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

Background

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

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

---

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

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

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

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

Fucα(1-2)Gal lectins exist in neurons

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

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

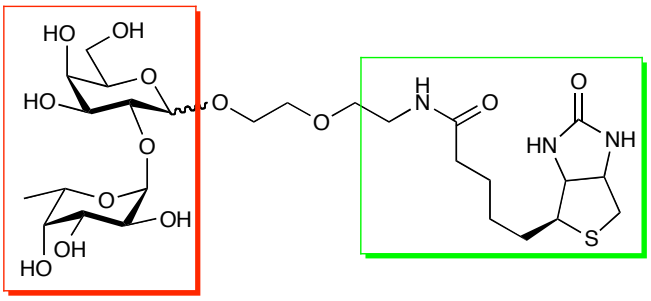

Figure 2.1. Fucα(1-2)Gal-biotin probe **1** was designed to mimic endogenous glycoproteins. The two main structural elements are a Fucα(1-2)Gal moiety (red) for protein recognition and a biotin moiety (green) for fluorescent labeling.

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

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

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

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

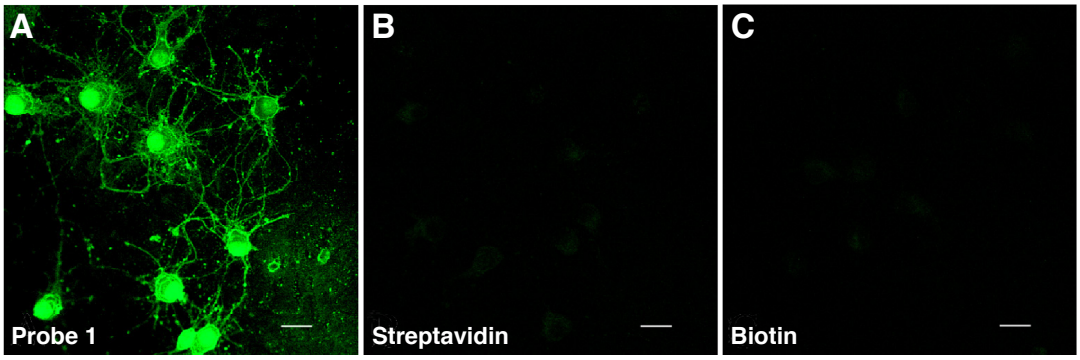

Figure 2.2. Fucα(1-2)Gal probe **1** binds to the cell surface of hippocampal neurons. A) Probe **1** (10 mM) binds selectively to neurons. B) Incubation of neurons with streptavidin dye conjugate produces no background signal. C) Incubation of neurons with biotin (10 mM) also produces no background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

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

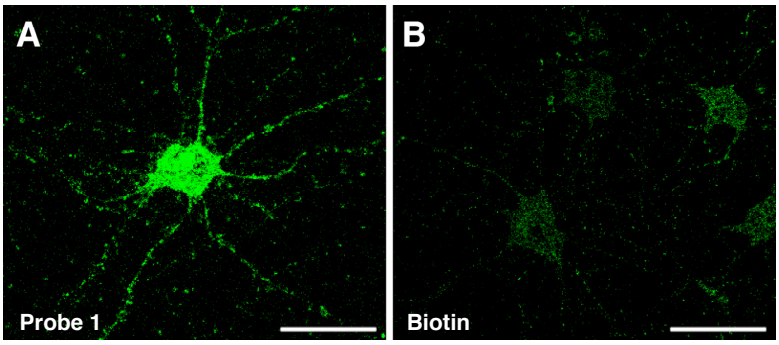

Figure 2.3. Fucα(1-2)Gal probe **1** binds to hippocampal neurons. Neurons were cultured for 23 DIV and then treated with 3 mM of (A) probe **1** or (B) biotin in the presence of 10 μM PAO. Scale bars, 45 μm

