

### **Chapter 3: Fucose $\alpha(1-2)$ Galactose-Containing Glycoproteins are Prevalent in the Brain and Regulate Neuronal Morphology<sup>\*†</sup>**

#### **Background**

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

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

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

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

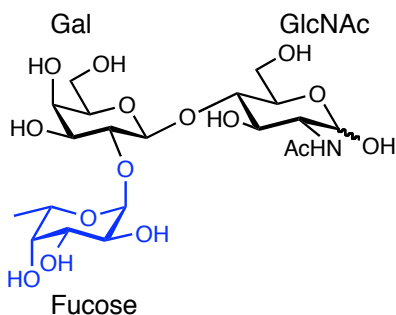
up to 3 hours after training and a 26% increase in tritium-labeled fucose levels 24 hours after the learning task.<sup>7, 8</sup> Addition of exogenous L-fucose or 2'-fucosyllactose (but not D-fucose, D-lactose, or 3-fucosyllactose) was also found to enhance LTP in hippocampal slices.<sup>9</sup> Furthermore,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

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

### **$\text{Fu}\alpha(1-2)\text{Gal}$ glycoproteins are enriched at synapses**

We previously demonstrated the presence of potential glycoproteins specific for the  $\text{Fu}\alpha(1-2)\text{Gal}$  disaccharide using the *Ulex europaeus* agglutinin I (UEA-I) lectin.<sup>12</sup> To allow for further characterization and identification of  $\text{Fu}\alpha(1-2)\text{Gal}$  glycoproteins, we obtained an antibody selective for  $\text{Fu}\alpha(1-2)\text{Gal}$  epitopes (Figure 3.1),<sup>6</sup> the mouse

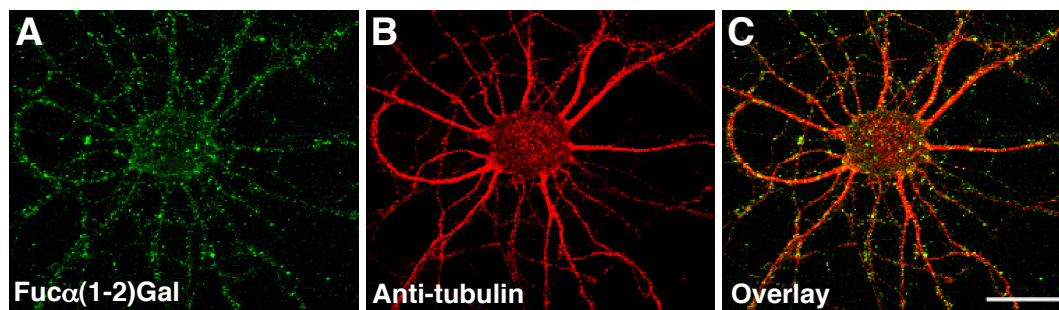
monoclonal antibody A46-B/B10, which was a generous gift from Dr. Uwe Karsten. Importantly, antibody A46-B/B10 has been shown to induce amnesia in animals<sup>5</sup>, suggesting that it recognizes one or more physiologically relevant epitopes.



**Figure 3.1.** Trisaccharide Fuc $\alpha$ (1-2)Gal $\beta$ (1-4)GlcNAc recognized by antibody A46-B/B10

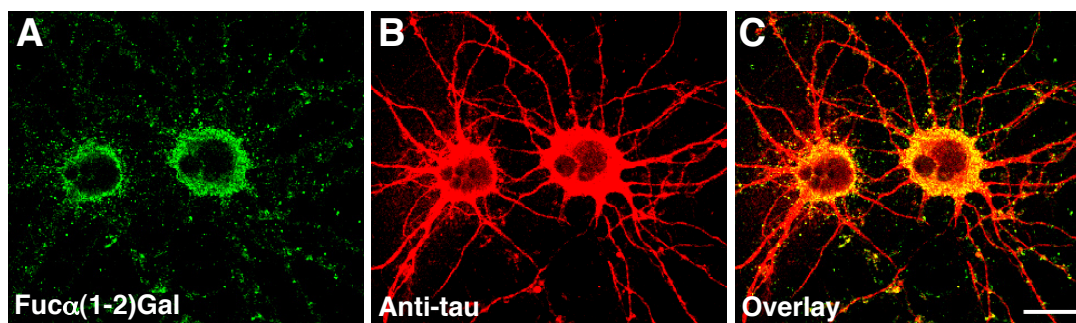
Fucose-containing glycoproteins are known to exist in the brain, but they have not been conclusively established to be present at synapses. Therefore, we sought to establish the location of Fuc $\alpha$ (1-2)Gal glycoproteins in neurons. Hippocampal neurons were cultured for 14 DIV to allow for synapse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

