Chapter 5: Investigation of Fucosylation by Metabolic Labeling with Alkynyl- and Azido-Fuc Derivatives

Background

The cell surface can be modified by abiotic functionalities to introduce unnatural sugars into cellular glycans, a process known as oligosaccharide engineering. This metabolic labeling with unnatural sugars has become an important tool to incorporate chemical probes into glycan chains within a cellular environment. These studies exploit the biosynthetic machinery within a cell to incorporate unnatural sugar analogues into glycoconjugates. The unnatural sugars often contain chemical functionalities not normally present within a cell such as the azide or alkyne groups, making them "bioorthogonal". Incorporation of such unnatural sugars enables the researcher to place a chemical handle on glycoconjugates of interest, which can then be reacted with other functional groups to install a tag or chemical reporter. Since azides and alkynes are not normally present on biomolecules within a cell, insertion of abiotic functional groups enables chemoselective labeling of glycoconjugates in a cellular context.

In addition to requiring a bioorthogonol chemical functionality, metabolic labeling necessitates a fast chemoselective ligation strategy that can be applied in a biological environment, at physiological pH and temperature. These reactions must be robust enough to avoid non-specific chemical reactions or metabolic side-effects. Such reactions include the Staudinger ligation of a triarylphosphine with an azide to form an amide bond (Figure 5.1A),[1, 2] Cu(I)-catalyzed [3+2] cycloaddition chemistry with an alkyne-azide pair via "click chemistry" (Figure 5.1B)[3-5], or strain-promoted cycloaddition

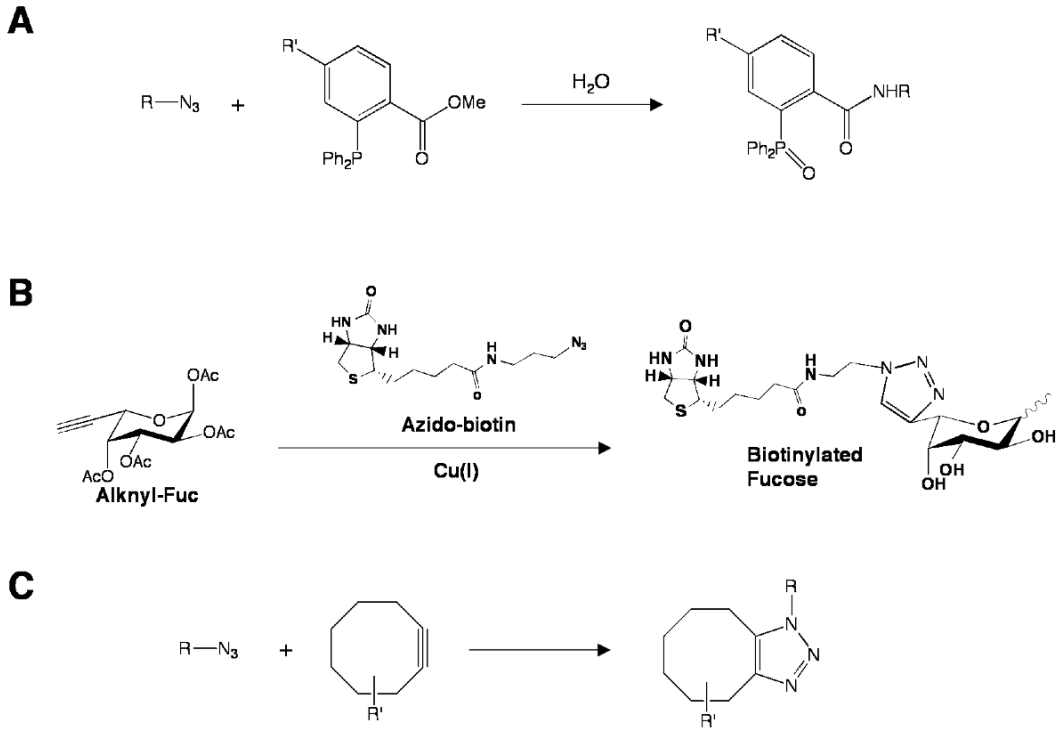

Figure 5.1. Biorthogonol chemical reactions. (A) Staudinger ligation. (B) Click chemistry reaction. (C) Strain-promoted-cycloaddition.

reactions between a cyclooctyne and azide which removes the necessity of a Cu(I) catalyst for click chemistry reactions (Figure 5.1C).[6]

The chemical labeling of glycan chains with unnatural sugars has played a key role in expanding the knowledge of sugar function. Recently, the Bertozzi and Wong groups independently demonstrated that alkynyl- or azido-containing fucose analogues (Figure 5.2) could be exploited to selectively label and image fucosylated glycans in mammalian cells.[7, 8] Their strategy exploits the fucose salvage pathway to convert unnatural fucose sugars into the corresponding GDP-fucose analogues, which then serve as donors for fucosyltransferases (Figure 5.3). Once the azido or alkynyl fucose analogue is incorporated into glycans, it can be reacted with fluorescent dyes, biotin or peptides via the Staudinger ligation or strain-promoted cycloadditions for incorporation of an azide, or

it can be reacted via click chemistry for incorporation of both azides and alkynyes. Bertozzi and co-workers synthesized fucose derivatives with azido groups at the C2, C4, and C6 positions.[7] Only the C6 azido fucose analogue (Figure 5.2) was successfully incorporated into the glycans of Jurkat cells, consistent with earlier observations that some fucosyltransferases tolerate substitutions at the C6 position of the pyranose ring. Wong and colleagues demonstrated that both azido and alkynyl-modified C6-fucose derivatives (Figure 5.2) could be incorporated into the glycans of hepatoma cells,

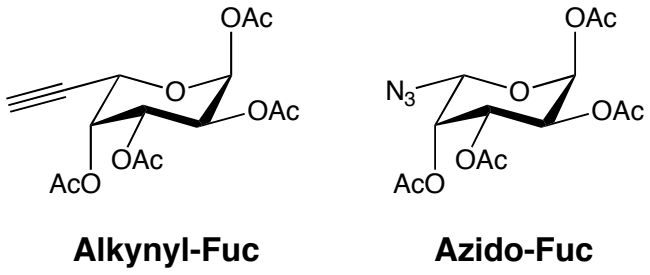

Figure 5.2. Chemical structures of alkynyl- and azido-Fuc with substitutions at the C6 position of the ring.

allowing for fluorescent imaging of fucosylated glycoconjugates.[8] Interestingly, the alkynyl fucose analogue was shown to be significantly less toxic to cells than the azido fucose analogue.[8]

While metabolic labeling has been extensively used in cell culture experiments for investigation of cancerous cell lines, an in-depth investigation of these compounds in other cell-types has not been performed. Thus, we envisioned using the azido- and alknyl-fucose analogues to investigate the role of fucosylation in the brain. The brain is especially challenging to study as neurons are post-mitotic, and fucose (Fuc) is present in low cellular abundance. Despite these challenges, we have successfully employed the

use of alkynyl- and azido-Fuc analogues for the investigation of protein fucosylation in the brain. We report that fucosylated glycoconjugates are present in neurons, and a number of glycoproteins can be modified by this technique. In particular, we developed a method to identify the Fuc glycoproteome from cultured cortical neurons by a gel-based LC-MS^2 approach. Recently, the Wong lab used metabolic labeling to identify the sialylated *N*-linked proteome in prostate cancer cells. Their approach uses a synthetic alkynyl-*N*-acylmannosamine (ManNAcyne) that can be utilized by the sialic acid biosynthetic machinery in an analogous manner as the fucose derivatizes.[9] They developed a glycoproteomic strategy for saccharide-selective glycoprotein identification (ID) and glycan mapping (GIDmap) that tags alkynyl-sialic acid-modified glycoproteins. These studies corroborate our finding that metabolic labeling can successfully be used for proteomics studies. We demonstrate that proteins such as the neural cell adhesion molecule (NCAM), the voltage-gated calcium channel alpha2/delta subunit (Cacna2d2), and the myristoylated-alanine rich protein kinase C substrate (MARCKS) proteins are fucosylated. Furthermore, we show that various neuronal substructures contain fucosyl glycans, such as the Golgi body, axons, and dendrites. Lastly, we are able to chemoselectively tag fucosylated glycans *in vivo,* which will enable future studies investigating fucosylation in living animals.

