Appendix I

Exploring Synapsin I Regulation and Fucosylation by Small Molecule Inhibitors

A1.1. Background

The synapsins are a multigene family of neuronal phosphoproteins involved in regulating neurotransmitter release and neuronal development.[1, 2] There are five known members of the family encoded by three distinct genes: synapsin I, synapsin II, and synapsin III.[3-6] They are the most abundant protein on synaptic vesicles and constitute ~ 1% of total brain protein.[7-9] The synapsins are composed of eight different domains termed A-H, and all synapsin members are homologous at the N-terminal domain, which contain the A, B, and C domains.[10] Domains A and C are the most conserved domains.[1, 11] Domain A contains the only phosphorylation site conserved in all synapsin isoforms, and phosphorylation of this site controls its association with synaptic vesicles. The site is a substrate for both protein kinase A (PKA) and the Ca^{2+}/calmodulin-dependent protein kinase I (CAMKI).[5, 10, 12] Phosphorylation of this site leads to dissociation from synaptic vesicles.[12] The C domain is also highly conserved, and binds ATP in all synapsin isoforms, however, no ATPase activity has been associated with this domain.[13] Thus, the function of ATP binding remains unknown. The C domain binds to other synapsin C domains, creating homo- and heterodimers, which are important for synaptic vesicle clustering.[14]

The synapsins have been most characterized for their role in regulating synaptic vesicle cycling, where they tether synaptic vesicles to the actin cytoskeleton.[15-21] Because of the homo- and heterodimerization of synapsins, this creates a meshwork of synaptic

vesicle pools as synapsins also interact with the synaptic vesicle membrane.[1, 22-25] The synapsins are reported to maintain the "reserve pool" of synaptic vesicles that contains a large portion of vesicles clustered away from the plasma membrane near the active zone of presynaptic terminals.[26-28] There is a small subset of synaptic vesicles, termed the "releasable pool", that are physically docked to the plasma membrane. Upon Ca^{2+} influx through voltage-gated Ca^{2+} channels, this pool of synaptic vesicles undergoes fusion with the plasma membrane, releasing neurotransmitter into the synaptic cleft.[29-32] Phosphorylation of synapsins leads to release from synaptic vesicles in the releasable pool in response to increased activity, which can then dock at the plasma membrane for further fusion events. Thus, the synapsins appear to regulate synaptic vesicle cycling, implicating them in the ability to modulate synaptic transmission.

In addition to their roles in regulating neurotransmitter release, the synapsins have also been reported to play a role in mediating neuronal growth as well as the formation and maintenance of synaptic contacts.[2] They are localized to the growth cones of developing cerebellar granule and hippocampal neurons.[33-36] In addition suppression of synapsins I, II, and III expression alters developmental morphology in cultured hippocampal neurons.[34, 37-39] In fact, hippocampal neurons cultured from synapsin I-deficient mice display stunted neurites and delayed synapsin formation.[37] We recently reported that fucosylation of synapsin I is involved in this process (Chapter 2), suggesting a novel mode of synapsin regulation.[40] Here, we further examine synapsin fucosylation with a panel of deoxy-galactose inhibitors, and demonstrate that only 2-dGal conclusively affects synapsin expression and fucosylation. In addition, we attempt to label synapsin I with ^{3}H-Fuc in transfected HeLa cells and cortical neurons. However, we were never

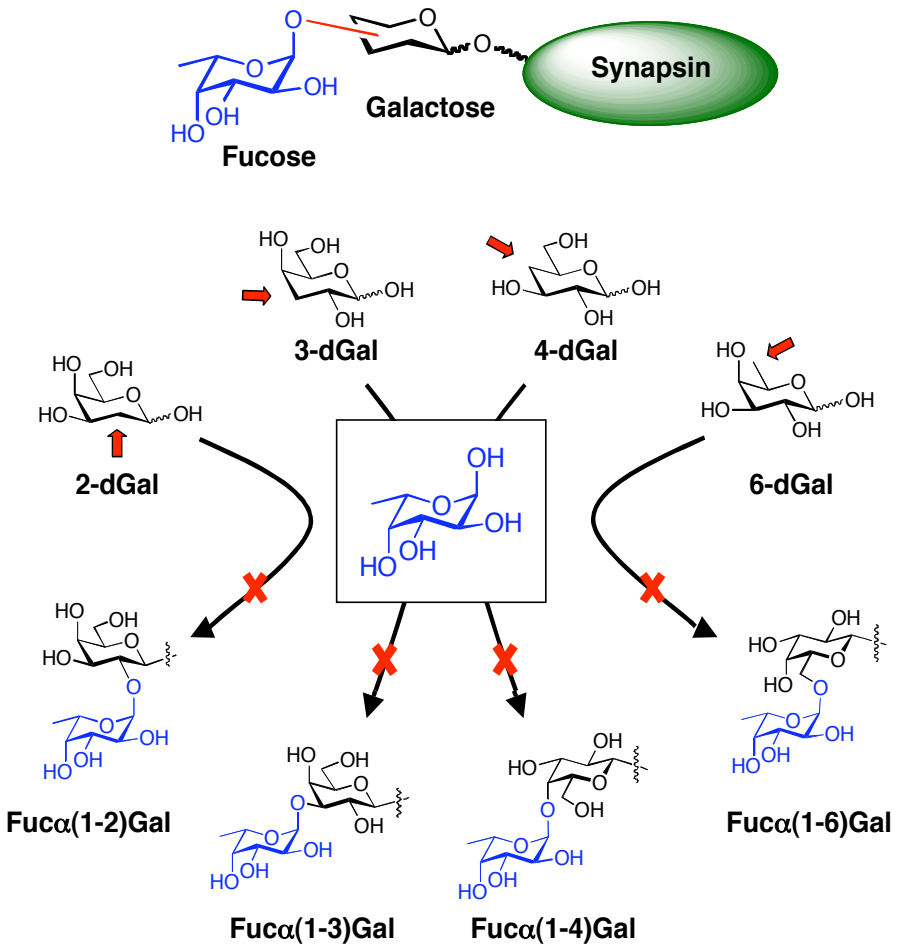


Figure A1.1. Strategy for mapping of Fuc-Gal linkages. Treatment of cells with different deoxy-Gal anlogues should inhibit the various Fuc-Gal linkages.

able to detect a fucosylated moiety through tritium labeling. Lastly, we examine inhibitors of glycosylation synthesis, and demonstrate that disruption of Golgi trafficking leads to loss of synapsin expression.

A1.2. Deoxy-Gal Analogues Can Map Fucose Linkages

Traditional methods of elucidating the structure of complex oligosaccharides are time-consuming and challenging. Large quantities of protein, sequential deglycosylation reactions using a series of exo- and endoglycosidases, and advanced MS or NMR

analyses are typically required.[41-43] Despite significant progress, many glycoproteins are not amenable to the established methods, and there is a significant need for new approaches to oligosaccharide linkage analysis.

