-Supporting Information-

A Role for Fucose α(1-2) Galactose Carbohydrates in Neuronal Growth

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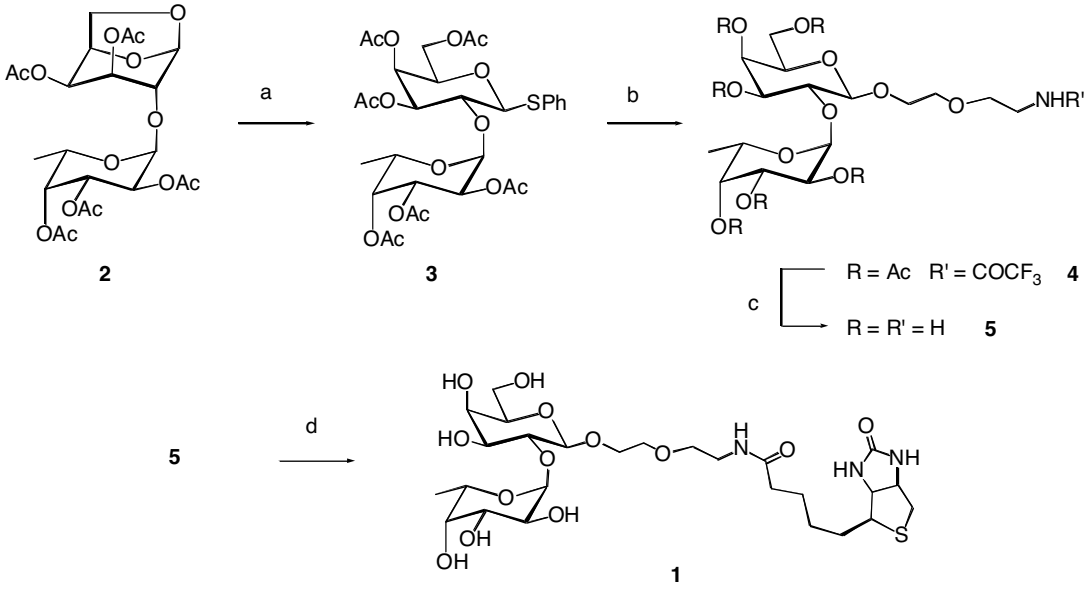
General Methods:

Chemicals were purchased from commercial suppliers and used as received. Lectins were purchased from Vector Labs (Burlingame, California), polymers from GlycoTech (Gaithersburg, Maryland), and tissue culture reagents from Gibco (Carlsbad, California). Unless stated otherwise, reactions were performed in flame-dried glassware under an argon environment, using freshly distilled solvents. Thin-layer chromatography (TLC) was carried out on glass sheets coated with Kieselgel 60 F_{254} Fertigplatten (Merck, Darmstadt, Germany). The plates were inspected by UV light and developed by treatment with a cerium ammonium molybdate stain followed by heating. 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 residual solvent or TMS as the internal standard. Data for ^{1}H are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant in Hz, and integration.

Experimental Details:

Scheme 1[a] Synthesis of Probe **1**.



[a]Conditions: (a) *i*.TMSSPh, ZnI_2 (76%) *ii*. Ac_2O, DMAP, C_5H_6N (quant.); (b) NIS-AgOTf, 2-(2-trifluoroacetamido ethoxy)ethanol, CH_2Cl_2 (68%); (c) K_2CO_3, MeOH (100%); (d) Et_3N, DMF, NHS-Biotin (60%).