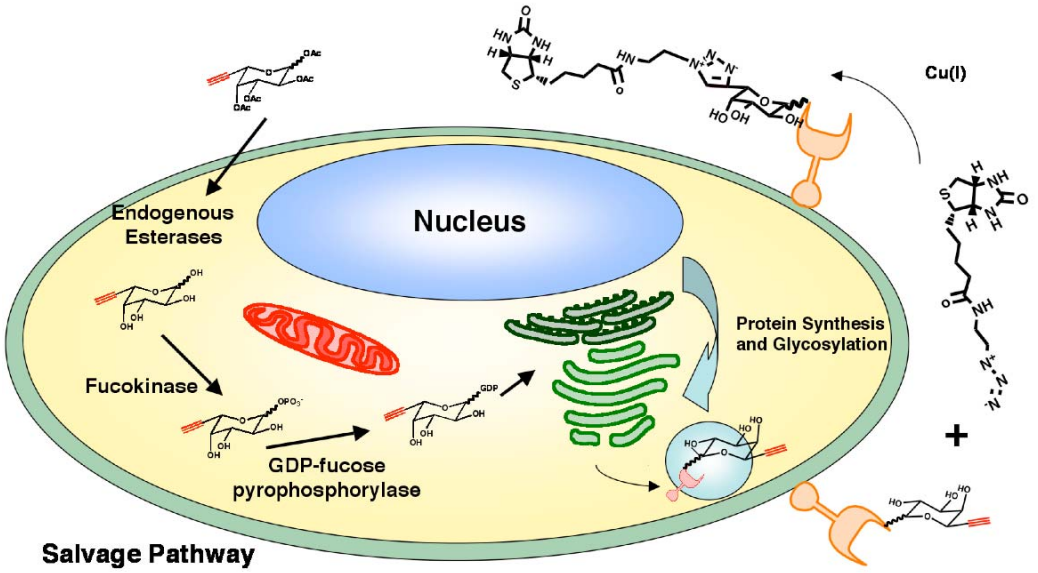

Figure 5.3. Alkynyl- or azido-Fuc analogues are metabolized via the fucose salvage pathway and can be incorporated into glycans on the cell surface.

Results

Alkynyl-Fucose Labels Glycoproteins in Cultured Neurons

We first examined the ability of alkynyl- and azido-Fuc analogues to chemoselectively label glycoconjugates in cultured cortical and hippocampal neurons in collaboration with the Wong lab. All synthetic molecules were provided by the Wong lab at the Scripps Research Institute. Cortical neurons were cultured for 1 day *in vitro* (DIV) and treated with either the acetylated alkynyl-Fuc, azido-Fuc or the Fuc analogue. After treatment, cell lysates were labeled by click chemistry with the corresponding azido- or alkynyl-biotin tag (Figure 5.1B) to determine the ability to selectively label individual glycoproteins. Neurons treated with the azido-Fuc derivative had an appreciable amount of cell death, consistent with the notion that the azido-Fuc compound

is more toxic to the cells than the alkynyl-Fuc sugar.[10] We observed significant labeling of fucosylated glycoproteins in both the alkynyl- and azido-Fuc treated cells (Figure 5.4). However, the azido-Fuc control exhibited labeling of proteins, whereas the alkynyl-Fuc control showed minimal signal by Western blotting. This suggests that the alkynyl-Fuc compound is more specific for labeling of fucosylated glycoproteins. We observed prominent bands at 50, 35, 30 and 28 kDa. The 50 kDa band was present in all samples, suggesting that the protein may be an endogenous biotinylated protein detected by streptavidin. Surprisingly, the 35, 30, and 28 kDa bands were present in the azido-Fuc control but not the alkynyl-Fuc control, suggesting that these proteins may be non-specifically labeled by the click reaction. In addition to these prominent bands, we

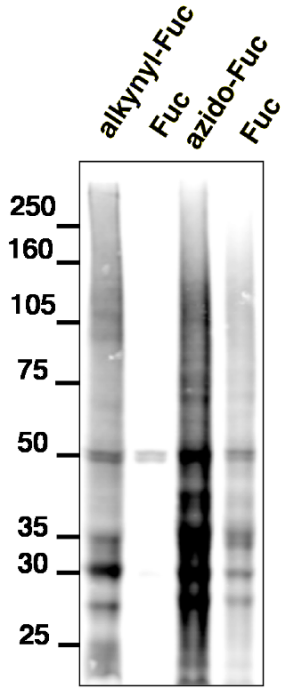

Figure 5.4. Alkynyl-Fuc labeling of glycoproteins is more specific than azido-Fuc in cultured cortical neurons. Neurons were cultured for 1 DIV and treated for three days with the alkynyl-Fuc, azido-Fuc, or Fuc derivatizes. Lanes 1 and 2 were click labeled with azido-biotin and lanes 3 and 4 were labeled with alkynyl-biotin, lysates were resolved by SDS-PAGE, and immunoblotted with streptavidin for visualization.

observe a number of fucosylated proteins between 50 and 250 kDa that appear to be specifically labeled in the alkynyl-Fuc lane, demonstrating the ability to selectively label individual glycoproteins. Due to the toxicity of the azido-Fuc compound and the extensive labeling of the control reaction, we turned our attention toward labeling with alkynyl-Fuc for further experiments.

Optimization of Click Reaction Conditions in Cortical Lysate

In order to effectively label fucosylated glycoproteins in neuronal lysates, we optimized the click chemistry conditions for the incorporation of alkynyl-fucose into cultured rat cortical neurons. These experiments are in collaboration with Chithra Krishnamurthy in the lab. Neurons at 8 DIV were treated with alkynyl-Fuc for 3 days. The cells were lysed at 11 DIV and chemoselectively labeled with the azido-biotin reporter using click chemistry. The original click reaction conditions used included 50 mM $CuSO_4$ as the source of copper catalyst, 2 mM sodium ascorbate as the Cu(II) reducing agent, 0.1 mM tristriazoleamine ligand (triazole) and 0.1 mM of the azido-biotin.[11] The reaction mixture was incubated for 2 h at room temperature and the extent of labeling was visualized by Western blotting with streptavidin. We examined the labeling reactions in absence of each of the reagents to examine selectivity of the alkyny-Fuc probe for labeling fucosylated glycoproteins (Figure 5.5). These click reaction conditions often gave indistinct labeling and caused extensive protein degradation of alkynyl-fucose-treated neuronal glycoproteins (Figure 5.6). We examined the source of Cu catalyst as well as the incubation conditions in order to optimize the conditions for

efficient labeling of fucosylated glycoconjugates with minimal non-specific background. Protein degradation was significantly decreased by incubation of the reaction at 4 ºC for 3h. We determined that CuBr was the optimal copper source under these conditions instead of the commonly used $CuSO_4$, consistent with previous reports (Figure 5.6).[8, 12] Because Cu(I) is the active copper species in the click reaction, using CuBr may have precluded the use of reducing agent. However, the use of CuBr with sodium ascorbate ensured the copper catalyst remained as the active Cu(I) species, due to possible endogenous oxidants in the lysate. This reaction gave much more distinct labeling of fucosylated glycoproteins than the use of $CuSO_4$, which seemed to induce protein degradation (Figure 5.6). However, as CuBr is insoluble in water, the freshly prepared suspension of CuBr needed constant agitation to ensure delivery of the correct amount of CuBr to the reaction mixture, which was determined to be 0.5 mM CuBr (data not shown).

