

Supporting Information for

Directing Neuronal Signaling through Cell-Surface Glycan Engineering

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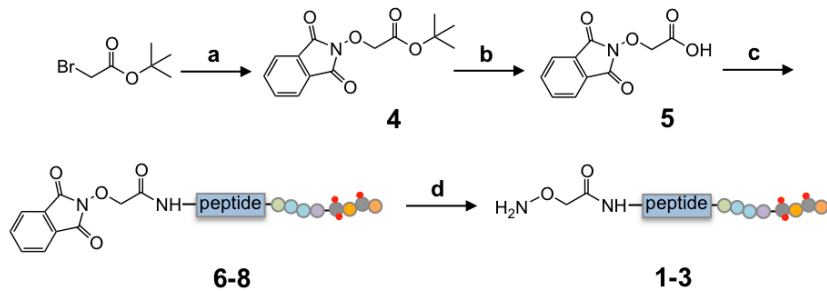
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Scheme S1. General synthesis of aminoxy-functionalized natural CS polysaccharides.^a



^a Reagents and conditions for generating aminoxy-functionalized CS polysaccharides. (a) *N*-hydroxyphthalimide (1.2 eq), NaHCO₃ (1.2 eq), DMF, 60 °C, 24 h, 88%; (b) TFA (3 eq), DCM, 1 h, 99%; (c) EDC (1.1 eq), sulfo-NHS (1.1 eq), 0.1 M MES, 0.3 M NaCl, pH 6.0, RT, 30 min, then NH₂-polysaccharide in 0.05 M NaHCO₃, RT, 12 h, 60%; (d) hydrazine monohydrate (3 eq), RT, 6 h, 92%.

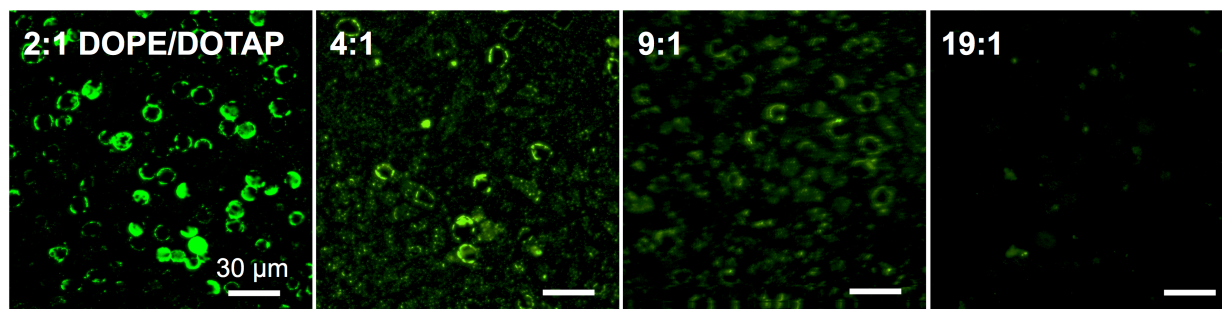


Figure S1. Optimization of the lipid ratio required for cell membrane fusion. Different DOPE:DOTAP lipid ratios (2:1, 4:1, 9:1, 19:1) were mixed and extruded with the same dodecanone concentration (10% *w/w*) to form liposome populations. The liposomes were then reacted with AF488-hyd (ddH₂O, 3 h, RT) and incubated with PC12 cells for 30 min, resulting in fusion and lipid molecule display in varying efficiencies on cell membranes. Rapid, efficient membrane fusion and subsequent lipid presentation was observed with liposomes having a 2:1 ratio of DOPE to DOTAP lipids, as visualized by fluorescence microscopy.