Phenyl 3,4,5-tri-*O*-(2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl)-1-thio-β-D-galactopyranoside (3): The following thioglycoside formation procedure was adapted from a method reported by Motawia and coworkers.[1] Known compound **2**[2] (1.80 g, 3.40 mmol), (phenylthio)trimethylsilane (2.0 mL, 10.4 mmol) and zinc iodide (3.30 g, 10.4 mmol) in CH_2Cl_2 (20.0 mL) were stirred at rt for 21 h. The mixture was diluted with EtOAc (120 mL) and washed successively with saturated aqueous $NaHCO_3$ (150 mL), water (3 x 50 mL) and brine (20 mL). The organic layer was then dried over sodium sulfate, filtered and concentrated *in vacuo*. The residue was dissolved in dry THF (15.0 mL) and 1M tetrabutylammonium fluoride in THF (6.0 mL) was added. After this reaction mixture

stirred for an additional 20 min at rt, the solvent was evaporated. Thereafter, the residue was redissolved in EtOAc (60 mL), washed with water (3 x 30 mL), saturated aqueous $NaHCO_3$ (30 mL) and brine (30 mL), dried over Na_2SO_4, then filtered and concentrated to afford the β–thioglycoside (1.6 g, 76%). To a solution of the β–thioglycoside (310 mg, 0.50 mmol) in dry pyridine (6.0 mL) was added acetic anhydride (3.0 mL). The reaction was allowed to stir at rt overnight. The solvent was evaporated and residual pyridine was azeotroped with toluene. Column chromatography (SiO_2, Hexane/EtOAc, 3:1) afforded **3** (330 mg, quant.). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.12 (d, 3H, *J* = 6.5 Hz, H-6'), 1.51 (s, 3H, OAc), 1.66 (2s, 6H, OAc), 1.76 (s, 3H, OAc), 1.87 (s, 3H, OAc), 1.93 (s, 3H, OAc), 3.41 (ddd, 1H, *J* = 0.8 Hz, 7.0 Hz, 7.3 Hz, H-5), 3.83 (dd, 1H, *J* = 7.7 Hz, H-1), 4.06 (dd, 1H, *J* = 7.3 Hz, 10.2 Hz, H-6b), 4.12 (dd, 1H, *J* = 7.7 Hz, *J* = 10 Hz, H-2), 4.13 (dd, 1H, *J* = 7.0 Hz, 10.2 Hz, H-6a), 4.62 (dq, 1H, *J* = 1.1 Hz, 6.5 Hz, H-5'), 5.19 (dd, 1H, *J* = 3.5 Hz, 10 Hz, H-3), 5.42 (dd, 1H, *J* = 0.8 Hz, 3.5 Hz, H-4), 5.44 (dd, 1H, *J* = 3.8 Hz, 11.0 Hz, H-2'), 5.60 (dd, 1H, *J* = 1.1Hz, 3.4 Hz, H-4'), 5.74 (d, 1H, *J* = 3.8, H-1'), 5.83 (dd, 1H, *J* = 3.4 Hz, 11.0 Hz, H-3'), 7.20-7.59 (m, 5H, Ph); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 15.9, 20.7 (3C), 20.8 (3C), 61.8, 65.8, 67.3, 67.9, 68.1, 70.1, 71.0, 73.1 (3C), 87.8, 98.2, 100.2, 127.7, 129.3, 131.7, 133.2, 169.9, 170.0, 170.2, 170.5, 170.6, 170.6. HRMS *m/z* Calculated for $C_{30}H_{38}O_{15}NaS$ $[M+Na]^+$ 693.1253. Found 693.1271.

2-(2-Trifluoroacetamido ethoxy)ethyl-3,4,5-tri-*O*-(2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl)-1-β-D-galactopyranoside (4): The following is a modified procedure from Kanie *et. al.*[3] Compound **3** (330 mg, 0.50 mmol) was azeotroped with toluene and then

