibroblasts were isolated by separating each embryo from the placenta and cutting away the brain and any dark red organs. The tissue was washed with ice cold CMF-HBSS, minced with sterile razor blades, and digested with trypsin as described above. Cells were dissociated from the tissue in 1 mL of CMF-HBSS by passing the tissue suspension through a P1000 pipet tip 20 times. The cells were diluted into DMEM supplemented with 10% FBS and penicillin/streptomycin (10,000 U/mL) and plated at the equivalent of one embryo per 10 cm plate in 10 mL media. Media was replaced the

following day, and cells were split 1:5 after confluent (3 to 5 days in culture). Cells were either maintained in culture for experiments or frozen in DMEM supplemented with 10% DMSO (Fisher) and 30% FBS and stored in liquid nitrogen until needed for culture.

HeLa Cell Culture. HeLa were cells grown in DMEM medium, supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 0.1 mg/mL streptomycin (Gibco). All transfections were carried out in antibiotic-free media. In all cases, cells were incubated in a 5% CO_2 humidified chamber at 37 °C.

Metabolic Incorporation of AzFuc and AlkFuc into Neuronal Proteins and CuAAC with alknyne-biotin. Neurons in culture were supplemented with 100 μM AlkFuc, AzFuc, or Fuc, by adding the appropriate volume of 400 mM stock solution into the culture media, for 3 d. For the specificity studies, tunicamycin (Sigma-Aldrich) in ethanol was added at a concentration of 25 μM to the indicated samples during the last 24 h of the metabolic labeling period, and was allowed to remain in the media through the metabolic labeling treatment. Cells were subsequently washed with PBS, pH 7.5, scraped off the plates, and lysed in boiling 1% SDS with sonication. Protein concentration was determined by BCA assay (Pierce), and lysate was subjected to CuAAC to tag metabolically labeled glycoproteins. Lysates in 1% SDS (2 μg/μL) were added into an equivalent volume of buffer containing 6% NP-40, 150 mM NaCl, 100 mM Na_2PO_4 and 2X Protease Inhibitor Cocktail (Roche, EDTA-free). Triazole ligand (0.1 mM), alkyne- or azido biotin (0.1 mM), $CuSO_4$ (50 mM), sodium ascorbate (20 mM) were added and the reaction buffer was incubated, rotating end over end, at 4 °C for 3 h. Labeled lysates were diluted with 4X sample buffer (200 mM Tris pH 6.8, 8% SDS, 400 mM DTT, 40% glycerol, 0.2% bromophenol blue), resolved on a NuPAGE 4-12% Bis-Tris gel (Life Technologies) and transferred to a polyvinylidene difluoride (PVDF) membrane (Milipore). The membrane was blocked in 3% BSA (Fisher) in TBST (50 mM Tris•HCl, 150 mM NaCl, 0.05% Tween

20, pH 7.4) for 1 h at rt, and blotted with streptavidin-Alexa Fluor 680 (0.01 µg/mL in TBST containing 3% BSA; Life Technologies) for 1 h at rt to visualize biotinylated proteins.

Purification of Biotin-Labeled Fucosylated Proteins. Biotinylated samples were prepared as described above, were precipitated using methanol/chloroform/water, and re-dissolved in boiling 1% SDS plus Complete™ protease inhibitors at a concentration of 2 mg/mL. The SDS was quenched with 1 volume of NETFD buffer (100 mM NaCl, 50 mM Tris•HCl pH 7.4, 5 mM EDTA, 6% NP-40) plus protease inhibitors. The samples were incubated with pre-washed streptavidin resin (Pierce; 100 µL/1 mg protein) for 2 h at 4 °C. The resin was washed thrice with 10 column volumes each of low salt buffer (0.1 M Na2HPO4 pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS), thrice with 10 column volumes each of high salt buffer (0.1 M Na_2HPO_4 pH 7.5, 0.5 M NaCl, 0.2% Triton X-100), once with 10 column volumes of 4 M urea, 1% SDS in PBS, and twice with 10 column volumes of 50 mM Tris•HCl pH 7.4. Captured protein was eluted in boiling elution buffer (6 M urea, 2 M thiourea, 30 mM biotin, 2% SDS, pH 12) for 15 min. The eluate was diluted 10 times with PBS and concentrated to 50 µL in Amicon-4mL Ultra concentrators (Millipore).