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

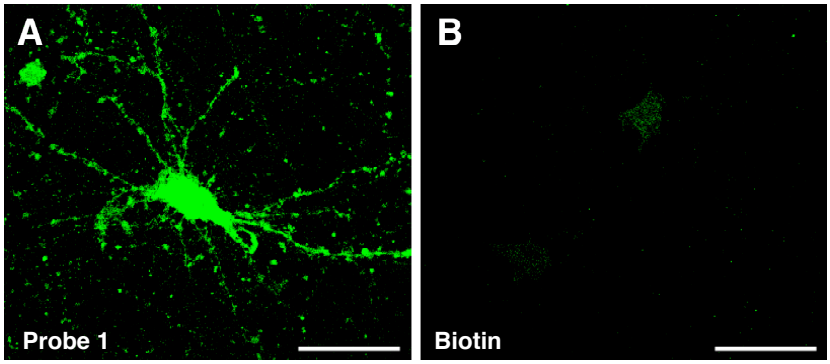

Figure 2.4. Lipid extraction does not alter labeling with Fucα(1-2)Gal probe **1**. Neurons were delipidated with $MeOH/CHCl_3$ prior to labeling with 3 mM of (A) probe **1** or (B) biotin in the presence of 10 μM PAO. Scale bars, 45 μm

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

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

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

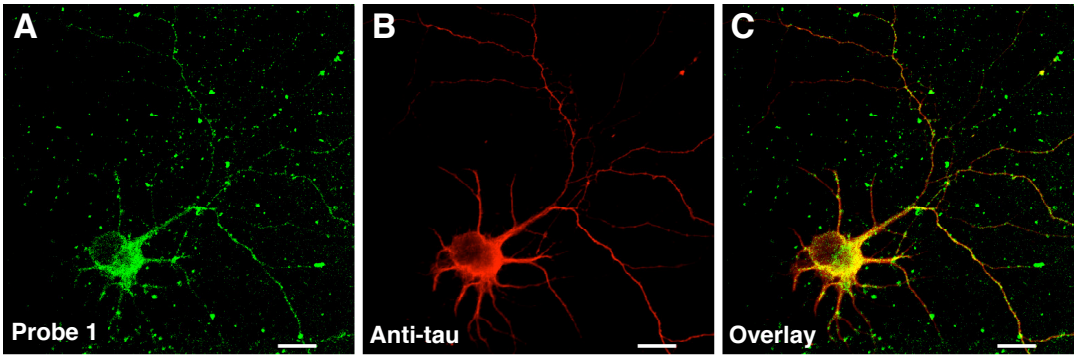

Figure 2.5. Costaining of neurons with Fucα(1-2)Gal probe **1** and an anti-tau antibody. A) Probe **1** staining (green). B) Tau antibody labels axons, dendrites, and cell bodies. C) Overlay of probe **1** and tau labeling (yellow indicates colocalization) shows that Fucα(1-2)Gal staining is distributed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

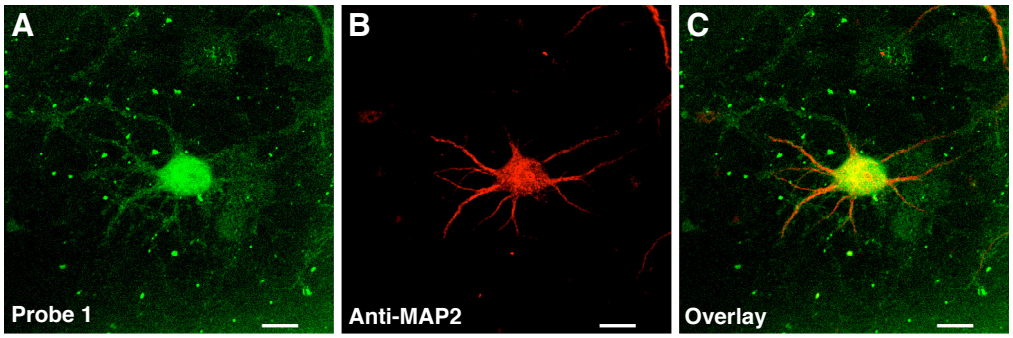

Figure 2.6. Costaining of neurons with Fucα(1-2)Gal probe **1** and a MAP2 antibody. A) Probe **1** staining (green). B) MAP2 labeling (red) shows dendrites and cell body. C) Overlay of probe **1** and MAP2 labeling (yellow indicates colocalization) shows that Fucα(1-2)Gal staining is distributed on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