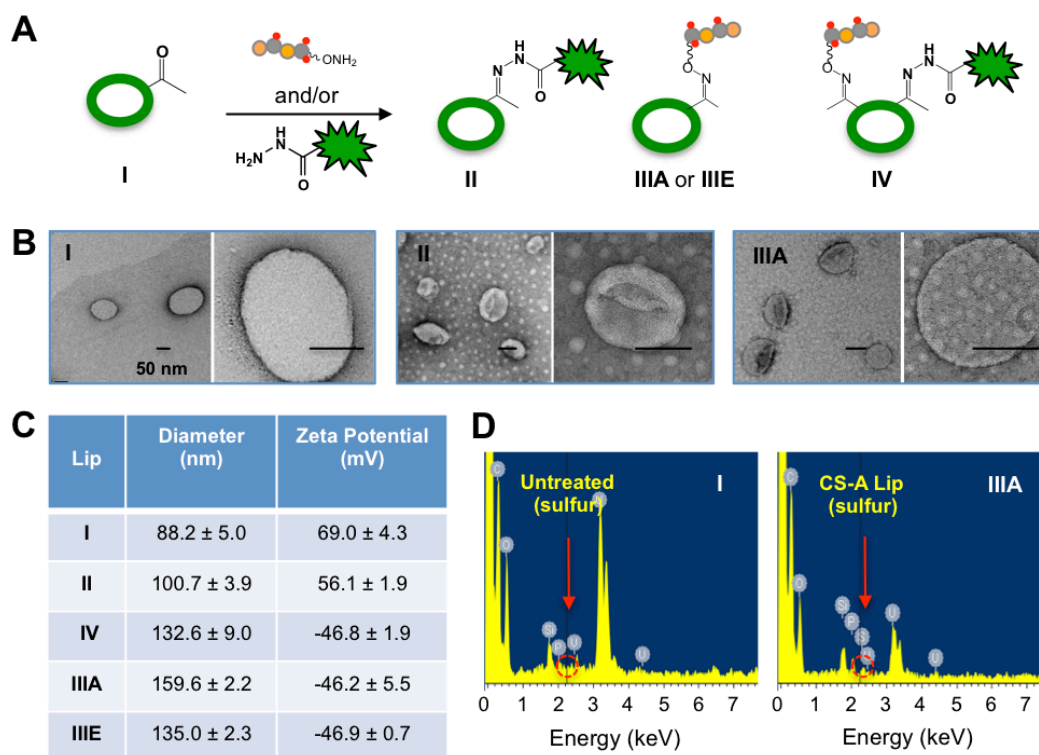


Figure S2. Biophysical characterization of the liposomes. Liposomal formation and molecule conjugation schematic with corresponding morphological, surface charge, size distribution, and elemental characterization. (A) Liposomes were formed with neutral DOPE, positively charged DOTAP, and dodecanone (at a ratio of 10:5:1). AF488-hydrazide, CS-A-aminooxy, and/or CS-E-aminooxy were then reacted with dodecanone-bearing liposomes (I), resulting in chemoselective hydrazone (II; IV) or oxime bond formation (IIIA (CSA); IIIE (CSE); IV). (B) Dodecanone-presenting liposomes (I) were functionalized with AF488-hydrazide (II) or CS-A-aminooxy polysaccharides (IIIA), negatively stained with uranyl acetate, and imaged using transmission electron microscopy. Images are depicted at both low and high magnification. (C) Dynamic light scattering revealed that the average liposomal hydrodynamic diameter increased when conjugated with the dye and/or polysaccharides, ranging from untreated to functionalized liposomes in the following order: I < II < IV < IIIE < IIIA. Similar size distributions were determined compared to those obtained by TEM analyses (B). The liposomal surface charge becomes negative when conjugated with CS-A and CS-E (IV, IIIE, and IIIA). (D) Energy dispersion spectroscopy analyses were performed on I and IIIA. As shown, sulfur was detected in the liposomes bearing CS-A (IIIA) when compared to untreated liposomes (I), indicating successful conjugation of CS-A-aminooxy polysaccharide.