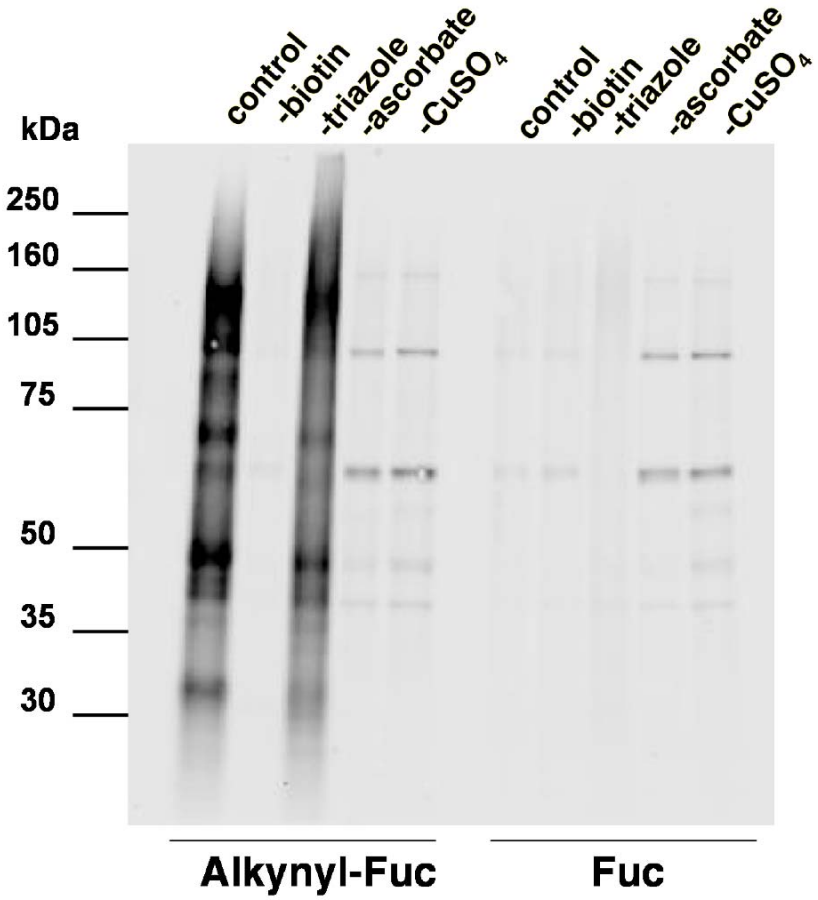

Figure 5.5. Testing the reactions parameters for the click chemistry with alkynyl-Fuc. Neuronal lysates were click labeled under the following conditions. Control reaction is all components needed for click chemistry including 50 mM $CuSO_4$, 2mM sodium ascorbate, 0.1 mM tristriazolamine (triazole), and 0.1 mM azido-biotin. Every other lane contains the components from the control reaction minus the reagent indicated above the lane. Image courtesy of C. Krishnamurthy.

Optimization of Fucosylated Glycoprotein Isolation by Streptavidin Affinity Chromatography

In addition to optimizing click chemistry conditions, fellow lab member Chithra Krishnamurthy developled a protocol for the purification of fucosylated glycoproteins by streptavidin chromatography. Neuronal lysates were cultured for 8 days and treated with

Fuc or alkynyl-Fuc for 3 more days. Lysates were click labeled with the azido-biotin reporter, and we attempted to isolate them by streptavidin affinity chromatography. Protein was first precipitated by a methanol/chloroform extraction to remove the excess biotin from the labeling reaction that would compete for binding sites on the streptavidin column. Protein was resolubilized and incubated with streptavidin resin for one hour at 4 °C. We optimized the binding and wash conditions and found that washing with 40 CV high salt buffer, 40 CV low salt buffer, 30 CV 4M Urea in 1% SDS, 40 CV phosphate buffered saline (PBS) and 40 CV of water was most effective in decreasing non-specific binding. We eluted bound proteins in 6 M urea, 2 M thiourea, 30 mM biotin, 2% SDS at pH 12 to achieve a quantitative elution.[13]

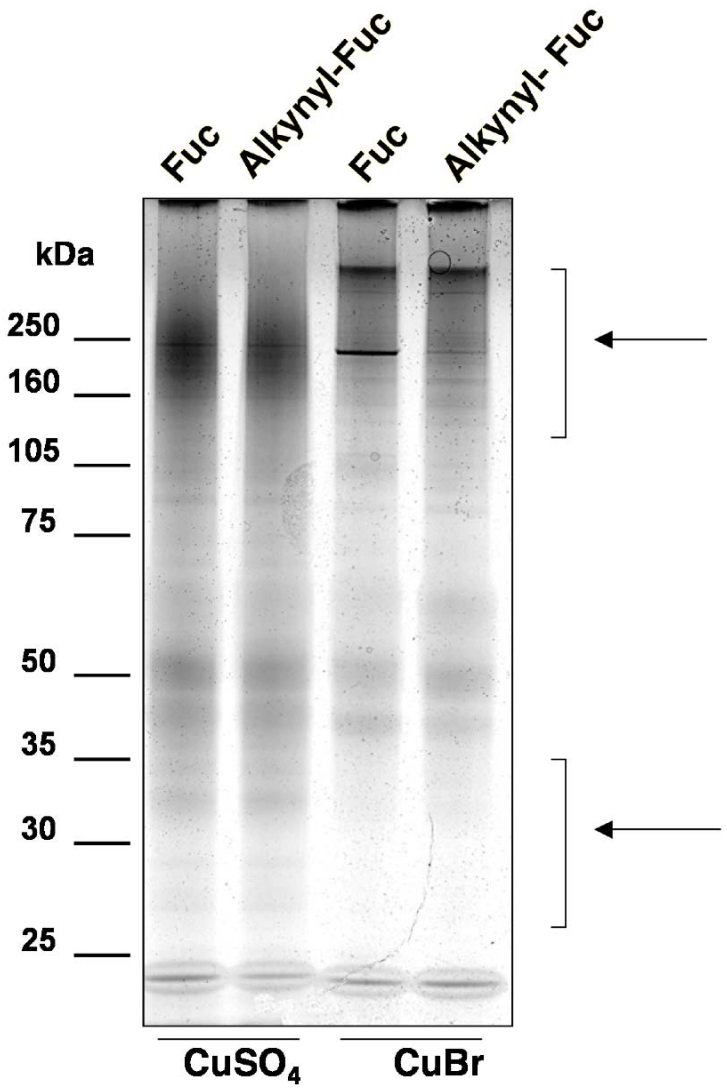

Figure 5.6. $CuSO_4$ induces non-specific protein degradation. Neuronal lysates were click labeled with either $CuSO_4$ or CuBr, than analyzed by silver stain to look at total protein levels. In both samples treated with the $CuSO_4$ catalyst, we observed extensive degradation of proteins between 105 and over 250 kDa as well as between 25 to ~32 kDa (as indicated by arrows). In contrast, proteins treated with the CuBr catalyst had labeling of distinct protein bands and no obvious smears of degraded proteins, suggesting that CuBr is the optimal catalyst. Arrows point to areas of protein degradation. Image courtesy of C. Krishnamurthy.

