Supplementary Information for

Quantitation of *O*-Glycosylation Stoichiometry and Dynamics using Resolvable Mass Tags

Jessica E. Rexach[1], Claude J. Rogers[1], Seok-Ho Yu[1], Jifang Tao[2], Yi E. Sun[2], Linda C. Hsieh-Wilson[1]‡

[1]*Division of Chemistry and Chemical Engineering and Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California 91125, USA.*

[2]*Neuropsychiatric Institute, Medical Retardation Research Center, University of California, Los Angeles, Los Angeles, CA 90095, USA.*

‡ To whom correspondence should be addressed. E-mail: lhw@caltech.edu

This file includes

Supplementary Methods

General reagents

Unless otherwise noted, reagents were purchased from the commercial suppliers Fisher (Fairlawn, NJ) and Sigma-Aldrich (St. Louis, MO) and were used without further purification.

Synthesis of aminooxy-functionalized PEG derivatives 2 and 3. To synthesize **2**, methoxypolyethylene glycol 2,000 propionic acid *N*-succinimidyl ester (Laysan; 0.1 mmol) was dissolved in DMF (3.5 ml), and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; 1 mmol) and *N*,*N*-diisopropylethylamine (4 mmol) were added. For the synthesis of **3**, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; 1 mmol), hydroxybenzotriazole (HOBt; 1 mmol), and *N*,*N*-diisopropylethylamine (4 mmol) were added to a solution of methoxypolyethylene glycol 5,000 propionic acid (Sigma-Aldrich; 0.1 mmol) in CH_2Cl_2 (3.5 ml). After stirring for 30 min at room temperature (RT), 1,2-diaminooxyethane dihydrochloride (1 mmol)[1] was added to each reaction mixture, and the solutions were allowed to stir for 2 d. The desired compounds were isolated by precipitation in ice-cold isopropyl alcohol to afford white solids (88% for **2** and **3)**. ^{1}H NMR of **2** (600 MHz, $CDCl_3$): δ = 3.71–3.58 (m, 244H), 3.56–3.54 (m, 4H), 3.38 (s, 3H). ^{13}C NMR of **2** (125 MHz, $CDCl_3$): δ = 70.7. ^{1}H NMR of **3** (600 MHz, $CDCl_3$): δ = 3.68–3.55 (m, 473H), 3.53–3.51 (m, 4H), 3.35 (s, 3H). ^{13}C NMR of **3** (125 MHz, $CDCl_3$): δ = 72.6, 72.0, 70.7, 70.4, 61.8, 59.1.

Chemoenzymatic labeling of purified CREB with PEG mass tags. $CREB_{mono}$ and $CREB_{co}$ were over-expressed and purified from *Sf9* cells as described[2]. $CREB_{co}$ (0.52 μg of a 1:5 mixture

of CREB:OGT) and $CREB_{mono}$ (0.13 μg per reaction) were incubated with Y289L GalT (0.1 mg ml^{-1}; expressed and purified as described[3]) and UDP-ketogalactose derivative **1** (500 μM; synthesized as reported[4]) in buffer containing 10 mM HEPES pH 7.9, 10 mM $MnCl_2$, 100 mM NaCl, Complete™ protease inhibitor cocktail (Roche) and 1 mM PMSF (8.3 μl total reaction volume) for 16 h at 4 °C. Negative controls were processed in parallel in the absence of **1**. The samples were diluted to 25 μl per reaction and dialyzed into denaturing buffer (5 M urea, 10 mM HEPES pH 7.5, 1 mM PMSF; 3 x 3h), after which the sample was acidified to pH 4.5 with 1.8 M NaOAc (final concentration 50 mM) and reacted with 4 mM of **2** or **3** (60 mM stock in H_2O) for 20-24 h at RT. Reactions were stopped by neutralization with 1 M HEPES pH 7.9 and boiling in SDS-PAGE sample loading dye (50 mM Tris pH 6.8, 100 mM DTT, 2% SDS, 0.05% bromophenol blue, 10% glycerol).

Testing for complete incorporation of the aminooxy-PEG derivative into CREB. $CREB_{mono}$ (0.13 μg per reaction) was subjected to chemoenzymatic labeling with **1** and Y289L GalT as described above to generate ketogalactose-modified $CREB_{mono}$. Previous studies have demonstrated that this enzymatic step proceeds quantitatively[4]. The resulting sample was dialyzed into denaturing buffer (5 M urea, 10 mM HEPES pH 7.5, 1 mM PMSF; 3 x 3h) and divided into two equal portions. One portion was incubated with **3** for 24 h at RT under $CREB_{mono}$ reaction conditions (see above). Subsequently, both portions of the sample were incubated for an additional 24 h at RT with 2.75 mM *N*-(aminooxyacetyl)-*N*'-(D-biotinoyl) hydrazine (30 mM aqueous stock, Dojindo) in the denaturing buffer described above. Samples were analyzed by blotting with streptavidin-IR800 (1:50,000; 1h RT incubation in Tris buffered saline with 0.1% Tween 20) and an anti-CREB antibody (Chemicon; 1:1000), the latter of which

was detected using an Alexa Fluor 680 goat anti-mouse secondary antibody.

Assessing the sensitivity of the mass tagging approach. A cell pellet of *Sf9* cells overexpressing OGT (p110) was lysed with boiling 1% SDS containing Complete™ protease inhibitor cocktail and 1 mM PMSF. Lysates were sonicated, boiled for 5 min, and centrifuged at 21,885 xg for 10 min to remove cellular debris. Protein concentrations were determined by the BCA assay (Pierce). The lysate (275 μg) was subjected to chemoenzymatic labeling with **1** and **2** (see **Methods**). Labeled lysates were subsequently diluted with unlabeled equivalent lysate (1:0, 3:1, 1:1, 1:3, 1:8, 0:1 ratio of labeled:unlabeled lysate) to generate six 100-μg standards with varying extents of PEG-labeled OGT (0-3.3%). Samples were precipitated with MeOH/$CHCl_3$/H_2O and re-suspended in gel loading buffer. A portion (25 μg) of each sample was analyzed by SDS-PAGE and immunoblotted with an anti-OGT antibody (DM-17). Western blots were visualized and quantified as described in the **Methods** section.

Validating detection of PEG-labeled protein across a range of stoichiometries. 293T cell lysate (450 μg in 1% SDS; see below for preparation) was subjected to chemoenzymatic labeling with **1** and Y289L GalT as described (see **Methods**). Labeled lysates were subsequently diluted with unlabeled equivalent lysate (1:0, 8:1, 3:1, 5:3, 1:1, 3:5, 1:3, 1:8, 0:1 ratio of labeled:unlabeled lysate) to generate eight 100-μg standards varying in their extent of ketogalactose incorporation into *O*-GlcNAc moieties (100, 87.5, 75, 62.5, 50, 37.5, 25, 12.5, and 0%). Each sample was incubated with **2** for 24 h at RT under standard reaction conditions. Samples were precipitated with MeOH/$CHCl_3$/H_2O and re-suspended in gel loading buffer. A

portion (25 μg) of each sample was analyzed by SDS-PAGE and Western blotting for Nup62. The glycosylation stoichiometry of Nup62 was quantified as described in the **Methods** section.