dissolved in CH_2Cl_2 (5.0 mL) and 2-(2-trifluoroacetamido ethoxy) ethanol (67 mg, 0.30 mmol) containing molecular sieves (4Å). After stirring for 10 min, the mixture was cooled to 20 °C. *N*-Iodosuccinimide (107 mg, 0.40 mmol), followed by silver triflate (108 mg, 0.40 mmol), was added. The reaction was allowed to warm to rt and proceeded for 2 h after addition of the silver triflate. After the reaction was complete, it was diluted with CH_2Cl_2 and filtered. The filtrate was washed with saturated aqueous Na_2SO_4, filtered and concentrated. Column chromatography (SiO_2, Hexane/EtOAc, 3:1) afforded **4** (210 mg, 68%, α:β = 1:3): only β was used. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.10 (d, 3H, *J* = 6.5 Hz, H-6'), 1.99 (s, 3H, OAc), 2.00 (s, 3H, OAc), 2.01 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.13 (s, 3H, OAc), 2.15 (s, 3H, OAc), 3.40 (ddd, 3H, *J* = 0.8 Hz, 7.0 Hz, 7.3 Hz, H-5, OCH_2), 3.55-3.78 (m, 6H,OCH_2), 3.88 (dd, 1H, *J* = 7.8 Hz, H-1), 3.97 (dd, 1H, *J* = 7.8 Hz, 10.2 Hz, H-6b), 4.02 (dd, 1H, *J* = 7.8, 10.0 Hz, H-2), 4.14 (dd, 1H, *J* = 7.8 Hz, 10.2 Hz, H-6a), 4.28 (m, 1H, H-3'), 4.50 (dq, 1H, *J* = 1.1 Hz, 6.5 Hz, H-5'), 5.00 (dd, 1H, *J* = 3.6 Hz, 10.0 Hz, H-3), 5.32 (dd, 1H, *J* = 0.5 Hz, 3.3 Hz, H-4), 5.31-5.42 (m, 3H, H-2', H-4', H-1'), 7.49 (bs, 1H, N-H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 16.3, 21.0 (2C), 28.7, 29.7, 45.5, 61.8, 65.0, 66.2, 67.9, 68.2 (2C), 69.4, 69.7, 70.3, 70.6, 71.0, 71.4, 97.9, 98.5, 128.6 (2C), 129.5 (2C), 130.1(2C), 132.4, 170.1, 170.2, 170.4. HRMS *m/z* Calculated for $C_{30}H_{42}F_3NO_{18}$ $[M+H]^+$ 762.2465. Found 762.2470.

(*N*- [6-([Biotinoyl]amino) ethoxy) ethyl -*O*-(α-L-fucopyranosyl)-(1-2)-β-D-galactopyranoside (1): The procedure for the preparation of **5** was adapted from Newman *et al*.[4] Compound **4** (0.50 g, 0.70 mmol) was dissolved in a 7% solution of K_2CO_3 in aqueous MeOH (2:5, *v:v*), and the reaction mixture was stirred for 12 h at rt.

The solution was neutralized with 1N HCl, and the organic layer was extracted with MeOH and concentrated to afford **5** (270 mg, quant.) as a white foam. Amine **5** (70 mg, 0.17 mmol) was dissolved in DMF (2.0 mL), and the pH was adjusted to 9 using TEA. *N*-Hydroxysuccinimido biotin (70 mg, 0.20 mmol) was added and the reaction stirred for 12 h at rt. The reaction mixture was concentrated to afford an off-white syrup. Preparative reversed-phase chromatography using a gradient of 0% to 50% aqueous 0.1 % TFA in CH_3CN over 60 min followed by a gradient of 50% to 100% CH_3CN over 60 min (retention time 40 min; flow rate 21 mL/min) afforded pure **1** (60 mg) in 60% yield. ^{1}H NMR (300 MHz, D_2O): δ 1.05 (d, 3H, *J* = 6.6 Hz, H-6'), 1.23-1.31 (m, 2H, CH_2), 1.41-1.60 (m, 4H, CH_2), 2.12 (t, 2H, *J* = 7.0 Hz, CH_2), 2.62 (d, 2H, *J* = 13.2 Hz, SCH_2), 2.84 (dd, 2H, *J* = 4.8 Hz, 12.6 Hz, CH_2), 3.17 (m, 4H, CH_2), 3.23 (dd, 2H, *J* = 4.8 Hz, *J* = 8.7 Hz, CH_2), 3.42-4.10 (m, 12H, H-2, H-3, H-4, H-5, H-6a, H-6b, H-2', H-3', H-4', H-5', CH), 4.45 (d, 1H, *J* = 7.8 Hz, H-1), 4.46 (m, 1H, CH), 5.08 (d, 1H, *J* = 2.2 Hz, H-1'); ^{13}C NMR (75 MHz, D_2O): δ = 15.6, 25.1, 27.7 (2C), 27.9, 35.4 (2C), 39.0, 39.7, 55.4, 60.2, 61.1, 62.1, 66.8, 67.2, 68.3, 68.8, 69.3, 69.4, 70.8, 71.7, 77.9, 98.2, 101.6, 165.3, 176.9. HRMS *m/z* Calculated for $C_{26}H_{45}N_3O_{13}S$ $[M+H]^+$ 640.2751. Found 640.2725.