Identification of the Fucose Proteome

After optimizing the click reaction and streptavidin isolation conditions, fellow lab member Chithra Krishnamurthy began experiments to identify the fucose proteome in neurons using LC/MS/MS. The approach involves labeling cortical neurons in culture with Fuc or alkynyl-Fuc, chemically tagging the cellular lysates with azido-biotin, and isolating biotinylated glycoproteins by streptavidin affinity chromatography. Lysates were then resolved by SDS-PAGE and silver stained to identify specific proteins present in the alkynyl-Fuc but not the control sample. Individual bands present were excised and subjected to in-gel tryptic digests and LC-MS/MS analysis (Figure 5.7). While there was some labeling in the control fucose column, we saw significantly more proteins eluted from the alkynyl-Fuc column. Specifically, we observed fucosylated glycoproteins at ~

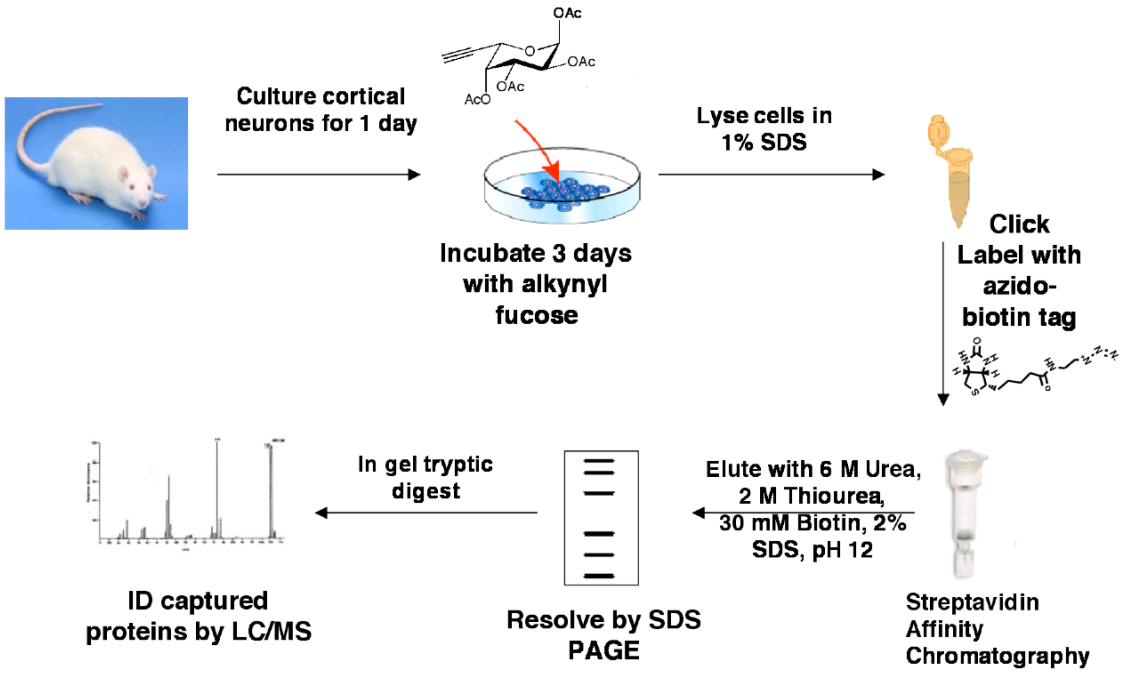

Figure 5.7. Strategy for the isolation and identification of alkynyl-Fuc-tagged glycoproteins from rat cortical neurons.

120, 110, 105, 90, 76, 75, 65, 60, 55, 45, 43, 40, 37, and 36 kDa (Figure 5.8). 17 bands from each lane were analyzed by LC-MS/MS in collaboration with Dr. Scott Ficarro and Dr. Eric Peters at the Genomics Institute of the Novartis Research Foundation. The proteins were searched against the NCBI non-redundant database and each individual sample was analyzed in Bioworks (Table 5.1). Proteins were considered putative Fuc-glycoproteins when we observed at least 3 unique peptides, which gives 99% probability that the protein is present in the sample. We observed significant protein degradation in the sample, as many of the proteins identified ran lower than their true molecular weights. This problem may be due to non-specific protein degradation induced by the click reaction and our harsh elution conditions. We are currently in the process of trying to resolve this phenomenon. Despite these problems, we successfully identified numerous fucosyl glycoproteins including cell adhesion molecules such as NCAM and the cell-fate determining protein Notch, two proteins that have previously been reported to be fucosylated. In addition, we identified proteins involved in ion transport and calcium signaling such as voltage-gated calcium channel alpha2/delta subunit and those involved in dendritic cell morphology such as several myristolated alanine-rich protein kinase C substrate (MARCKS) proteins.

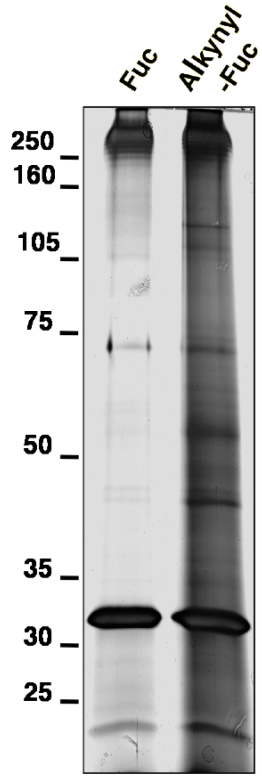

Figure 5.8. Silver stain of proteins isolated from alkynyl-Fuc and Fuc labeled cortical neurons by streptavidin affinity chromatography. Eluted proteins were resolved by SDS-PAGE. The alkynyl-Fuc sample contains significantly more labeling than the control-Fuc sample.

Protein	MW	Accession	Function
Nesprin 1	**1010433**	**29839561**	**Nuclear scaffold protein**
Similar to Low Density Lipoprotein Receptor-Related Protein	**504556**	**109482078**	**Regulates lipid metabolism and neural development**
Homeotic Discs 1	**331123**	**73622271**	**Histone methylation; maintain active transcription**
Type 3 Inositol 1,4,5-Triphosphate Receptor	**303842**	**483831**	**Intracellular calcium release channels; regulates calcium signaling**
Notch Homolog 4	**205936**	**46237578**	**Cell fate assignment and pattern formation in development**

Golgi Sialoglycoprotein MG-160	**134504**	**17376711**	**Trafficking and processing of fibroblast growth factor (FGF)**
NCAM L1	**126395**	**109462307**	**Cell adhesion***
Voltage-gated Calcium Channel Alpha2/Delta Subunit	**124551**	**1905817**	**Ion channel, calcium signaling***
Neural Cell Adhesion Molecule (NCAM)	**117508**	**817988**	**Cell adhesion***
Similar to Vacuolar Proton Translocating ATPase 116 Kda Segment	**95061**	**109473276**	**Acidification of synaptic vesicles; neurotransmitter release**
Potassium Channel	**93560**	**3929231**	**Ion channel**
Cell Adhesion Molecule	**75679**	**2181948**	**Cell adhesion**
Hsc70-ps1	**70884**	**56385**	**Synaptic vesicle cycling, uncoats clathrin-coated vesicles**
Similar to Cadherin-like 24	**66351**	**94398073**	**Desmosome adhesion**
Elongation Factor 1-alpha	**50072**	**74204203**	**Translation regulation**
Lysosomal Membrane Glycoprotein 1 (LAMP1)	**43941**	**6981144**	**Cellular protein degradation**
14-3-3 Gamma Isoform	**35074**	**74215924**	**Regulation of cell signaling cascades**
Similar to Myristoylated Alanine-Rich C-Kinase Substrate	**30186**	**109510177**	**Maintain dendritic spine morphology**
Myristoylated Alanine Rich Protein Kinase C Substrate	**29643**	**6678768**	**Maintain dendritic spine morphology**
14-3-3 Eta Isoform	**28288**	**83754700**	**Regulation of cell signaling cascades**
AMP-activated Protein Kinase, Noncatalytic Gamma-1 Subunit Isoform 2	**28267**	**47132575**	**Cell signaling***
Growth Associated Protein 43 (GAP-43)	**23589**	**8393415**	**Axonal growth, development and plasticity***
MARCKS-like 1	**19835**	**51858596**	**Maintain dendritic spine morphology**

Table 5.1. Proteins identified from the alkynyl fucose proteome arranged by molecular weights with their functions. Starred proteins were identified in the Fucα(1-2)Gal proteome from chapter 3.