We envisioned exploiting a series of deoxy sugar analogues to map the structure of the oligosaccharide present on synapsin. Our approach stems from the observation that 2-dGal can inhibit protein fucosylation by competing with D-galactose for incorporation into the oligosaccharide chain.[44, 45] Upon cellular uptake, 2-dGal is converted via the Leloir pathway to the 1-phosphate analogue, which is subsequently converted to the activated uridyl diphosphate (UDP) sugar.[46] Based upon these observations, we postulated that 3-dGal, 4-dGal and 6-dGal might also be converted into UDP sugars in sufficient quantity to compete with D-galactose. We have previously demonstrated in Chapter 2 that 2-dGal is an efficient substrate for metabolism through the Leloir pathway. While the conversion efficiencies of other deoxy sugars via this pathway have not been systematically examined in mammalian cells, *in vitro* studies using purified enzymes have suggested that substitution at the various positions should be tolerated, with the C4 position least favored.[47-49] Subsequent incorporation of the deoxy analogues into glycoproteins would inhibit formation of the corresponding fucose-galactose disaccharide and permit mapping of the precise linkage (Figure A1.1).

We evaluated the relative ability of a series of 2-, 3-, 4- and 6-deoxy-galactose analogues to inhibit formation of the glycosidic linkage on synapsin Ia. The 3-dGal and 4-dGal analogues were synthesized by Stacey Kalovidouris and Callie Bryan using procedures reported by Lowary *et al.*[50] HeLa cells were pre-treated for 1 h with the deoxy analogues at the concentrations indicated in Figure A1.2. After pre-incubation,

cells were transiently transfected with synapsin Ia, α(1-2)fucosyltransferase H enzyme (FUT1), and β-galactosidase. FUT1 has been shown to catalyze the formation of Fucα(1-2)Gal linkages on glycoproteins bearing the type 2 Lewis antigen[51] and thus was employed to potentially enhance fucosylation of synapsin. β-Galactosidase was used to normalize each sample for transfection efficiency. Cell lysates were resolved by SDS-PAGE, and the fucosylation and protein levels of synapsin were measured by

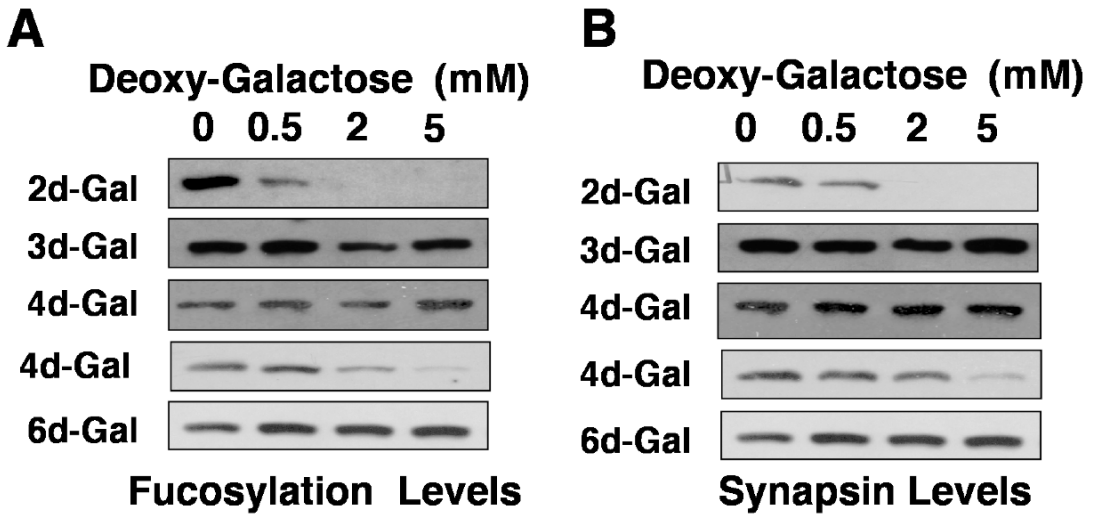


Figure A1.2. Deoxy-Gal treatment can potentially be used to determine oligosaccharide linkages. HeLa cells were treated with deoxy-Gal analogues at the indicated concentrations. Fucosylation levels were determined by Western blotting with antibody A46-B/B10 (A) and expression levels were determined by immunoblotting with anti-synapsin I (B).

immunoblotting.

As anticipated, treatment of mammalian cells with 2-dGal had a dramatic effect on the fucosylation levels (Figure A1.2A) and expression of synapsin (Figure A1.2B). In contrast, 3-dGal and 6-dGal had no observable effect on the fucosylation or expression level of synapsin. In some experiments, we saw no effect with 4-dGal treatment. However, in other experiments, incubation of cells with 4-dGal at higher concentrations

produced effects similar to those observed for 2-dGal in experiments. Thus, the results of 4-dGal treatment are inconclusive.

A1.3. 2-dGal Treatment Affects Synapsin I Deletion Mutants and S579A Synapsin

Once we established that deoxy-Gal sugars can potentially be used to determine oligosaccharide linkages, we sought to address the specificity of 2-dGal treatment using synapsin I deletion mutants and the fucosylation-deficient S579A synaspin I construct. HeLa cells were pre-treated with 2-dGal for 1 h and then transfected with Flag-synapsin

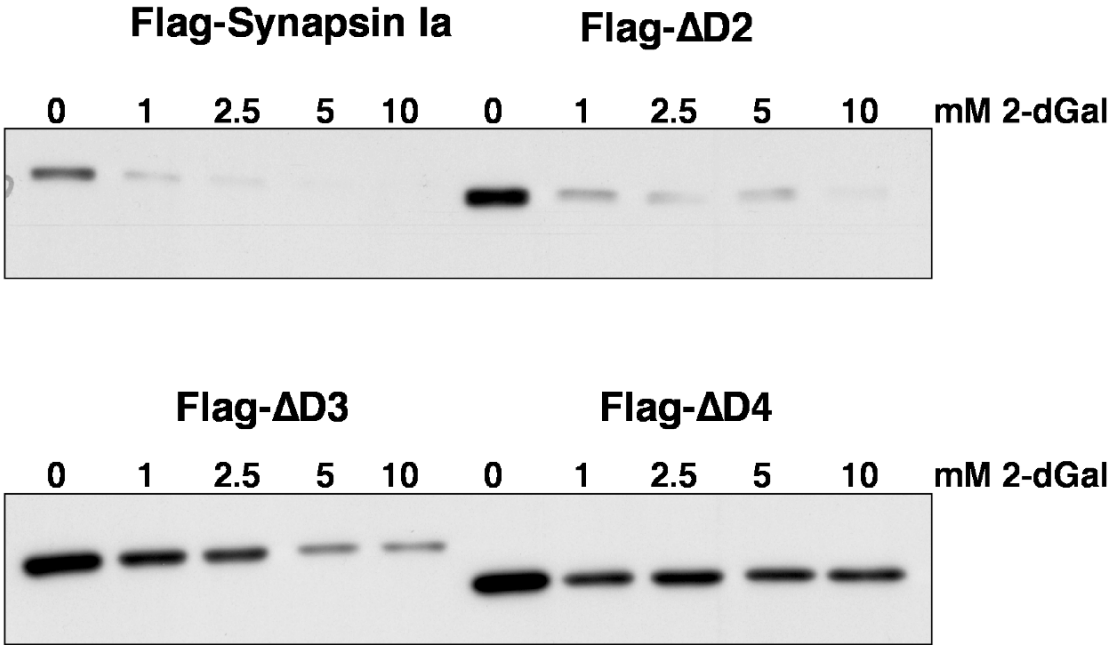


Figure A1.3. Deoxy-Gal affects Synapsin Ia deletion mutants. HeLa cells were transfected with the indicated constructs and treated with increasing concentrations of 2-dGal as indicated above. Flag-synapsin I, Flag-ΔD2, and Flag-ΔD3 exhibited increasing degradation by Western blot analysis with increasing concentrations of 2-dGal. In contrast, the Flag-ΔD4 mutant, which does not contain a Fucα(1-2)Gal epitope, exhibited an initial decrease in protein expression, followed by stability to increasing concentrations of 2-dGal.