TAMRA Labeling of Neuronal cultures and Purification of TAMRA-Labeled Fucosylated Proteins. Neurons in culture were supplemented with 100 µM AzFuc or Fuc, by adding the appropriate volume of 400 mM stock solution into neuronal growth meida, for 3 d. Cells were subsequently washed with PBS, pH 7.5, scraped off the plates, and lysed in 1% SDS with limited sonication. The samples were not boiled to avoid decomposition of the azide tag. Protein concentration was determined by BCA assay (Pierce) and lysates in 1% SDS (4 µg/µL) were subjected to copper catalyzed alkyne-azido cycloaddition utilizing reagents available from the ClickIT kit (Life Technologies). Subsequent to labeling, labeled samples were precipitated and resolubilized as described above at a concentration of 2 mg/mL. The SDS was quenched with 1 volume of NETFD buffer plus protease inhibitors. The samples were incubated with anti-TAMRA

antibody (Life Technologies) at 1 mg/mL(check) for 5 h at 4 °C, rotating end over end. The samples were subsequently incubated with pre-equilibrated Protein A/G beads (Pierce) for 1 h at 4 °C, rotating end over end, after which the beads were washed thrice with 1 mL binding buffer, and once with 1 mL of ice-cold 50 mM Tris, pH 7.5. Captured protein was eluted in 2X boiling sample buffer for 5 min.

LC-MS/MS Analysis. The eluate was resolved on a 10% acrylamide-SDS gel and visualized by silver stain. The bands were cut and destained and subsequently subjected to reduction with DTT, alkylation with iodoacetamide, and overnight tryptic disgestion. The resulting extracted enzymatic digest solutions were taken to dryness, and the samples were reconstituted with 20 µL 0.1 M HoAC, and their analyses were started immediately on an Orbitrap Velos, using a true nanoLC system. A "top 20 data dependent" experiment was performed on each reconstituted sample, meaning a full MS scan with high mass accuracy was taken followed by tandem MS of the top twenty m/z values. The samples were run with single peptide standards injected between each sample run to minimize carry over between samples, and two standards were injected after ever 10 samples. Samples were run such that a control band was always run before its sample counterpart, and pairs of bands of widely varying MW were run in varying order to help further distinguish hits specific to the AlkFuc lane. Upon manual inspection of the data, the system appeared to perform fine, as verified by the consistent retention time and signal of the standard run after reach gel band sample. All samples were searched by Mascot against the SwissProt database (version 57.15) with full tryptic specificity, allowing for +57 Da static modification of cysteine (iodoacetamide labeling) and +16 Da variable modification of methionine (oxidation that occurs as an artifact of processing). Precursor mass accuracies of +/- 25 ppm were used a second minimal criterion for peptide identification acceptance. Protein identification were accepted if three peptides each having a 95% certainty level together combined to give a protein identification with a 99.9% certainty, as defined by Peptide/Protein Prophet in Scaffold 2.0.

Western Blotting for Parallel Identification of AlkFuc Labeled Proteins. The purified, labeled lysates, as described in the main text Material and Methods, was resolved on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked in 5% milk (BioRad) in TBST (50 mM Tris•HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) for 1 h at rt. Primary antibodies in 5% milk in TBST were added overnight at 4 °C at the following concentrations: mouse anti-NCAM monoclonal antibody (Abcam) at 1 μg/mL, mouse anti-synapsin I ascites (Synaptic Systems) at 0.1 μg/ml, mouse anti-munc18-1 (Synaptic Systems) at 0.1 μg/mL, or mouse p44 MAPK monoclonal antibody (Cell Signaling) at 1:2000 dilution. Membranes were washed with TBST, incubated with the appropriate Alexa Fluor 680-conjugated (Invitrogen) or IR800-conjugated (Rockland) secondary antibody, and visualized using a LiCOR Odyssey Imaging System.