In addition to the gel labeling approach, we attempted to use the GID-map strategy developed by the Wong laboratory which involves an off-bead tryptic digest followed by elution of bound peptides with PNGase F, an enzyme that cleaves off *N*-linked sugars.[9] The eluate was analyzed by multidimensional protein identification (MudPit) MS. However, despite their success with this methodology for identifying sialic acid glycoproteins,[9] we were never able to successfully isolate fucosyl glycoproteins in significant quantities over the control column. This likely reflects the lower abundance of fucosylated relative to sialylated glycoproteins. This also suggests that our gel-based approach may be the optimal experiment to identify and isolate fucosylated glycoproteins. Due to the low abundance of fucosylated peptides and the high abundance of non-specific proteins, the wash conditions were not stringent enough for us to see differences in the alkynl-Fuc versus Fuc control when using the GID-map approach (data not shown).

Alkynyl-Fuc Labels Different Neuronal Substructures

We next investigated the ability to chemoselectively label fucosylated glycoconjugates in cultured hippocampal neurons for fluorescence microscopy. Cells were treated with 50 μM alkynyl-Fuc at 8 days *in vitro* (DIV) for 3 more days. Cells were fixed, permeabilized, and subjected to click labeling conditions with azido-biotin. Fucosylated glycoconjugates were visualized by fluorescence microscopy with streptavidin conjugated to fluorescein. We optimized the labeling conditions by testing the reaction at different temperatures and for different lengths of time. Labeling at 4 °C overnight provided the most specific labeling (data not shown) and all subsequent hippocampal cultures were labeled under these parameters. We observed extensive staining of the cell body, as well as labeling of neuronal processes in the alkynyl-Fuc

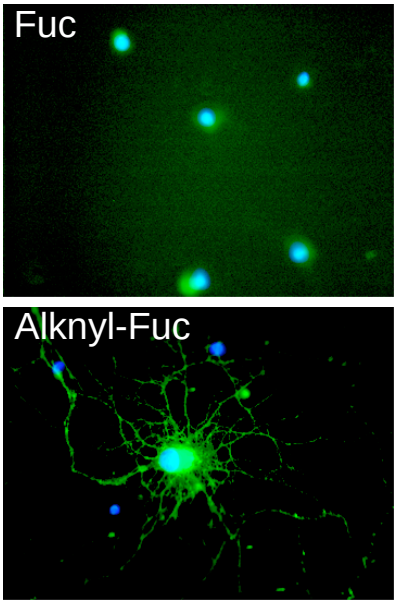

Figure 5.9. Metabolic labeling with alknyl-Fuc in labels the cell body and neuronal processes of hippocampal cultures. Hippocampal neurons were cultured for 8 DIV and treated with alkynyl-Fuc or Fuc for 3 more days. Cells were fixed, permeabilized, and click labeled with azido-biotin. Fucosylated proteins were visualized with streptavidin conjugated to alexa fluor 488. Nuclei were stained with DAPI.

treated neurons (Figure 5.9). There was minimal background in the control, mostly localized to the cell body. Thus, we are capable of specifically labeling fucosylated glycoproteins in the cell body as well as along neuronal processes of hippocampal cultures.

Identification of Neuronal Substructures Labeled by Alkynyl-Fuc

We next sought to address the neuronal localization of fucosylated glycoconjugates in developing hippocampal neurons in culture. We first examined the colocalization of the known fucosylated glycoprotein NCAM (the neural cell adhesion

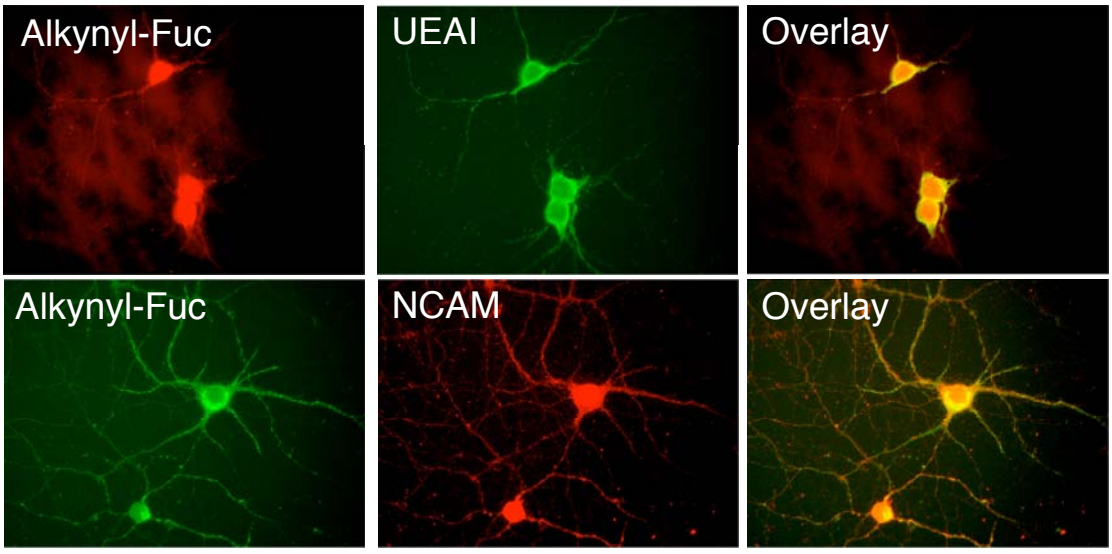

Figure 5.10. Staining with alkynyl-Fuc labels physiologically relevant epitopes. 11 DIV cultures were treated for 3 days with alkynyl-Fuc or Fuc (14 DIV). The neurons were click labeled with azido biotin, and stained with streptavidin (red, top panel) and UEAI conjugated to fluorescein. In the bottom panels, cells were labeled with streptavidin (green and fucosylated NCAM (red)). Both have some extent of colocalization suggesting that alkynyl-Fuc is labeling fucosylated glycans in hippocampal neurons.

molecule), and the fucose binding lectin UEAI to determine whether we are labeling physiologically relevant glycoconjugates. There was some colocalization between NCAM and alkynyl-Fuc, as well as extensive colocalization with UEAI (Figure 5.10), suggesting that we are chemically labeling fucosyl glycans. We examined young neurons that have begun developing axons, as well as older neuronal cultures that have functional synapses. Cells were treated for 3 days with alkynyl-Fuc or Fuc, then fixed, permeabilized, and chemoselectively labeled with a biotin reporter using click chemistry. We investigated the localization of fucosylated glycans with the axonal marker tau, the dendritic marker map2, and the Golgi marker giantin by confocal fluorescence microscopy (Figure 5.11). We observed extensive overlay of the alkynyl-Fuc probe with the Golgi marker giantin, suggesting that most fucosyl glycans reside in the Golgi apparatus (Figure 5.11A). In addition, we identified specific labeling of both axons (Figure 5.11B) and dendrites (Figure 5.11C) in 4 DIV neuronal cultures. We observed similar labeling of older neuronal cultures (data not shown), suggesting that fucosylated glycoconjugates are localized in these neuronal substructures throughout development.