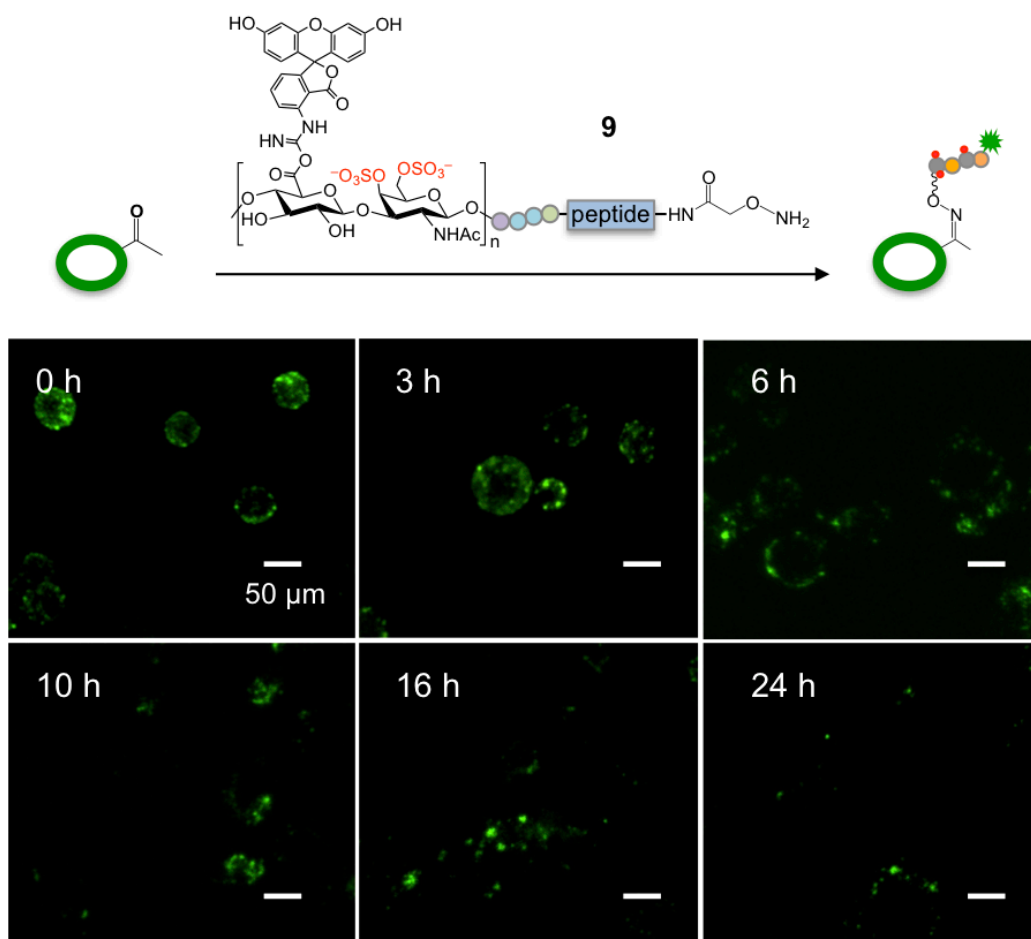


Figure S3. Membrane turnover of exogenous lipid-conjugated CS-E molecules. Dodecanone-containing liposomes were first reacted with fluorophore-conjugated aminoxy-CS-E, incubated with PC12 cells for 30 min, and then imaged using live cell fluorescence microscopy at 0 to 24 h after fusion. Fluorescence was primarily observed at the cell membrane after initial CS-E insertion. After 3 h and 6 h in culture, internalization of lipid-bound CS-E was observed, with increased loss in signal by 10 h, as cells appeared to be spreading out on the substrate. By 16 h after liposome incubation, weak fluorescence was observed around the cell periphery, indicating that the ligand-conjugated lipids had completely diffused from the membrane or were internalized.