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

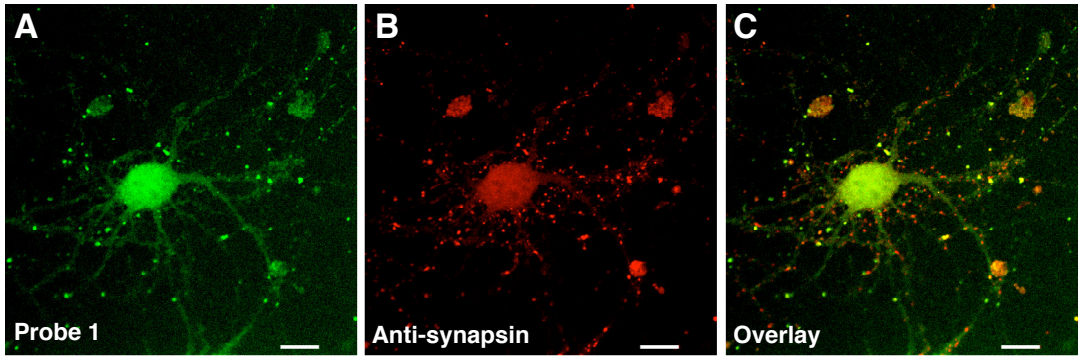

Figure 2.7. Costaining of neurons with Fucα(1-2)Gal probe **1** and an anti-synapsin antibody. A) Probe **1** staining (green). B) Synapsin antibody (red) labels presynaptic terminals. C) Overlay of probe **1** and synapsin labeling (yellow indicates colocalization) shows that Fucα(1-2)Gal staining does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

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

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

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

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

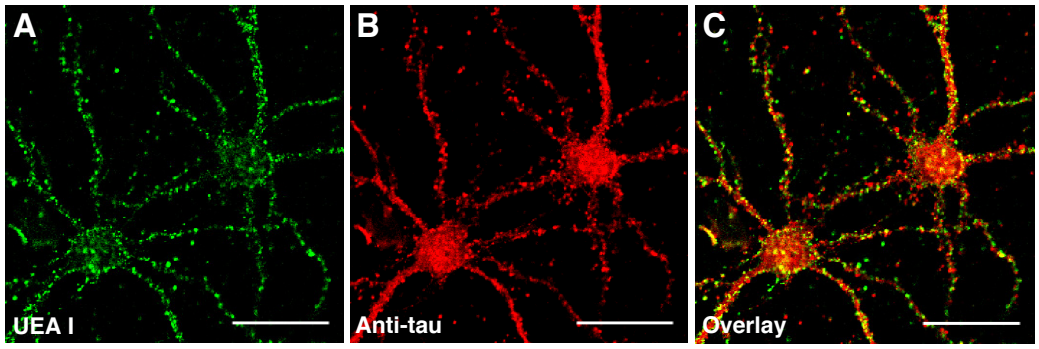

Figure 2.8. Costaining of neurons with UEA-I lectin and an anti-tau antibody. Neurons were stained with (A) fluorescein-conjugated UEA-I lectin and (B) an anti-tau antibody in the presence of 10 μm PAO. C) Overlay of both images (yellow indicates colocalization). UEA-I lectin labels neurons on the cell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