In collaboration with Chithra Krishnamurthy, we examined alkynyl-Fuc labeling in mature 14 DIV hippocampal neurons. We observed the strongest labeling of fucose within the Golgi compartment of mature 14 DIV hippocampal neurons (data not shown). In addition, we examined the colocalization of fucose to synapses with the synaptic marker synapsin. There was some colocalization to synapses indicated by yellow puncta; however, the majority of alkynyl-Fuc-labeled protein did not appear to be highly localized to synapses (Figure 5.12).

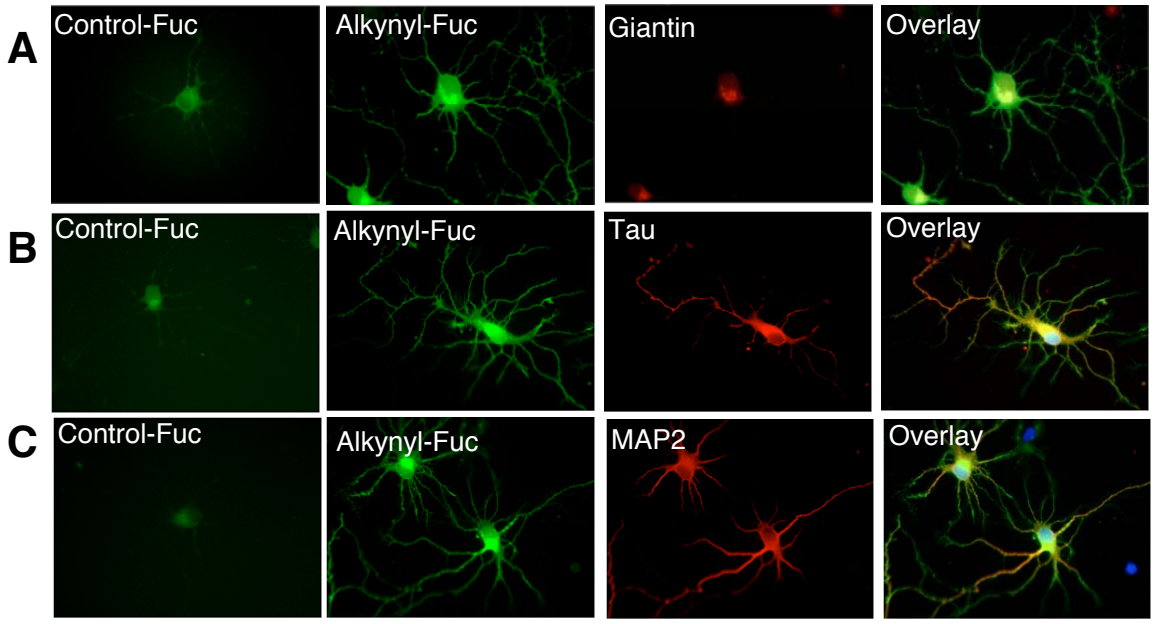

Figure 5.11. Alkynyl-Fuc glycoproteins are localized along axons and dendrites, as well as in the Golgi apparatus. Hippocampal cultures were treated at 1 DIV for 3 days with Fuc or alkynyl-Fuc. Cells were biotyinylated with azido biotin via click chemistry and detected with streptavidin conjugates to fluorescein (green) Cells were costained with (A) giantin (red, Golgi marker), (B) tau (red, axonal marker), and (C) map2 (red, dendritic marker).

In Vivo Labeling of Fucosylated Glycans

Having demonstrated the ability to chemoselectively label fucosyl oligosaccharides *in vitro*, we recently began exploring the potential to label fucosylated glycans *in vivo*. Such experiments significantly decrease the amount of molecule needed per experiment and expand our ability to monitor fucosylation in living animals. Since fucose and other sugars do not cross the blood-brain barrier, we needed to develop a protocol involving direct injection into the brain. We began our pilot studies in mouse pups, in which the Fucα(1-2)Gal epitope is highly up-regulated (Chapter 3). Using a stereotaxic device and a microinjector, we injected alkynyl-Fuc and Fuc into the

forebrain of early postnatal mouse pups using coordinates 2 mm posterior to Bregma, 2 mm lateral to midline, and 2 mm ventral to the top of the skull. We next determined the optimal amount of fucose for injections. Notably, only minute quantities (2 μL) were needed for injections, minimizing the amount of molecule needed per experiment. After injection, the cortices were removed, lysed, and click labeled with azido-biotin. Lysates were resolved by SDS-PAGE and visualized by blotting with streptavidin conjugated to a fluorophore (Figure 5.13). We were able to detect specific labeling of fucosylated glycoconjugates in the alkynyl-Fuc-injected animals, whereas there was very little labeling in control-Fuc-injected animals. However, we are still in the process of optimizing the gel procedure. All non-specific labeling occurred between 25 – 50 kDa, and may represent endogenous biotinylated proteins. In addition, the two prominent bands at 50 and ~40 kDa were present in the control sample. We observed a number of fucosylated glycoproteins between 50 and over 250 kDa that were only labeled in the alkynyl-Fuc-injected animals, suggesting that we can successfully incorporate the alkynyl-Fuc tag into fucosyl oligosaccharides *in vivo* via metabolic labeling. We are currently optimizing the *in vivo* injection procedures for immunohistochemical experiments. We observe strong background fluorescence in the control injections (data not shown), and are working towards a method for clean labeling. Immunohistochemical studies will enable us to investigate the subcellular distribution of fucosyl-oligosaccharides in the brain, and monitor changes in their distribution in response to learning paradigms in living animals.

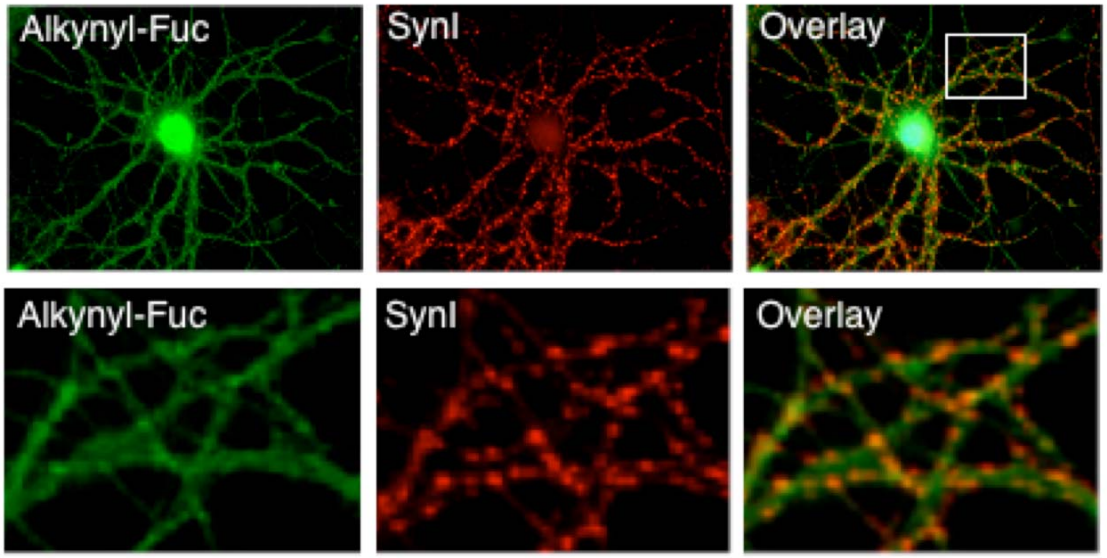

Figure 5.12. Alkynyl-Fuc glycoproteins are not highly localized to synapses. Hippocampal neurons were cultured for 11 days and treated with Fuc or alkynyl-Fuc for 3 days (14 DIV). Cells were click labeled with azido-biotin and visualized with streptavidin (green), or synapsin I (red). Bottom panels are equivalent in size to the white box in the top panels.