I, Flag-Synapsin I ΔD2, Flag-Synaspin I ΔD3, Flag-Synapsin I ΔD4, or Flag-S579A-Synapsin I. 24 h post-transfection, cells were harvested and resolved by SDS-PAGE and blotted with an anti-Flag antibody to examine protein expression levels. As expected, the Flag-Synapsin I, Flag-Synapsin ΔD2, and Flag-Synapsin ΔD3, which all contain Fucα(1-2)Gal moieties, exhibited loss of synapsin expression with increasing concentrations of 2-dGal (Figure A1.3). Interestingly, the loss of synaspin I expression was reduced with greater truncations of the D-domain with the Flag-Synapsin I ΔD3 construct being the most stable to 2-dGal treatment. Surprisingly, the Flag-Synapsin I ΔD4 construct, which lacks a Fucα(1-2)Gal moiety, had a modest reduction in synapsin I expression after treatement with 1 mM 2-dGal, then exhibited stable expression with increasing concentrations of 2-dGal (Figure A1.3). This initial decrease in synapsin expression may suggest that there is another glycan lacking a Fucα(1-2)Gal that 2-dGal affects in this construct, or may reflect some non-specific effects of 2-dGal treatment. However, after the initial decrease in synapsin expression, the a Flag-Synapsin I ΔD4 construct exhibited no further decrease in expression, consistent with a role for 2-dGal in affecting synapsin I fucosylation and expression via the C-terminal region.

We next explored whether mutation of the putative fucosylation site at Ser 579 rescued synapsin I from 2-dGal-mediated degradation. Mutation of this site to Ala should abolish glycosylation, thus 2-dGal should have no effect on degradation of synapsin if mediated by the Fucα(1-2)Gal epitope at this site. Interestingly, the Flag-S579A-Synapsin exhibited significant loss of expression upon increasing concentrations of 2-dGal (Figure A1.4), similar to Flag-Synapsin degradation levels. This suggests that 2-dGal-mediated synapsin I degradation is not mediated via this fucosylation site. We

have evidence that a second site of Fucα(1-2)Gal glycosylation exists in synapsin I (Chapter 2), thus perhaps this alternative glycosylation site is responsible for 2-dGal-induced degradation. Experiments are currently underway to identify this alternate site of glycosylation, and when identified similar experiments will be performed to determine if this site is responsible for synapsin I stability.

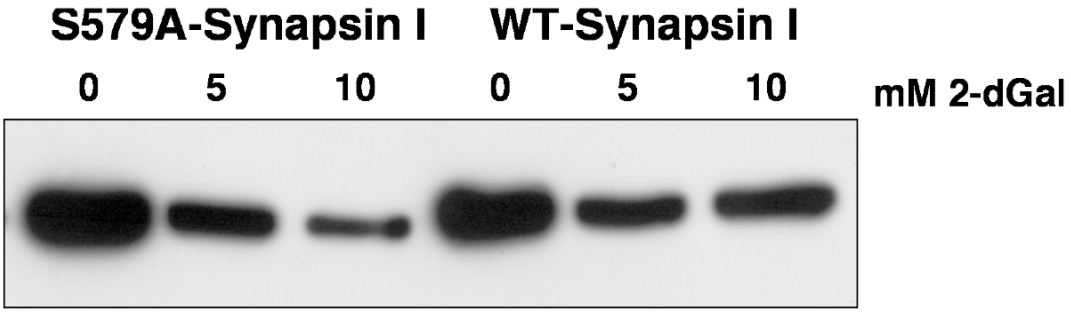


Figure A1.4. S579A-Synapsin I is degraded by 2-dGal treatment. HeLa cells were transfected with Flag-Synapsin I or Flag-S579A-Synapsin I and treated with 2-dGal as indicated above. Lysates were analyzed by immunoblotting with and anti-Flag antibody.

A1.4. Flag_S579A-Synapsin may have a Defect in Localization to Synaptic Terminals.

Since S579A-Synapsin I did not appear to be involved in synapsin I degradation from defucosylation with 2-dGal, we next examined whether this fucosylation site might be involved in protein targeting. Wild-type C57BL/6 neurons were transfected at 0 DIV with GFP-Synapsin I or GFP-S579A-Synapsin I and grown for 14 DIV to establish functional synapses. We observed a significant decrease in the amount of synapsin localized to synapses by fluorescence microscopy (Figure A1.5). The data suggest that fucosylation of this site may be involved in synaptic targeting. However, more experiments need to be performed to confirm the result.

A1.5. Examination of Synapsin Fucosylation with ^{3}H-Fucose

Tritium labeling is the traditional method to detect sugars on glycoconjugates. However, tritium is a weak beta emitter, and exposure times can take weeks to months to detect the sugar. Despite these drawbacks, we explored the ability to label synapsin I with ^{3}H-Fuc in both transfected Chinese hamster ovary (CHO) cells and neuronal cell cultures. CHO cells were transfected with empty vector (pcDNA 3.1) as a control, with synapsin Ia, or co-transfected with synapsin Ia and FUT1. FUT1 increases the expression of synapsin I in transfected cells (Figure A1.6), and we hoped to enhance synapsin fucosylation with this enzyme. However, despite multiple attempts, we were never able to significantly immunoprecipitate synapsin I from these cells (Figure A1.6). In addition, radiolabeling was very weak of endogenous CHO fucosylated proteins, even after a 2-week exposure time (data not shown). This suggests that radiolabeling of transfected CHO cells was not the optimal system to detect ^{3}H-Fuc on synapsin I, or significantly longer exposure times would be required.

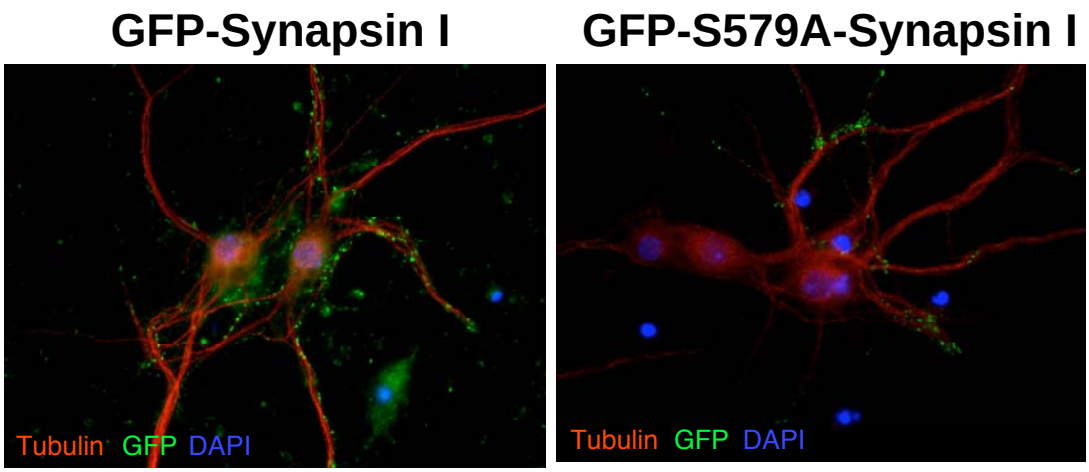


Figure A1.5. GFP-S579A-Synaspin has a defect in localization to synapses. C57BL/6 cortical neurons were transfected at 0 DIV with GFP-synapsin I or GFP-S579A-Synapsin Ia and cultured for 14 DIV, then examined by fluorescence microscopy.