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



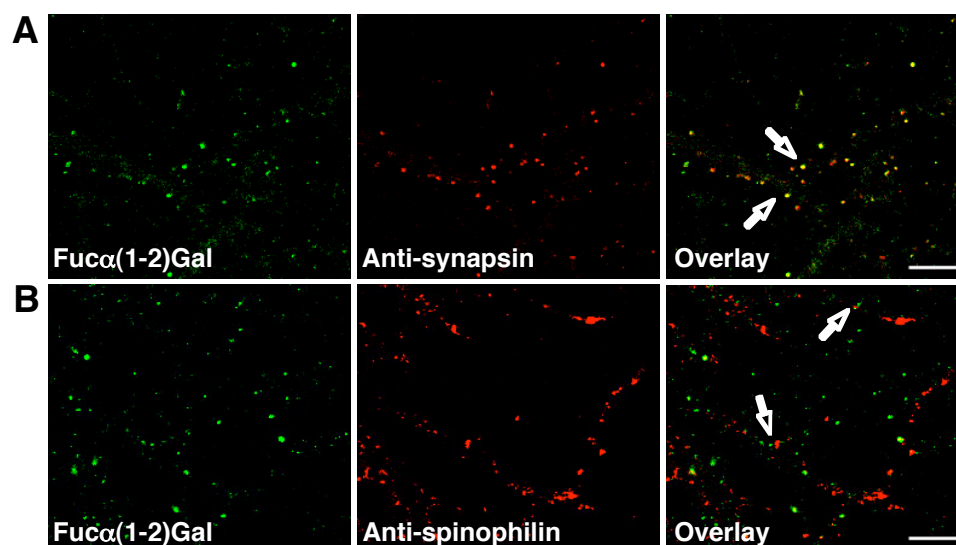
**Figure 3.2.** Costaining of neurons with Fuca(1-2)Gal antibody A46-B/B10 and an anti-tubulin antibody. A) A46-B/B10 antibody staining (green). B) Anti-tubulin labeling shows axons, dendrites, and cell bodies. C) Overlay of A46-B/B10 antibody and anti-tubulin labeling (yellow indicates colocalization)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

To confirm that the Fuca(1-2)Gal antibody was labeling glycoproteins rather than simply interacting with the lipid membrane, neurons were delipidated<sup>14</sup> prior to treatment with antibody A46-B/B10. Specifically, neurons cultured for 20 days were treated with a methanol/chloroform (MeOH/CHCl<sub>3</sub>) mixture followed by coimmunostaining with antibody A46-B/B10 and an anti-tau antibody. Indeed, lipid extraction prior to immunostaining does not diminish the labeling of neurons, and we see staining of the cell body and neurite processes (Figure 3.3),<sup>13</sup> confirming the labeling of glycoproteins rather than glycolipids.



**Figure 3.3.** Lipid extraction of cellular membranes does not abolish staining with antibody A46-B/B10. Neurons were delipidated with MeOH/CHCl<sub>3</sub> before staining with (A) antibody A46-B/B10 (green) and (B) an anti-tau antibody (red). C) Overlay of images (yellow indicates colocalization). Scale bar, 25  $\mu$ m