Discussion

Metabolic labeling has emerged as an important chemical tool to enhance our understanding of glycobiology. Toward this end, multiple chemical probes have been synthesized for incorporation into various glycoconjugates including such sugars as fucose, sialic acid, and mannose. These chemical probes exploit the biosynthetic machinery of the cell to install bioorthoganol chemical functionalities onto glycoproteins of interest. These versatile chemical tools have previously been investigated in cultured cell lines. Here, we explored the ability to monitor fucose in the brain. Tagging fucose in neurons will facilitate a molecular level understanding of the role that fucosyl oligosaccharides play in learning and memory. Investigation of neurons is especially

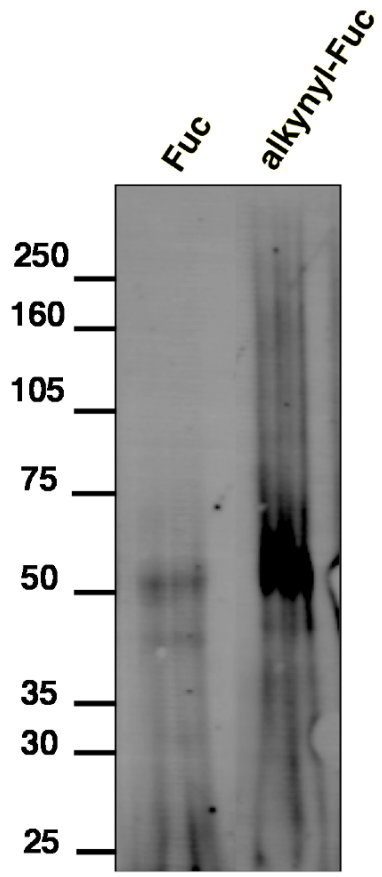

Figure 5.13. Intracranial administration of alkynyl-Fuc leads to direct alkynyl incorporation into fucosyl glycoproteins *in vivo*. Postnatal day 3 mice were injected unilatrerally with Fuc or alkynyl-Fuc and tissue was harvested 2 days post-injection. Lysates were click labeled with azido-biotin, resolved by SDS-PAGE and probed with streptavidin conjugated to a fluorophore. There was significant labeling in the alkynyl-Fuc lane when compared to Fuc injected animals. In particular, there was significant labeling of fucosylated glycoproteins between 50 and over 250 kDa, suggesting that intracranial drug administration can label fucosylated glycoproteins *in vivo*.

challenging to study as the cells are post-mitotic and fucose is present in low cellular abundance. Here, we explored the ability to metabolically label fucosylated glycans in cultured neuronal cells and *in vivo*. In addition, we explored the fucosylated proteome in cultured cortical neurons.

Metabolic labeling with alkynyl-Fuc analogues in cultured cortical neurons enabled selective labeling of fucosylated glycans as visualized by Western blotting and

fluorescence microscopy. Fucosylated glycoproteins were highly localized to the Golgi apparatus of both developing and mature neuronal cultures. However, there was also some background staining of the Golgi body in the control click reaction, suggesting that some of the Golgi labeling is non-specific (data not shown). The terminal Golgi apparatus contains the fucosyltransferases necessary for fucosylation of glycoconjugates, thus extensive labeling of the Golgi body was expected and is consistent with previous studies of cancerous cell lines. In addition, we examined the localization of alkynyl-Fuc labeled glycoconjugates in developing hippocampal neurons. Fucosylated glycoconjugates were present in both the axons and dendrites of young and mature neuronal cultures. Interestingly, in older neuronal cultures with functional synapses, we did not observe strong localization to synaptic compartments of the cell, in contrast to our previous studies that Fucα(1-2)Gal glycoproteins are highly localized to presynaptic terminals (Chapter 2).[14] This suggests that the predominant glycoproteins labeled may not be Fucα(1-2)Gal, but are likely fucose present in other linkages. We examined the binding of lectin UEAI and found alkynyl-Fuc treated cells bound with lower avidity than the control, most likely due to the change of a methyl group on Fuc to the bulky alkynyl-group, consistent with a previous report.[10] There was also some colocalization between alkynyl-Fuc labeling and UEAI in the cell soma and along neuronal processes, suggesting that we are labeling physiologically relevant epitopes. We observed that NCAM, a protein previously been reported to be fucosylated[15-17] and identified in proteomics studies done in this report, colocalized strongly with alkynyl-Fuc labeling. We did not observe complete colocalization, suggesting that we are labeling NCAM as well as other fucose-containing glycoproteins within mature neuronal cultures. Cumulatively, these

data suggest that fucosylated glycans can be metabolically labeled *in vitro* in neuronal cultures. In addition, we observe alkynyl-Fuc staining along both axons and dendrites, which suggests that the modified glycoconjugates are being successfully trafficked along neuronal processes.

We next explored the fucosylated proteome in cultured cortical neurons through metabolic labeling with alkynyl-Fuc. We identified 23 fucosyl glycoproteins, some of which are novel and some that have been previously characterized. Identification of known fucosyl glycoproteins validates the metabolic labeling approach to label fucosylated glycoproteins. We identify NCAM and NCAM L1, which are cell adhesion molecules involved in cell adhesion, neuronal migration, axonal fasciculation, and synaptogenesis.[18-21] Both of these proteins were identified as Fucα(1-2)Gal glycoproteins in Chapter 3 of this thesis. We also identified the voltage-gated calcium channel alpha2/delta subunit which was previously identified as a putative Fucα(1-2)Gal glycoprotein (Chapter 3). In addition, we identify the notch homolog 4 protein as a fucosylated glycoprotein. The notch family of proteins is involved in cell fate determination and differentiation.[22] Notch is known to be fucosylated directly on serines of epidermal growth factor repeats known as *O*-fucosylation.[23] Fucosylation of notch modulates its interaction with ligands, and can have important consequences for cell fate determination.[24, 25]

Interestingly, we also identify several novel cytosolic fucosylated proteins, such as several members of the MARCKS family of proteins and GAP-43. The MARCKS family of proteins regulates dendritic spine morphology and are myristoylated, so they can be membrane bound.[26-31] These proteins influence cell morphology, cell motility,

and are important for the maintenance of dendritic spines and synaptic plasticity.[32] GAP-43 is palmitoylated, and can also interacts with membranes.[33, 34] It is involved in regulating axonal growth, guidance, development, and plasticity.[35, 36] GAP-43-deficient animals display defects in motor skills and sensory impairments.[37] Identification of cytosolic proteins that associate with membranes supports a role for fucosylation in development and other nervous system functions, and supports the notion that soluble cytosolic proteins may be fucosylated like synapsin I.

Ion transport and calcium signaling proteins were identified such as the voltage-gated calcium channel alpha2/delta subunit and the type 3 inositol 1,4,5, triphosphate receptor. The voltage-gated calcium channel alpha2/delta subunit has been shown to bind the drug pregabalin, which helps prevent seizure activity, reduce pain-related behaviors, and anxiety disorders.[38] The type 3 inositol 1,4,5, triphosphate receptor regulates calcium currents important for GABAergic signaling.[39] Interestingly, the Huntingtin protein influences neuronal calcium signaling through this channel which may affect glutamate-induced Ca^{2+} signals leading to neuronal dysfunction and apoptosis.[40] Identification of these proteins suggests that fucose may be involved in various neuropathological processes and disease progression.