Culturing of embryonic rat cortical neurons and 293T cells. Cortical neurons from E18 Long Evans rats were cultured as described[5]. Cells were plated on poly-DL-ornithine- and fibronectin- (Sigma) coated dishes. Neurons were maintained for up to 5 days in vitro (DIV) in Basal Medium Eagle (Sigma) containing 5% fetal bovine serum, penicillin (100 U ml^{-1}), streptomycin (100 mg ml^{-1}), glutamine (2 mM), and B27 serum-free supplement (Invitrogen) and treated as described in the **Methods**. For all neuronal depolarization experiments, basal neuronal activity was silenced by incubation with tetrodotoxin (TTX; 1 μM, added 2-4 h before treatment). HEK 293T cells were cultured in high glucose DMEM (Gibco) containing 10% fetal bovine serum, penicillin (100 U ml^{-1}), and streptomycin (100 mg ml^{-1}).

Preparation of cell and tissue lysates. Cell cultures were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4) and rapidly lysed with boiling 1% SDS containing Complete™ protease inhibitor cocktail and 1 mM PMSF. Lysates were sonicated to shear DNA, boiled for 5 min, and centrifuged at 21,885 xg for 10 min. Protein concentrations were determined using the BCA assay.

For experiments studying MeCP2 phosphorylation at Ser-80, nuclear extracts were prepared using the Qiagen Qproteome cellular fractionation kit following the manufacturer's protocol in order to remove contaminating immunoreactive bands. All buffers were supplemented with 10 μM PUGNAc (*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino *N*-phenylcarbamate, Toronto Research Chemicals) and phosphatase inhibitors (1 mM $NaVO_3$, 20

mM NaF and 0.5 mM Na_2MoO_4), and the resulting nuclear fraction was precipitated by the MeOH/$CHCl_3$/H_2O method and resuspended in 1% SDS containing Complete™ protease inhibitor cocktail.

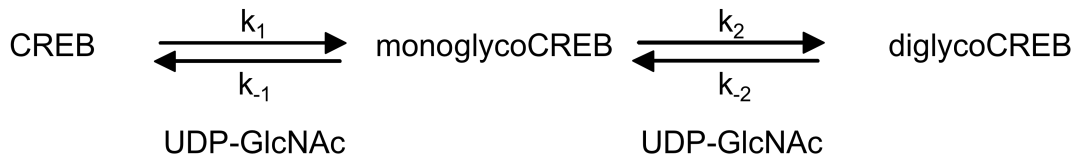
For kinetic studies of mono- and diglycosylation of CREB, crude nuclear extracts were prepared from cell cultures to enrich the sample. Cell cultures were washed twice with ice-cold PBS and lysed in ice-cold cytoplasmic lysis buffer (10 mM HEPES, 10 mM KCl, 2 mM $MgCl_2$, 0.5% NP-40, 0.2 mM Na_3VO_4, 0.5 mM NaF, 50 mM GlcNAc, 10 μM PUGNAc, protease inhibitor cocktail, and 1 mM PMSF). Lysates were centrifuged at 4,000 xg for 5 min at 4 °C, and the supernatant was discarded. The crude nuclear pellets were then lysed with an equal volume of 2% SDS with protease inhibitors, sonicated, boiled for 5 min, and centrifuged at 21,885 xg for 10 min.

To label CREB from tissue samples, tissues from adult Sprague Dawley rats were finely minced with a razor and homogenized using a Kontes all glass Dounce homogenizer (B type pestle) in ice-cold cytoplasmic lysis buffer (described above). Lysates were centrifuged at 4,000 xg for 5 min at 4 °C, and the supernatant was discarded. Pellets were washed once with 20 mM HEPES, 25% glycerol, 2 mM $MgCl_2$, 1 mM EDTA, 50 mM GlcNAc and protease inhibitors, and centrifuged again at 4,000 xg for 5 min at 4 °C. The crude nuclear pellets were then lysed with an equal volume of 2% SDS with protease inhibitors, sonicated, boiled for 5 min, and centrifuged at 21,885 xg for 10 min. Supernatants were collected and protein concentrations determined using the BCA assay.

General procedure for immunoblotting. Immunoblotting was performed using standard methods[6]. The following primary antibodies were used: CREB (5432, Chemicon, 1:1000),

CREB (06-863, Upstate, 1:500), pS133 CREB (Millipore, 1:1000), OGT (a kind gift from Prof. Gerald Hart, Johns Hopkins University, 1:1000), OGT (DM-17, Sigma, 1:1000), GRASP-55 (BD Transduction Laboratories, 1:1000), OGA (a kind gift from Prof. Sidney Whiteheart, University of Kentucky, 1:1000), Nup62 (BD Transduction Laboratories, 1:1000), Sp1 (Upstate, 1:1000), synapsin (a kind gift from Profs. Paul Greengard and Angus Nairn; note that this antibody detects synapsin Ia, IIa and IIIa. Synapsin Ia migrates slightly above synapsin IIa), MeCP2 (Upstate, 1:500), MeCP2 (Hu antibody, generated by Drs. Keping Hu and Weidong Wang), and pS80 MeCP2[7]. Pan *O*-GlcNAc levels were detected with the anti-*O*-GlcNAc antibody CTD110.6 (Covance, 1:2000). The following secondary antibodies were used: IRDye 800 goat anti-rabbit (Rockland Immunochemicals) and Alexa Fluor 680 goat anti-mouse (Molecular Probes). For quantification of *O*-GlcNAc stoichometry, we recommend testing multiple antibodies.

Measuring the relative glycosylation rates of mono- and diglycosylated CREB. Neuro2a cells were treated GlcN for 0, 15, 30, 120 or 360 minutes. Crude nuclear extracts were prepared, and each sample (100 μg) was subjected to chemoenzymatic labeling with PEG derivative **2** as described in the **Methods**. Following resolution by SDS-PAGE and gel transfer, the membranes were immunoblotted with an anti-CREB antibody. For each sample, the percentage of monoglycosylated or diglycosylated CREB, minus the initial amount of each glycoform (at t = 0), was quantified and plotted as a function of time. The apparent rate constants were approximated as follows:



If we assume that the UDP-GlcNAc concentration remains constant at a given time point and thus does not contribute significantly to the relative rates of mono- versus diglycosylation and that the reverse reactions under conditions of GlcN stimulation are small (i.e. $k_1 >> k_{-1}$ and $k_2 >> k_{-2}$), then

$$\frac{d[\text{monoglycoCREB}]}{dt} = k_1[\text{nonglycoCREB}] - k_2[\text{monoglycoCREB}]$$

$$\frac{d[\text{diglycoCREB}]}{dt} = k_2[\text{monoglycoCREB}]$$

And the relative rate of formation of monoglycosylated CREB to diglycosylated CREB,

$$\frac{\text{Relative rate(mono)}}{\text{Relative rate(di)}} = \frac{\frac{d[\text{monoglycoCREB}]}{dt}}{\frac{d[\text{diglycoCREB}]}{dt}}$$

