Chapter 2: Protein Fucosylation Regulates Synapsin I Expression and Neuronal Morphology[1]

Background

Experimental studies suggest that protein fucosylation can be regulated in response to synaptic activity. Both task-specific learning and LTP have been shown to induce the fucosylation of proteins at the synapse.[1, 2] Furthermore, the activity of fucosyltransferases, enzymes involved in the transfer of fucose to glycoproteins, has also been demonstrated to increase substantially during synaptogenesis[3] and upon passive avoidance training in animals.[4] Together, these studies suggest that protein fucosylation may be a highly regulated process in the brain and may contribute to neuronal development and synaptic plasticity. Despite these intriguing observations, little is known about the molecular mechanisms by which Fucα(1-2)Gal sugars influence neuronal communication. Moreover, no Fucα(1-2)Gal glycoproteins had been characterized from the brain, and the precise roles of the this sugar in regulating the structure and function of neuronal proteins was unknown.

[1] Portions of this chapter were taken from Murrey, HE,; Gama, CI,; Kalovidouris, SA,; Luo, WI,; Driggers, EM,; Porton, B,; and Hsieh-Wilson, LC,; Protein fucosylation regulates synapsin Ia/Ib expression and neuronal morphology in primary hippocampal neurons. *Proc. Natl. Acad. Sci. USA* **2006,** 103, (1), 21-26.

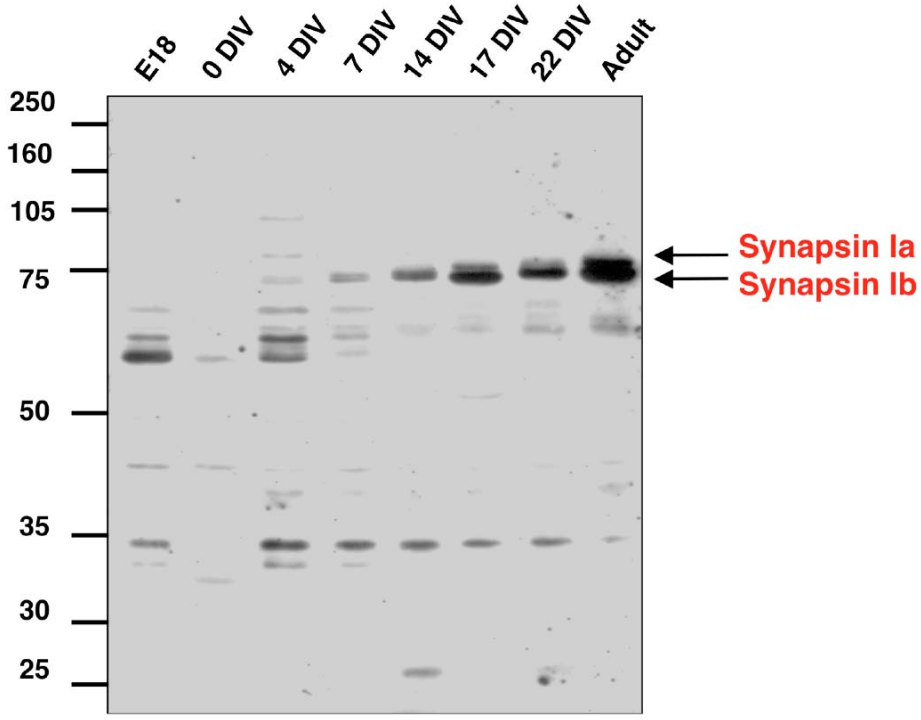

Figure 2.1. Fucα(1-2)Gal glycoproteins are present at different developmental days *in vitro* (DIV). Expression of fucosylated glycoproteins changes during the course of neuronal development. Synapsins Ia and Ib the predominant glycoproteins expressed in mature neuronal cultures and adult hippocampus.

Here, we show that Fucα(1-2)Gal carbohydrates are expressed on several glycoproteins during neuronal development. We demonstrate that synapsin Ia and Ib are the predominant Fucα(1-2)Gal glycoproteins expressed in the adult rat hippocampus. We also present molecular insights into the function of the Fucα(1-2)Gal epitope in regulating neuronal proteins, demonstrating that fucosylation increases the cellular half-life of synapsin in cells and modulates neurite outgrowth. Our studies suggest important

roles for the Fucα(1-2)Gal epitope in the regulation of synaptic proteins and the morphological changes that may underlie synaptic plasticity.

Expression of Fucα(1-2)Gal on Glycoproteins in the Hippocampus

We investigated whether Fucα(1-2)Gal glycoproteins are present in the hippocampus, a brain structure important for spatial learning and memory.[5] Cell lysates from adult rat hippocampus, embryonic day 18 (E18) hippocampus, and cultured embryonic hippocampal neurons were analyzed by Western blotting using an antibody (A46-B/B10) selective for Fucα(1-2)Gal.[6] Antibody A46-B/B10 has been shown to induce amnesia in animals,[6, 7] suggesting that it recognizes one or more physiologically relevant epitopes. We found that the Fucα(1-2)Gal epitope is present on several different proteins during neuronal development (Figure 2.1). In E18 hippocampal tissue, three major glycoproteins of approximately 35, 60, and 65 kDa are prominently observed, whose expression is significantly reduced in the adult hippocampus. In contrast, glycoproteins of approximately 73 and 75 kDa are found in mature cultured neurons and in adult brain tissue. Interestingly, expression of Fucα(1-2)Gal is observed on multiple proteins in developing neurons cultured for 4 and 7 days *in vitro* (DIV), periods when axons, dendrites and functional synapses are being formed, suggesting a putative role for these glycoproteins in the regulation of these processes. Thus, expression and/or fucosylation of Fucα(1-2)Gal glycoproteins appears to change dramatically during the course of neuronal development.

Fucα(1-2)Gal Is Enriched at Synapses

In collaboration with Cristal Gama, we next investigated the subcellular localization of Fucα(1-2)Gal sugars in neurons. Hippocampal neurons were cultured for 14 DIV to allow for synapse formation and were subsequently fixed, permeabilized and co-immunostained with antibody A46-B/B10 and an antibody against the neuronal marker tubulin. The Fucα(1-2)Gal epitope exhibited a punctate pattern consistent with enriched localization to neuronal synapses (Figure 2.2A). To examine whether the sugar was present at pre- or postsynaptic terminals, neurons were co-immunostained for Fucα(1-2)Gal and the presynaptic marker synapsin I or the postsynaptic marker spinophilin. We observed Fucα(1-2)Gal labeling in a subpopulation of the synapses (58 ± 2%; n = 350), overlapping with synapsin-positive puncta (Figure 2.2B) and generally apposing spinophilin-positive puncta (Figure 2.2C). Membrane de-lipidation did not alter the immunostaining pattern, which confirms the staining of glycoproteins rather than glycolipids (data not shown). These findings indicate that Fucα(1-2)Gal sugars are enriched on glycoproteins in presynaptic nerve terminals. The lack of complete co-localization of synapsin I-positive puncta with antibody A46-B/B10 may suggest that only a certain subset of synapses contain Fucα(1-2)Gal glycoproteins.

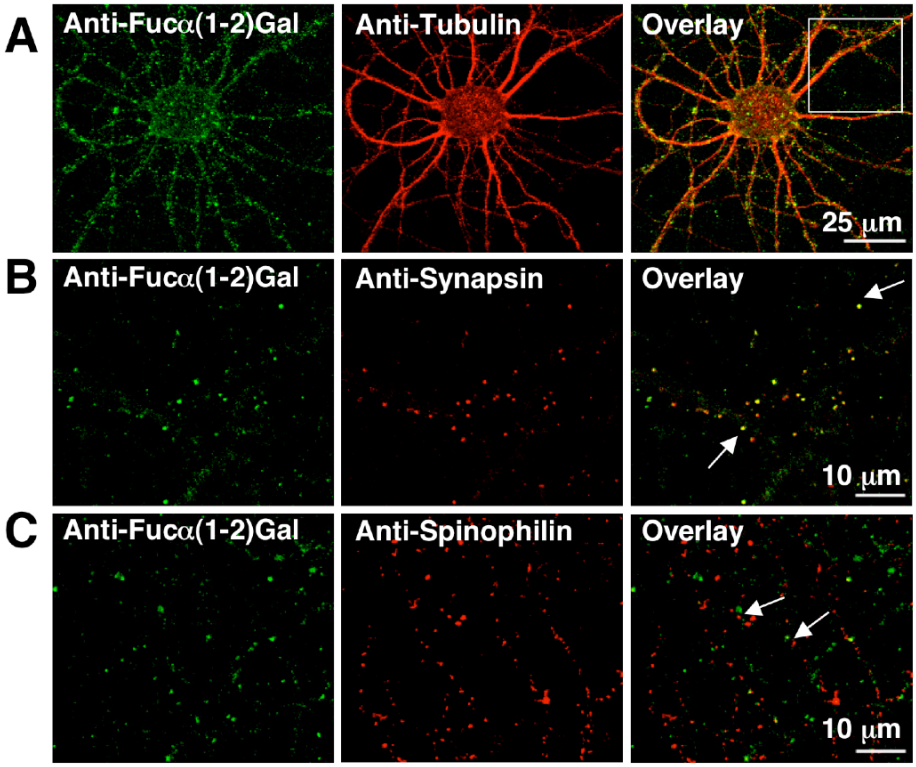

Figure 2.2. Fucα(1-2)Gal glycoproteins are enriched in presynaptic nerve terminals. Co-immunostaining of 14 DIV neurons with antibody A46-B/B10 (green) and tubulin (red, A), the presynaptic marker synapsin I (red, B), and the postsynaptic marker spinophilin (red, C). B and C are equivalent in area to the white rectangle in A. White arrows point to overlap of Fucα(1-2)Gal with synapsin (B) or apposition to the postsynaptic marker spinophilin (C). Image courtesy of Cristal Gama.