We next turned our attention towards radiolabeling cultured cortical neurons. We first attempted to inhibit fucosylation of endogenous proteins with 2-dGal treatment. Cortical neurons were cultured for 2 DIV, then treated with 15 mM 2-dGal and 200 μCi of ^{3}H-Fuc for 2 more days. Lysates were resolved by SDS-PAGE, and either immunoblotted with antibody A46-B/B10 or exposed to film for tritium detection. We observed complete loss of fucosylated bands detected by the Fucα(1-2)Gal antibody after treatment with 2-dGal (data not shown). However, we did not observe any difference in tritium labeling of fucosylated glycoproteins, suggesting that the majority of fucose is incorporated into other linkages in neurons (data not shown). We attempted to label older neuronal cultures in which synapsin expression is prominent, however, we again had problems with the immunoprecipitation and results were inconclusive.

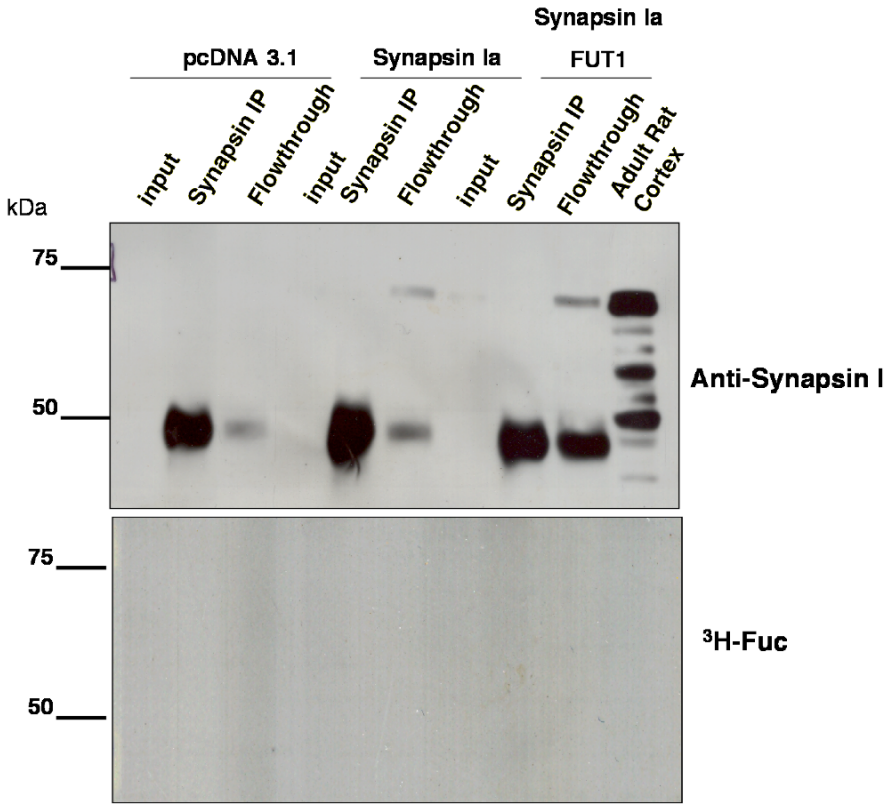


Figure A1.6. Autoradiography fails to detect a fucosylated glycan on synaspin I. Western blot of immunoprecipitated synapsin I (top panel) and audioradiography of immunoprecipitates (bottom panel) from CHO cells transfected as indicated above. Input is 10% of lysate used for immunoprecipitation, synapsin IP is immunoprecipitated synapsin, and flowthrough is 20% of lysate after immunoprecipitation. Co-transfection with FUT1 enhances synapsin I expression, lanes 4 and 7. No synapsin is detected in IP lanes.

A1.6. Synapsin I Expression is Reduced by Inhibitors of Golgi Trafficking

Synapsin I is a cytosolic protein, thus the mechanism of glycosylation is unknown. There is no signal sequence on synapsin to enter the endoplasmic reticulum (ER) for glycosylation reactions, which led us to postulate that perhaps synapsin I is fucosylated in the cytosol via a novel mechanism. We examined synapsin expression in response to the small molecule inhibitor Brefeldin A (Figure A1.8) that inhibits protein translocation from the ER to the Golgi in cultured hippocampal neurons. Neurons were

cultured for 6 DIV, then treated for 1 day with Brefeldin A. Cells were lysed and resolved by SDS-PAGE followed by immunoblotting for synapsin I, Fucα(1-2)Gal, *N*-cadherin as a control for a known glycoprotein, and tubulin as a control for a known cytosolic protein. We observed a loss of the Fucα(1-2)Gal signal as recognized by antibody A46-B/B10, consistent with a loss of glycosylation by inhibiting Golgi trafficking (Figure A1.9A). We also observed a decrease in molecular weight of the glycosylated N-cadherin protein, consistent with a loss of glycosylation (Figure A1.9B). As expected, we saw no effects on the cytosolic protein tubulin (Figure A1.9B). We were surprised to discover a decrease in synapsin I expression when we inhibited Golgi trafficking (Figure A1.9C). This may suggest that synapsin I is glycosylated via the Golgi apparatus.

A1.7. Discussion

An understanding of the role of Fucα(1-2)Gal carbohydrates in neuronal communication has been hampered by the lack of information regarding their molecular level functions. Here, we further investigated synaspin I fucosylation through different mechanisms. We attempted tritium labeling of synapsin, yet were unsuccessful due to immunoprecipitation problems. We also examined the ability to map Fuc-Gal linkages using a panel of deoxy-Gal substrates. We consistently observed an effect on synapsin fucosylation and expression with 2-dGal. This is consistent with formation of a Fucα(1-2)Gal linkage on synapsin I. On occasion, we also saw effects on synapsin I expression with higher concentrations of 4-dGal. While the results with 4-dGal are inconclusive, we have demonstrated in Chapter 2 that there is not a Fucα(1-4)Gal epitope on synapsin I

through enzymatic deglycosylation with an α(1-3,4)-fucosidase. If 4-dGal affects synapsin I expression and fucosylation, this may suggest an important role for the C4 position of galactose, presumably elsewhere in the oligosaccharide chain. Alternatively, 4-dGal could inhibit enzymes of the Leloir pathway, thus preventing fucosylation of synapsin I. The observation that higher concentrations of 4-dGal are required to abolish glycosylation is consistent with previous reports demonstrating that 4-deoxy analogues are poor substrates for galactosyltransferases and enzymes of the Leloir pathway.[49, 52] Nonetheless, the 4-dGal analogue may be a competent substrate in mammalian cells. Further experiments are currently underway to confirm incorporation of 4-dGal into the oligosaccharide chain.