Solving for the ratio of the rate constants,

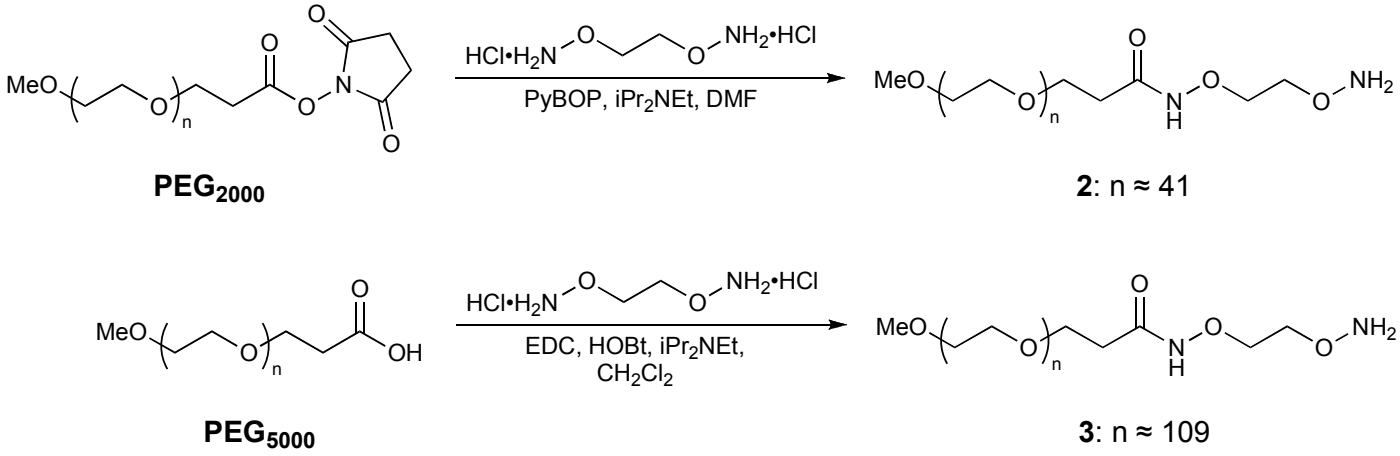
$$\frac{k_1}{k_2} = \frac{[\text{monoglycoCREB}] \times \text{relative rate(mono : di)} + [\text{monoglycoCREB}]}{[\text{nonglycoCREB}]}$$

The relative rate of formation of monoglycosylated CREB to diglycosylated CREB (relative rate (mono:di) = 4.0) was calculated as the ratio of the initial rates of formation of each species, which was the slope of the linear fit across the first three time points in the graph. From this value, the ratio of the rate constants for mono- and diglycosylation of CREB (k_1/k_2) was approximated at 15 min and 30 min to be 2.9 and 3.3, respectively, and the average value of 3.1 was reported.

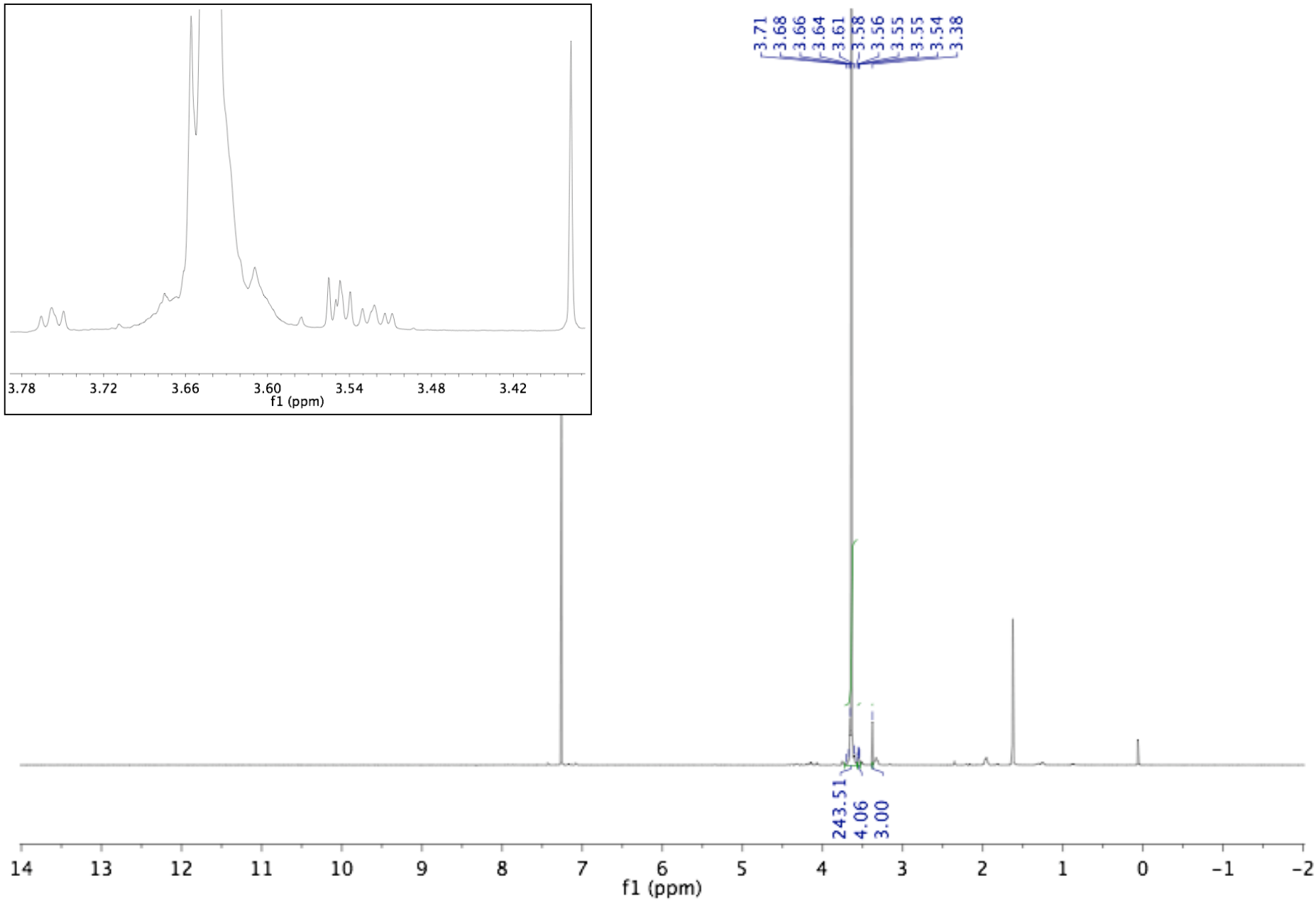
References

1. Shirayev, A., Lin, P.K.T. & Moiseev, I.K. *Synthesis-Stuttgart* **1**, 38-40 (1997).
2. Lamarre-Vincent, N. & Hsieh-Wilson, L.C. *J. Am. Chem. Soc.* **125**, 6612-6613 (2003).
3. Ramakrishnan, B. & Qasba, P.K. *J. Biol. Chem.* **277**, 20833-20839 (2002).
4. Khidekel, N.*, et al. J. Am. Chem. Soc.* **125**, 16162-16163 (2003).
5. Tao, X., Finkbeiner, S., Arnold, D.B., Shaywitz, A.J. & Greenberg, M.E. *Neuron* **20**, 709-726 (1998).
6. Clark, P.M.*, et al. J. Am. Chem. Soc.* **130**, 11576-11577 (2008).
7. Tao, J.*, et al. Proc. Natl. Acad. Sci. U S A* **106**, 4882-4887 (2009).