Synapsin Ia and Ib Are the Major Fucα(1-2)Gal Glycoproteins in the Hippocampus

We next sought to identify neuronal glycoproteins modified by the Fucα(1-2)Gal epitope. Attempts to purify Fucα(1-2)Gal glycoproteins from brain extracts using antibody A46-B/B10 were unsuccessful due to the relatively weak binding affinity of the antibody for the carbohydrate epitope (data not shown). In collaboration with Wen-I. Luo, we circumvented these challenges, by identifying potential glycoproteins using a

combination of subcellular fractionation, gel electrophoresis, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Adult rat hippocampal lysates were enriched in synaptic proteins using standard subcellular fractionation procedures. The crude synaptosomal fractions were resolved by 1-D or 2-D gel electrophoresis and 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. Proteins of interest were identified by immunoblotting and the corresponding bands were excised from a Coomassie-stained gel, digested with trypsin, and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). MS analysis revealed three potential Fucα(1-2)Gal-containing glycoproteins: synapsin Ia, synapsin Ib, and *N*-ethylmaleimide-sensitive factor (NSF). Eleven measured peptides matched the masses calculated from the National Center for Biotechnology Information (NCBI) non-redundant database for both synapsins Ia and Ib with greater than 50 ppm accuracy, and the unmodified matching peptides covered 11.2% of the amino acid sequence (data not shown). For NSF, 24 peptides were detected within 50 ppm, which provided 29.7% overall sequence coverage (data not shown).

To establish whether synapsin Ia/ Ib and NSF were indeed recognized by antibody A46-B/B10, each protein was immunoprecipitated and examined by Western blotting with antibody A46-B/B10 in collaboration with Wen-I. Luo. Upon immunoprecipitation, synapsins Ia and Ib were specifically detected by the antibody, whereas NSF was not recognized (Figure 2.3A). In addition, loss of the fucosylated bands corresponding to synapsins Ia and Ib was observed by Western blot analysis of adult hippocampal lysates

from synapsin I-deficient mice, confirming that synapsin Ia and Ib are the predominant Fucα(1-2)Gal glycoproteins (Figure 2.4). Treatment of purified synapsin I with PNGaseF and EndoH, enzymes that cleave *N*-linked oligosaccharides from proteins, did not abolish the interaction with antibody A46-B/B10, which suggests that the Fucα(1-

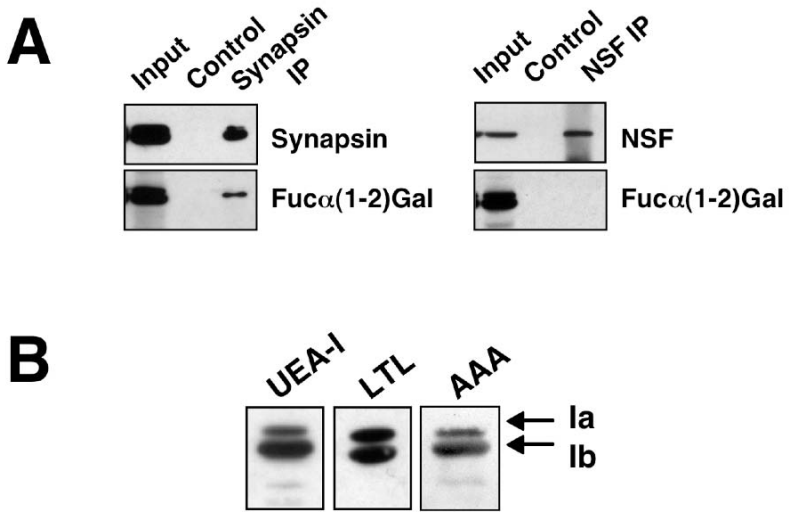

Figure 2.3. Synapsins Ia and Ib are Fucα(1-2)Gal glycoproteins. (A) Immunoprecipitation of synapsin and NSF demonstrates that synapsin is specifically recognized by antibody A46-B/B10. (B) Lectin immunoblotting with fucose-specific lectins confirms the presence of a fucosyl oligosaccharide on synapsins Ia and Ib. Figure 2.3 (A) courtesy of Wen-I. Luo.

2)Gal moiety is present on an *O*-linked glycan (data not shown). Together, these studies suggest that synapsin Ia and Ib are glycosylated with an *O*-linked fucosyl oligosaccharide.

Characterization of the Carbohydrate Structure on Synapsin

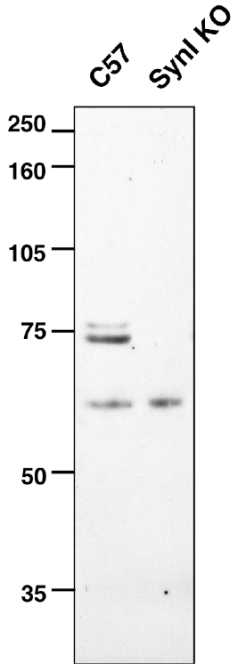

Figure 2.4. Loss of the fucosylated bands at 75 and 73 kDa in synapsin I KO mice confirms that synapsin I is recognized by antibody A46-B/B10.

Having identified the synapsins, we turned our attention to establishing the presence of the Fucα(1-2)Gal epitope. As an independent confirmation to recognition by antibody A46-B/B10, we examined the ability of α-L-fucose-specific lectins to bind to synapsin. *Lotus tetragonolobus* lectin (LTL) and *Ulex europeaus* agglutinin lectin (UEA-I) have been reported to interact strongly with terminal Fucα(1-2)Gal carbohydrates, whereas *Anguilla anguilla* lectin (AAA) prefers Fucα(1-3)Gal carbohydrates and interacts only weakly with Fucα(1-2)Gal.[8] Consistent with the presence of a Fucα(1-2)Gal moiety on synapsin, both LTL and UEA-I readily detected synapsins Ia and Ib (Figure 2.3B). However, AAA also recognized synapsin, indicating that lectins cannot be used to determine the nature of the fucose-galactose linkage on synapsin.

As fucosidases have been shown to hydrolyze specific glycosidic linkages, we next treated synapsins Ia and Ib with an α-(1-2)-fucosidase or an α-(1-3,4)-fucosidase

from *Xanthomonas manihotis*. Rapid deglycosylation of synapsin was observed upon treatment with the α-(1-2)-fucosidase (Figure 2.5). In contrast, the α-(1-3,4)-fucosidase, which hydrolyzes both Fucα(1-3) and Fucα(1-4) linkages, had no effect on the fucosylation levels of synapsin, even after 6 h of treatment (Figure 2.5 and data not shown). Together, these results provide strong evidence that synapsin Ia and Ib are covalently modified by the critical Fucα(1-2)Gal epitope.

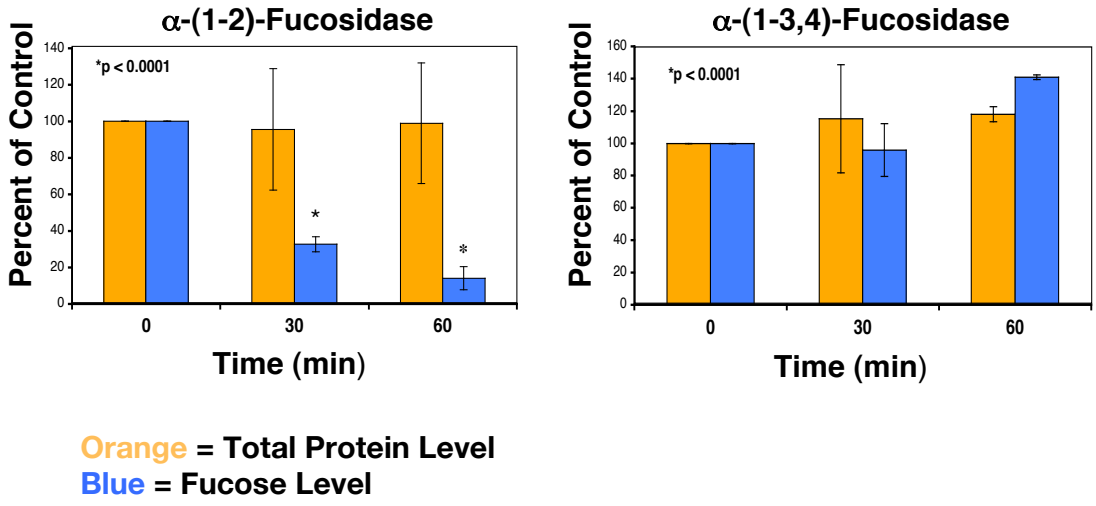

Figure 2.5. Treatment of bovine synaspin I with an α-(1-2)-fucosidase rapidly decreases synapsin I fucosylation levels as determined by immunoblotting and densitometry analysis with anti-synapsin and antibody A46-B/B10 (left panel). In contrast, an α-(1-3,4)-fucosidase failed to remove fucose from synapsin I (right panel), confirming the presence of a Fucα(1-2)Gal epitope on synapsin.

Synapsin I Is Fucosylated in Various Subcellular Compartments

We next investigated the extent of fucosylation on neuronal synapsin I. Subcellular fractions of rat forebrain lysates were analyzed for the levels of fucosylated or total synapsin (Figure 2.6A). Fucosylated synapsin was present in all subcellular fractions containing synapsin. Moreover, the relative level of fucosylated synapsin to

total synapsin was equivalent in the fractions examined. Quantitative analysis revealed that the membrane-associated to soluble ratio (LP2:LS2) of fucosylated synapsin was similar to that of synapsin (39:1 and 38:1 for fucosylated synapsin and synapsin, respectively; Figure 2.6B). These results suggest that neuronal synapsin is extensively fucosylated in various subcellular compartments and that fucosylation does not alter the subcellular localization of synapsin.