Biochemical Validation of the Proteins MARCKS, cacna2d21, and synaptotagmin 1: MARCKS, cacna2d1, and synaptotagmin 1 (syt1) cDNA was purchased from Openbiosystems and cloned into a pCMV mammalian vector with a C-terminal 3X FLAG peptide. Syt1 was truncated to the first 140 amino acids (37) to limit hydrophobic interactions between the syt1 lipid binding domains and reagents or resin. Proteins were expressed in primary P3 Fx-/- mouse embryonic fibroblasts or HeLA cells with Lipofectamine 2000 (Life Technologies) and concurrently treated with either 100 μM AlkFuc or Fuc. 24 h subsequent to transfection and treatment, cells were lysed in 1% SDS and labeled with azido-biotin via CuAAC as described in the main text Material and Methods. Labeled material was precipitated with MeOH/$CHCl_3$, resolubilized in 1 % SDS, and affinity purified over FLAG M2 resin (Sigma Aldrich). The purified, labeled material was resolved on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked in 5% milk (BioRad) in TBST (50 mM Tris•HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4) for 1 h at rt. Mouse anti-FLAG antibody (Sigma Aldrich) was added at 0.5 μg/mL in 5% milk in TBST for 1 h at rt. Membranes were washed with TBST, incubated with

IR800 conjugated goat anti-mouse secondary antibody, or Alexa Fluor 680 conjugated streptavidin, and visualized using a LiCOR Odyssey Imaging System.

***In Vivo* Labeling by AlkFuc in Post-Natal Mice Pups.** All procedures were approved by IACUC and animals were handled according to the IACUC guidelines. For injection into neonatal rat pups, individual animals P1-P8 were removed from the dam and cryogenically anesthetized by placing them in a latex sleeve and gently submerging them in an ice bath until they appeared anesthetized. A tail pinch was used to determine if the anesthesia was sufficient. The skin on the head at the site of injection was cleaned with chlorhexidine. The skull of the rat pup is cartilaginous at this age, and thus injections can proceed without the need of a surgical incision. The pup was injected with a Hamilton syringe using a 33-gauge needle attached to a microinjector. The compounds were injected based on stereotaxic coordinates previously published, and were injected at 0.1 μl/min for a total volume of 1-2.5 μL unilaterally into the cortex. As a control, Fuc was injected into the hippocampus. After insertion of the needle, a 1 min resting period preceded the injection. The injection needle was withdrawn over a 2 min period. The puncture wounds were sealed with surgical glue. Pups were tattooed to identify AlkFuc and Fuc injected animals by using a 29-gauge needle to inject a small quantity of tattoo ink into one of the digits or footpad. After injection the pups were warmed on a water circulating heating pad until responsive. They were returned to the dam and maintained on a heat pad until the pups begin nursing. The rump of each pup was exposed to a small amount of urine from the dam to mask any odors that may be associated with the handling and injection procedure. Pups were observed for 4-6 hours post-surgery, and any pups that did not appear to be nursing by lack of a milk spot, or appeared cold or dehydrated were euthanized immediately. The pups were euthanized 1-3 days post-injection by CO_2, and the cortex was isolated. For pain relief, the dam of the injected pups was given 2 mg/kg ketoprofen subcutaneously just prior to the surgery in order that the pup received the analgesic and anti-inflammatory effects of the drug through nursing. The pups

were not treated post-operatively for pain relief as there is no information on a safe dosage to be administered directly to neonates, and the use of such drugs may induce aberrant behavior in the pups and can increase the chance of cannibalization.

Immunohistochemistry of Metabolically Labeled Mouse Brains. The brains of mouse pups were removed and immersion-fixed overnight in 4% paraformaldehyde in PBS at 4 °C. The following day, the solution was replaced with an ice-cold solution of 15% sucrose in PBS at 4 °C until the brains sank, followed by 30% sucrose in PBS. The brain tissue was mounted in OCT medium (Tissue Tek) and frozen in a dry ice/MeOH bath. Adult mice were perfusion fixed and the brains dissected, followed by immersion in 15-30% sucrose, as described above. Frozen brains were stored at -80 °C until they were processed for sectioning. Fixed tissues were cryogenically sliced on a Leica CM1800 cryostat in coronal or sagittal sections (20 μm sections for P3 pups and 50 μm sections for adult brain). Sections were dried at 37 °C for 20 min and then blocked in 10% donkey serum and 0.3% Triton X-100 in PBS for 1 h at rt. Sections were then subjected to CuAAC, and were incubated in 25 mM $CuSO_4$, 10 mM sodium ascorbate, 0.05 mM triazole, and 0.05 azido-biotin in PBS overnight at 4 °C. Subsequent to CuAAC labeling, sections were incubated with Alexa Fluor 488 conjugated streptavidin (1:1000 in 2% donkey serum and 0.1% Triton-X-100 in PBS, Sigma) for 1 h at rt. After staining, slices were mounted in Vectashield containing 40,6-diamidino-2-phenylindole (DAPI, Vector Laboratories) and fluorescence imaged on a Nikon T2000 with a 5X or 10x objective.