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

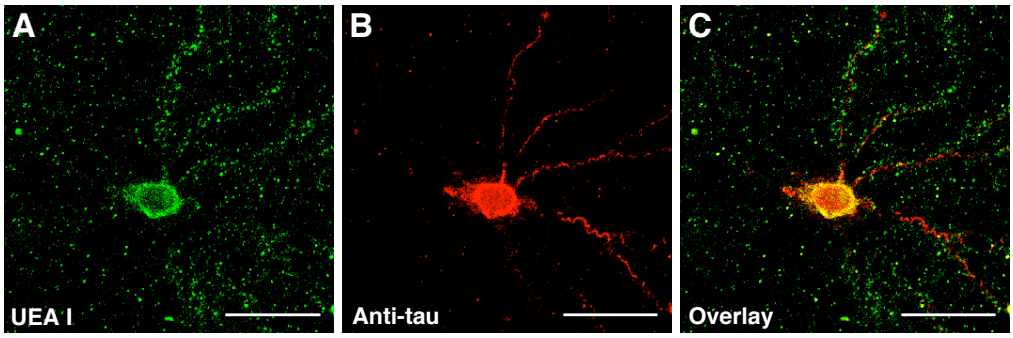

Figure 2.9. Lipid extraction using MeOH/$CHCl_3$ did not diminish UEA-I lectin labeling. Costaining of neurons with (A) UEA-I lectin (green) and (B) anti-tau antibody (red) in the presence of 10 μM PAO after lipid extraction. C) Overlay of both images (yellow indicates colocalization). Scale bars, 45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

Fucα(1-2)Gal carbohydrates modulate neuronal outgrowth

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

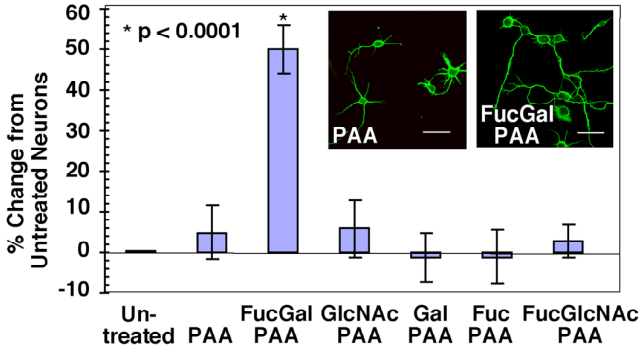

Figure 2.10. Fucα(1-2)Gal promotes neuronal growth. Neurite outgrowth was quantified by measuring the longest neurite per cell after treatment with 130 μM of the indicated compounds. Error bars represent SEM from 100 total neurons in two separate experiments.

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

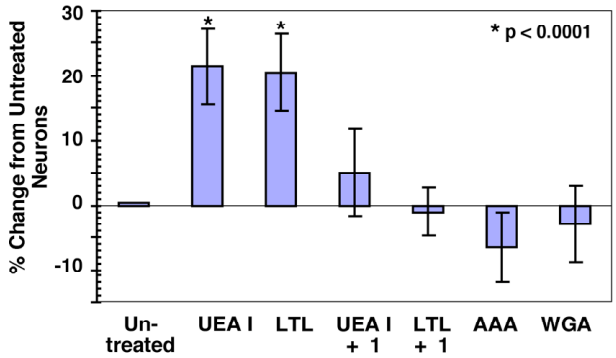

Figure 2.11. Only the Fucα(1-2)Gal-selective lectins UEA I and LTL stimulate neuronal growth. Neurite outgrowth was quantified after treatment with 3.7 μM of the indicated lectins. Error bars represent SEM from 100 total neurons in two separate experiments.

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

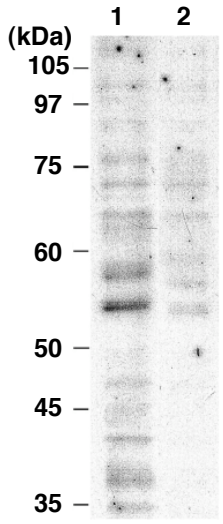

Figure 2.12. Treatment with 2-dGal diminishes the expression of the Fucα(1-2)Gal epitope on glycoproteins. Neurons were treated for 4 days with or without 30 mM 2-dGal. Protein lysates were then analyzed by Western blotting using antibody A46-B/B10. Lane 1: Untreated neurons. Lane 2: Neurons treated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