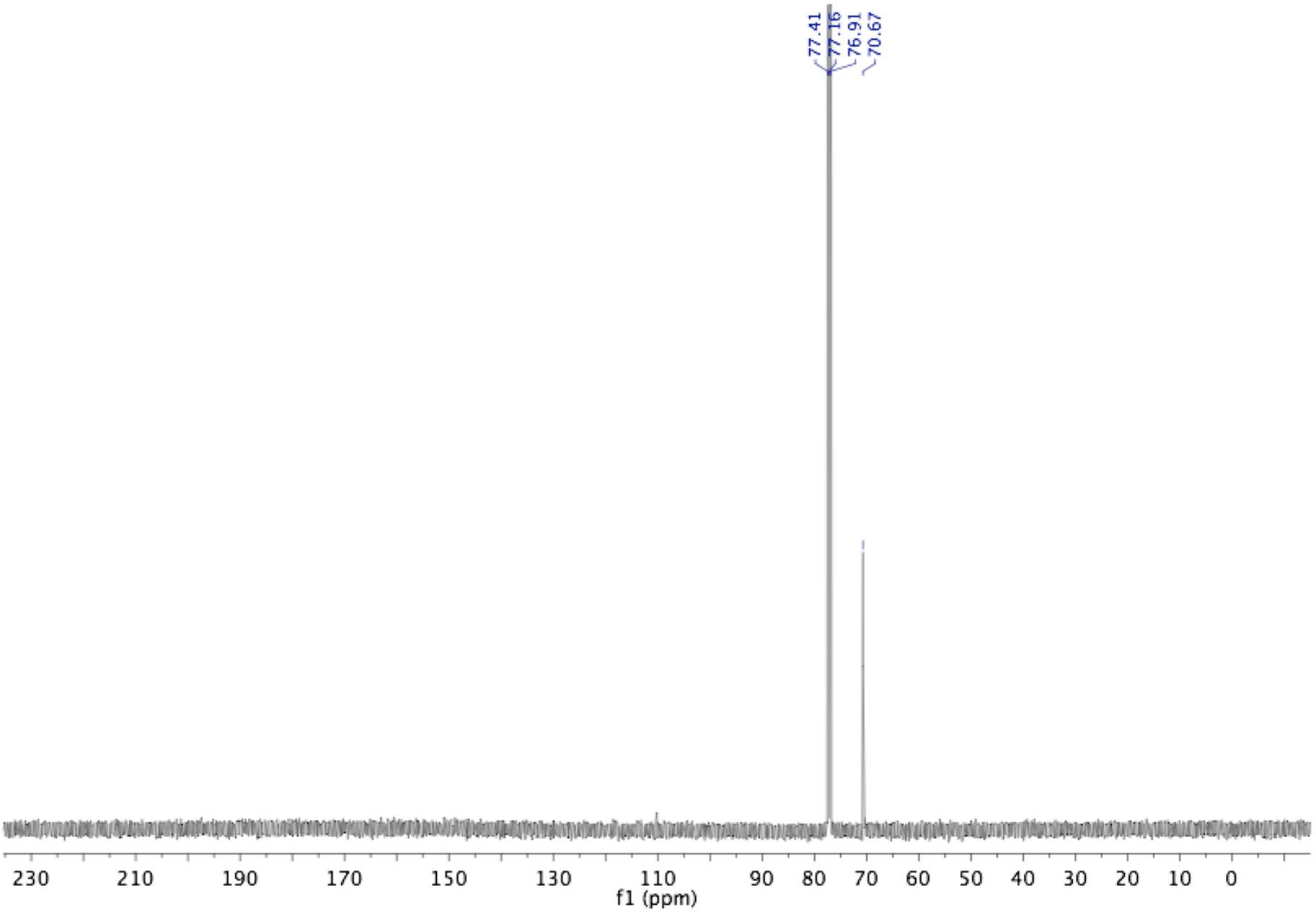
Supplementary Figures



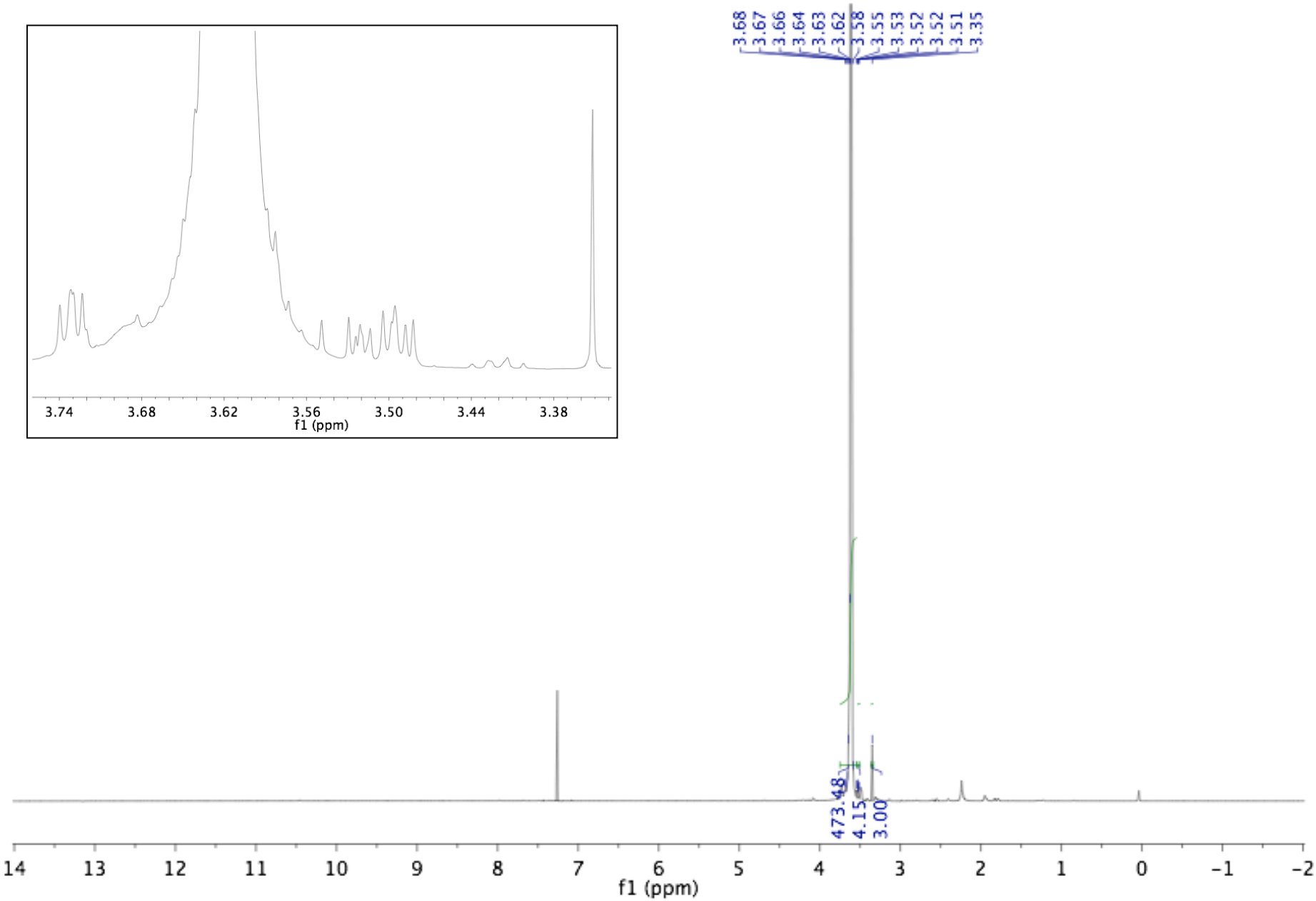
Supplementary Scheme 1. Synthesis of aminooxy-functionalized PEG derivatives **2** and **3**. See **Supplementary Methods** for experimental details.