***In Vivo* Labeling by AlkFuc in Adult Mice.** All procedures were approved by IACUC and animals were handled according to the IACUC guidelines. Adult $Fx^{-/-}$ mice were maintained on normal rodent chow supplemented with 0.5 to 1% Fucose (Sigma). One week prior to surgery, animals were placed on a normal rodent diet to minimize levels of endogenous fucose. The animals were weighed, anesthetized under isoflurane, given and the appropriate amount of an analgesic (Ibuprofen, 2 mg/kg) via subcutaneous injection, and immobilized on a stereotaxic device. The crown of the head was

shaved and sterilized, and a fine dentist drill was used to drill through the skull. Microcannulas were implanted on one hemisphere at the coordinates (2 mm, 2 mm, 2 mm) to access the hippocampus, and 2 uL of 1 mM alkynyl fucose was injected via a Hamilton syringe with a 33 G needle. The wound was sealed using the surgical adhesive VetBond and the microcannula was capped using a removable cap. The animal was taken off the isoflurane, and placed on a covered heating pad in a new cage, with food and water easily accessible. The animals were typically mobile and responsive within 5 min. After recuperating for 2 d, the animals were once again anesthetized with isofluorane and immobilized on the stereotaxic device to deliver a second dose of alkynyl fucose. Subsequent to the injection, the microcannula was capped and the animal was allowed to recover as mentioned above. A third and final dose was administered in the same way 2 d later. One day following to the final dose of AlkFuc, and seven days subsequent to the surgery, the animals were humanely euthanized and the brain dissected. The hippocampus was removed and lysed in 1% boiling SDS with sonication, and the protein quantitated with the Pierce BCA assay. Lysates treated with both Fuc and AlkFuc were biotinylated via CuAAC as described in the main text Materials and Methods. Half the labeled sample was subjected to PNGaseF digest (NEB) according to reagent protocols subsequent to CuAAC labeling, and labeled lysates were analyzed by Western as described above.

References

1. Rabuka D, Hubbard SC, Laughlin ST, Argade SP, & Bertozzi CR (2006) A Chemical Reporter Strategy to Probe Glycoprotein Fucosylation. *Journal of the American Chemical Society* 128(37):12078-12079.

2. Hsu TL*, et al.* (2007) Alkynyl sugar analogs for the labeling and visualization of glycoconjugates in cells. *Proceedings of the National Academy of Sciences of the United States of America* 104(8):2614-2619.

3. Hanson SR*, et al.* (2007) Tailored Glycoproteomics and Glycan Site Mapping Using Saccharide-Selective Bioorthogonal Probes. *Journal of the American Chemical Society* 129(23):7266-7267.

4. Laughlin ST & Bertozzi CR (2007) Metabolic labeling of glycans with azido sugars and subsequent glycan-profiling and visualization via Staudinger ligation. *Nature protocols* 2(11):2930-2944.

5. Khidekel N*, et al.* (2003) A Chemoenzymatic Approach toward the Rapid and Sensitive Detection of O-GlcNAc Posttranslational Modifications. *Journal of the American Chemical Society* 125(52):16162-16163.

6. Shao J & Tam JP (1995) Unprotected Peptides as Building Blocks for the Synthesis of Peptide Dendrimers with Oxime, Hydrazone, and Thiazolidine Linkages. *Journal of the American Chemical Society* 117(14):3893-3899.