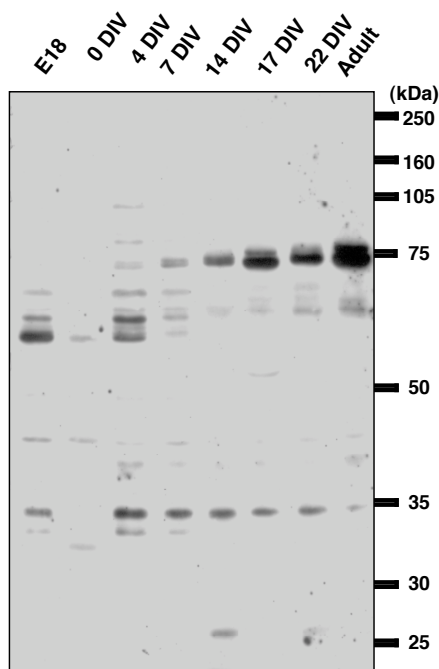
The punctate staining pattern observed suggested that  $\text{Fuca}\alpha(1-2)\text{Gal}$  glycoproteins were present at synapses. To validate this result, we compared the subcellular localization of the  $\text{Fuca}\alpha(1-2)\text{Gal}$  glycoproteins to that of synapsin I and spinophilin. Synapsin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



**Figure 3.4.** Costaining of neurons cultured for 14 DIV with  $\text{Fuca}\alpha(1-2)\text{Gal}$  antibody A46-B/B10 and either an anti-synapsin antibody or an anti-spinophilin antibody. A) A46-B/B10 antibody staining (green) colocalizes with anti-synapsin staining (red), suggesting that  $\text{Fuca}\alpha(1-2)\text{Gal}$  glycoproteins are enriched at presynaptic terminals. B) A46-B/B10 antibody staining (green) does not colocalize with anti-spinophilin staining (red), suggesting that  $\text{Fuca}\alpha(1-2)\text{Gal}$  glycoproteins are mostly apposed to postsynaptic terminals. Arrows indicate colocalization in (A) and apposition in (B). Scale bars, 10  $\mu\text{m}$

### Expression of Fuc $\alpha$ (1-2)Gal on glycoproteins is developmentally regulated

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



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

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

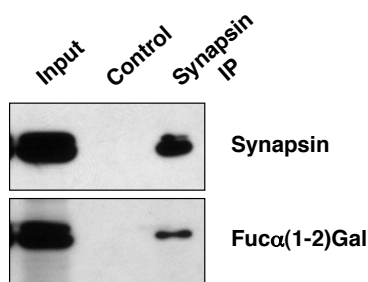
glycoproteins is drastically reduced in the adult hippocampus. Significantly, the major Fuc $\alpha$ (1-2)Gal glycoproteins of 73 and 75 kDa found in mature cultured neurons and adult brain tissue are distinct from those in embryonic tissue. Interestingly, expression of Fuc $\alpha$ (1-2)Gal is observed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

### **Synapsin Ia and Ib are the major Fuc $\alpha$ (1-2)Gal glycoproteins in the hippocampus**

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

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

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



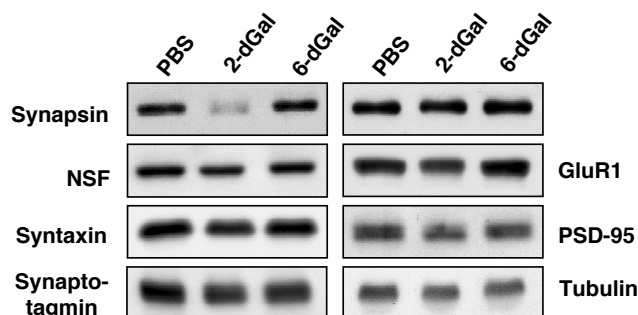
**Figure 3.6.** Synapsins Ia and Ib are  $\text{Fuc}\alpha(1-2)\text{Gal}$  glycoproteins. Immunoprecipitated synapsin I is detected by antibody A46-B/B10. Input, lysate used for immunoprecipitation; Control, immunoprecipitation in the absence of antibody; Synapsin IP, immunoprecipitated synapsin. *Upper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