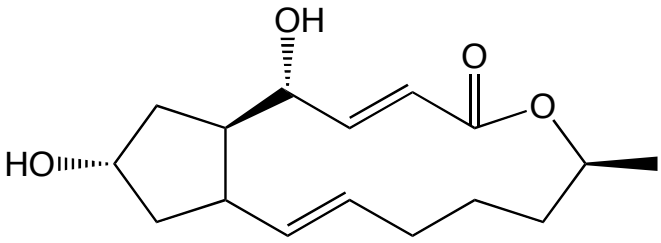


Figure A1.7. Chemical structure of Brefeldin A.

Disruption of the glycosidic linkage in cells led to synapsin Ia turnover. We also observed significant degradation of synapsin I ΔD mutants that contain a Fucα(1-2)Gal moiety. Surprisingly, we observed a modest decrease of synapsin I expression in the deletion mutant ΔD4, which does not contain a Fucα(1-2) epitope as determined by Western blotting with antibody A46-B/B10 or UEA1 lectin (Chapter 2). This suggests there may be some non-specific effects of 2-dGal treatment on synapsin I degradation. However, despite this initial decrease in synapsin I degradation, the construct was stable

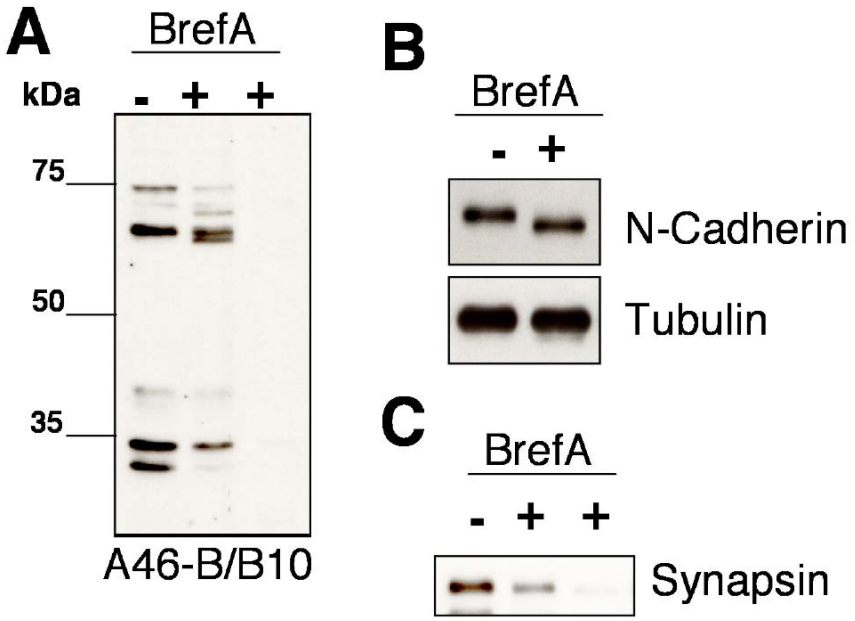


Figure A1.8. Treatment of 6 DIV cortical neurons with the Golgi trafficking inhibitor Brefeldin A for 1 DIV leads to loss of synapsin I expression (C) and expression of the Fucα(1-2)Gal epitope on glycoproteins (A). Treatment of N-cadherin with Brefelding A leads to loss of glycan expression and a molecular weight shift whereas treatment of tubulin has no effect (B). Cells were treated with 0.5 to 1 μg Brefeledin A.

to higher concentrations of 2-dGal, suggesting that defucosylation of synapsin I fucosylation sites is involved in 2-dGal-mediated synaspin degradation. We next tested the identified fucosylation site at Ser 579 for its response to 2-dGal treatment. We observed degradation of S579A-Synapsin I similar to wild-type synapsin I, suggesting that this site is not involved in defucosylation-induced protein degradation, and that it is likely due to another Fucα(1-2)Gal moiety in synapsin I protein.

We examined the ability of GFP-S579A-Synapsin I to target to presynaptic terminals. While S579A synapsin I was able to target to some terminals as indicated by the green punctate staining in figure A1.5 (right panel), we observed significantly less synapsin I-positive puncta than the wild-type counterpart. This may suggest a problem with S579A synapsin in localization to presynaptic terminals, and would implicate

fucosylation of this site in synapsin I targeting. This data is a preliminary result, and further experiments need to be performed to determine whether the result is not an artifact. In addition, we wish to explore these findings in synapsin I-deficient neurons, to determine if transfection of S579A-Synanpsin can rescue neurite outgrowth deficits.

Cumulativley, these results suggest that the Fucα(1-2)Gal carbohydrate may regulate the expression levels of synapsin I *in vivo*. Interestingly, the targeting of synapsins to presynaptic terminals involves complex interactions within the domains of synapsin and between its different isoforms (Ia/Ib, IIa/b, IIIa-d).[53] As the Fucα(1-2)Gal epitope is not found on synapsin II and III family members (Chapter 2),[40] glycosylation could influence the differential expression of synapsin I isoforms, as well as the targeting of different synapsin isoforms to the synapse. Alterations in synapsin expression or targeting via fucosylation could affect the clustering of synaptic vesicles, the size of the synaptic vesicle reserve pool, and neurotransmitter release.

Interestingly, synapsin I is a cytosolic protein, and the mechanism of glycosylation is unknown. We observed a significant decrease in synapsin I expression in response to inhibition of ER to Golgi trafficking, suggesting that synapsin I either travels through the normal glycosylation pathway, or interacts with the Golgi in such a manner that disruption of trafficking affects synapsin expression. If synapsin is glycosylated via a Golgi-dependent mechanism, than this would represent a novel pathway for glycosylation as synapsin I does not contain an ER translocation signal sequence. Future studies will address the impact of synapsin fucosylation on neurotransmitter release, neuronal morphology, and protein targeting. A comprehensive

molecular level understanding of Fucα(1-2)Gal carbohydrates should continue to reveal new insights into the diverse mechanisms of neuronal communication.

A1.8 Materials and Methods

General

Chemicals were purchased from Sigma-Aldrich unless otherwise stated. All reactions were performed in glass round bottom flasks (Pyrex) under an argon atmosphere. Thin-layer chromatography (TLC) was carried out on glass sheets coated with Kieselgel 60 F_{254} Fertigplatten (Merck, Darmstadt, Germany), and plates were inspected by UV light and developed by treatment with a cerium ammonium molybdate stain. Column chromatography was carried out using silica gel 60 (ICN Silitech 32-63 D, 60 Å). High resolution fast atom bombardment mass spectra (FAB-MS) were obtained on a Jeol JMS-600H spectometer and low resolution electrospray mass spectra (ES-MS) were acquired on a PE Sciex API 365 LC/MS/MS Triple Quadrupole mass spectrometer with a proton nanospray source. ^{1}H and ^{13}C NMR spectra were recorded using a Varian Mercury 300 spectrometer with the solvent or TMS as the internal standard. The chemical shifts are expressed on the scale in parts per million (ppm). The following abbreviations are used to explain the observed multiplicities: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet; br, broad.