Treatment of Hippocampal Neurons with PAA Polymers and Lectins:

Hippocampal neurons were cultured using a procedure modified from Goslin and Banker.[5] Briefly, the hippocampi of embryonic day 18 (E18) rats were dissected and transferred to 4.5 mL of ice-cold Calcium and Magnesium Free-Hank's Balanced Salt Solution (CMF-HBSS). Trypsin (2.5%, no EDTA) was added to 5 mL, and the tissue was incubated for 15 min at 37 °C. The trypsin solution was removed and the tissue

sample washed three times with 5 mL of CMF-HBSS. Cells were then dissociated from the tissue in 1 mL of CMF-HBSS by passing through a P1000 pipet tip twenty times. The cells were counted, diluted into Minimal Eagle's Medium (MEM) plus 10% fetal bovine serum, and seeded on poly-DL-ornithine-coated glass coverslips (Carolina Biological) at a density of 75 cells/mm^2 (100 μL/coverslip) for 30 min. After this time, 500 μL of supplemented Neurobasal medium (47.5 mL Neurobasal medium without L-glutamine; 0.5 mL L-glutamine (200 mM); 0.5 mL penicillin/streptomycin (10,000 U/mL); 0.5 mL antibiotic-antimycotic (100X stock), 1.0 mL B-27 serum-free supplement (50X stock); 50 μL of 0.5 M kynurenic acid in 1 N NaOH) was added to each coverslip. The use of kynurenic acid to block glutamate receptor ion channels and enhance neuronal health and survival is standard protocol for neuronal cultures.[5] The cultures were incubated in 5% CO_2 at 37 °C for 20 h. The media was then removed, and a 50 μL solution of carbohydrate-PAA conjugate (1.3 μM, 13 μM, or 130 μM) in PBS or a 50 μL solution of lectin (3.7 μM) in HEPES was added to supplemented Neurobasal medium (450 μL) on each coverslip. For the competition experiments, a 50 μL solution of the lectin (3.7 μM) and probe **1** (1.5 mM) in HEPES was preincubated at 4 °C for 12 h and was then added to supplemented Neurobasal medium (450 μL) on each coverslip. The cultures were incubated for 24 h in 5% CO_2 at 37 °C and analyzed as described below.

Immunocytochemistry of Hippocampal Neuronal Cultures:

The hippocampal neurons on the coverslips were rinsed one time with PBS, fixed in 4% paraformaldehyde for 20 min at rt, washed twice with PBS, permeabilized in 0.3% Triton X-100 for 5 min at rt, and washed twice with PBS. Non-specific binding was blocked by

incubating with 3% BSA for 1 h at rt and then rinsing once with PBS. Cells were then incubated with anti-tau antibodies (rabbit polyclonal, 1:600; Sigma) in 3% BSA for 2 h at rt. Excess antibody was rinsed away 5 times with PBS. The secondary antibody, anti-rabbit IgG AlexaFluor 488 (1:600; Molecular Probes), was added for 1 h at 37 °C in 3% BSA. Excess secondary antibody was washed off 5 times with PBS. The coverslips were mounted onto glass slides using Vectashield mounting medium (Vector Labs) and sealed with clear nail polish. Cells were imaged using a Zeiss Axiovert 100M inverted laser microscope (Biological Imaging Center in the Beckman Institute at Caltech). Images were captured with LSM Pascal software using a 40X plan-neofluar oil objective and an excitation wavelength of 488 nm or 568nm.