### **Fucosylation regulates synapsin expression in neurons and neurite outgrowth**

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



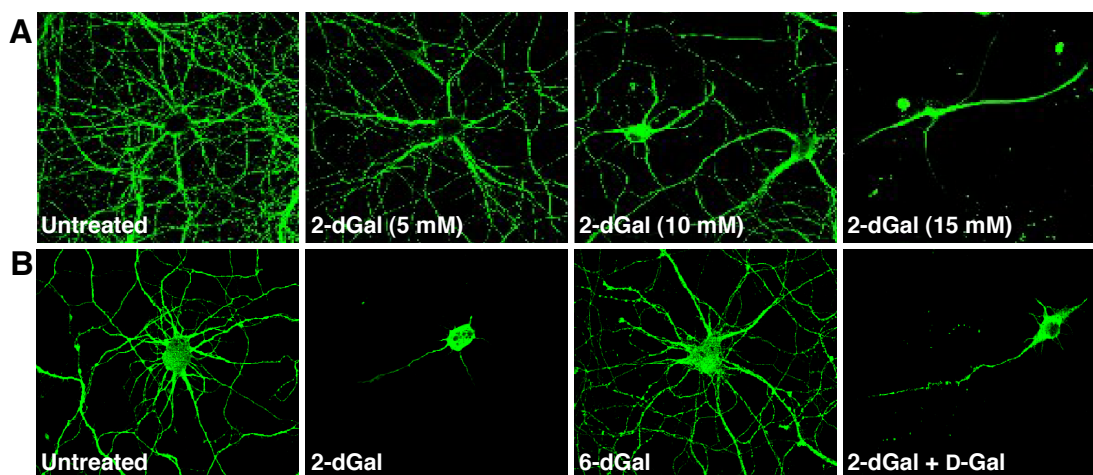
the effects of 2-dGal were specific to synapsin, as the expression of other synaptic proteins was unchanged by the 2-dGal treatment.



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

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

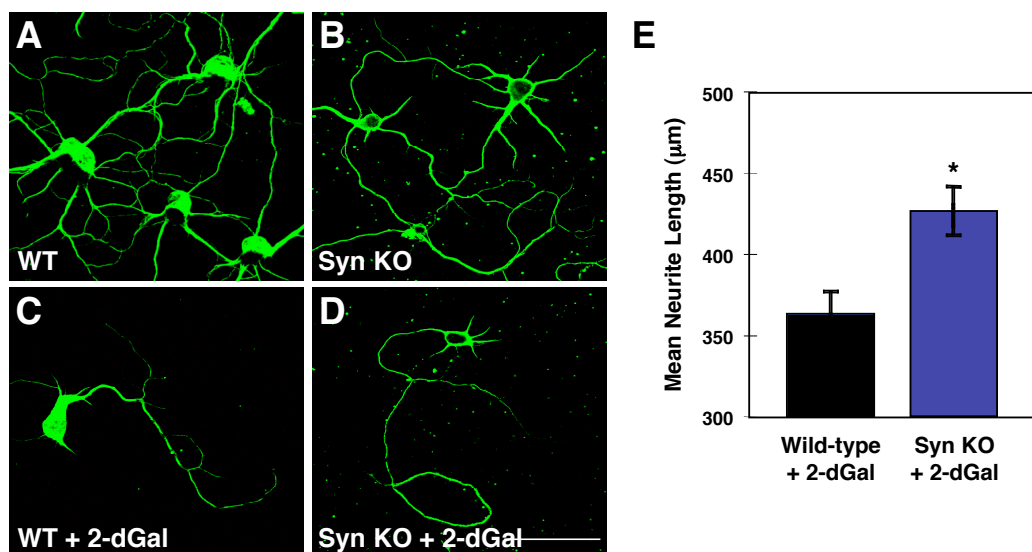
and disrupts neuronal connections, suggesting that Fuc $\alpha$ (1-2)Gal is important for maintaining neuronal plasticity.



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

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

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



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

## Discussion

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

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

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

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

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

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

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

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

### Experimental Procedures for Chapter 3

#### *Buffers and Reagents:*

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

#### *Embryonic Hippocampal Dissection:*

Embryonic tissue was dissected as described in Chapter 2.

#### *Neuronal Cultures and Immunocytochemistry:*

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

*De-lipidation of Neurons with MeOH/CHCl<sub>3</sub> Prior to Immunostaining with Antibody A46-B/B10:*

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

*Adult Hippocampal Dissection and Lysis:*

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

*Western Blotting:*

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



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

*Treatment of Cells with Deoxy-Galactose Analogues:*

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

*Morphometric Analysis:*

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

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