Treatment of Mammalian Cells with Deoxy-Galactose Analogues

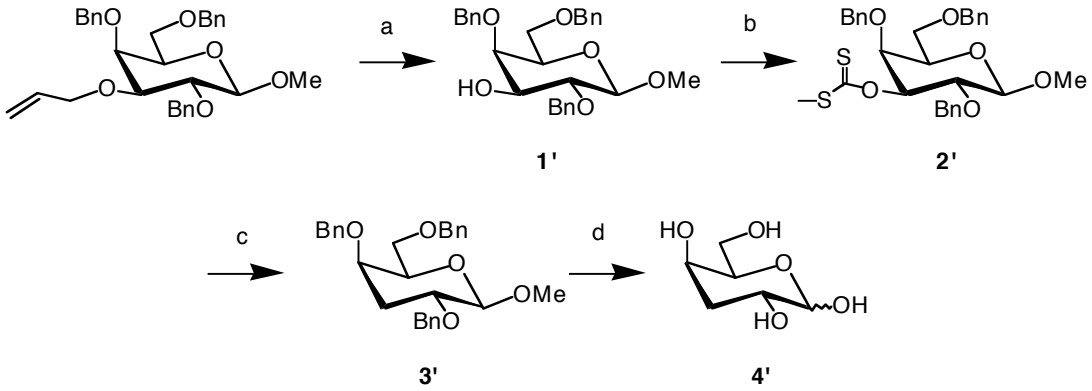
HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were seeded at 6×10^{5} cells per

60 mm dish and incubated at 37 °C/ 5% CO_2 for 24 h. The cells were then pretreated with the deoxy-galactose analogues (0.5 - 50 mM) for 1h at 37 °C/ 5% CO_2 and transfected at ~60% confluence without changing the media with pcDNA3.1-SynIa, FUT1,[21] and pSV-β-galactosidase (Promega) using Lipofectamine 2000 (Invitrogen) for deoxy-Gal analogues. Cells were transfected with pCMV-Flag-Synapsin Ia, pCMV-Flag-Synapsin ΔD2, pCMV-Flag-Synapsin ΔD3, pCMV-Flag-Synapsin Δ4, or pCMV-Flag-S579A-Synapsin I for 2-dGal experiments. After 20-22 h, the cells were harvested, resuspended in PBS (1 mL per plate), 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). Equivalent amounts of transfected protein were loaded onto SDS-PAGE gels and analyzed by Western blotting.

Synthesis of 3-dGal and 4-dGal

Syntheses were performed by Stacey Kalovidouris and Callie Bryan. The synthetic methods used to prepare 3-dGal and 4-dGal were analogous to those reported by Lowary *et al.*[1] and are described below.

Scheme A1.1 Synthesis of 3-deoxy-D-galactose.



[a]Conditions: (a) methyl 3-*O*-allyl-2,4,6-tri-*O*-benzyl-β-D-galactopyranoside, EtOH, benzene, H_2O, $(Ph_3P)_3RhCl$, DABCO, Hg_2O, $HgCl_2$ (61%) (b) THF, NaH, imidazole, CS_2, MeI (58%) (c) toluene, tributylstanane, AIBN (40%) (d) *i.* H_2, $Pd(OH)_2/C$, CH_2Cl_2, MeOH *ii.* H_2O, H^+ (50%).

Methyl 2,4,6-tri-*O*-benzyl-β-D-galactopyranoside (1'): A solution of methyl 3-*O*-allyl-2,4,6-tri-*O*-benzyl-β-D-galactopyranoside[2] (600 mg, 1.2 mmol), tris(triphenylphosphine)rhodium(I) chloride (160 mg, 0.20 mmol), and 1,4 diazabicyclo[2.2.2]octane (60 mg, 0.54 mmol) in EtOH (10 mL), benzene (4.5 mL) and water (1.5 mL) was refluxed for 20 h. Upon evaporation of the solvents, the resulting residue was dissolved in a solution of acetone (9 mL) and water (1 mL). Mercuric oxide (10 mg) and mercuric chloride (1.5 g) were added to the solution. After 12 h of stirring at rt, the mixture was diluted with CH_2Cl_2 and washed sequentially with a saturated solution of KI, water and then brine. Purification by SiO_2 column chromatography (5:1 hexanes / EtOAc) of the organic layer gave 340 mg of a white solid (61%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.12 (s, 3H, CH_3), 3.40 (d, 1H, *J* = 4.5 Hz, 3-OH), 3.55 (d, 1H, *J* = 7.5 Hz, H-2), 3.59-3.70 (m, 4 H, H-3, H-5, H-6, H-6'), 3.85 (d, 1H, *J* = 3.5 Hz, H-4), 4.33 (d, 1H, *J*=7.5 Hz, H-1), 4.43-4.50 (m, 6H, $PhCH_2$), 7.20-7.40 (m, 15H, Ph), ^{1}H-NMR (300 MHz, $CDCl_3$) in the literature:[3,4] δ = 1.12 (s, 3H, CH_3), 3.40 (d, 1H, *J* = 4.5 Hz, 3-OH), 3.55 (d, 1H, *J* = 7.5 Hz, H-2), 3.59-3.70 (m, 4 H, H-3, H-5, H-6, H-6'), 3.85 (d, 1H, *J* = 3.5 Hz, H-4), 4.33 (d, 1H, *J*=7.5 Hz, H-1), 4.43-4.50 (m, 6H, $PhCH_2$), 7.20-7.40 (m, 15H, Ph).

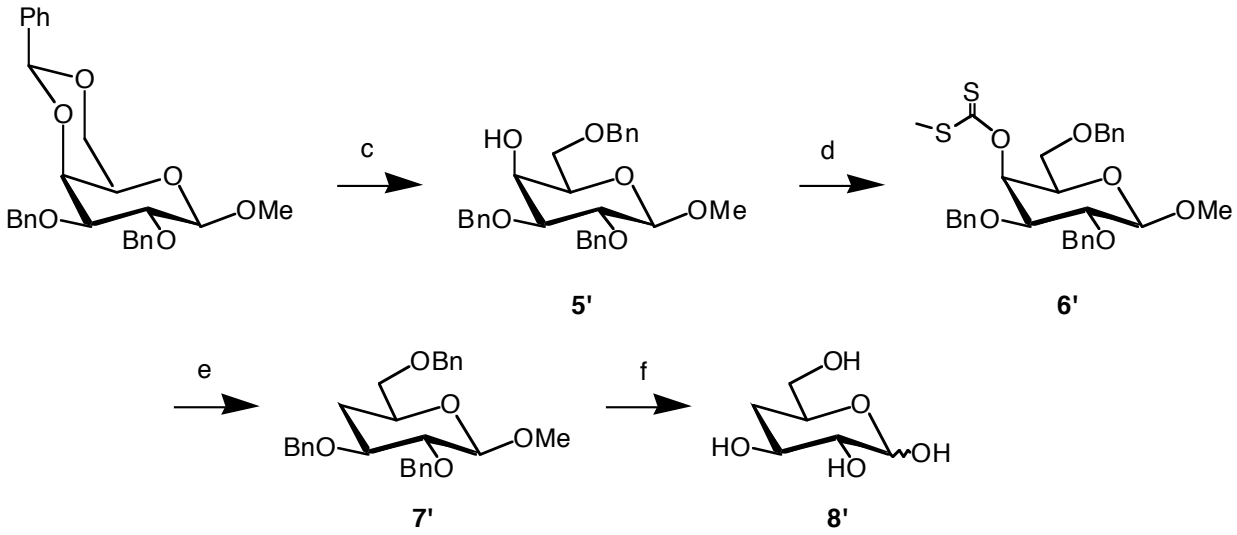
Methyl 2,4,6-tri-*O*-benzyl-3-O-[(methylthio)thiocarbonyl)]-β-D-galactopyranoside (2'): Sodium hydride, 60% dispersion in oil, (88 mg, 2.1 mmol) and imidazole (5.0 mg, 0.07 mmol) were added to a solution of **1'** dissolved in THF (5 mL) and this solution stirred for 1 h. After this time, CS_2 (450 μL, 0.07 mmol) was added and the solution stirred for an additional hour. MeI (130 μL, 0.02 mmol) was subsequently added, and the reaction mixture was stirred for 12 h. The solvent was evaporated *in vacuo*, and the resulting residue was subjected to SiO_2 column chromatography (5:1 hexanes / EtOAc) to yield 234 mg of an oil (58%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.12 (s, 3H, CH_3), 2.54 (s, 3H, SCH_3) 3.55-3.58 (m, 2H, H-6, H-6'), 3.68-3.71 (m, 1H, H-5), 3.95-4.01 (m, 1H, H-2), 4.18 (d, 1H, *J* = 3.0 Hz, H-4), 4.43-4.50 (m, 3H, $PhCH_2$, H-1), 4.65-4.71 (m, 4H, $PhCH_2$), 5.75 (d, 1H, *J* = 3.0 Hz, H-3), 7.20-7.45 (m, 15H, Ph). ^{1}H-NMR (300 MHz, $CDCl_3$) in the literature:[5] δ = 1.12 (s, 3H, CH_3), 2.54 (s, 3H, SCH_3) 3.55-3.60 (m, 2H, H-6, H-6'), 3.68-3.73 (m, 1H, H-5), 3.95–4.00 (m, 1H, H-2), 4.18 (d, 1H, *J*= 3.0 Hz, H-4), 4.43-4.50 (m, 3H, $PhCH_2$, H-1), 4.65-4.71 (m, 4H, $PhCH_2$), 5.75 (d, 1H, *J* = 3.0 Hz, H-3), 7.20-7.45 (m, 15H, Ph).