The ability to label fucose *in vivo* opens new avenues where we can monitor fucosylation in living animals. Using intracranial injections, we detected numerous glycoproteins from *in vivo* alkynyl-Fuc labeling between 50 and over 250 kDa. We are currently attempting to identify the fucosylated proteome from these *in vivo* labeling studies. We are also developing methods for immunohistological analysis of brain slices, with the future intent of training animals in learning and behavioral paradigms, then

monitoring changes in localization or synthesis of fucosylated glycoproteins. These studies will help elucidate the proteins involved in learning and memory consolidation. While click chemistry using a copper catalyst is not amenable to *in vivo* labeling, Bertozzi and colleagues have developed a method to click label glycoproteins *in vivo* utilizing copper free chemistry.[41] They developed difluorinated cyclooctyne (DIFO) reagents to activate the alkyne and eliminate the copper catalyst, which allows click chemistry to work in living animals. They have recently demonstrated the ability to label zebrafish *in vivo*,[42] suggesting that we could potentially use these reagents to monitor fucosylation dynamics in living mice. Such studies will reveal molecular insights into learning and memory that have been unattainable by conventional biochemical approaches.

Cumulatively, our studies reveal exciting new insights into the molecular mechanisms that govern fucosyl oligosaccharides. We demonstrated that fucosylated glycoproteins are found along both axons and dendrites, as well as in the Golgi apparatus. Identification of proteins involved in regulating neuronal morphology and dendritic spine numbers suggest important roles for fucose in the molecular events that may underlie synaptic plasticity. Identification of the fucosyl proteome enables our investigation of fucose function in the nervous system. We hope to pursue metabolic labeling of fucose in living animals to investigate the roles these individual proteins may play in memory consolidation, development, and synaptic plasticity.

Materials and Methods

Embryonic Hippocampal Dissection

Timed-pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Kingston, Mass) and housed at the Caltech laboratory animal facilities. Timed-pregnant rats at embryonic day 18 (E18) were euthanized in accordance with proper IACUC protocols. Neurons were cultured as described in Chapter 2.

Embryonic Cortical Dissection

Timed-pregnant rats (E18) were dissected as described above. After the brain was removed from the decapitated embryos, the cortices were cut out and the meninges removed. The cortices were placed in ice-cold HBSS and trypsinized with 0.25% trypsin for 15 minutes at 37 °C and triturated to dissociate individual cells. Cortical neurons were evenly plated out on ten 10-cm petri dishes, coated with poly-D-ornithine., in 5 mL of DMEM. After 30 minutes, the DMEM was removed and replaced with supplemented neurobasal media.

Click Labeling Conditions and Protein Precipitation

Cortical neurons were lysed by sonication in boiling 1% SDS and the protein quantified with the BCA Protein Assay (Pierce). The cell lysate was neutralized with an equal volume of neutralization buffer (6% NP-40, 100 mM Na_2PO_4, 150 mM NaCl) and labeled at 2 mg/mL protein with 0.1 mM azido-biotin (5 mM stock in DMSO, stored at -20 °C), 0.1 mM tristriazoleamine catalyst (5 mM stock in DMSO, stored at -20 °C), 2 mM sodium ascorbate (100 mM stock in water, freshly prepared) and 0.5 mM CuBr (50

mM stock in water, freshly prepared) in a microtube. The reaction tube was allowed to rotate at 4 °C overnight. The protein was precipitated using the SDS-PAGE Clean Up Kit (GE Healthcare) to remove excess reagents and resolubilized in boiling 1% SDS, then normalized to 2 mg/mL.

Isolation by Streptavidin Column, Resolution SDS-PAGE gel and Tryptic Digest

Streptavidin resin (Pierce) was equilibrated in a 1:1 neutralization buffer: 1% SDS solution. Labeled lysate was neutralized with an equal volume of neutralization buffer and allowed to incubate with 100 μL of streptavidin resin, rotating, overnight at 4°C. After binding, the resin was washed with 40 column volumes (CV) of Low Salt Buffer (0.1 M Na_2PO_4 pH 7.5/ 0.15 M NaCl/ 1% Triton-X100/ 0.5% sodium deoxycholate/ 0.1% SDS), 40 CV of High Salt Buffer (0.1 M Na_2PO_4 pH 7.5/ 0.5 M NaCl/ 0.2% Triton X-100), 30 CV of 4 M Urea/ 1% SDS in H_2O, 40 CV of PBS and 40 CV of H_2O in 2 mL BioSpin Chromatography Columns (BioRad). Alternative wash conditions used were 40 CV Low Salt Buffer, 40 CV High Salt Buffer, 30 CV 2% SDS in PBS, 30 CV 8M Urea in H_2O, 30 CV 1 M KCl and 40 CV H_2O. After washing, streptavidin resin was removed and incubated in a microtube, rotating at RT, in one CV of elution buffer (6 M urea, 2 M thiourea, 30 mM biotin, 2% SDS, pH 12) for 15 minutes. The resin was then boiled for 15 minutes in the elution buffer, vortexing every 5 minutes. The tube was spun down and the eluate was removed. The eluate was diluted 10 times with PBS and concentrated to 50 μL in Amicon-4mL Ultra concentrators (Millipore).

Tryptic Digest

The eluate was resolved on a 10% acrylamide-SDS gel and visualized by silver stain. The gel was destained and the bands cut out before the in gel tryptic digest as described in Chapter 3. The resulting tryptic peptides were acidified in 0.1% HOAc before MS analysis. A tryptic digest was preformed in solution on the eluate as described previously. The tryptic peptides were acidified with 0.1% HOAc before MS analysis.

Western Blotting

Labeled lysates were resolved on a 4-12% acrylamide-SDS gel and proteins were transferred to PVDF membrane (Millipore) for 2 h.

Immunocytochemistry of Hippocampal Neuronal Cultures treated with Fucose and Alkynyl-fucose

After 11 days in culture, hippocampal neurons on coverslips were treated with 50 μM fucose or alkynyl-fucose for three days. At 14 days in culture, media was aspirated and cells were rinsed once with PBS, fixed in 4% paraformaldehyde for 20 minutes at room temperature, washed twice with PBS, permeabilized in 0.3% Triton X-100 for five minutes at room temperature and washed twice with PBS. Non-specific binding was blocked with 3% BSA in PBS for 1 h at room temperature and then the coverslips were rinsed once with PBS. The click reaction was carried out on the coverslip with 100 μL of 0.05 mM azido-biotin, 0.05 mM tristriazoleamine catalyst, 1 mM sodium ascorbate and 0.5 mM $CuSO_4$ in PBS added to the top of each coverslip, overnight at 4 °C. After rinsing twice with PBS, primary antibody: anti-synapsin (rabbit, 1:100; Sigma), anti-

PSD-95 (mouse, 1:250; Affinity BioReagents), anti-giantin (Santa Cruz, 1:100), anti-NCAM (mouse, 1:100, Sigma) and UEAI conjugated to fluorescein (50μL/mL, Sigma) was added in 3% BSA in PBS, overnight at 4 °C. After the coverslips were washed three times with PBS, fluorophore conjugated secondary antibodies (goat anti-rabbit; 1:500 and goat anti-mouse; 1:500) were added in 3% BSA in PBS for one hour at 37 °C. Alkynyl-fucose was detected with streptavidin conjugated to AlexaFluor 488 (1:100; Molecular Probes) or AlexaFluor 546 (1:100, Molecular Probes) added together with the secondary antibodies. The coverslips were washed three times with PBS and mounted onto slides with Vectashield with DAPI (Vector Labs) and sealed with clear nail polish. Cells were then subjected to fluorescence and confocal microscopy.

In Vivo Labeling of Fucosylated Glycans in Mice

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 appear anesthetized. A toe 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, unmodified L-fucose will also be injected into the hippocampus.

After insertion of the needle, a one-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 alkynyl-fucose vs. control fucose injections 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 they began moving. They were returned to the dam where they will be 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, dehydrated, or exhibited neurological symptoms 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 hopes that the pup receives 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.

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