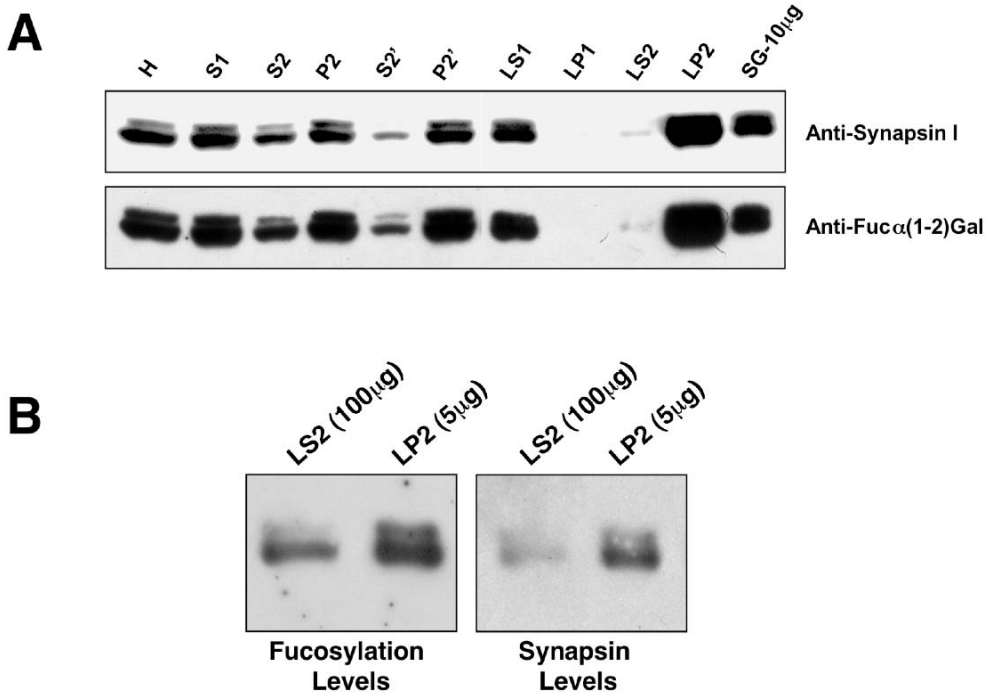

Figure 2.6. (A) Synapsin I is fucosylated in all subcellular compartments. 100 μg of total fractions were loaded H=homogenate, S1, S2, S2' are soluble fractions, P2=insoluble fraction, P2'=crude synaptosomes LS1=soluble fraction from synaptosome lysis, LP1=insoluble fraction from synaptosome lysis, LS2=synaptosol, LP2=crude synaptic vesicles, SG=synaptic vesicles after sucrose gradient purification (B) Densitometry analysis of soluble (LS2) versus membrane-associated (LP2) suggests that synapsin I is fucosylated to equivalent degrees in these subcellular fractions.

Inhibiting Synapsin Fucosylation Significantly Decreases Its Cellular Half-Life

To investigate the impact of fucosylation on the functional properties of synapsin, we inhibited the fucosylation of synapsin in cells using 2-dGal. 2-dGal has previously been shown to prevent the fucosylation of glycoproteins.[9] Upon cellular uptake, 2-dGal is converted via the Leloir pathway to the corresponding activated uridyl diphosphate (UDP) analogue (Figure 2.7A).[9, 10] UDP-2-deoxy-galactose competes with UDP-galactose for incorporation into glycan chains and thereby terminates the chain by preventing formation of the Fucα(1-2)Gal linkage.[9]

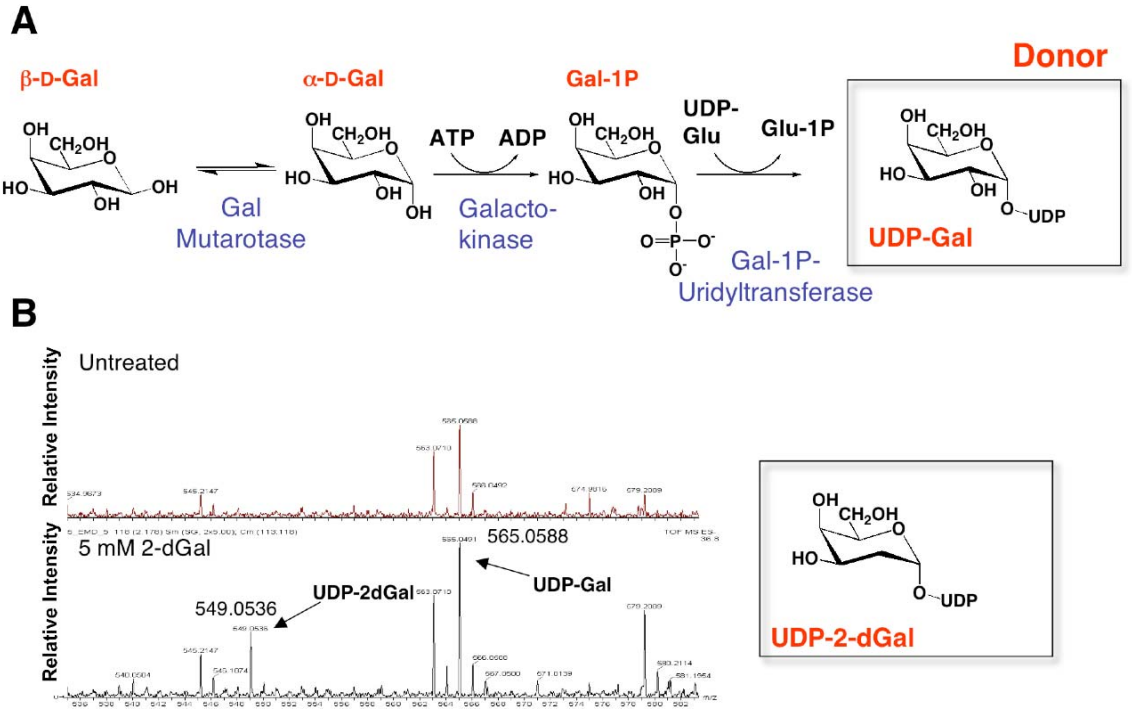

Figure 2.7. (A) Leloir pathway for galactose metabolism. (B) UDP-2-dGal is formed in cells treated with 2-dGal as determined by LC-MS.

We first established that incubation of HeLa cells with 2-dGal leads to the biosynthesis of UDP-2-deoxy-galactose. Sugars were extracted from HeLa cells treated with 2-dGal, and analyzed by liquid chromatography MS (LC-MS). We observed significant formation of UDP-2-deoxy-galactose by LC-MS analysis of sugar extracts,

demonstrating that 2-dGal is an efficient unnatural substrate for the Leloir pathway enzymes (Figure 2.7B). We next investigated the effects of 2-dGal on synapsin I expressed in HeLa cells. Cell lysates containing equivalent amounts of transfected

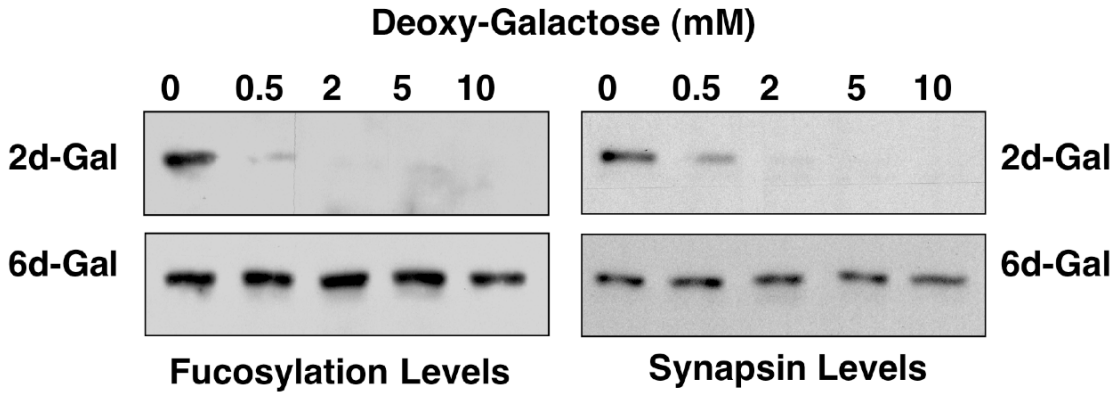

Figure 2.8. Treatment of HeLa cells expressing synapsin I with 2-dGal affects synapsin fucosylation and expression levels (top panels). Treatment with 6-dGal has no effect (bottom panels).

protein were resolved by SDS-PAGE, and the fucosylation and protein levels of synapsin were measured by immunoblotting. Consistent with the presence of a Fucα(1-2)Gal epitope on synapsin, 2-dGal had a dramatic effect on the fucosylation level of synapsin (Figure 2.8). Surprisingly, the 2-dGal treatment also led to a significant decrease in the level of synapsin protein. The effects appear to be specific to 2-dGal, as treatment with other deoxy-galactose sugars, including 6-deoxy-D-galactose (6-dGal), had no effect on either the fucosylation or protein levels of synapsin (Figure 2.8 and data not shown). Thus, 2-dGal was found to specifically affect synapsin levels and fucosylation specifically through the C2 position of galactose.

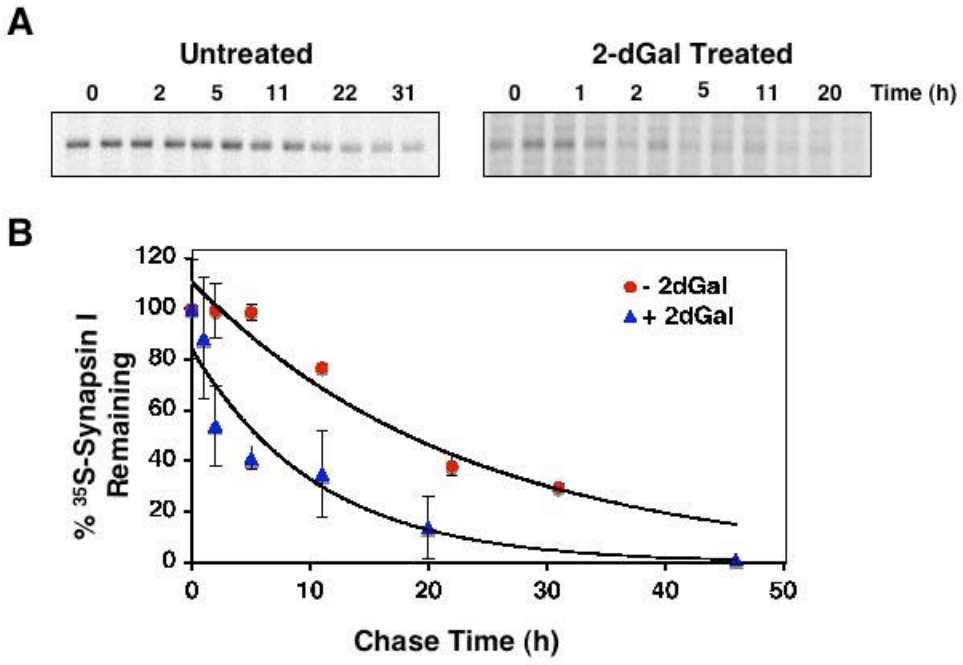

Figure 2.9. Synapsin I defucosylation decreases its cellular half-life. (A) ^{35}S-labeled synapsin I after indicated chase periods, samples are in duplicate. (B) Graphical representation of densitometry analysis of synapsin I remaining.