Methyl 2,4,6-tri-*O*-benzyl-3-deoxy-β-D-galactopyranoside (3'): To a solution of **2'** (230 mg, 0.42 mmol) in toluene was added tributylstannane (0.37 mL, 1.2 mmol) followed by AIBN (51 mg, 0.31 mmol). This solution was refluxed for 1 h, evaporated *in vacuo*, then purified by SiO_2 column chromatography (10:1 hexanes / EtOAc) to afford 75 mg of an oil (40%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.12 (s, 3H, CH_3), 1.46 (ddd, 1H, *J* = 14.5 Hz, *J* = 2.4 Hz, H-3') 2.36 (ddd, 1H, *J* = 14.5 Hz, *J* = 4.0 Hz, H-3), 3.62-3.68 (m, 5H, H-2, H-4, H-5, H-6, H-6'), 4.36 (d, 1H, *J*= 11.0 Hz, $PhCH_2$), 4.38 (d, 1H, *J*

= 8 Hz, H-1), 4.65-4.70 (m, 5H, $PhCH_2$), 7.20-7.45 (m, 15H, Ph). ^{1}H-NMR (300 MHz, $CDCl_3$) in the literature:[6] ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.12 (s, 3H, CH_3), 1.46 (ddd, 1H, *J* = 14.5 Hz, *J* = 2.4 Hz, H-3') 2.36 (ddd, 1H, *J* = 14.5 Hz, *J* = 4.0 Hz, H-3), 3.60-3.70 (m, 5H, H-2, H-4, H-5, H-6, H-6'), 4.36 (d, 1H, *J*= 11.0 Hz, $PhCH_2$), 4.38 (d, 1H, *J* = 8 Hz, H-1), 4.65-4.70 (m, 5H, $PhCH_2$), 7.20-7.45 (m, 15H, Ph).

3-Deoxy-D-galactose (4'): A solution of **3'** (40 mg, 0.09 mmol), $Pd(OH)_2$/C (40 mg), CH_2Cl_2 (1 mL) and MeOH (4 mL) was stirred at rt under a H_2 atmosphere for 12 h. The mixture was filtered through Celite, evaporated, and then the 12 h reaction and filtration steps were repeated to afford methyl-3-deoxy-β-D-galactoside. The galactoside was then re-dissolved in water (20 mL), Dowex 50X-8 was added to the aqueous suspension, and the reaction mixture was left to reflux for 12 h. After this time, the mixture was filtered, and the filtrate was lyophilized to yield 7.0 mg (50%) of a fluffy amorphous solid. Selected ^{1}H-NMR (300 MHz, D_2O): δ = 4.55 (d, 1H, *J* = 8.0 Hz, H-1 β-pyranose), 5.18 (d, 2H, *J* = 4.0 Hz, H-1 of α-pyranose and β-furanose), 5.25 (d, 1H, *J* = 1.0 Hz, H-1 of β-furanose) ^{1}H-NMR (250 MHz, D_2O) in the literature:[7] δ = 4.55 (d, 1H, *J* = 8.0 Hz, H-1 β-pyranose), 5.18 (d, 2H, *J* = 4.0 Hz, H-1 of α-pyranose and β-furanose), 5.25 (d, 1H, *J* = 1.0 Hz, H-1 of β-furanose).

Scheme A1.2 Synthesis of 4-deoxy-D-galactose.



[a]Conditions: (a) PhCHO, CH_2Cl_2, $ZnCl_2$ (65%) (b) DMF, benzyl bromide, NaH (quant.) (c) $NaCNBH_3$, methyl orange indicator, ethereal HCl, 3 Å MS (75%) (d) THF, NaH, imidazole, CS_2, MeI (83%) (e) toluene, tributylstanane, AIBN (60%) (f) *i.* H_2, $Pd(OH)_2/C$, CH_2Cl_2, MeOH *ii.* H_2O, H^+ (80%).

Methyl 2,3,6-tri-*O*-benzyl-4-deoxy-β-D-galactopyranoside (5'): $NaCNBH_3$ (265 mg, 4.00 mmol) and methyl orange indicator were added to methyl 2,3-di-*O*-benzyl-4,6-*O*-benzylidene-β-D-galactopyranoside (180 mg, 0.40 mmol) dissolved in THF (3.0 mL) containing 3 Å molecular sieves (100 mg). The reaction mixture was cooled to 0 °C and ethereal HCl (~ 1.5 mL of a 1M diethyl ether solution) was added until a red color persisted. The reaction was left to stir rt for 2 days. At that point, the mixture was

quenched with $NaHCO_3$, diluted with CH_2Cl_2 (100 mL) and washed with water (20 mL) and brine (20 mL). The organic layer was then dried, filtered, evaporated and purified by SiO_2 column chromatography (4:1 hexanes / EtOAc) to afford 130 mg (75 %) of a white solid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.12 (s, 3H, CH_3), 2.45 (d, 1H, *J* = 2.0 Hz, 4-OH), 3.55-3.57 (m, 2 H, H-2, H-5), 3.59 (d, 1 H, *J* = 7.1 Hz, H-3), 3.70 (d, 1H, *J* = 10 Hz, H-6), 3.79 (d, 1H, *J* = 10 Hz, H-6'), 3.99 (d, 1H, *J*=2.0 Hz, H-4), 4.21 (d, 1H, *J* = 8.0 Hz, H-1), 4.43-4.50 (m, 6H, $PhCH_2$), 7.20-7.40 (m, 15H, Ph). ^{1}H-NMR (300 MHz, $CDCl_3$) in the literature:[8] δ = 1.12 (s, 3H, CH_3), 2.45 (d, 1H, *J* = 2.0 Hz, 4-OH), 3.55-3.57 (m, 2 H, H-2, H-5), 3.59 (d, 1 H, *J* = 7.1 Hz, H-3), 3.70 (d, 1H, *J* = 10 Hz, H-6), 3.79 (d, 1H, *J* = 10 Hz, H-6'), 3.99 (d, 1H, *J*=2.0 Hz, H-4), 4.21 (d, 1H, *J* = 8.0 Hz, H-1), 4.43-4.50 (m, 6H, $PhCH_2$), 7.20-7.40 (m, 15H, Ph).