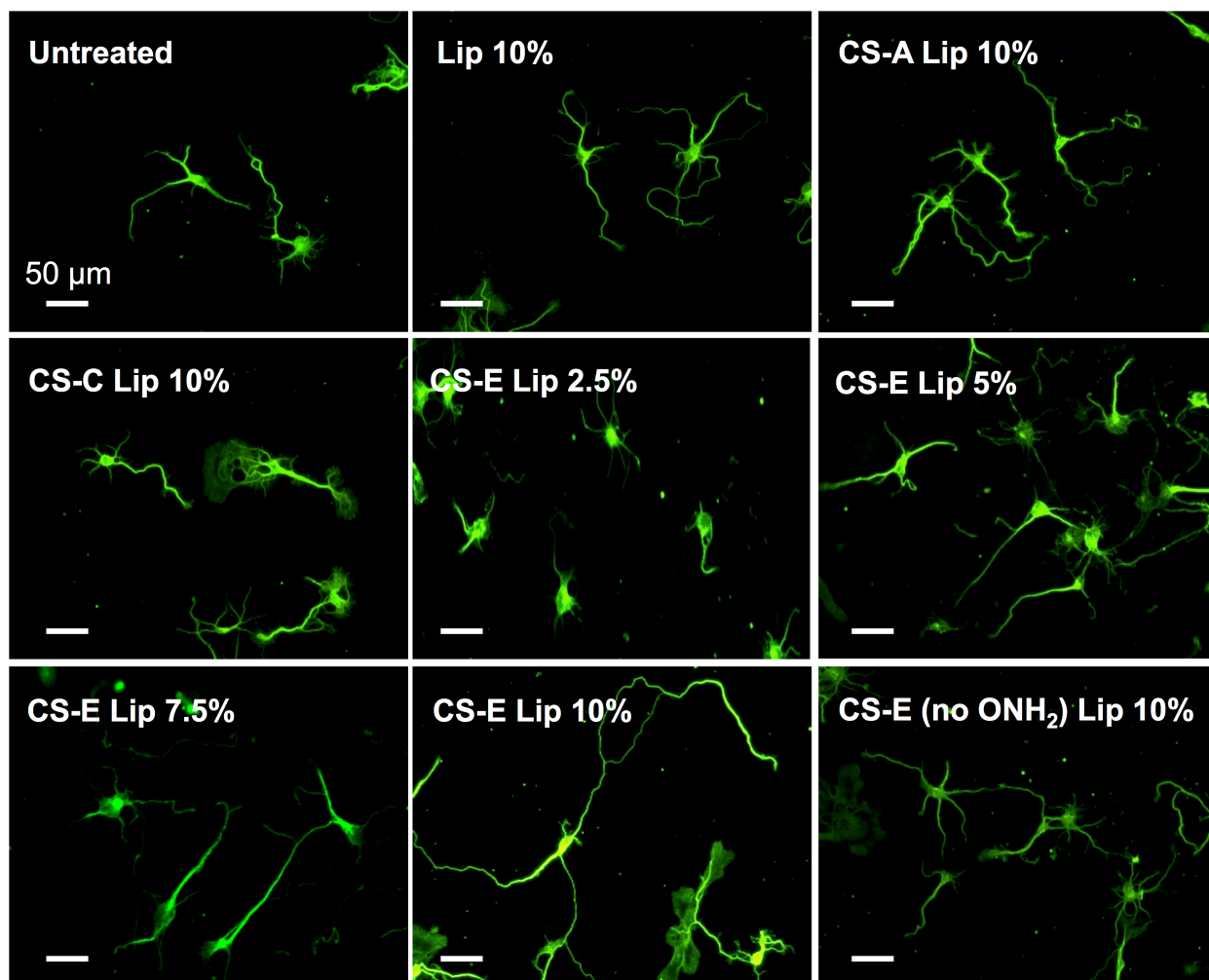


Figure S4. Presentation of CS-E polysaccharides on neuronal cell surfaces stimulates neurite outgrowth. Representative images of neurite outgrowth for neurons treated with unmodified liposomes or liposomes displaying CS-A, CS-C, and CS-E polysaccharides with varied liposomal dodecanone concentrations (2.5-10%) for 24 h. As controls, neurons were incubated with liposomes (Lip 10%) or liposomes that were incubated with CS-E polysaccharides lacking the aminoxy functionality (CS-E (no ONH₂) Lip 10%). Neurons were immunostained with a mouse anti- α -tubulin antibody and imaged using confocal fluorescence microscopy.

Materials and Methods

Chemicals, biochemical reagents, cell lines, and animals. All chemicals and reagents were of analytical grade, obtained from Sigma Aldrich (St. Louis, MO), and used without further purification unless specified. Chondroitin sulfate (CS)-A, -C, and -E polysaccharides and chondroitinase ABC (ChABC) were purchased from Seikagaku Corporation (Tokyo, Japan). Lipids, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All secondary antibodies (Alexa Fluor (AF) 680 goat anti-rabbit, AF680 goat anti-mouse, and AF488 goat anti-mouse), AF488 hydrazide sodium salt (AF488-hydrazide), and cell culture media and reagents were obtained from Life Technologies Corporation (Carlsbad, CA). Adherent pheochromocytoma cells (PC12 cells; CRL-1721.1), horse serum (HS), and F-12K media (30-2004) were obtained from American Type Culture Collection (Manassas, VA). Accutase (AM105) was received from Innovative Cell Technologies, Inc. (San Diego, CA). Nerve growth factor (NGF) was obtained from R&D Systems, Inc. (Minneapolis, MN). Primary antibodies against phospho-Akt (Ser473) (4060) and total Akt (pan) (4691) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Sprague-Dawley rats were obtained from Charles River Laboratories, Inc. (Hollister, CA).

Synthesis of aminoxy-conjugated CS polysaccharides.

tert-Butyl 2-((1,3-dioxoisindolin-2-yl)oxy)acetate (4). To a solution of *N*-hydroxyphthalimide (3.03 g, 18.6 mmol, 1.20 eq) in DMF (30 mL) was added sodium bicarbonate (1.56 g, 18.6 mmol, 1.20 eq). The mixture was stirred at 60 °C for 1 h, after which *tert*-butyl 2-bromoacetate (2.77 mL, 15.5 mmol) was added. The reaction mixture was stirred for 16 h at 60 °C. The mixture was then diluted with DCM (30 mL), extracted with 1 M NaHCO₃ until the aqueous layer became colorless, washed with ddH₂O (2 x 25 mL) and brine (1 x 25 mL), dried over MgSO₄, and concentrated to afford a yellow solid **4**. The product was purified by silica flash chromatography (Hex:EtOAc, 85:15) to afford a white solid **4** (3.76 g, 88%). ¹H NMR (500 MHz, CDCl₃): δ 1.48 (s, 9H, CH₃), 4.71 (s, 2H, CH₂), 7.75-7.76 (m, 2H, *J* = 5 Hz, Ar-H), 7.84-7.85 (m, 2H, *J* = 5 Hz, Ar-H).