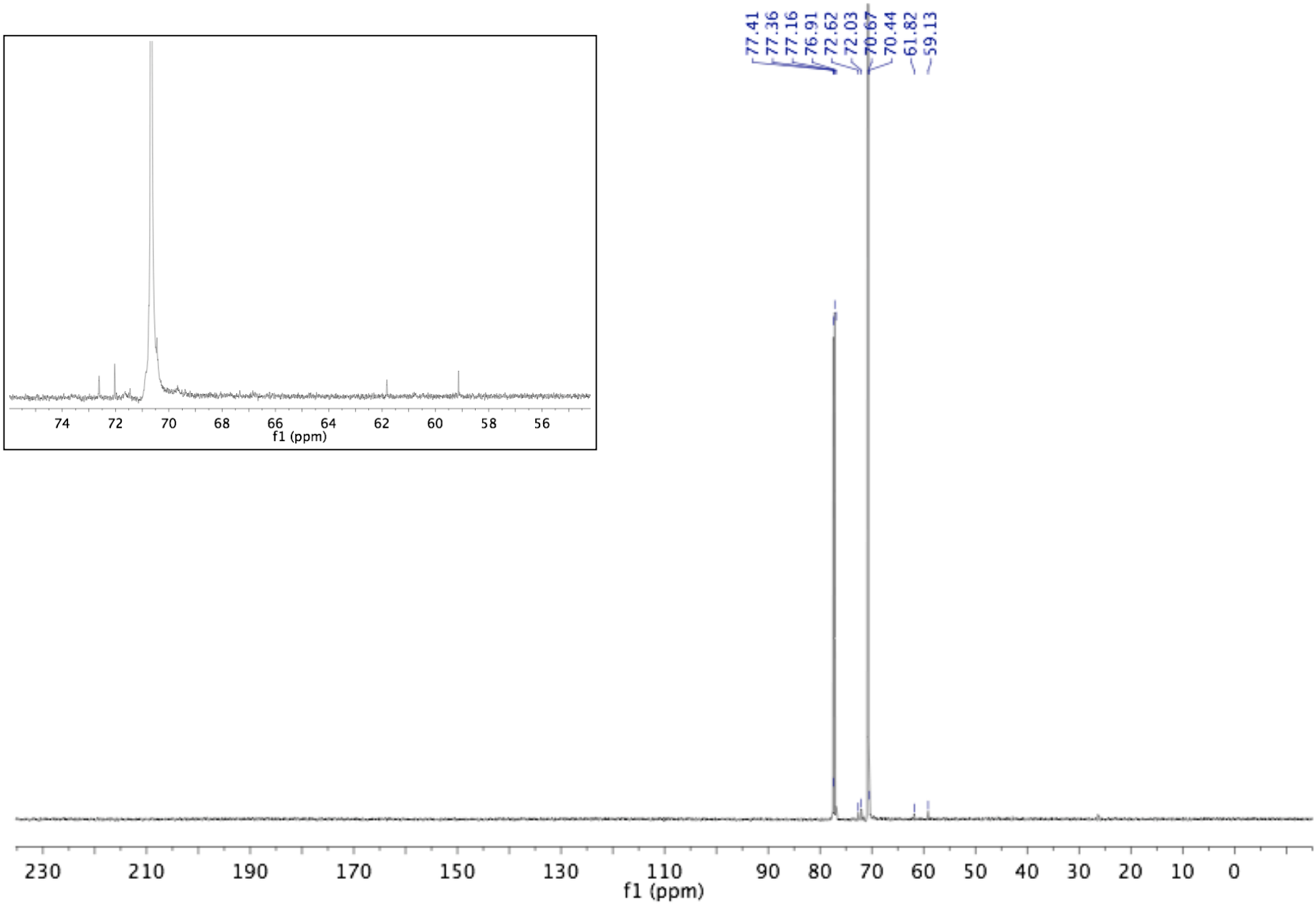
Supplementary Figure 1. ^{1}H NMR of aminooxy-PEG derivative **2** (600 MHz, $CDCl_3$)



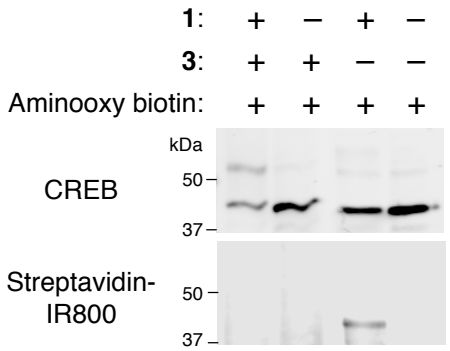
Supplementary Figure 2. ^{13}C NMR of aminooxy-PEG derivative **2** (125 MHz, $CDCl_3$).



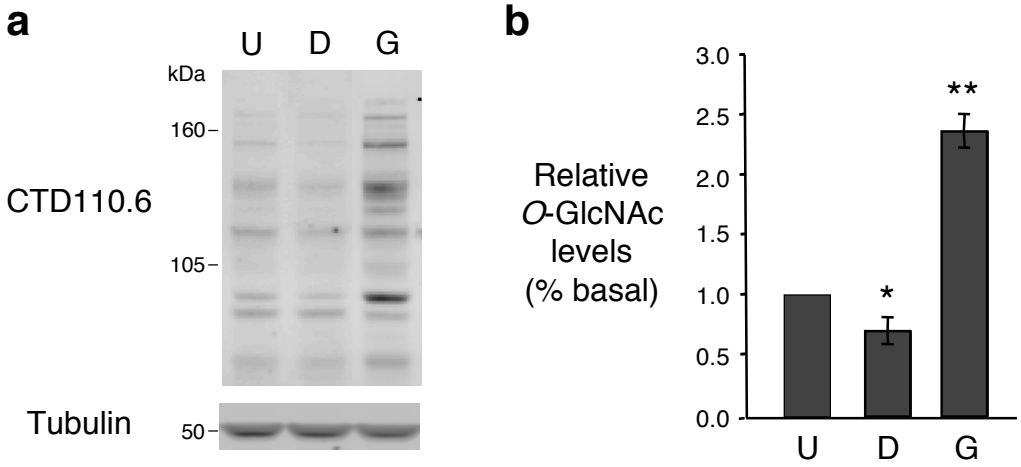
Supplementary Figure 3. ^{1}H NMR of aminooxy-PEG derivative **3** (600 MHz, $CDCl_3$).



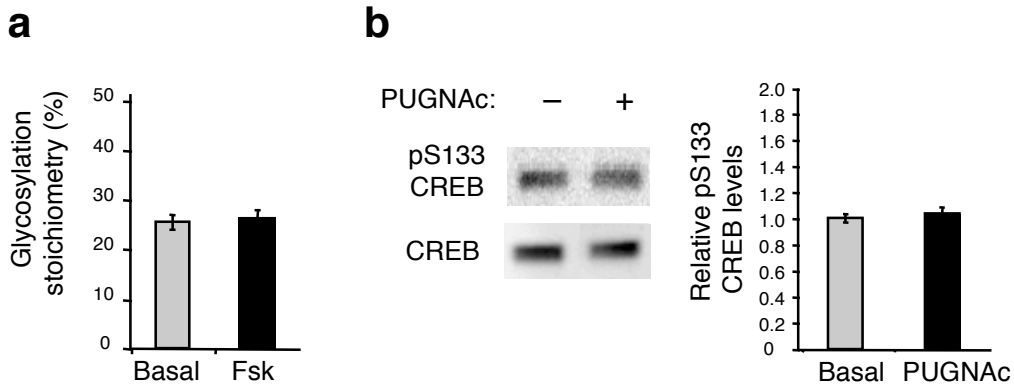
Supplementary Figure 4. ^{13}C NMR of aminooxy-PEG derivative **3** (125 MHz, $CDCl_3$).