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

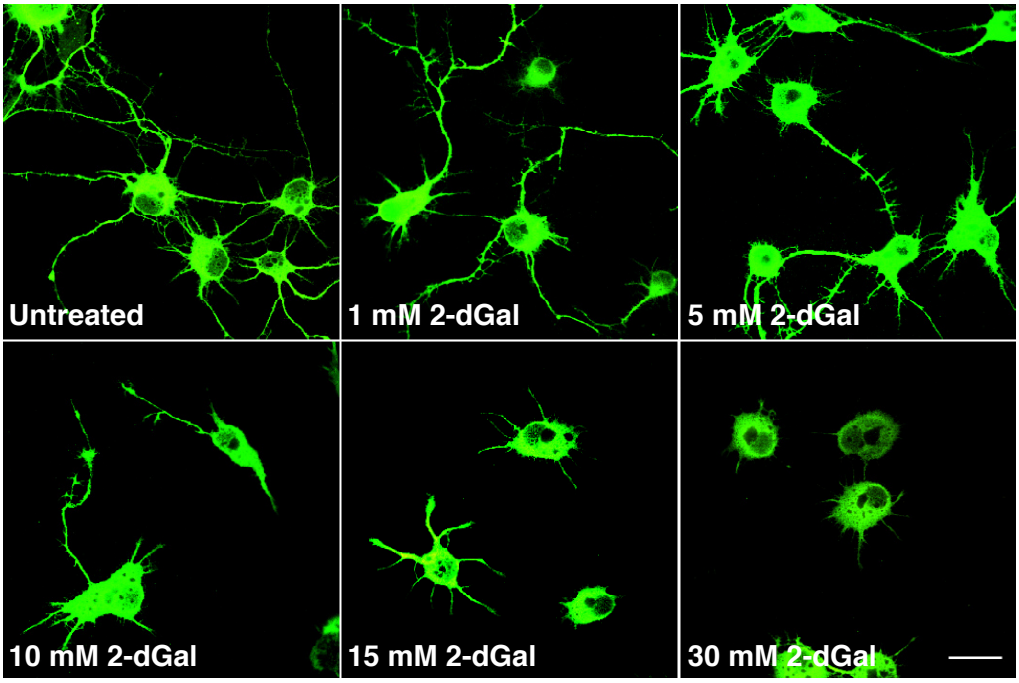

Figure 2.13. Hippocampal neurons treated with varying concentrations of 2-dGal exhibit increasing defects in neuronal growth. After 1 day in culture, neurons were treated with the specified concentrations of 2-dGal for 2 days, followed by immunostaining with anti-tau antibodies.

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

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

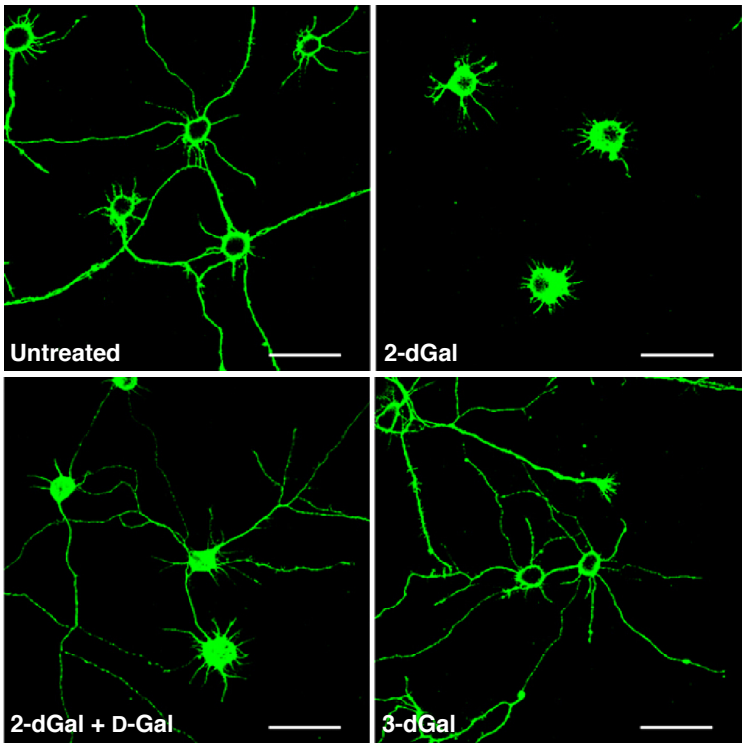

Figure 2.14. Treatment of hippocampal neurons with 2-dGal (15 mM), but not 3-dGal (15 mM), for 2 days inhibits neuronal growth. The effects of 2-dGal can be reversed by subsequent treatment with D-Gal (75 mM) for an additional 2 days. Neurons were immunostained with anti-tau antibodies.