2-((1,3-Dioxisoindolin-2-yl)oxy)acetic acid (5). Compound **5** was synthesized as previously described.¹ Briefly, TFA (2 mL) was slowly added to a solution of **4** (0.51 g, 1.8 mmol) in DCM (6.0 mL). The mixture was stirred at RT for 2 h under Ar. The reaction was then concentrated and azeotroped with DCM (3 x 5 mL) to afford a white solid **5** (0.40 g, 99%). ¹H NMR (500 MHz, CDCl₃): δ 4.88 (s, 2H, CH₂), 7.85-7.87 (m, 2H, *J* = 10 Hz, Ar-H), 7.93-7.94 (m, 2H, *J* = 10 Hz, Ar-H).

2-((1,3-Dioxisoindolin-2-yl)oxy)-conjugated CS polysaccharides (6-8). To a buffered solution containing 0.1 M MES and 0.5 M NaCl (pH 6.0), **5** (5.0 mg, 0.023 mmol), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC; 4.2 mg, 0.027 mmol, 1.2 eq), sulfo-*N*-hydroxysuccinimide (sulfo-NHS; 7.5 mg, 0.035 mmol, 1.5 eq) were added, and the mixture was stirred at RT for 30 min. Concurrently, CS-E, CS-A, or CS-C (4.0 mg, ~70 μmol, ~0.5 eq) was stirred in 0.05 M NaHCO₃ for 30 min. The polysaccharide amine solution was then added to the small molecule acid mixture and allowed to react for 12 h at RT under Ar. The mixture was then purified via dialysis against ddH₂O for 48 h (6 x 8 h) using a molecular weight membrane of 35,000 Da, followed by gel filtration chromatography (G-25). Fractions containing the polysaccharide were lyophilized to afford white solids **6-8**. For **6 CS-E**: ¹H NMR (500 MHz, CDCl₃): δ 2.04 (s, 3H, CH₃), 3.28-3.49 (m, 2H, CH), 3.51-4.35 (m, 9H, CH, CH₂OSO₃⁻), 4.42-4.67 (m, 1H, CH), 7.48 (m, 2H, Ar-H), 7.58 (m, 2H, Ar-H). 64% yield. For **7 CS-A**: ¹H NMR (500 MHz, CDCl₃): δ 2.03 (s, 3H, CH₃), 3.40 (br s, 1H, CH), 3.64 (br s, 1H, CH), 3.72-3.95 (m, 6H, CH), 4.03 (br s, 2H, CH), 4.51-4.63 (m, 2H, CH), 7.62 (m, 1H, Ar-H), 7.77 (m, 1H, Ar-H). 60% yield. For **8 CS-C**: ¹H NMR (500 MHz, CDCl₃): δ 2.02 (s, 3H, CH₃), 3.39 (br s, 1H, CH), 3.64 (br s, 1H, CH), 3.72-4.06 (m, 6H, CH, CH₂OSO₃⁻), 4.12-4.27 (m, 3H, CH), 4.57 (br s, 1H, CH), 7.62 (m, 2H, Ar-H), 7.75 (m, 2H, Ar-H). 78% yield.

Aminoxy-conjugated CS polysaccharides (1-3). Hydrazine monohydrate (1 μL, 0.031 mmol, 4.3 eq) was added dropwise to a solution of **6-8** (4.0 mg, ~70 μmol) in ddH₂O (4 mL), and the mixture was stirred for 3 h at RT under Ar. The solution was then purified via dialysis against ddH₂O for 48 h (6 x 8 h) using a molecular weight membrane of 35,000 Da, followed by gel filtration chromatography (G-25). Fractions containing the polysaccharide were lyophilized to afford white solids **1-3**. For **1 CS-E**: ¹H NMR (600 MHz, CDCl₃): δ 2.04 (s, 3H, CH₃), 3.38 (br s, 1H, CH), 3.56-3.85 (m, 5H, CH), 3.86-4.33 (m, 4H, CH, CH₂OSO₃⁻), 4.42-4.67 (m, 2H, CH).

For **2 CS-A**: ^1H NMR (500 MHz, CDCl_3): δ 2.04 (s, 3H, CH_3), 3.39 (br s, 1H, CH), 3.61 (br s, 1H, CH), 3.69-3.87 (m, 6H, CH), 4.08 (br s, 2H, CH), 4.46-4.64 (m, 2H, CH). For **3 CS-C**: ^1H NMR (500 MHz, CDCl_3) δ 2.03 (s, 3H, CH_3), 3.38 (br s, 1H, CH), 3.61 (br s, 1H, CH), 3.69-4.06 (m, 5H, CH, $\text{CH}_2\text{OSO}_3^-$), 4.15-4.27 (m, 3H, CH), 4.46-4.65 (m, 2H, CH).