Morphometric Analysis:

All experiments were performed in duplicate. For each experiment, 50 randomly selected cells were analyzed per coverslip using similar quantitative analysis techniques reported by Schmid *et al.*[6] Briefly, only neurites longer than ~10 μm and not in contact with other cells were measured using NIH Image 1.62 software. The mean neurite lengths were compared among the different conditions by the ANOVA test using the statistical analysis program StatView (SAS Institute Inc., Cary, NC).

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

Hippocampal neuronal cultures were prepared as described above and maintained at 37 °C, 5% CO_2 in supplemented Neurobasal medium. After 23 days in culture, the medium

was replaced, and neurons were treated with the endocytosis inhibitor phenylarsine oxide[7] (PAO; 4 μL in DMSO, final concentration 10 μM) and either probe **1** (24 μL in PBS, final concentration 3 mM), biotin (24 μL in PBS, final concentration 3 mM), or fluorescein-conjugated UEA I lectin (4 μL, 1:100 final dilution) in supplemented Neurobasal medium (400 μL final volume) for 1 h at 37 °C, 5% CO_2. After 1 h, neurons were rinsed 2 times with PBS, fixed in 4% paraformaldehyde for 20 min at rt, washed 2 times with PBS, permeabilized in 0.3% Triton X-100 for 5 min at rt, and washed 2 times with PBS. Non-specific binding was blocked with 3% BSA for 1 h at rt and then rinsed one time with PBS. Anti-tau antibody (rabbit polyclonal, 1:600; Sigma) was added in 3% BSA for 2 h at rt and the excess antibody rinsed off 5 times with PBS. Probe **1** was detected with streptavidin conjugated to AlexaFluor 488 (1:200; Molecular Probes), and anti-tau was detected with a secondary antibody conjugated to AlexaFluor 568 (goat anti-rabbit IgG, 1:600; Molecular Probes). Both dye-conjugated streptavidin and secondary antibodies were added in 3% BSA for 1 h at 37 °C and the excess reagent washed off 5 times with PBS. Coverslips were then mounted onto slides with Vectashield, sealed, and imaged using confocal laser microscopy.

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

To confirm that probe **1** was binding specifically to proteins rather than interacting with the membrane lipids, neurons were delipidated following the protocol of Yavin and Yavin.[8] Briefly, after 23 days in culture, cells were rinsed once with PBS then exposed to MeOH/$CHCl_3$ (1/2 by vol) for 15 min at –80 °C. This procedure fixes the cells to the

coverslip and extracts cellular lipids. After removing the MeOH/$CHCl_3$ mixture, coverslips were dried at rt then incubated with 3% BSA for 1 hr at rt. After one rinse with PBS, neurons were treated with the endocytosis inhibitor phenylarsine oxide[7] (PAO; 4 μL in DMSO, final concentration 10 μM) and either probe **1** (24 μL in PBS, final concentration 3 mM), biotin (24 μL in PBS, final concentration 3 mM), or fluorescein-conjugated UEA I lectin (4 μL, 1:100 final dilution) in supplemented Neurobasal medium (400 μL final volume) for 1 h at 37 °C, 5% CO_2. Excess reagents were rinsed off 5 times with PBS before incubating with anti-tau antibody (1:600) for 2 h at rt. Cells were washed 5 times with PBS and then treated with streptavidin conjugated to AlexaFluor 488 (1:200) and secondary antibody conjugated to AlexaFluor 568 (goat anti-rabbit IgG, 1:600) in 3% BSA for 1 h at 37 °C. Cells were then rinsed 5 times with PBS before mounted onto slides and analyzed by confocal laser microscopy. De-lipidation did not alter the staining of either **1** or UEA I lectin (Figures S1 and S2).