Based on these results, we postulated that fucosylation might be critical for modulating the half-life and turnover of synapsin in cells. We conducted pulse-chase experiments of synapsin Ia expressed in HeLa cells in the presence or absence of 2-dGal. Cells were pulse labeled with ^{35}S- L-cysteine and ^{35}S- L-methionine and then incubated for various times in the absence of radioisotopes. After the indicated chase times, synapsin Ia was immunoprecipitated from the cell lysates. A relatively long half-life of 18 h was observed for synapsin Ia (Figure 2.9), consistent with previous studies of endogenous synapsin I in cultured hippocampal neurons ($t_{1/2} \approx 20$ h) [11]. In contrast, treatment of the cells with 2-

dGal led to a dramatic reduction in synapsin half-life to 5.5 h. These results indicate that de-fucosylation of synapsin induces its degradation in cells.

Synapsin Degradation Is Mediated by the Calcium-Dependent Protease Calpain

To investigate the molecular mechanisms responsible for synapsin turnover, cells expressing synapsin were treated with various inhibitors of protein degradation in the presence or absence of 2-dGal. Specifically, we used the lysosomal inhibitors bafilomycin A1 and ammonium chloride, the proteasome inhibitor MG132, and two inhibitors of the calcium-dependent protease calpain. With the exception of MG132, the inhibitors had minimal effects on synapsin expression levels in the absence of 2-dGal (Figure 2.10). As before, 2-dGal treatment of the cells significantly reduced the levels of synapsin expression. Notably, inhibition of the protease calpain using a calpain inhibitor

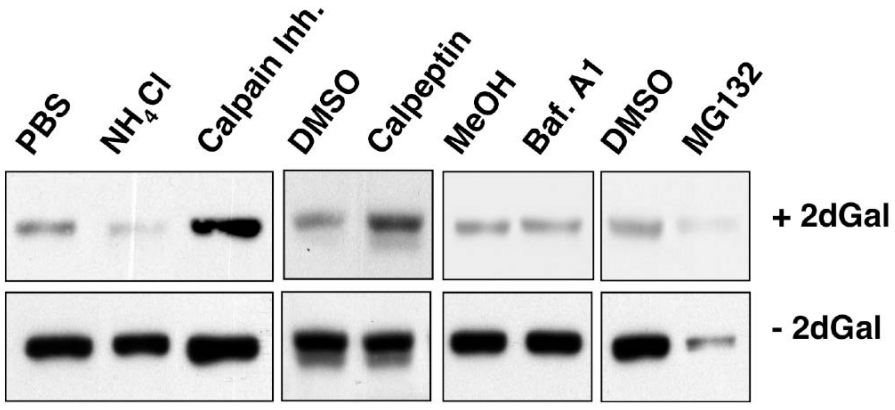

Figure 2.10. Inhibition of the calcium-activated protease calpain with calpain inhibitor peptide or calpeptin rescues synapsin from degradation by 2-dGal. The lysosome was inhibited with ammonium chloride and bafilomycin A1. The proteasome was inhibited with MG132.

peptide or calpeptin rescued the effects of 2-dGal, significantly attenuating the loss of synapsin, whereas the lysosomal and proteasomal inhibitors could not rescue synapsin from degradation. These data suggest that fucosylation protects synapsin from rapid degradation mediated at least in part by the Ca^{2+}-activated protease calpain.

Fucosylation Modulates the Expression of Synapsin in Neurons and Neurite Outgrowth

To examine the effects of 2-dGal on synapsin fucosylation in neurons, neurons were cultured for 7 DIV to allow for adequate expression of synapsin and subsequently treated with either 2-dGal or 6-dGal for 3 days. 2-dGal dramatically reduced the expression of synapsin I in cultured neurons (Figure 2.11A). Importantly, the effects of

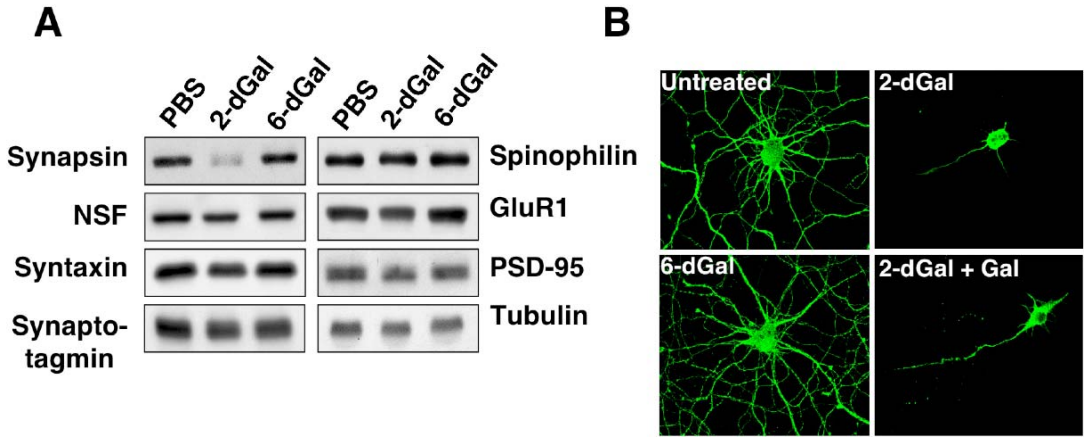

Figure 2.11. (A) 2-dGal reduces synapsin I expression whereas 6-dGal has no effect. Other pre- and postsynaptic proteins, as well as tubulin, are unaffected by 2-dGal treatment. (B) E18 cortical neurons were cultured for 7 DIV and treated with 2-dGal or 6-dGal for 3 days. 2-dGal led to a dramatic collapse of synapses and reduction in neurite length (top panels) whereas 6-dGal had no effect (bottom, left panel). The effects were partially rescued by subsequent treatment with Gal. Figure 2.11 (B) courtesy of C. Gama.

2-dGal appear to be selective as treatment with another deoxy sugar, 6-dGal, did not alter the expression of synapsin. Moreover, the effects of 2-dGal were specific to synapsin as

the expression of other synaptic proteins, including NSF, synaptotagmin, syntaxin, PSD-95, the AMPA receptor GluR1 subunit, and spinophilin, was unchanged by the 2-dGal treatment.

As the synapsins play important roles in neuronal development and synaptogenesis,[12, 13] we next investigated whether 2-dGal might influence neuronal growth and morphology in collaboration with Cristal Gama. Hippocampal neurons were cultured for 7 DIV as above to establish functional synapses and subsequently incubated for 3-5 days with 2-dGal at various concentrations. Treatment with 2-dGal induced a

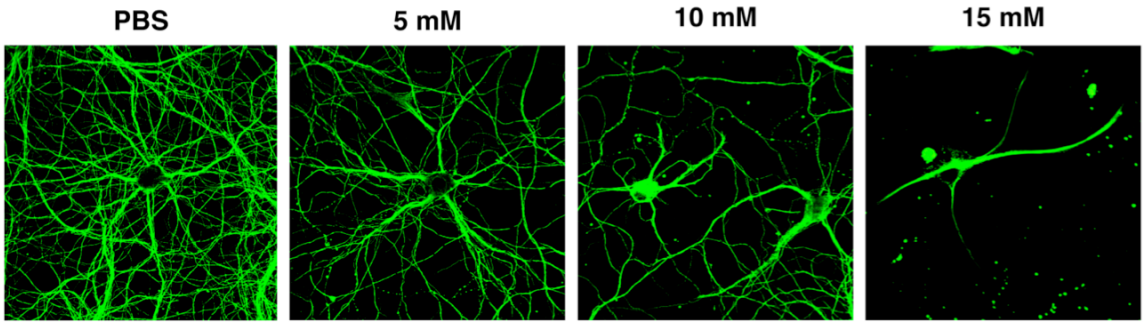

Figure 2.12. Concentration-dependence of 2-dGal on neuronal morphology. Neurons were cultured for 7 DIV and treated for 3 days with the indicated amounts of 2-dGal. Image courtesy of C. Gama.

dramatic retraction of neurites and collapse of synapses, whereas 6-dGal had no effect (Figure 2.11B). The effects of 2-dGal could be partially rescued by subsequent incubation of the neurons with D-Gal, which is expected to re-establish the Fucα(1-2)Gal linkage (Figure 2.11B, 2.06 ± 0.14 fold rescue; $n = 50$; $p < 0.0001$). These results suggest that disruption of the Fucα(1-2)Gal linkage on neuronal glycoproteins has a profound impact on neurite outgrowth and neuronal morphology.

One potential mechanism by which 2-dGal might influence neuronal morphology is by regulating the function and/or expression of synapsin in presynaptic terminals.

Notably, the phenotypic effects of 2-dGal on neurite outgrowth at 5 mM concentration are similar to deletion of the synapsin I gene, which results in retarded neurite outgrowth and delayed synapse formation[12] (Figure 2.12). However, because other neuronal proteins bear the Fucα(1-2)Gal modification (Figure 1A), these proteins might also contribute to the morphological effects observed upon de-fucosylation.