Fluorophore-conjugated aminoxy-CS-E (9). The protocol for conjugating aminoxy-CS-E with fluoresceinamine was adapted from Glabe *et al.*² To a stirring solution of **1** (1.00 mg, 14.29 μmol) in ddH_2O (1 mL) was added cyanogen bromide (1.00 mg, 0.00094 mmol, excess) in ddH_2O (0.3 mL). The pH of the reaction was adjusted to 11.0 using 0.2 M NaOH, which was stirred for 10 min at RT. The mixture was then desalted on a disposable PD-10 sephadex column (17-0851-01, GE Healthcare Biosciences, Pittsburgh, PA) using 0.2 M sodium borate buffer (pH 8.0). The CS-E fractions were pooled (3 mL) and immediately stirred with fluoresceinamine (1.00 mg, 0.0029 mmol, excess) (Sigma Aldrich Co., St. Louis, MO) in the dark overnight (12 h). The mixture was concentrated and purified on a G-25 sephadex column in the dark. The CS-E fractions were again pooled, flash frozen, and lyophilized to afford an orange solid **9**.

Liposome formation. A solution of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, 120 μL , 10 mg/mL in CHCl_3), dodecanone (48 μL , 10 mg/mL in CHCl_3 , 0.1% *w/w*), and 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP, 64 μL , 10 mg/mL in CHCl_3 , 0.5% *w/w*) were thoroughly mixed and then dried with a stream of argon followed by high vacuum for 12 h. The dried lipids were reconstituted in 1.16 mL ddH_2O (2 mg/mL total lipids) and hydrated for 30 min. The mixture was then sonicated for 2 min and extruded (11 passes through the membrane) to form large unilaminar vesicles using a mini-extruder (Avanti Polar Lipids, Inc.; Alabaster, AL) according to the manufacturer's instructions.

Liposome conjugation. AF488-hydrazide-presenting liposomes: Liposomes (100 μL , 2.24 mM dodecanone) were incubated with AF488-hydrazide (12.8 μL , 1.75 mM, 0.1:1 hydrazide:ketone) for 3 h at RT in the dark overnight at a stoichiometric ratio to label approximately 10% of the available ketones. This ratio was chosen after method optimization to conserve carbohydrate material but maintain a significant amount of cell labeling. CS-aminoxy-tethered liposomes: Liposomes (100 μL , 2.24 mM dodecanone) were reacted with CS-A/C/E-aminoxy polysaccharides **1-3** (~20 μL , 33.3/31.7/25.6 μM) for 3 h at RT in the dark overnight. Mixed-displaying liposomes: Liposomes (100 μL , 2.24 mM dodecanone) were reacted with CS-A/C/E-

aminoxoy polysaccharides (~20 μ L, 33.3/31.7/25.6 μ M) for 4 h at RT in the dark, followed by AF488-hydrazide at a stoichiometric ratio to label approximately 10% of the available ketones.

Dynamic light scattering (DLS). Ketone-presenting (**I**) and AF488-hydrazide- (**II**), mixed AF488/CS-A-ONH₂- (**IV**), CS-A-ONH₂- (**IIIA**), and CS-E-ONH₂- (**IIIE**) conjugated liposomes were generated as described above and diluted (1:20) with 1.5 mM KCl. The size distributions of these liposomal samples were then determined using a DynaPro NanoStar (Wyatt Technologies; Santa Barbara, CA) DLS instrument. The data are represented as the mean liposomal hydrodynamic diameter \pm S.E.M. with eight to ten measurements conducted per experiment (n = 3).

Zeta Potential analyses. Ketone-presenting (**I**) and AF488-hydrazide- (**II**), mixed AF488/CS-A-ONH₂- (**IV**), CS-A-ONH₂- (**IIIA**), and CS-E-ONH₂- (**IIIE**) conjugated liposomes were generated as described above, diluted (1:20) with 1.5 mM KCl, and subjected to zeta potential analyses using a ZetaPALS (Brookhaven Instruments Corp., Holtsville, NJ) analyzer. The data are represented as the mean zeta potential \pm S.E.M. with three to ten measurements conducted per experiment (n = 3).