Discussion

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

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

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

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

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

Experimental Procedures for Chapter 2

Buffers and Reagents:

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

Embryonic Hippocampal Dissection:

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

Hippocampal Neuronal Cultures:

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

Immunocytochemistry of Hippocampal Neuronal Cultures:

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

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

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

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

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

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

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

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

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

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

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

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

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

Confocal Laser Microscopy:

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

References

1. Helenius, A. & Aebi, M. Intracellular functions of N-linked glycans. *Science* **291**, 2364 – 2369 (2001).

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

3. Holt, C.E. & Dickson, B.J. Sugar codes for axons? *Neuron* **46**, 169 – 172 (2005).

4. Rose, S.P.R. Glycoproteins and memory formation. *Behav. Brain Res.* **66**, 73 – 78 (1995).

5. McCabe, N.R. & Rose, S.P.R. Passive-avoidance training increases fucose incorporation into glycoproteins in chick forebrain slices in vitro. *Neurochem. Res.* **10**, 1083 – 1095 (1985).

6. Zanetta, J.-P., Reeber, A., Vincendon, G. & Gombos, G. Synaptosomal plasma membrane glycoproteins. II. Isolation of fucosyl-glycoproteins by affinity chromatography on the *Ulex Europeus* lectin specific for L-fucose. *Brain Res.* **138**, 317 – 328 (1977).

7. Krusius, T. & Finne, J. Structural features of tissue glycoproteins. Fractionation and methylation analysis of glycopeptides derived from rat brain, kidney and liver. *Eur. J. Neurosci.* **78**, 369 – 379 (1977).

8. Bullock, S., Potter, J. & Rose, S.P.R. Effects of the amnesic agent 2-deoxygalactose on incorporation of fucose into chick brain glycoproteins. *J. Neurochem.* **54**, 135 – 142 (1990).

9. Krug, M., Jork, R., Reymann, K., Wagner, M. & Matthies, H. The amnesic substance 2-deoxy-D-galactose suppresses the maintenance of hippocampal LTP. *Brain Res.* **540**, 237 – 242 (1991).

10. Lorenzini, C.G.A., Baldi, E., Bucherelli, C., Sacchetti, B. & Tassoni, G. 2-deoxy-D-galactose effects on passive avoidance memorization in the rat. *Neurobiol. Learn. Mem.* **68**, 317 – 324 (1997).

11. Rose, S.P.R. & Jork, R. Long-term memory formation in chicks is blocked by 2-deoxygalactose, a fucose analog. *Behav. Neural Biol.* **48**, 246 – 258 (1987).

12. Jork, R., et al. Monoclonal antibody specific for histo-blood group antigens H (types 2 and 4) interferes with long-term memory formation in rats. *Neurosci. Res. Commun.* **8**, 21 – 27 (1991).

13. Karsten, U., et al. A new monoclonal antibody (A46-B/B10) highly specific for the blood group H type 2 epitope: Generation, epitope analysis, serological and histological evaluation. *Br. J. Cancer* **58**, 176 – 181 (1988).

14. Krug, M., Wagner, M., Staak, S. & Smalla, K.H. Fucose and fucose-containing sugar epitopes enhance hippocampal long-term potentiation in the freely moving rat. *Brain Res.* **643**, 130 – 135 (1994).

15. Matthies, H., Staak, S. & Krug, M. Fucose and fucosyllactose enhance in-vitro hippocampal long-term potentiation. *Brain Res.* **725**, 276 – 280 (1996).