To examine the relative contribution of synapsin I to the effects of 2-dGal, neurons were cultured from synapsin I-deficient or wild-type mice for 2 days in collaboration with Cristal Gama, treated in the presence or absence of 2-dGal for 3 days and examined by confocal fluorescence microscopy. Top panels (Figure 2.13A) are representative images of untreated wild-type and synapsin I-deficient neurons after 5 days in culture, respectively. Neurons from wild-type mice treated with 2-dGal had shorter neurites relative to their untreated wild-type counterparts (left panels in Figure 2.13A). The

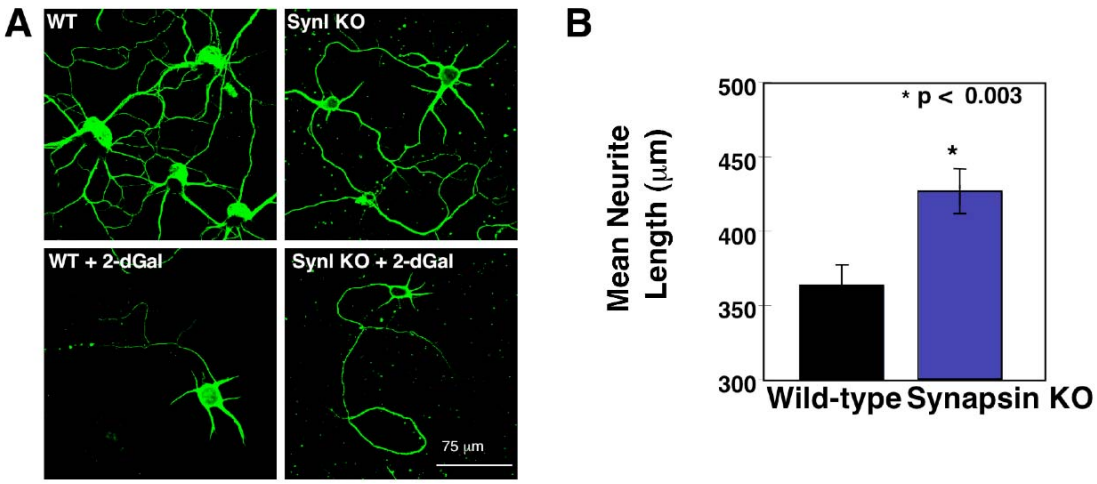

Figure 2.13. 2-dGal induces less neurite retraction in synapsin I KO neurons. (A) Neurons from synaspin I KO or wild-type neonatal pups were cultured for 2 DIV and treated for 3 days with PBS (top panels) or 2-dGal (bottom panels). (B) Quantification of neurite lengths of 2-dGal treated wild-type and synaspin I hippocampal neurons.

effects of de-fucosylation at a concentration of 15 mM 2-dGal were more pronounced than elimination of the synapsin I gene (Figure 2.13A). Finally, treatment with 2-dGal induced a more dramatic neurite retraction in wild-type relative to synapsin-deficient neurons (bottom panels in Figure 2.13A). Although the length and extensive overlap among 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 longer neurites than wild-type neurons upon treatment with 2-dGal (bar graph in Figure 2.13B).

Synapsin I Is the Only Synapsin Gene Fucosylated

Synapsin I is a member of a multigene family of neuronal phosphoproteins that regulate neurotransmitter release.[14] They are encoded by three genes termed synapsin I, II, and III with different splice variants creating distinct isoforms.[15]

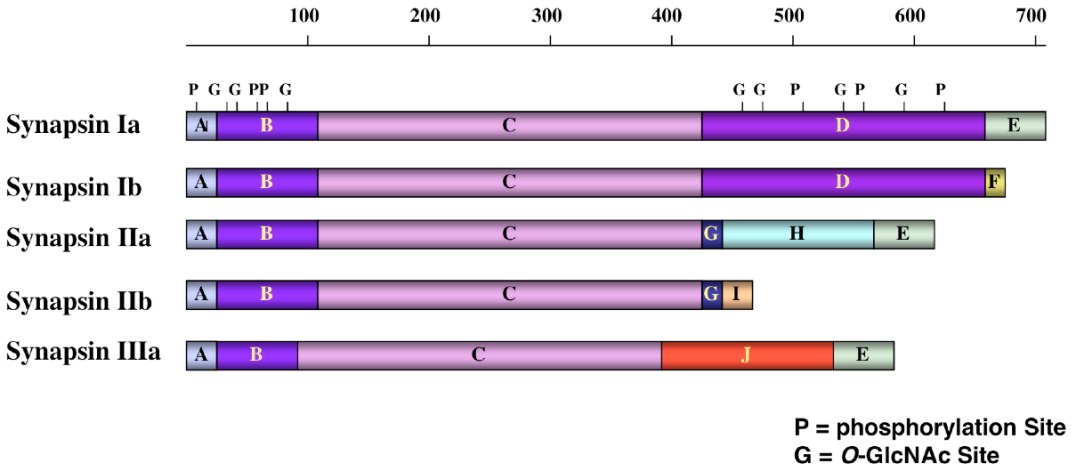

Figure 2.14. Domain organization of the synapsin gene family.

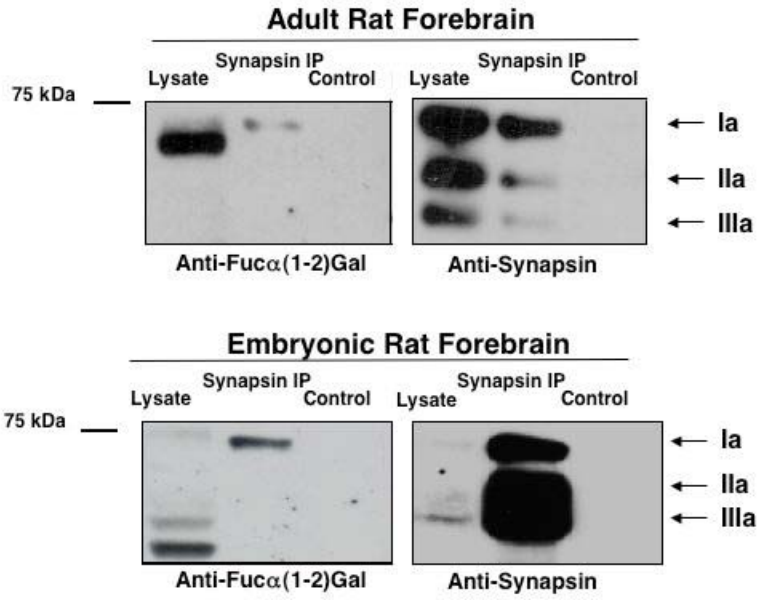

Figure 2.15. Only synapsin I is fucosylated. The synapsins were immunoprecipitated from adult (top panels) and embryonic (bottom panels) rat forebrain. Immunoprecipitates were proved with antibody A46-B/B10 (left panels) and an anti-synapsin (right panels) antibody.

Synapsins Ia and Ib are both splice variants of the same gene. The synapsins are highly conserved at the N-terminal region in domains A-C, and differ at the C-terminal domains (Figure 2.14). Domain C, which is the most conserved domain amongst all synapsin isoforms, is important for interaction with synaptic vesicles, via insertion of a small peptide directly into the lipid bilayer of the vesicles.[16] Due to the extensive sequence conservation between genes, we next investigated whether other synapsin family members were fucosylated. Synapsins Ia, IIa, and IIIa were immunoprecipitated with antibody G304,[17] which recognizes the E domain of the three synapsin genes, from rat

forebrain lysates of adult and embryonic rats. In adult rats, expression of synapsin Ia is highest, and in embryonic tissues, expression of synapsins IIa and IIIa is more prominent. Immunoprecipitates were probed with antibody A46-B/B10 to determine whether other synapsins were fucosylated. Synapsin Ia was fucosylated in both embryonic and adult rat brain as indicated by the band present in the immunoprecipitated lanes. Surprisingly, neither synapsins IIa or IIIa were found to be fucosylated. This is most evident from the blot of embryonic lysates, where synapsins IIIa and IIa have greater expression, but no bands were detected by the anti-Fucα(1-2)Gal antibody. (Figure 2.15). Due to the high degree of sequence conservation at the N-terminal regions of the of synapsin gene family, these data implicate the D domain of synapsin I as containing the potential Fucα(1-2)Gal epitope.

Synapsin I Contains 1.5 - 3.2 Fucosylation Sites

To gain insight into the stoichiometry of fucosylation, we compared synapsin I purified from bovine brain against a fucosylated bovine serum albumin (BSA) standard (Figure 2.16). 2'-Fucosyllactose (Fucα(1-2)Galβ(1-4)GlcNAc) was conjugated to BSA using reductive amination chemistry, and an epitope density of approximately 3.0 ± 0.8 moles of fucose per BSA molecule was determined using the Habeeb assay.[18] Comparison of the relative binding of antibody A46-B/B10 to synapsin I versus this standard revealed a stoichiometry of approximately 1.5 - 3.2 Fucα(1-2)Gal epitopes per synapsin molecule.

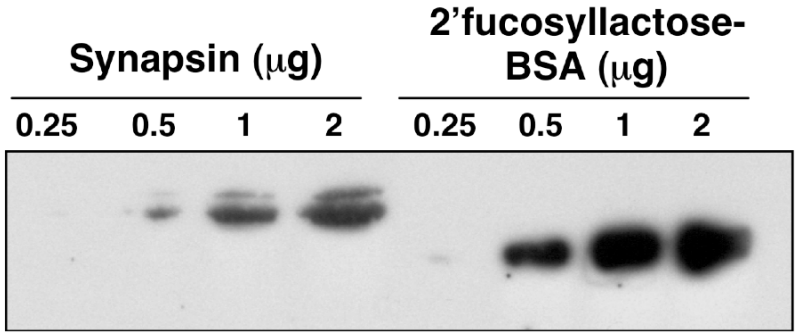

Figure 2.16. Bovine synapsin I and BSA conjugated to 2'fucosyllactose were resolved by SDS-PAGE and examined by densitometry analysis to determine the extent of synapsin I fucosylation. Analysis reveals a stoichiometry of 1.5 – 3.2 Fucα(1-2)Gal epitopes per synapsin molecule.