Transmission electron microscopy (TEM) and energy dispersion spectroscopy (EDS). Dodecanone-containing liposomes were prepared and conjugated with AF488-hydrazide and CS-A-aminoxoy polysaccharide as described above. A drop of each liposome sample was placed on a TEM copper/carbon grid for 30 s, which was then blotted using filter paper. A drop of 2% uranyl acetate was then added to each sample for 5 min followed by blotting with filter paper. The grid was then rinsed with ddH₂O and dried. TEM and elemental analyses of each liposome sample were performed using an FEI Tecnai F30UT (300 kV) equipped with a high angle annular dark field detector, an Oxford energy dispersion X-ray detector, and an AMT CCD camera.

Cell culture. PC12 cells were cultured on collagen-coated (10 μ g/mL) tissue culture plates in F-12K media supplemented with 6.5% HS, 6.5% FBS, and 1x penicillin/streptomycin (P/S) at 37 $^{\circ}$ C with 5% CO₂. Hippocampal and cortical neurons were cultured in Neurobasal media supplemented with B27, 20 mM GlutaMAX, and 1x P/S at 37 $^{\circ}$ C with 10% CO₂.

Cell-surface remodeling. AF488-hydrazide-bearing liposomes were diluted 1:100 with the appropriate cell culture medium and incubated with PC12 cells or hippocampal neurons for 30-45 min. The medium was evacuated, and the cells were washed three times with 0.5 mL of sterile 1x phosphate buffered saline (PBS, Sigma-Aldrich). Fresh medium (0.5 mL) was then added, and the cells were imaged live under a Zeiss LSM 5 Pascal inverted confocal microscope (Carl Zeiss Microscopy; Thornwood, NJ). For labeling differentiated PC12 cells with CS-E, PC12 cells were cultured on collagen-coated tissue culture plates and stimulated with NGF (100 μ M, 256-GF; R&D, Minneapolis MN) to induce differentiation. After 4 d of NGF treatment, the cells were treated with 1 U/mL ChABC (1:1 activation buffer (0.4 M Tris/HCl pH 8.0, 0.4 M NaOAc, 0.1% BSA)/complete medium) for 2 h (37 °C, 5% CO₂). The cells were then washed with PBS (4 x 0.5 mL), and medium that was supplemented with CS-E-functionalized liposomes (diluted 1:100) was added for 30-45 min. After fusion, the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min, stained with a monoclonal mouse anti-CS-E antibody^{2,3} (1:250 in 1% BSA/0.1% Triton X-100/PBS; 4°C, 12 h), followed by an AF488-conjugated goat anti-mouse antibody (1:1000 in 1% BSA/0.1% Triton X-100/PBS; RT, 1 h), and imaged using a Zeiss LSM 5 Pascal inverted confocal microscope.

Immunocytochemistry. For imaging, cells were fixed with 4% PFA in PBS (15 min), rinsed with PBS twice, permeated, and blocked with a solution containing 1% BSA and 0.1% Triton X-100 (20 min), incubated with a monoclonal mouse anti-CS-E antibody (1:250; 4 °C, 12 h), washed with PBS (2 x 5 min), incubated with an anti-mouse AF488 antibody (1:1000; RT, 1 h), washed with PBS (2 x 5 min), and imaged under a Zeiss LSM 5 Pascal inverted confocal microscope.

Flow cytometry. Dodecanone-containing liposomes were prepared as mentioned above with the exception that the % w/w of dodecanone to DOPE was varied (0, 5, 10, 15, and 20%). PC12 cells were cultured until ~80% confluent and then detached from the tissue culture plate using TrypLE (Life Technologies, Inc.). The cells were twice washed with cold PBS (500 μ L) and spun down (1,400 x g, 2.5 min). For AF488-hydrazide labeling, the cells were resuspended and incubated on ice in a buffered solution (45 mM BSA, 10 mM HEPES, and DNase I (0.5 U/mL) in HBSS at pH 7.4) containing 0, 5, 10, 20% w/w ketone-hydrazide conjugated liposomes for 30 min (diluted 1:100). The dye concentration was adjusted per condition according to the ketone concentration in the liposomes at a stoichiometric ratio to label approximately 10% of the available ketones.

After labeling, the cells were again twice washed with cold PBS (500 μ L) and spun down (1,400 x g, 2.5 min). After resuspension in the buffer listed above, the cells were filtered through a 40 μ m-filtered FACS tube, and subjected to flow cytometric analyses using a FACSCalibur flow cytometer (BD Biosciences; San Jose, CA). For CS-E labeling, PC12 cells were incubated with a monoclonal mouse anti-CS-E antibody (1:250) for 1 h followed by two wash cycles, incubated with an AF488-conjugated goat anti-mouse antibody (1:1000) for 1 h on ice, washed again twice, and subjected to flow cytometric analyses. The labeled samples were referenced to untreated cells. As controls, PC12 cells were separately incubated with liposomes without the ketone and hydrazide-AF488 or the AF488-conjugated goat anti-mouse antibody. Each sample was run in duplicate, and each sample set was performed twice.