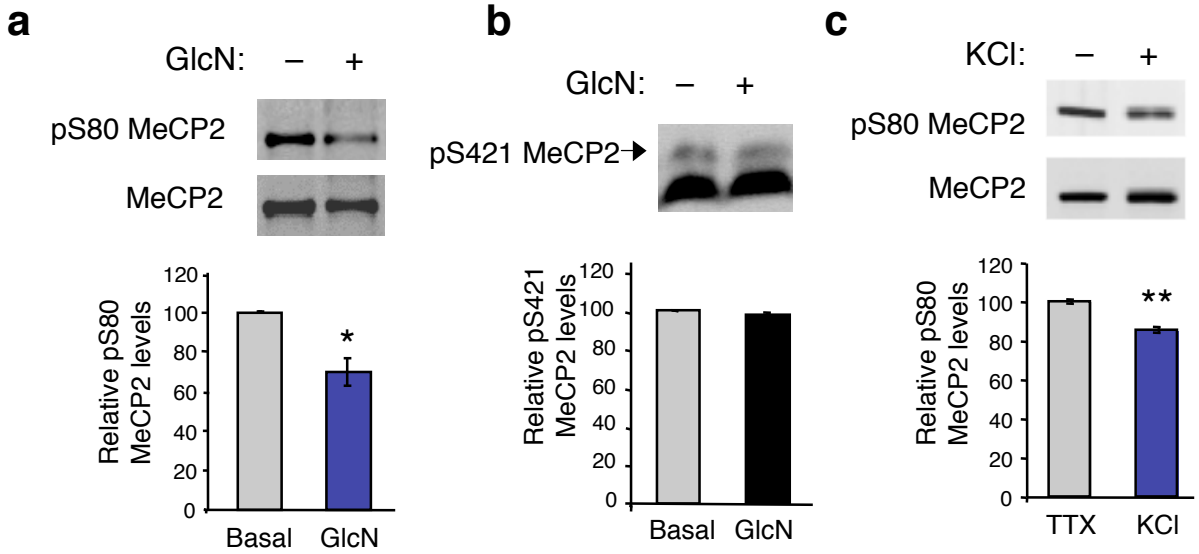
Supplementary Figure 5. The PEG-labeling reaction proceeds to completion. $CREB_{mono}$ was chemoenzymatically labeled with UDP-ketogalactose derivative **1** and Y289L GalT, followed by reaction with aminooxy-functionalized PEG **3**. The protein was then reacted with an aminooxy biotin derivative to cap unreacted ketone groups, resolved by SDS-PAGE, and immunoblotted simultaneously with an anti-CREB antibody and streptavidin-IR800. Biotin incorporation was detected only in the control reaction lacking **3**. **1** was excluded as a control for selectivity.



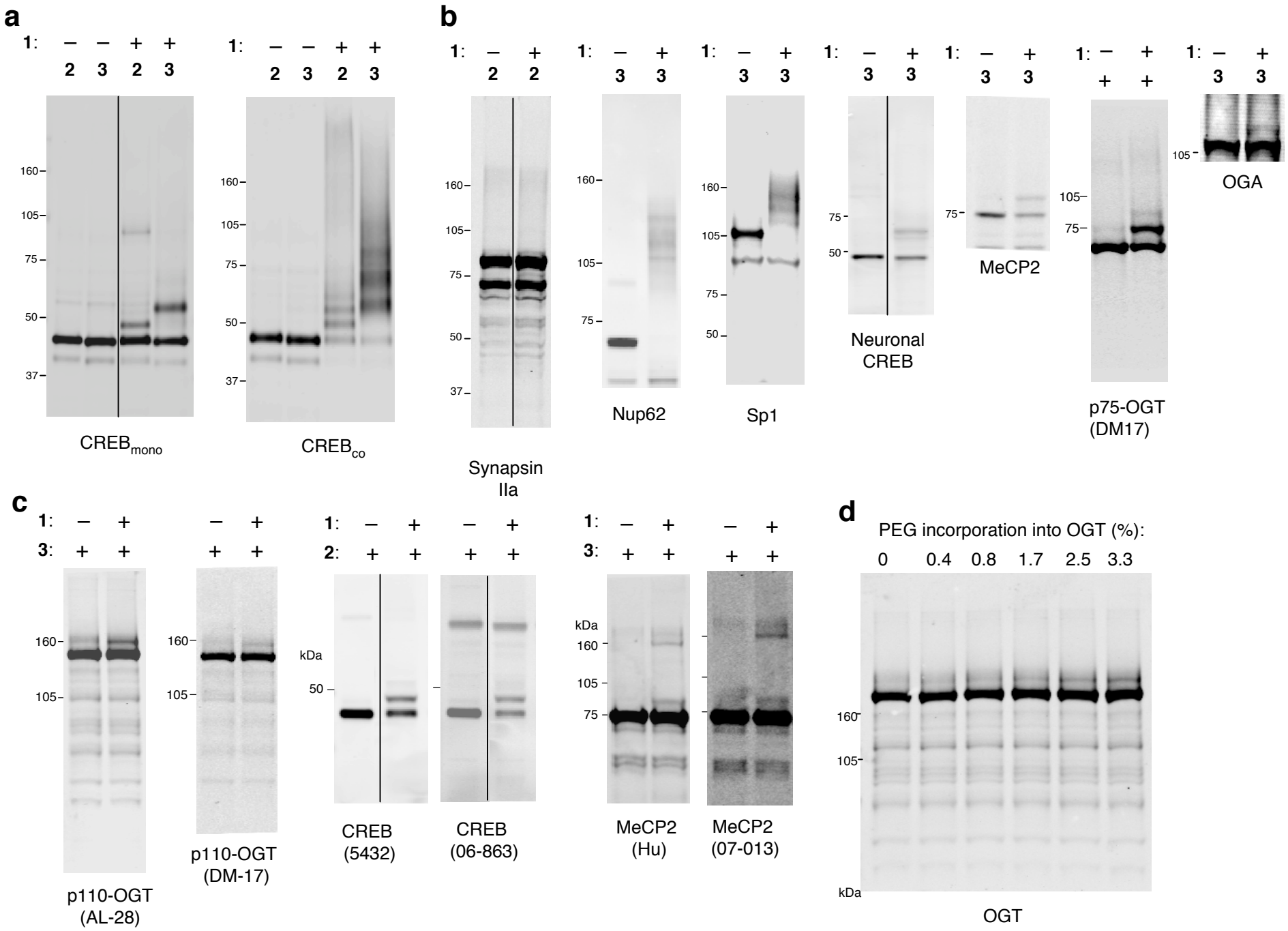
Supplementary Figure 6. Treatment of neurons with DON (D) or GlcN (G) leads to changes in overall *O*-GlcNAc glycosylation levels, as detected by a general *O*-GlcNAc antibody (CTD110.6). This antibody recognizes an incomplete subset of *O*-GlcNAc-modified proteins and does not detect glycosylated CREB. (**a**) DON decreased overall *O*-GlcNAc levels by 0.7 ± 0.1-fold, while GlcN increased overall *O*-GlcNAc levels by 2.4 ± 0.1-fold. Cortical rat neurons were cultured for 5 days and treated with either DON, GlcN, or vehicle (U) for 6 h. Samples were resolved by SDS-PAGE and immunoblotted with the anti-*O*-GlcNAc antibody. (**b**) Quantification of the changes in overall glycosylation levels from $n = 3$ samples. Data were plotted with respect to the *O*-GlcNAc levels in untreated cells and represent mean ± s.e.m. Statistical analysis was performed using the Student's *t*-test. * $P = 0.04$, ** $P = 0.006$ relative to vehicle control.