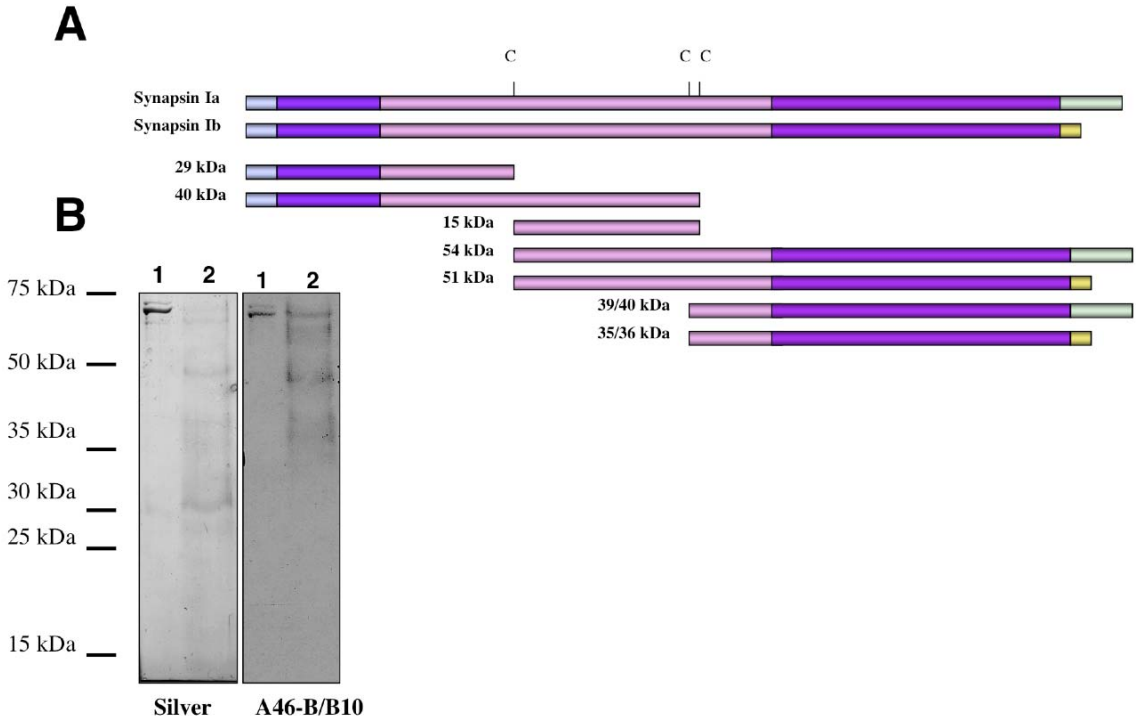

Figure 2.17. NTCB cleavage of purified synapsin I. Bovine synapsin I was cleaved with NTCB under alkaline conditions. (A) Expected fragments observed after NTCB cleavage. (B) Left panel is a silver stain of undigested (lane 1) and digested (lane 2) to observe all peptide fragments. Right panel is a Western blot to demonstrate fragments labeled by the Fucα(1-2)Gal antibody.

Synapsins Ia and Ib are Fucosylated in Domain D via an *O*-Linkage

As immunoprecipitation studies implicated the D domain of synapsin I as containing a Fucα(1-2)Gal moiety, we sought to address whether this was the case. Purified bovine synapsins Ia and Ib were subjected to cleavage by the small molecule reagent 2-nitro-5-thiocyanobenzoic acid (NTCB). This reagent will cleave proteins after cysteine residues under alkaline conditions.[19] Synapsins Ia and Ib contain 3 cysteine residues at amino acids 222, 360, and 370. Figure 2.17 shows a map of synapsins Ia and Ib cleavage products obtained after NTCB treatment. Products from the cysteines at positions 360 and 370 resulted in peptides indistinguishable by SDS-PAGE, and thus are represented as one peptide in the figure. After NTCB treatment, proteins were separated by SDS-PAGE, and either transferred to PVDF for immunoblotting, or stained with silver

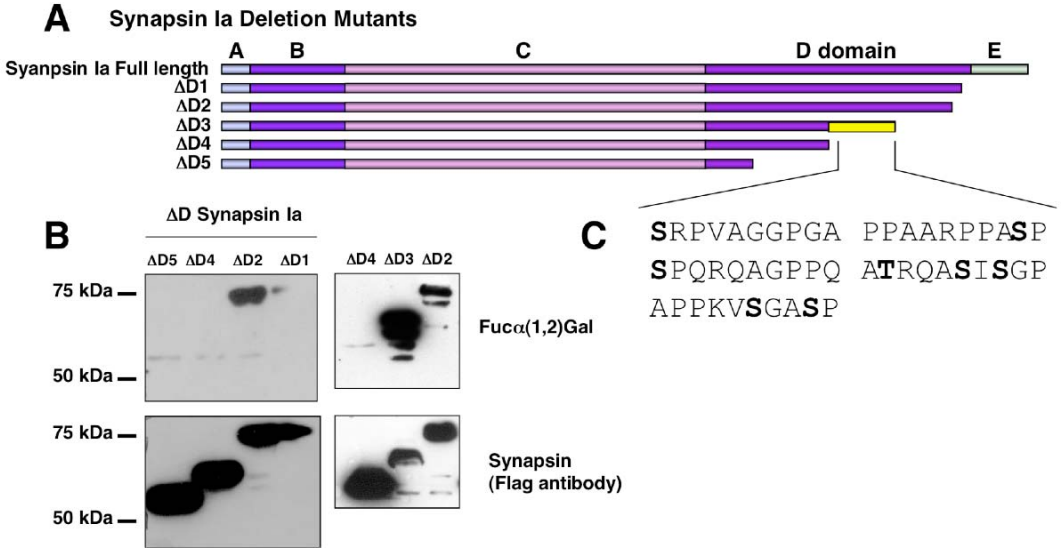

Figure 2.18. (A) Deletion mutants created from the Flag-synapsin I construct. (B) Western blot anlaysis reveals that the Fucα(1-2)Gal antibody recognizes domains ΔD3, ΔD2, and ΔD1 (top panels) but not domains ΔD4 and ΔD5. (C) Sequence of amino acids in the region narrowed down by deletion mutagesis that should contain a Fucα(1-2)Gal moiety.

nitrate to identify all cleavage fragments. Transferred proteins were immunoblotted with antibody A46-B/B10. The smallest fragments recognized by the Fucα(1-2)Gal antibody were the 39/40 kDa fragment of synapsin Ia and the 35/36 kDa fragment corresponding to synapsin Ib (data not shown). These fragments contain a short segment of domain C, along with domain D in its entirety, confirming the presence of a Fucα(1-2)Gal epitope in domain D of synapsins Ia and Ib.

To identify the region of glycosylation within the D domain, we created a series of deletion mutants of this region (Figure 2.18A). We first subcloned the cDNA of synapsin I into the pFLAG-CMV vector (Sigma) to place an N-terminal Flag tag on the protein. The construct allows for expression in heterologous host cells, and the Flag-tag facilitates immunoprecipitation and Western blot analyses. We next created truncation mutants by PCR, and subsequently expressed the deletion mutants in HeLa cells to examine fucosylation levels with antibody A46-B/B10. All truncation mutants were successfully expressed, and the Fucα(1-2)Gal-specific antibody was able to detect fucose on deletion mutants ΔD1 - ΔD3 (Figure 2.18B). The antibody did not detect any fucose on deletion mutants ΔD4 - ΔD5. This suggests that there is at least one Fucα(1-2)Gal epitope between residues 531 and 658. As there are no asparagines residues within this region, the modification is attached via an *O*-linkage to either a serine or threonine residue within this domain (Figure 2.18C).

Synapsin I Contains a Putative Fucα(1-2)Gal Site at Ser579

To determine the exact site of fucosylation, we created a series of point-mutants corresponding to serine and threonine residues within amino acids 531-590 of the Flag-synapsin I construct. The Flag-synapsin I point mutants were transiently expressed in HeLa cells and examined for their expression and recognition by Fucα(1-2)Gal-specific

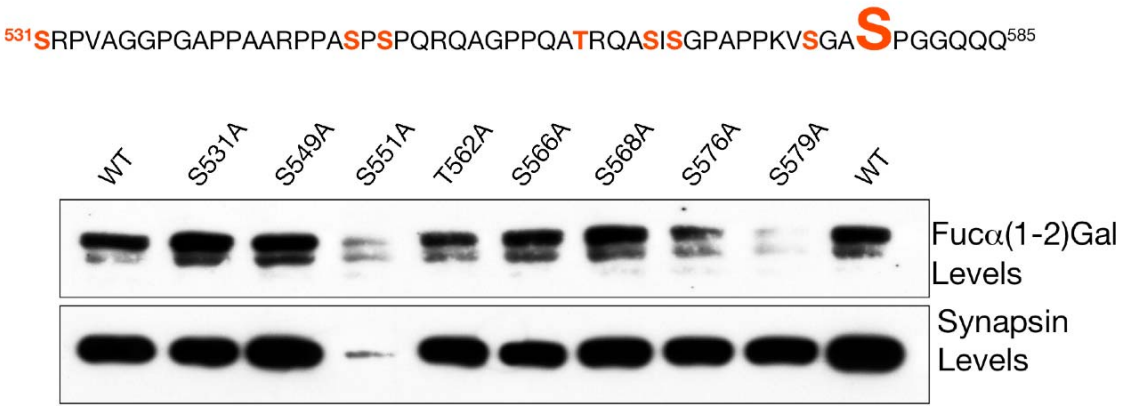

Figure 2.19. Identification of synapsin I Fucα(1-2)Gal sites by site-directed point mutagenesis. Only Ser579 has an appreciable decrease in fucosylation levels as detected with antibody A46-B/B10.

antibody A46-B/B10. All point mutants were capable of being expressed, however the S531A mutant did not express as well as the other point mutants (Figure 2.19). However, we observed a significant decrease in fucosylation of point mutant S579A, whereas fucosylation of other mutants appeared to be unaffected. There was still some detection by antibody A46-B/B10 of the S579A mutant, suggesting the presence of another site of fucosylation consistent with densitometry analysis. These data suggest that synapsin I contains a putative Fucα(1-2)Gal moiety at Ser 579, and another site of fucosylation may exist in the protein.

To determine whether another fucosylation site exists in synaspin I, we created a series of double mutants using the S579A construct, of all Ser and Thr residues between

amino acids 531 and 705. Furthermore, despite our data suggesting that the Fucα(1-2)Gal moiety is on an *O*-linked glycan in the D domain, we also created double mutants of three Asn residues at amino acids 661, 668, and 671 as well as Ser and Thr residues in the E domain of synapsin I. All mutants were capable of being expressed, and all mutants appeared to be recognized by antibody A46-B/B10 to a similar degree. To date we have not been able to identify a second site with the double mutants (data not shown). However, experiments are still in progress to identify the sites.

UEA1 Lectin Recognizes a Different Fucα(1-2)Gal Moiety on Synapsins Ia and Ib than Antibody A46-B/B10

To facilitate our identification of fucosylation sites, we employed lectin immunoblotting with UEA1. Surprisingly, UEA1 recognizes the S579A mutant as well as wild-type synapsin I, suggesting that UEA1 lectin recognizes a different epitope on synapsin I. To identify the region that UEA1 interacts with, we used the ΔD deletion mutants to narrow down the site of fucosylation. Interestingly, UEA1 only interacts with deletion mutants ΔD1 and ΔD2 and not with ΔD3, which antibody A46-B/B10 interacts with (data not shown). This suggests that UEA1 recognizes a Fucα(1-2)Gal moiety between amino acids 591 and 659. We are currently exploring the ability of UEA1 lectin to bind to synapsin I double mutants, however, results have been inconclusive to date.