16. Sukumar, R., Rose, S.P.R. & Burgoyne, R.D. Increased incorporation of [H-3]fucose into chick brain glycoproteins following training on a passive-avoidance task. *J. Neurochem.* **34**, 1000 – 1006 (1980).

17. Kalovidouris, S.A., Gama, C.I., Lee, L.W. & Hsieh-Wilson, L.C. A role for fucose alpha(1-2) galactose carbohydrates in neuronal growth. *J. Am. Chem. Soc.* **127**, 1340 – 1341 (2005).

18. Goslin, K., Asmussen, H. & Banker, G. in Culturing Nerve Cells, 2nd ed. (G. Banker & K. Goslin, Eds.) 251 – 281 (MIT Press, Cambridge, 1998).

19. Nouel, D., et al. Differential binding profile and internalization process of neurotensin via neuronal and glial receptors. *J. Neurosci.* **17**, 1795 – 1803 (1997).

20. Yavin, E. & Yavin, Z. Cell surface and cytoskeletal antigens in cerebral cell cultures after chloroform-methanol delipidation. *J. Neurosci. Res.* **9**, 229 – 237 (1983).

21. Papasozomenos, S.C. & Binder, L.I. Phosphorylation determines two distinct species of Tau in the central nervous system. *Cell Mot. Cytoskeleton* **8**, 210 – 226 (1987).

22. Huber, G. & Matus, A. Differences in the cellular distributions of two microtubule-associated proteins, Map1 and Map2, in rat brain. *J. Neurosci.* **4**, 151 – 160 (1984).

23. Fletcher, T.L., Cameron, P., De Camilli, P. & Banker, G. The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. *J. Neurosci.* **11**, 1617 – 1626 (1991).

24. Alonso, E., Saez, F.J., Madrid, J.F. & Hernandez, F. Lectin histochemistry shows fucosylated glycoconjugates in the primordial germ cells of Xenopus embryos. *J. Histochem. Cytochem.* **51**, 239 – 243 (2003).

25. Wei, A., Boy, K.M. & Kishi, Y. Biological evaluation of rationally modified analogs of the H-type-II blood group trisaccharide. A correlation between solution conformation and binding affinity. *J. Am. Chem. Soc.* **117**, 9432 – 9436 (1995).

26. Sears, P. & Wong, C.H. Carbohydrate mimetics: A new strategy for tackling the problem of carbohydrate-mediated biological recognition. *Angew. Chem. Int. Ed.* **38**, 2300 – 2324 (1999).

27. Sacchettini, J.C., Baum, L.G. & Brewer, C.F. Multivalent protein-carbohydrate interactions. A new paradigm for supermolecular assembly and signal transduction. *Biochemistry* **40**, 3009 – 3015 (2001).

28. Tiunova, A.A., Anokhin, K.V. & Rose, S.P.R. Two critical periods of protein and glycoprotein synthesis in memory consolidation for visual categorization learning in chicks. *Learn. Mem.* **4**, 401 – 410 (1998).

29. Jork, R., et al. Identification of rat hippocampal glycoproteins showing changed fucosylation following 2-deoxy-D-galactose-induced amnesia in a brightness discrimination task. *Neurosci. Res. Commun.* **5**, 105 – 110 (1989).

30. Luscher, C., Nicoll, R.A., Malenka, R.C. & Muller, D. Synaptic plasticity and dynamic modulation of the postsynaptic membrane. *Nat. Neurosci.* **3**, 545 – 550 (2000).

31. Trachtenberg, J.T., et al. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature* **420**, 788 – 794 (2002).

32. Matthies, H., Kretlow, J., Smalla, K.H., Staak, S. & Krug, M. Glycosylation of proteins during a critical time window is necessary for the maintenance of longterm potentiation in the hippocampal CA1 region. *Neuroscience* **91**, 175 – 183 (1999).

33. Glass, W.F., Briggs, R.C. & Hnilica, L.S. Use of lectins for detection of electrophoretically separated glycoproteins transferred onto nitrocellulose sheets. *Anal. Biochem.* **115**, 219 – 224 (1981).