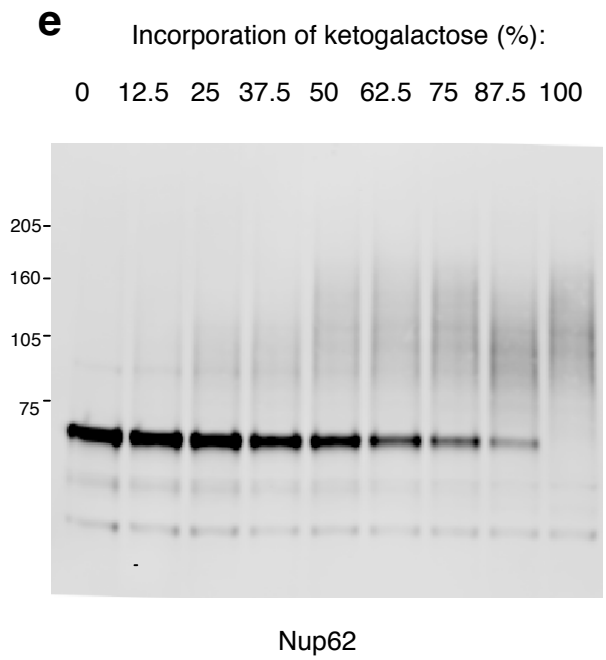


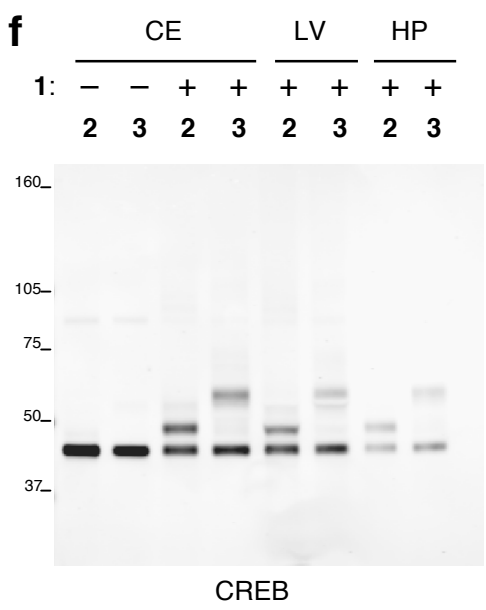
Supplementary Figure 7. (**a**) Fsk treatment of 293T cells does not change the stoichiometry of CREB glycosylation. Quantification of glycosylation stoichiometry in **Fig. 3a** for basal and Fsk samples ($n = 6$) [25.4 ± 1.5% (basal) and 26.1 ± 1.6% (Fsk); $P = 0.7$]. 293T cells were treated with vehicle (basal) or Fsk (10 mM, 25 min). Samples underwent chemoenzymatic *O*-GlcNAc labeling with PEG mass tags, were resolved by SDS-PAGE, and immunoblotted for CREB. (**b**) PUGNAc treatment does not affect overall levels of pS133 CREB phosphorylation. Quantification is shown in the bar graph to the right for $n = 4$. [1.0 ± 0.03-fold (basal) and 1.0 ± 0.05-fold (PUGNAc); $P = 0.5$]. 293T cells were treated with vehicle (basal) or PUGNAc (100 mM, 6 h). Samples were resolved by SDS-PAGE and immunoblotted for pS133 CREB and CREB. Phosphorylation levels were corrected for total CREB levels and plotted relative to the basal phosphorylation levels. Data represent mean ± s.e.m. Statistical analysis was performed using the Student's *t*-test.