Conclusions

Increasing evidence has linked synaptic activity with changes in the levels of protein fucosylation in the brain. For instance, both task-specific learning and LTP have

been shown to enhance protein fucosylation at the synapse.[1, 2] Moreover, the activity of fucosyltransferases increases substantially during synaptogenesis[3] and upon passive avoidance training in animals.[4] Together, these studies suggest that protein fucosylation may relate to the dynamic regulation of synaptic proteins. Studies have implicated a particular carbohydrate, Fucα(1-2)Gal, in cognitive processes such as learning and memory. Although Fucα(1-2)Gal has been postulated to covalently modify synaptic glycoproteins, the identity of such proteins has remained elusive. In this study, we identify synapsins Ia and Ib as the major Fucα(1-2)Gal glycoproteins in maturing neuronal cultures and the adult rat hippocampus. Our results provide the first molecular insights into the functions of Fucα(1-2)Gal and demonstrate that these carbohydrates play a critical role in the regulation of synaptic proteins.

The synapsins are a family of neuron-specific phosphoproteins that are associated with synaptic vesicles, and are homologous at their *N*-terminal region.[14] Studies indicate that these proteins regulate multiple aspects of neuronal function including neurotransmitter release by regulating the supply of releasable vesicles during periods of high activity,[14, 26] and controlling synaptic vesicle dynamics in developing neurons via a cAMP-dependent pathway.[27] Accordingly, synapsin knock-out mice show reduced numbers of synaptic vesicles within nerve terminals and exhibit significant alterations in neurotransmitter release and synaptic depression.[14, 28] The synapsins have also been implicated in diverse aspects of neuronal development, including axonal outgrowth, nerve terminal development, synapse formation, and synapse maintenance.[12, 29, 30]

Our studies indicate that fucosylation of synapsin critically impacts its expression and turnover in presynaptic nerve terminals. The potential to modulate the expression

level of synapsin is expected to have important consequences for neuronal function. For instance, the addition of exogenous synapsin I to embryonic *Xenopus* spinal neurons has been shown to accelerate structural and functional maturation of neuromuscular synapses, including the early compartmentalization of synaptic vesicles into nerve terminals and a mature form of quantal secretion.[31, 32] Conversely, reduction in synapsin expression levels also has profound effects: synapsin-deficient mice exhibit significant delays in axonal extension, neuronal differentiation, and synapse formation.

We found that de-fucosylation of synapsin promoted its degradation by calcium-activated calpain proteases, which are a family of non-lysosomal, neutral cysteine proteases. These observations corroborate and extend recent findings suggesting a role for calpain in the regulation of both synaptic transmission and neuronal morphology.[33, 34] Interestingly, calpain has been suggested to be critical for refilling depleted vesicle stores in sensory motor synapses of *Aplysia* via a mechanism involving the cleavage of potential substrates such as synapsin.[35] In addition, calpain was found to degrade SNAP-25 (synaptosomal-associated protein of 25 kDa), a protein essential for neurotransmitter release, in a calcium-dependent manner.[36] The proteolytic activity of calpain has also been shown to induce cytoskeletal rearrangements, leading to both facilitation and inhibition of neurite outgrowth.[34, 37, 38] Although further studies will be necessary to understand more fully the mechanisms of calpain-mediated synapsin degradation, our results suggest an expanded role for this protease family in presynaptic nerve terminals and reveal that fucosylation has profound effects on the half-life of synapsin, possibly preventing synapsin from calcium-activated degradation.

In this study, we investigated the role of synapsin fucosylation on neuronal growth and morphology. 2-dGal, a small molecule inhibitor of Fucα(1-2)Gal linkages, served as a valuable tool to de-fucosylate synapsin and dissect the role of the carbohydrate in modulating 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. We believe that the extent of neurite retraction in synapsin-deficient mice is less pronounced because the primary target of 2-dGal, synapsin I, is missing. Indeed, the bar graph shown in Figure 2.13 likely represents a lower estimate of the contribution of synapsin as neurites from synapsin-deficient neurons are shorter than those from wild-type neurons prior to treatment with 2-dGal. Based on these results, we propose that de-fucosylation may disrupt synapsin function, leading to its degradation and neurite retraction. Although further studies will be needed to resolve whether synapsin fucosylation stimulates or inhibits neurite outgrowth, these results strongly support the notion that synapsin fucosylation plays a role in modulating neuronal growth and morphology.

Our data also implicate other Fucα(1-2)Gal glycoproteins in the regulation of neuronal morphology. We demonstrate that Fucα(1-2)Gal carbohydrates are not limited to synapsin, but are found on several additional proteins in developing neurons. Expression of the sugar and/or these glycoproteins changes dramatically during the course of neuronal development. We found that disruption of synapsin fucosylation contributed, but was not fully sufficient, to account for the striking neurite retraction induced by 2-dGal. For instance, 2-dGal had stronger effects on neurite outgrowth at

high concentrations relative to deletion of the synapsin I gene, suggesting that 2-dGal may disrupt the fucosylation of other Fucα(1-2)Gal glycoproteins that influence neuronal morphology. Moreover, 2-dGal was still capable of inducing partial neurite retraction in synapsin-deficient neurons and young cultured neurons where synapsin expression is low.[20] Thus, Fucα(1-2)Gal sugars appear to modulate the functions of multiple proteins involved in neuronal morphology and exert their effects via several distinct molecular mechanisms.

Finally, our findings may shed light on behavioral and electrophysiological studies implicating Fucα(1-2)Gal in long-term memory storage. Alterations in neuronal morphology, such as dynamic changes in dendritic spine number and shape, occur during memory consolidation and LTP.[39, 40] Future studies will investigate whether Fucα(1-2)Gal sugars and their associated glycoproteins contribute to structural remodeling events that underlie synaptic plasticity.

Materials and Methods

Neuronal Cultures and Immunocytochemistry. Hippocampal and cortical neurons were cultured and immunostained as described previously.[20] Synapsin I knockout mice [12] were generously provided by Drs. H.T. Kao and P. Greengard (The Rockefeller University, New York, NY). Antibody A46-B/B10[6] was a generous gift from Dr. U. Karsten (Max-Delbruck 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 (1:5000; Molecular Probes) and anti-spinophilin (1:10000; [21]) antibodies

were added in 3% BSA for 2 h at 37 °C. Goat anti-mouse IgM-AlexaFlour 488 or goat anti-rabbit IgG AlexaFluor 568 (1:250; Molecular Probes) was added for 1 h at 37 °C in 3% BSA.

Adult Rat Hippocampal Dissection, Lysis, and Immunoprecipitation. The hippocampi of adult male Sprague-Dawley rats (100-150 g) were homogenized in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.2% sodium deoxycholate, 1% NP-40 supplemented with protease inhibitors with a glass Dounce homogenizer and sonicated briefly. Supernatants were clarified by centrifugation at 12,000× g for 10 min, and protein concentrations were determined using the BCA protein assay method (Pierce). Immunoprecipitations were performed at approximately 2 mg/mL lysate using an anti-synapsin (Chemicon) or anti-NSF (Synaptic Systems) antibody.

Synaptic Vesicle Purification from Adult Rat Brain. Synaptic vesicles were purified using a sucrose density gradient as described previously.[22]

Treatment of Cells with Deoxy-Galactose Analogues. Rat neuronal cultures were treated after 7 days as described.[20] Neuronal cultures from C57BL/6 E16 mice were treated after 7 DIV with 2-dGal (0, 5, 10, or 15 mM) in PBS for 5 days. Neurons from C57BL/6 and synapsin I knockout P0 mice were cultured for 2 days and then treated for 3 days with 15 mM 2-dGal. HeLa cells were seeded at 6×10^5 cells per 60 mm dish in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and incubated at 37 °C / 5% CO_2 for 24 h. After pretreatment with the deoxy-galactose analogues (0.5 - 10 mM) for 1 h, the cells were transfected at ~60% confluence with the plasmid pCMV-FLAG-Synapsin Ia and pSV-β-galactosidase (Promega) using Lipofectamine 2000 (Invitrogen). After 22 h, the cells were harvested, resuspended in

PBS, and either lysed in 1% boiling SDS (70% of the cells) or analyzed for transfection efficiency using a β-galactosidase assay (30% of the cells). For synapsin degradation experiments, cells were treated with 2-dGal or PBS as above, followed by treatment 4 h post-transfection with bafilomycin A1 (100 nM, Acros), MG132 (5 μM, Sigma), ammonium chloride (25 mM, Fisher), calpain inhibitor peptide (33 μM, Sigma), or calpeptin (2 μM, Alexis Biochemicals). Cells were lysed as above after 15 h of treatment, resolved by SDS-PAGE, and analyzed by immunoblotting with chemiluminescence detection (Pierce).

Pulse-Chase Analysis. HeLa cells were transfected and treated with 2-dGal as above with the following modifications. Cells were transfected at ~95% confluence with pCMV-FLAG-Synapsin Ia. Sixteen hours post-transfection, cells were washed with PBS and starved in DMEM (without L-cysteine and L-methionine) supplemented with 10% FCS for 1 h. Cells were pulse labeled with 100 μCi/mL Easytag™ Express Protein Labeling Mix (Perkin Elmer) for 1 h, washed with DMEM, and chased in conditioned medium collected from the cells prior to the starvation period. Cells were lysed in 1% boiling SDS and neutralized with an equal volume of NETFD (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 6% NP-40 supplemented with protease inhibitors). Synapsin was immunoprecipitated using anti-FLAG M2 affinity agarose (Sigma), resolved by SDS-PAGE, fixed with destain, treated with Amplify (Amersham), and dried. ^{35}S-labeled protein was measured using a Typhoon 8600 Variable Mode Imager (Molecular Dynamics, Amersham).