7. Agard NJ, Baskin JM, Prescher JA, Lo A, & Bertozzi CR (2006) A Comparitive Study of Bioorthogonal Reactions with Azides. *ACS chemical biology* 1(10):644-648.

8. Kohn M & Breinbaur R (2004) The Staudinger ligation - A gift to chemical biology. *Angewandte Chemie* 43(24):3106-3116.

9. Rostovtsev VV, Green LG, Fokin VV, & Sharpless KB (2002) A Stepwise Huisgen Cycloaddition Process: Copper(I)-Catalyzed Regioselective "Ligation" of Azides and Terminal Alkynes. *Angewandte Chemie* 41(14):2596-2599.

10. Agard NJ, Prescher JA, & Bertozzi CR (2004) A Strain-Promoted [3 + 2] Azide−Alkyne Cycloaddition for Covalent Modification of Biomolecules in Living Systems. *Journal of the American Chemical Society* 126(46):15046-15047.

11. Hubbard SC, Boyce M, McVaugh CT, Peehl DM, & Bertozzi CR (2011) Cell surface glycoproteomic analysis of prostate cancer-derived PC-3 cells. *Bioorganic & medicinal chemistry letters* 21(17):4945-4950.

12. Zaro BW, Hang HC, & Pratt MR (2013) Incorporation of unnatural sugars for the identification of glycoproteins. *Methods in molecular biology* 951:57-67.

13. Al-Shareffi E*, et al.* (2013) 6-Alkynyl fucose is a bioorthogonal analog for O-fucosylation of epidermal growth factor-like repeats and thrombospondin Type-1 repeats by protein O-fucosyltransferases 1 and 2. *Glycobiology* 23(2):188-198.

14. Krusius T & Finne J (1977) Structural Features of Tissue Glycoproteins: Fractionation and Methylation Analysis of Glycopeptides Derived from Rat Brain, Kidney, and Liver. *European Journal of Biochemistry* 78:369-379.

15. Laughlin ST & Bertozzi CR (2009) Imaging the glycome. *Proc. Natl Acad. Sci. USA* 106(1):12-17.

16. Kleene R & Schachner M (2004) Glycans and neural cell interactions. *Nature reviews. Neuroscience* 5(3):195-208.

17. Hsu T-L & Wong CH (2007) Personal Communication.

18. Khidekel N*, et al.* (2007) Probing the dynamics of O-GlcNAc glycosylation in the brain using quantitative proteomics. *Nat Chem Biol* 3(6):339-348.

19. Rybak JN, Scheurer SB, Neri D, & Elia G (2004) Purification of biotinylated proteins on streptavidin resin: a protocol for quantitative elution. *Proteomics* 4(8):2296-2299.

20. Wuhrer M*, et al.* (2003) Localization of defined carbohydrate epitopes in bovine polysialylated NCAM. *Biochimie* 85(1-2):207-218.

21. Liedtke S*, et al.* (2001) Characterization of N-glycans from mouse brain neural cell adhesion molecule. *Glycobiology* 11(5):373-384.

22. Pestean A*, et al.* (1995) Identification of the *Ulex europeaeus* agglutinin-I-binding protein as a unique glycoform of the neural cell adhesion molecule in the olfactory sensory axons of adult rats. *Neuroscience Letters* 195(2):117-120.

23. Laux T*, et al.* (2000) GAP43, MARCKS, and CAP23 Modulate PI(4,5)P2 at Plasmalemmal Rafts, and Regulate Cell Cortex Actin Dynamics through a Common Mechanism. *J. Cell Biol.* 149(7):1455-1472.

24. Meiri KF, Pfenninger KH, & Willard MB (1986) Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a compoenent of growth cones and coresponds to pp46, a major polypeptide of subcellular fraction enriched in growth cones. *Proc. Natl. Acad. Sci. USA* 83:3537-3541.

25. Sheu FS, McCabe BJ, Horn G, & Routtenberg A (1993) Learning selectively increases protein kinase C substrate phosphorylation in specific regions of the chick brain. *Proc. Natl. Acad. Sci. USA* 90(7):2705-2709.

26. Strittmatter SM VT, Fishman MC (1992) GAP-43 as a plasticity protein in neuronal form and repair. *J. Neurobio.* 23(5):507-520.