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

Hippocampal neurons were plated on poly-DL-ornithine-coated glass coverslips as described above. After one day in culture, the medium was replaced with fresh medium, and the small molecules were added. A dose-response experiment was initially performed to determine the minimum concentration of 2-dGal needed to elicit an effect. Neurons were treated with varying concentrations of 2-dGal (1, 5, 10, 15, or 30 mM in 25 μL PBS with 475 μL of supplemented Neurobasal medium) for 2 days before immunostaining with anti-tau antibodies as described above (Figure S3). Importantly, no

cellular toxicity was observed at concentrations up to 30 mM 2-dGal, as demonstrated by trypan blue staining, adherence of the cells to the coverslip, and healthy cellular morphology. A concentration of 15 mM was used in subsequent experiments as it produced a strong effect on neurite outgrowth. Cells were treated as above under 4 different conditions: (1) incubation with 15 mM 2-dGal for 2 days, (2) incubation with 15 mM 3-deoxy-D-galactose for 2 days, (3) incubation with 15 mM 2-dGal for 2 days followed by incubation with 75 mM D-galactose for 2 days, or (4) no treatment for 2 days. After adding the small molecules, cultures were incubated at 37 °C, 5% CO_2, then washed once with PBS, and immunostained with the anti-tau antibody as described above.

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

In addition to cells plated on coverslips, hippocampal neurons were grown in 30 mm dishes and treated with or without 30 mM 2-dGal (25 μL in PBS with 475 μL supplemented Neurobasal medium). After 4 days, cells were harvested with 2.5% trypsin, lysed with 1% boiling SDS with protease inhibitors, and cell lysates probed by Western blotting using the anti-Fucα(1-2)Gal antibody A46-B/B10.[9] Protein concentrations of the neuronal lysates were determined using the BCA Protein Assay (Pierce). Equal amounts of total protein were resolved by 10% SDS-PAGE, and proteins were transferred to PVDF membrane (Millipore) in 20 mM Tris-Cl pH 8.6/ 120 mM glycine/ 20% methanol. Western blots were blocked for 1 h with 3% periodated BSA[10] and rinsed with TBST (50 mM Tris-Cl pH 7.4/ 150 mM NaCl/ 0.1% Tween-20). Blots

were incubated with anti-Fucα(1-2)Gal antibody A46-B/B10[9] (0.5 μg/mL; gift from Dr. U. Karsten, Max-Delbruck Centre for Molecular Medicine, Berlin-Buch, Germany) in TBST overnight at 4 °C with constant rocking, then rinsed and washed twice for 10 min with TBST. Immunoreactivity was visualized by incubation with a horseradish peroxidase conjugated goat anti-mouse antibody (1:2500; Pierce) in TBST for 1 h followed by a rinse and four washes of 20 min with TBST. Blots were visualized by chemiluminescence using ECL reagents (Amersham) on X-Omat R film (Kodak). Treated neurons exhibited significantly reduced levels of the Fucα(1-2)Gal epitope on several glycoproteins (Figure S4).

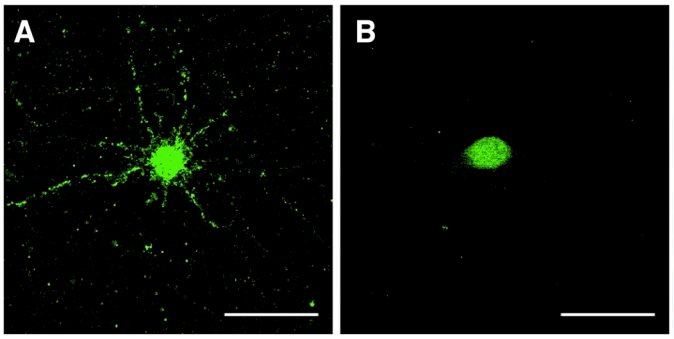


Figure S1. Lipid extraction of neurons does not alter the labeling with probe **1**. E18 hippocampal neurons (23 days in vitro) were de-lipidated with MeOH/$CHCl_3$ prior to labeling with 3 mM of (A) **1** or (B) biotin in the presence of 10 μM PAO. Scale bars represent 45 μm.