Molecular Cloning. The synapsin Ia gene was subcloned into the vector pFLAG-CMV (Sigma) using the restriction enzymes KpnI and BglII to place an N-terminal

FLAG tag on the protein. Synapsin I deletion mutants were created by PCR mutagenesis from the pFLAG-CMV-Synapsin Ia construct with the following antisense primers: ΔD1 5'-CGAGGTACCTCAGGTCAGAGACTGGGATTTG-3'; ΔD2 5'-CGAGGTACCTCAGAG-CTGGGGGTGCGG-3'; ΔD3 5'-CGAGGTACCTCACGTGACTGGCGGCCTTGA-3'; ΔD4 5'-CGAGGTACCTCACTGGCGCTGAGGTCCTGG-3'. Each fragment was created with the sense primer: 5'-AAATGTCGTAATAACCCCGCCCCCGTTGAACGC-3'. The fragments were subcloned into the pFLAG-CMV vector with KpnI and BglII.

Synapsin I point mutants were created by site directed mutagenesis of the Flag-Synapsin I construct using the QuickChange strategy (Stratagene) with the indicated primer pairs. Mutant S531A sense primer 5'-AGGCCGCCAGGCACGGCCAGTGG-3' and the antisense primer 5'-CCACTGGCCGTGCCTGGCGGCCT-3'. Mutant S549A sense primer 5'-CCCGCCGGCCGCCCCATCTCCAC-3' and the antisense primer 5'-GTGGAGATGGGGCGGCCGGCGGG-3'. Mutant S551A sense primer 5'-GGCCTCCCCAGCTCCACAGCGTC-3' and the antisense primer 5'-GACGCTGTGGAGCTGGGGAGGCC-3'. Mutant T562A sense primer 5'-CCCCACAGGCTGCCCGTCAGGCATC-3' and the antisense primer 5'-GATGCCTGACGGGCAGCCTGTGGGG-3'. Mutant S566A sense primer 5'-CCCGTCAGGCAGCTATCTCTGGTCCAG-3' and the antisense primer 5'-CTGGACCAGAGATAGCTGCCTGACGGG-3'. Mutant S568A sense primer 5'-GTCAGGCATCTATCGCTGGTCCAGCTCCA-3' and the antisense primer 5'-TGGAGCTGGACCAGCGATAGATGCCTGAC-3'. Mutant S576A sense primer 5'-CACCGAAGGTCGCAGGAGCCTCACC-3' and the antisense primer 5'-

GGTGAGGCTCCTGCGACCTTCGGTG-3’. Mutant S579A sense primer 5’-CTCAGGAGCCGCACCCGGAGGGC-3’ and the antisense primer 5’-GCCCTCCGGGTGCGGCTCCTGAG-3’.

Synapsin I double mutants were created on the S579A-Flag-Synapsin I construct. A second round of QuickChange was performed with all the aforementioned primers and the indicated new primer pairs. Mutant S579A/S603A sense primer 5’-CATTCGTCAGGCCGCCCAGGCAGGTC-3’ and the antisense primer 5’-GACCTGCCTGGGCGGCCTGACGAATG-3’. Mutant S579A/T611A sense primer 5’-CGGACCTCGCGCTGGCGGACCC-3’ and the antisense primer 5’-GGGTGGCCCAGCGCGAGGTCCG-3’. Triple mutant S579A/T615A/T616A sense primer 5’-GGGCCACCCGCCGCACAGCAGCCCCGG-3’ and the antisense primer 5’-CCGGGGCTGCTGTGCGGCGGGTGGCCC-3’. Mutant S579A/S622A sense primer 5’-GCCCCGGCCCGCCGGCCCAGGTC-3’ and the antisense primer 5’-GACCTGGGCCGGCGGGCCGGGGC-3’. Mutant S579A/T631A sense primer 5’-GCTGGACGTCCCGCCAAACCACAG-3’ and the antisense primer 5’-CTGTGGTTTGGCGGGACGTCCAGC-3’. Mutant S579A/S640A sense primer 5’-GCTCAGAAACCCGCCCAGGATGTGCC-3’ and the antisense primer 5’-GGCACATCCTGGGCGGGTTTCTGAGC-3’. Mutant S579A/S662A sense primer 5’-CCCAGCTCAACAAAGCCCAGTCTCTGACC-3’ and the antisense primer 5’-GGTCAGAGACTGGGCTTTGTTGAGCTGGG-3’. Mutant S579A/S664A sense primer 5’-CAAATCCCAGGCTCTGACCAATGCCTTC-3’ and the antisense primer 5’-GAAGGCATTGGTCAGAGCCTGGGATTTG-3’. Mutant S579A/T666A sense primer 5’-CCCAGTCTCTGGCCAATGCCTTCAACC-5’ and the antisense primer 5’-

CGTTGAAGGCATTGGCCAGAGACTGGG-3’. Mutant S579A/S680A sense primer 5’-GCCCAGGCCCGGCCTTAGCCAGG-3’ and the antisense primer 5’-CCTGGCTAAGGCCGGGCCTGGGC-3’. Mutant S579A/S682A sense primer 5’-GGCCCAGCCTTGGCCAGGATGAGG-3’ and the antisense primer 5’-CCTCATCCTGGCCAAGGCTGGGCC-3’. Mutant S579A/T691A sense primer 5’-GGTGAAAGCTGAGGCCATCCGCAGCCTG-3’ and the antisense primer 5’-CAGGCTGCGGATGGCCTCAGCTTTCACC-3’. Mutant S579A/S693A sense primer 5’-GAGACCATCCGCGGCCTGAGGAAG-3’ and the antisense primer 5’-CTTCCTCAGGCCGCGGATGGTCTC-3’. Mutant S579A/S697A sense primer 5’-GCAGCCTGAGGAAGGCTTTCGCCAGC-3’ and the antisense primer 5’-GCTGGCGAAAGCCTTCCTCAGGCTGC-3’. Mutant S579A/S700A sense primer 5’-GAAGTCTTTCGCCGGCCTCTTCTCCG-3’ and the antisense primer 5’-CGGAGAAGAGGCCGGCGAAAGACTTC. Mutant S579A/S705A sense primer 5’-CCAGCCTCTTCGCCGACTGAGGTACC-3’ and the antisense primer 5’-GGTACCTCAGTCGGCGAAGAGGCTGG-5’.

Asn point mutants were created on the S579A-Flag-Synapsin construct with the indicated primers. Mutant S579A/N661A sense primer 5’-CCCCCAGCTCGCCAAATCCCAGTC-3’ and the antisense primer 5’-GACTGGGATTTGGCGAGCTGGGGG-3’. Mutant S579A/N668A sense primer 5’-CCAGTCTCTGACCGCTGCCTTCAACCTTCC-3’ and the antisense primer 5’-GGAAGGTTGAAGGCAGCGGTCAGAGACTGG-3’. Mutant S579A/N671A sense primer 5’-CCAATGCCTTCGCCCTTCCAGAGCC-3’ and the antisense primer 5’-GGCTCTGGAAGGGCGAAGGCATTGG-3’.

Western Blotting. PVDF membranes were blocked for 1 h at rt in 5% milk/TBST (50 mL, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) except when used with antibody A46-B/B10, which was blocked in 3% periodated BSA in PBS.[23] Primary antibodies were added overnight at 4 °C at the following concentrations: anti-synapsin (Chemicon) at 0.5 μg/mL, anti-synapsin (G143) [24] at 0.2 μg/mL, anti-NSF (Synaptic Systems) at 1:1000, anti-synaptotagmin 1 (Synaptic Systems) at 1:3000, anti-spinophilin[21] at 1:5000, anti-syntaxin (Synaptic Systems) at 1:3000, anti-PSD-95 (Synaptic Systems) at 1:3000, anti-tubulin (Sigma) at 1:50,000, anti-GluR1 (Santa Cruz) at 1:1000, and A46-B/B10 at 0.5 μg/mL in TBS. Membranes were washed with TBST, incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Pierce), and visualized by chemiluminescence (Pico Chemiluminescent Substrate, Pierce).

Characterization of Synapsin with Lectins and Glycosidases. Bovine synapsin Ia/b (500 ng) was resolved by SDS-PAGE, transferred to PVDF membrane, and detected by blotting with horseradish peroxidase-conjugated lectins according to the manufacturer's protocol (EY Laboratories). For glycosidase analyses, purified bovine synapsin Ia/b (100 ng) was denatured in boiling 1% SDS for 1 min and neutralized by an equivalent volume of NETFD buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 6% NP-40 supplemented with protease inhibitors). Reactions were performed in 50 μL fractions using α-(1-2)-fucosidase or α-(1-3,4)-fucosidase (400 μU/100 ng synapsin; Calbiochem) in 50 mM sodium acetate buffer at pH 5.0 at 37 °C. Samples were resolved by SDS-PAGE and analyzed by immunoblotting and densitometry with NIH Image 1.62 software. Statistical analysis was performed using StatView (SAS Institute Inc.).

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 1.52 software, and mean neurite lengths were compared by the ANOVA test using the statistical analysis program Stat View 4.0.

Extraction of UDP-Gal and UDP-2-dGal from Cells. HeLa cells were treated in the presence or absence of 2-dGal (5 mM) as described above. The sugar nucleotides were extracted as reported previously,[25] and the samples (100 μL) were diluted with ice-cold isopropanol (900 μL), and centrifuged at 13,000 rpm for 15 min at 4 °C. The resulting supernatant was evaporated to dryness, dissolved in 100 mM HEPES, pH 8.5 (200 μL), and extracted with chloroform (200 μL). The aqueous phase was removed, evaporated to dryness, re-dissolved in 0.1 mM aqueous triethyammonium acetate (TEAA), pH 7.4 (20 μL), 15 μL of which was injected directly onto the LC-MS. Chromatography was performed using a C18-capillary column (Waters Xterra MS-C18 CapLC column (300 μm I.D. × 15 cm)). The chromatographic profile was from 95% solvent A (8.3 mM triethylamine/100 mM hexafluoroisopropanol, pH 7.4) to 55% methanol in 10 min at a flow rate of 5 μL/min; the UDP compounds eluted in 95% solvent A. MS analysis was performed on a Waters Q-ToF-API US (Milford, MA), and negative mode ESI data was collected at 1 s/scan, from 400-2000 *m/z*. Spectra were processed only by smoothing (5 × 2 Savitsky-Golay). Identical sets of MS scans were summed from each chromatographic trace to enable comparison between samples.

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