27. Widmer F & Caroni P (1993) Phosphorylation-site mutagenesis of the growth-associated protein GAP- 43 modulates its effects on cell spreading and morphology. *J. Cell Biol.* 120(2):503-512.

28. Matus A (2005) MARCKS for Maintenance in Dendritic Spines. *Neuron* 48(1):4-5.

29. Robert KM*, et al.* (2005) Effect of myristoylated alanine-rich C kinase substrate (MARCKS) overexpression on hippocampus-dependent learning and hippocampal synaptic plasticity in *MARCKS* transgenic mice. *Hippocampus* 15(5):675-683.

30. Zwiers H, Verhaagen J, van Dongen CJ, de Graan PN, & Gispen WH (1985) Resolution of rat brain synaptic phosphoprotein B-50 into multiple forms by two-dimensional electrophoresis: evidence for multisite phosphorylation. *J. Neurochem.* 44(4):1083-1090.

31. Theis T*, et al.* (2013) Functional Role of the Interaction between Polysialic Acid and Myristoylated Alanine-rich C Kinase Substrate at the Plasma Membrane. *J. Biol. Chem.* 288(9):6726-6742.

32. Murrey HE*, et al.* (2009) Identification of the plasticity-relevant fucose-alpha(1-2)-galactose proteome from the mouse olfactory bulb. *Biochemistry* 48(30):7261-7270.

33. Walz A, Monbaerts P, Greer CA, & Treloar HB (2006) Disrupted compartmental organization of axons and dendrites within olfactory glomeruli of mice deficient in the olfactory cell adhesion molecule, OCAM. *Mol. Cell. Neurosci.* 32:1-14.

34. Godenschwege TA, Kristiansen LV, Uthaman SB, Hortsch M, & Murphey RK (2006) A conserved role for Drosophila Neuroglian and human L1-CAM in central-synapse formation. *Curr. Biol.* 16(1):12-23.

35. Palladino MJ, Bower JE, Kreber R, & Ganetzky B (2003) Neural Dysfunction and Neurodegeneration in *Drosophila* Na^+/K^+ ATPase Alpha Subunit Mutants. *J. Neurosci.* 23(4):1276-1286.

36. Rizo J & Sudhof TC (2002) Snares and munc18 in synaptic vesicle fusion. *Nat. Rev. Neurosci.* 3(8):641-653.

37. Han W*, et al.* (2004) N-Glycosylation Is Essential for Vesicular Targeting of Synaptotagmin 1. *Neuron* 41(1):85-99.

38. Fukuda M (2002) VAMP-2/synaptobrevin binding to synaptotagmin I promotes O-glycosylation of synaptotagmin I. *J. Biol. Chem.* 277(33):30351-30358

39. Murrey HE*, et al.* (2006) Protein fucosylation regulates synapsin Ia/Ib expression and neuronal morphology in primary hippocampal neurons. *Proc. Natl. Acad. Sci. U. S. A.* 103(1):21-26.

40. Mayer ML & Armstrong N (2004) Structure and function of glutamate receptor ion channels. *Ann. Rev. Phys.* 66:161-181.

41. Smith PL*, et al.* (2002) Conditional control of selectin ligand expression and global fucosylation events in mice with a targeted mutation at the FX locus. *J. Cell Biol.* 158(4):801-815.

42. Sletten EM & Bertozzi CR (2009) Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. *Angew. Chem. Int. Ed. Engl.* 48(38):6974-6998.

43. Del Amo DS*, et al.* (2010) Biocompatible copper(I) catalysts for *in vivo* imaging of glycans. *J. Amer. Chem. Soc.* 132(47):16893-16899.

44. Chang PV*, et al.* (2010) Copper-free click chemistry in living animals. *Proc. Natl. Acad. Sci. U. S. A.* 107(5):1821-1826.

45. Laughlin ST, Baskin JM, Amacher SL, & Bertozzi CR (2008) In Vivo Imaging of Membrane-Associated Glycans in Developing Zebrafish. *Science* 320(5876):664-667.

46. Goslin K & Banker G (1991) *Culturing Nerve Cells*, eds Banker G & Goslin K (MIT Press, Cambridge, MA), pp 251-281.