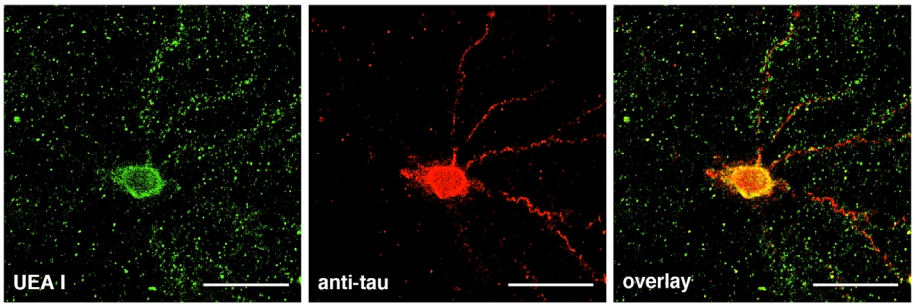


Figure S2. Co-staining of E18 hippocampal neurons (23 days in vitro) with UEA I lectin (1:100; green) and anti-tau antibody (1:600; red) in the presence of 10 μM PAO following lipid extraction. An overlay of both images is shown in yellow. Scale bars represent 45 μm.

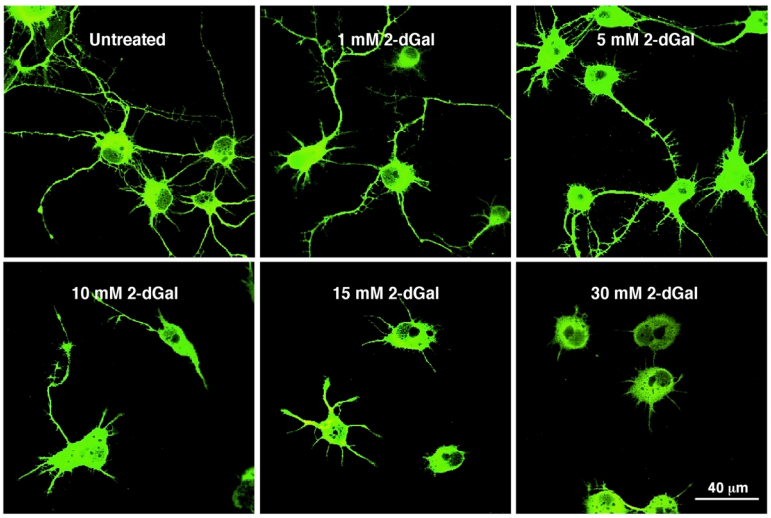


Figure S3. Treatment of E18 hippocampal neurons with varying concentrations of 2-dGal. After 1 day in culture, neurons were treated with the specified concentrations of 2-dGal for 2 days, followed by immunostaining with anti-tau antibodies. Scale bar represents 40 μm.

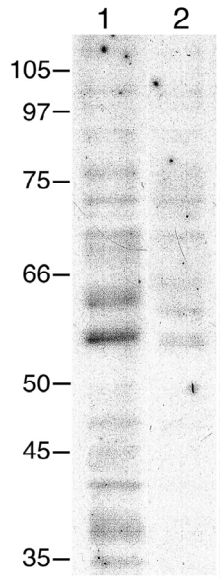


Figure S4. Treatment of E18 hippocampal neuronal cultures with 2-dGal diminishes expression of the Fucα(1-2)Gal epitope on glycoproteins. After a 4-day incubation with or without 30 mM 2-dGal, protein lysates were analyzed by Western blotting using an anti-Fucα(1-2)Gal antibody. Lane 1: Untreated neurons (75 μg total protein). Lane 2: Neurons treated with 30 mM 2-dGal (75 μg total protein).

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