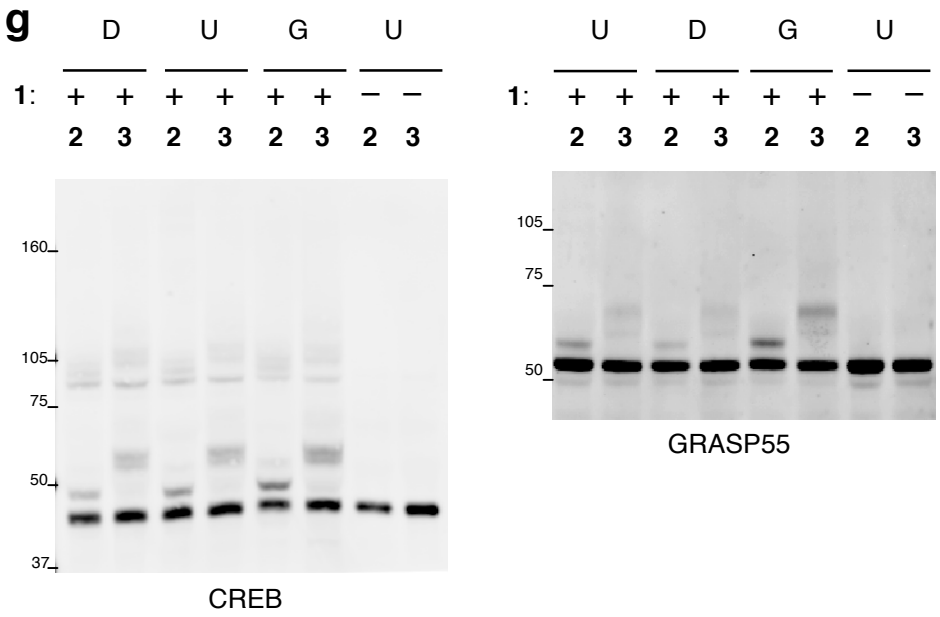


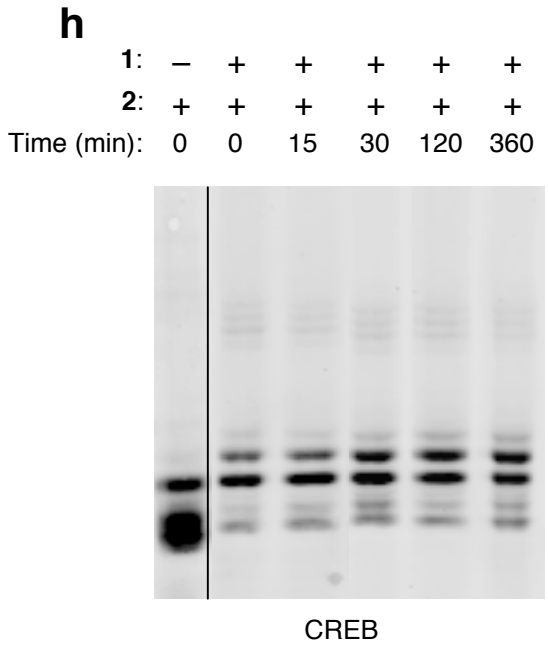
Supplementary Figure 8. GlcN treatment of neurons decreases pS80, but not pS421 levels, on MeCP2. KCl-induced depolarization also decreases pS80. (**a**) Following GlcN treatment, pS80 MeCP2 levels decreased by 30.1 ± 6.9% as measured by immunoblotting with a pS80-specific MeCP2 antibody ($n = 4$). (**b**) However, GlcN treatment left MeCP2 phosphorylation at Ser-421 unchanged ($n = 6$). Cortical rat neurons were cultured for 5 days and treated with GlcN for 6 h. (**c**) KCl-induced neuronal depolarization decreases pS80 MeCP2 levels by 15.2 ± 1.6% as measured by immunoblotting with a pS80-specific MeCP2 antibody ($n = 8$). Cortical rat neurons were cultured for 5 days and depolarized by addition of KCl to the culture media (55 mM, 2 h). Samples were resolved by SDS-PAGE and immunoblotted for MeCP2 and pS80 MeCP2. pS421 MeCP2 runs at slightly higher MW on SDS-PAGE gels[7]. Phosphorylation levels were corrected for total MeCP2 levels and plotted relative to the basal phosphorylation levels in each case. Data represent mean ± s.e.m. Statistical analysis was performed using the Student's *t*-test. * $P = 0.03$, ** $P = 0.00007$

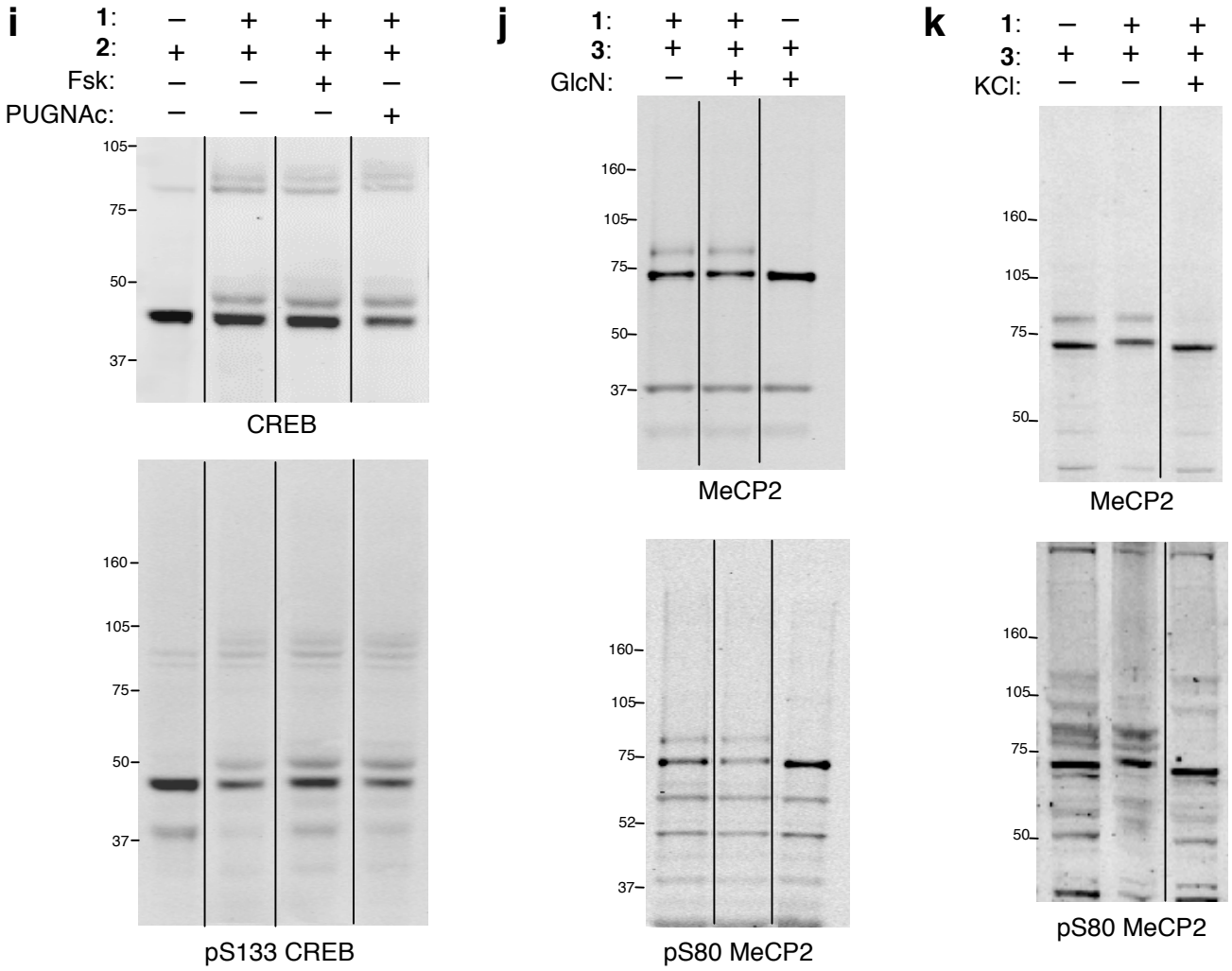

a
1: – – + +
2 3 2 3
160
105
75
50
37
CREB mono
CREB co
b
Synapsin IIa
Nup62
Sp1
Neuronal CREB
MeCP2
p75-OGT (DM17)
OGA
c
1: – +
3: + +
2: + +
kDa
p110-OGT (AL-28)
p110-OGT (DM-17)
CREB (5432)
CREB (06-863)
MeCP2 (Hu)
MeCP2 (07-013)
d
PEG incorporation into OGT (%):
0 0.4 0.8 1.7 2.5 3.3
OGT


e
Incorporation of ketogalactose (%):
0 12.5 25 37.5 50 62.5 75 87.5 100
205
160
105
75
Nup62


f
CE
LV
HP
1: – – + + + + + +
2 3 2 3 2 3 2 3
160
105
75
50
37
CREB


g
D
U
G
U
1: + + + + + + – –
2 3 2 3 2 3 2 3
160
105
75
50
37
CREB
U
D
G
U
1: + + + + + + – –
2 3 2 3 2 3 2 3
105
75
50
GRASP55


h
1: – + + + + +
2: + + + + + +
Time (min): 0 0 15 30 120 360
CREB



Supplementary Figure 9. Full-length Western blots of corresponding images in **Figures 1-4**. (**a**) **Figure 1b** Western blots. (**b**) **Figure 1c** Western blots. (**c**) **Figure 2a** Western blots. (**d**) **Figure 2b** Western blots. (**e**) **Figure 2c** Western blots. (**f**) **Figure 3a** Western blot. CE = cerebellum, LV = liver, HP = hippocampus. (**g**) **Figure 3b** Western blots. U = untreated, D = DON, GlcN = Glucosamine. (**h**) **Figure 3c** Western blots. (**i**) **Figure 4a** Western blot. Fsk = Forskolin. (**j**) **Figure 4d** Western blot. GlcN = Glucosamine. (**k**) **Figure 4g** Western blot. Refer to the figure legends for details. The antibodies used for immunoblotting are indicated below each gel. Black vertical lines indicate where lanes that were not directly adjacent in the gel were juxtaposed by vertically slicing the image.