Methyl 2,3,6-tri-*O*-benzyl-4-O-[(methylthio)thiocarbonyl)]-β-D-galactopyranoside (6'): Sodium hydride, 60% dispersion in oil, (88 mg, 2.1 mmol) and imidazole (5.0 mg, 0.07 mmol) were added to a solution of **5'** dissolved in THF (5 mL) and this solution stirred for 1 h. After this time, CS_2 (450 μL, 0.07 mmol) was added and the solution stirred for an additional hour. MeI (130 μL, 0.02 mmol) was subsequently added, and the reaction mixture was stirred for 12 h. The solvent was evaporated *in vacuo*, and the resulting residue was subjected to SiO_2 column chromatography (5:1 hexanes / EtOAc) to yield 334 mg of an oil (83%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.12 (s, 3H, CH_3), 2.58 (s, 3H, SCH_3), 3.53-3.57 (m, 4H, H-2, H-3, H-6, H-6'), 3.76-3.82 (m, 1H, H-5), 4.35 (d, 1H, *J* = 7.5 Hz, H-1), 6.48 (d, 1H, *J*= 3.0 Hz, H-4), 4.43-4.44 (m, 2H, $PhCH_2$,), 4.65-4.72 (m, 4H, $PhCH_2$), 7.20-7.45 (m, 15H, Ph). ^{1}H-NMR (300 MHz, $CDCl_3$) in the literature:[5]

δ = 1.12 (s, 3H, CH_3), 2.58 (s, 3H, SCH_3), 3.53-3.60 (m, 4H, H-2, H-3, H-6, H-6'), 3.76-3.85 (m, 1H, H-5), 4.35 (d, 1H, *J* = 7.5 Hz, H-1), 6.48 (d, 1H, *J*= 3.0 Hz, H-4), 4.43-4.45 (m, 2H, $PhCH_2$,), 4.65-4.72 (m, 4H, $PhCH_2$), 7.20-7.45 (m, 15H, Ph).

Methyl 2,3,6-tri-*O*-benzyl-4-deoxy-β-D-galactopyranoside (7'): To a solution of **6'** (230 mg, 0.42 mmol) in toluene was added tributylstannane (0.37 mL, 1.2 mmol) followed by AIBN (51 mg, 0.31 mmol). This solution was refluxed for 1 h, evaporated *in vacuo*, then purified by SiO_2 column chromatography (10:1 hexanes / EtOAc) to afford 112 mg of an oil (60%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.12 (s, 3H, CH_3), 1.43 (t, 1H, *J* = 13.0 Hz, H-4') 2.12 (t, 1H, *J* = 13.0 Hz, H-4), 3.68-3.72 (m, 5H, H-2, H-3, H-5, H-6, H-6'), 4.21 (d, 1H, *J* = 8.0 Hz, H-1), 4.53 (d, 1H, *J*= 11.0 Hz, $PhCH_2$), 4.65-4.70 (m, 5H, $PhCH_2$), 7.20-7.45 (m, 15H, Ph). ^{1}H-NMR (300 MHz, $CDCl_3$) in the literature: [6, 9] δ = 1.12 (s, 3H, CH_3), 1.43 (t, 1H, *J* = 13.0 Hz, H-4') 2.12 (t, 1H, *J* = 13.0 Hz, H-4), 3.68-3.72 (m, 5H, H-2, H-3, H-5, H-6, H-6'), 4.21 (d, 1H, *J* = 8.0 Hz, H-1), 4.53 (d, 1H, *J*= 11.0 Hz, $PhCH_2$), 4.65-4.70 (m, 5H, $PhCH_2$), 7.20-7.45 (m, 15H, Ph).

4-Deoxy-D-galactose (8'): A solution of **7'** (40 mg, 0.09 mmol), $Pd(OH)_2$/C (40 mg), CH_2Cl_2 (1 mL) and MeOH (4 mL) was stirred at rt under a H_2 atmosphere for 12 h. The mixture was filtered through Celite, evaporated, and then the 12 h reaction and filtration steps were repeated to afford methyl-3-deoxy-β-D-galactoside. The galactoside was then re-dissolved in water (20 mL), Dowex 50X-8 was added to the aqueous suspension, and the reaction mixture was left to reflux for 12 h. After this time, the mixture was filtered, and the filtrate was lyophilized to yield 11 mg (80%) of a fluffy amorphous solid.

Selected ^{1}H-NMR (300 MHz, D_2O): δ = 3.49-3.54 (m, 3H, α-pyranose, α-/β-furanose), 4.57 (d, 1H, *J* = 7.2 Hz, H-1 β-pyranose). ^{1}H-NMR (250 MHz, D_2O) in the literature: [10-12] δ = 3.49-3.55 (m, 3H, α-pyranose, α-/β-furanose), 4.57 (d, 1H, *J* = 7.2 Hz, H-1 β-pyranose).

Transfection of Cortical Neurons and ^{3}H-Fucose Labeling

HeLa cells were maintained as described above. Cells were pretreated with 200 μCi of ^{3}H-Fucose (Sigma), then transfected with pcDNA 3.1 Synapsin Ia as described above. Cells were lysed in 1% boiling SDS and protein concentrations were determined with the BCA Assay (Pierce). Cortical neurons were prepared as described in Chapter 2. Neurons were grown on 100 mM dishes pre-coated with poly-DL-lysine (Sigma) which were cultured for either 2 DIV or 5 DIV, than treated with 15 mM 2-dGal or PBS + 200 μCi of ^{3}H-Fucose for 2 more days. Cell lysates were neutralized in NETFD and 1 mg of total lysate was immunoprecipitated with 5 μg of antibody G304. Immunoprecipitates were washed 3 × 1% NP-40. Lysates and immunoprecipitates were split 10% for Western blotting and 90% for autoradiography, then resolved by SDS-PAGE and transferred to PVDF membranes for Western blotting. Membranes were immunoblotted as described in Chapter 2 with antibody A46-B/B10 or G304. For autoradiography, gels were immersed in Amplify (Amersham). And then dried on a Bio-Rad Gel Dryer. Gels were exposed to autoradiographic film (Amersham) for 2 days up to 3 weeks at -80 °C.

Treatment of Neurons with Brefeldin A

Cortical neurons were prepared as described previously (Chapter 2) and cultured in 100 mm dishes coated with poly-DL-lysine. Neurons were cultured for 6 DIV than treated with 0.5 or 1 μg Brefeldin A or a vehicle control for 1 DIV.

A1.9. References

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