Cell internalization assay. Liposomes were reacted with **9** as mentioned above for 3 h at RT in the dark. The liposome stock solution was diluted 1:100 with HBSS (45 mM BSA, 10 mM HEPES, pH 7.4) and added (400 μ L) to PC12 cells for 30 min on ice in the dark. After labeling, the cells were diluted with PBS (500 μ L), washed (3 x 500 μ L wash/centrifuge cycles), and added to Roswell Park Memorial Institute (RPMI) medium without phenol red. The cells were imaged live at 0, 3, 6, 10, 16 and 24 h after labeling under a Zeiss 700 LSM confocal microscope.

Neurite outgrowth assays. Hippocampal neurons from E18 Sprague-Dawley rats were dissected as previously described³ and plated at 2,000 cells/well on poly-lysine-coated 96-well plates (BD Biosciences; San Jose, CA) in 50 μ L of complete DMEM (10% FBS, 1% P/S) for 30 min, followed by 150 μ L of complete Neurobasal (B27, 20 mM GlutaMAX, 1% P/S). After 48 h, CS-A, -C, and -E-conjugated ketone liposomes (10% w/w dodecanone; 2.5-10% ketone for CS-E dose-dependent studies) were added (diluted 1:100 in 200 μ L of Neurobasal) in complete Neurobasal. The medium was replaced after 30 min to remove the remaining liposomes and unreacted CS. Due to endocytosis of CS after approximately 6-10 h, cells were treated with polysaccharide-displaying liposomes again at 8 and 16 h, followed by medium replacement 30 min after addition. At each time point, the cells were fixed with 4% PFA (20 min), washed with PBS (2 x 5 min), permeabilized with 1% BSA and 0.1% Triton X-100 in PBS (15 min), washed with PBS (2 x 5 min), incubated with a mouse α -tubulin antibody (Sigma-Aldrich; St. Louis, MO; 1:1000) at 4 $^{\circ}$ C for 12 h, washed with PBS (2 x 5 min), incubated with a AF488-conjugated goat anti-mouse antibody (1:1000) at RT for 1 h, washed with PBS (2 x min), and imaged under

a Zeiss LSM 5 Pascal inverted confocal microscope as previously described.⁴ Each well was reconstructed in Adobe Illustrator after capturing a 9 x 9 grid of 2034-pixel images. Four to five wells were blindly subjected to MetaMorph (Molecular Devices; Sunnyvale, CA) neurite outgrowth software analyses to calculate the mean outgrowth per neuron. As controls, untreated neurons, neurons subjected to ketone liposomes without polysaccharide, and neurons treated with ketone liposomes (+CS-E without an aminoxy group) were assayed for outgrowth. The data are represented as the mean outgrowth percentage (\pm S.E.M.) with $n = 7$ ($n > 200$ cells per assay).

Stimulation assays. Cortical neurons from E18 Sprague-Dawley rats were dissected as previously described⁵ and plated at 5×10^6 cells per poly-ornithine-coated (Sigma Aldrich; St. Louise, MO) 10-cm plate in complete DMEM (10% FBS, 1% P/S) for 30 min, followed by complete Neurobasal (B27, 20 mM glutaMAX, 1% P/S). After 7 d, CS-C- and CS-E-conjugated ketone liposomes (10% w/w dodecanone) were added to the complete Neurobasal for 30 min. The medium was then replaced with fresh Neurobasal that contained 50 ng/mL NGF (256-GF; R&D Systems, Minneapolis, MN), and the neurons were stimulated for 0, 10, 30, and 60 min. At each time point, the medium was evacuated, the cells were scraped and collected in cold PBS (1 mL), centrifuged (4 °C, 3,000 x g, 2:30 min), and homogenized in lysis buffer (50 mM Tris/HCl pH 7.4, 250 mM mannitol, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM Na₂VO₄, 1% Triton X-100, and 1x complete protease inhibitors (Roche) by 10 passages through a 26-gauge 3/8" needle on ice. The lysates were then cleared (4 °C, 16,000 x g, 15 min), and the total protein concentration was determined for each sample using the BCA assay (Thermo Scientific Pierce; Rockford, IL). Western blot analyses were performed as previously described⁴, blotting separately with a rabbit anti-phospho-Akt(Ser473) antibody and a rabbit anti-Akt antibody at 1:1000 in 5% BSA. Akt activation was calculated by determining the ratio of phospho-Akt to total Akt for each time point and condition normalized to α -tubulin staining. Relative Akt activation was reported with respect to the untreated values (+NGF, no liposomes).

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