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Dynamic *O*-GlcNAc modification regulates CREB-mediated gene expression and memory formation

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The transcription factor cyclic AMP–response element binding protein (CREB) is a key regulator of many neuronal processes, including brain development, circadian rhythm and long-term memory. Studies of CREB have focused on its phosphorylation, although the diversity of CREB functions in the brain suggests additional forms of regulation. Here we expand on a chemoenzymatic strategy for quantifying glycosylation stoichiometries to characterize the functional roles of CREB glycosylation in neurons. We show that CREB is dynamically modified with an O-linked β-*N*-acetyl-D-glucosamine sugar in response to neuronal activity and that glycosylation represses CREB-dependent transcription by impairing its association with CREB-regulated transcription coactivator (CRTC; also known as transducer of regulated CREB activity). Blocking glycosylation of CREB alters cellular function and behavioral plasticity, enhancing both axonal and dendritic growth and long-term memory consolidation. Our findings demonstrate a new role for O-glycosylation in memory formation and provide a mechanistic understanding of how glycosylation contributes to critical neuronal functions. Moreover, we identify a previously unknown mechanism for the regulation of activity-dependent gene expression, neural development and memory.

CREB controls gene expression programs that underlie diverse neuronal processes, ranging from neural development and survival to complex adaptive behaviors such as long-term memory and drug addiction[1–3]. Extensive studies have focused on the importance of protein phosphorylation in regulating CREB activity in the nervous system[4–8]. Phosphorylation of Ser133, a key regulatory site in the protein, leads to recruitment of the coactivator CREB-binding protein (CBP) and activation of CREB-mediated transcription[9]. However, phosphorylation is not always sufficient to stimulate CREB-dependent transcription[6,10,11], suggesting that there are additional, undiscovered mechanisms for the complex coordination of CREB activity.

O-glycosylation of proteins by the monosaccharide β-*N*-acetyl-D-glucosamine (*O*-GlcNAc) is a dynamic, inducible post-translational modification with striking similarities to phosphorylation[12–14]. Attachment of this simple glycan to serine or threonine residues occurs on more than 1,000 proteins, including proteins involved in transcription and translation, signal transduction, cell cycle progression and synaptic plasticity[12–16]. The abundance of *O*-GlcNAc glycosylation in the brain[13,15,16] and the fact that it shares many features with phosphorylation—a key regulator of cell signaling, synaptic plasticity, and learning and memory[17]—suggests critical roles for *O*-GlcNAc in the nervous system. Indeed, changes in the overall amount of *O*-GlcNAc have been shown to modulate long-term potentiation[18], calcium influx via inositol phosphate channels[19] and neurite branching[20]. The overall amount of *O*-GlcNAc glycosylation was also inversely related to the amount of phosphorylation on the protein tau[21], implicating *O*-GlcNAc glycosylation in the pathogenesis of Alzheimer's disease. However, a mechanistic understanding of how *O*-GlcNAc glycosylation contributes to neuronal processes and higher-order brain functions is unclear.

A major challenge in understanding the biological roles of *O*-GlcNAc has been the difficulty of detecting and studying this modification. Much like phosphorylation, *O*-GlcNAc glycosylation is chemically and enzymatically labile, often substoichiometric and subject to complex cellular regulation[12–14]. In addition, *O*-GlcNAc glycosylation has several unique features that renders it even more difficult to study than phosphorylation. For instance, phosphorylation has only three major forms (phosphorylated serine, threonine or tyrosine), whereas *O*-GlcNAc represents one of hundreds of different cellular glycans (for example, mucin polysaccharides, glycosaminoglycans and so on)[22]. Traditional methods for *O*-GlcNAc detection, such as radiolabeling with tritiated sugars, are not as sensitive as detection of phosphorylation with ^{32}P-labeled ATP. Moreover, site-specific *O*-GlcNAc antibodies are rare and notoriously difficult to generate[13], in contrast to phosphorylation state–specific antibodies, which are widely used. Finally, the fact that there is only one *O*-GlcNAc transferase gene (*OGT*)[12,23] complicates genetic approaches toward elucidating the precise roles of the modification because knocking down or inhibiting OGT may have broad pleiotropic effects.

Recently, we developed a new chemoenzymatic method for detecting *O*-GlcNAc–modified proteins and quantifying *in vivo* glycosylation[24]. In this method, the *O*-GlcNAc–modified residues on proteins are labeled with a polyethylene glycol (PEG) mass tag to shift the molecular weight of the glycosylated species. By immunoblotting the labeled cell lysate for proteins of interest, one can quantify glycosylation stoichiometries *in vivo* and establish whether proteins are mono-, di- or multiply *O*-GlcNAc glycosylated. In addition, different post-translationally modified subpopulations can be rapidly visualized by immunoblotting with antibodies specific for phosphorylation or some other modification. As such, the approach provides a direct readout of whether phosphorylation and *O*-GlcNAc glycosylation are mutually exclusive on proteins of interest (that is, follow a 'yin-yang' model) or whether they exist together on the same molecule. Here we use this chemoenzymatic mass-tagging method in combination with

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biochemical, molecular, cellular and neurobiological approaches to understand the role of *O*-GlcNAc glycosylation in regulating the transcription factor CREB. We demonstrate that glycosylation at Ser40 inhibits both basal and activity-induced CREB-mediated transcription and serves to regulate important neuronal functions, including neurite outgrowth and memory consolidation.

RESULTS

CREB is highly glycosylated in neurons at Ser40

Previously, we demonstrated that CREB is modified with *O*-GlcNAc in mammalian cells and the rat forebrain[24,25]. To evaluate the role of O-glycosylation in regulating the function of CREB, we quantified the stoichiometry of CREB glycosylation in neurons using our chemoenzymatic approach[24]. Proteins with terminal GlcNAc sugars were selectively labeled with a 2,000-Da PEG mass tag[24] and immunoblotted with a CREB-specific antibody to visualize the glycosylated species (**Fig. 1a**). We found that a large fraction of CREB (44–48%) was monoglycosylated in both cultured cortical neurons and various brain regions of adult mice. Unlike other post-translational modifications on CREB, which are largely undetectable in unstimulated neurons, the proportion of CREB that was glycosylated was high under basal conditions and was comparable to the amount of Ser133-phosphorylated CREB in activated PC12 cells[26].

To map the glycosylation sites, CREB was expressed in Neuro2a cells, immunoprecipitated and subjected to electron transfer dissociation (ETD)-MS analysis. In addition to the region of glycosylation identified previously (Thr259-Ser260-Thr261)[25], *O*-GlcNAc glycosylation was mapped to Ser40 and Thr227 or Thr228 (**Fig. 1b** and **Supplementary Results**, **Supplementary Fig. 1**). To determine the major site of glycosylation, we expressed both Flag-tagged wild-type CREB and CREB with various alanine substitutions at putative glycosylation sites in cultured cortical neurons and measured their relative levels of glycosylation under basal conditions using our chemoenzymatic approach. Mutation of Ser40 to alanine (S40A) led to a large reduction in CREB glycosylation compared to wild-type CREB (56.1 ± 3.1%; **Fig. 1c**), whereas mutation of both Thr227 and Thr228 (T227A T228A) led to a smaller decrease in glycosylation (35.9 ± 9.6%). CREB glycosylation was also decreased by mutation of Thr259, Ser260 and Thr261 (T259A S260A T261A; 13.6 ± 8.5%), and simultaneous mutation of all potential sites (A6 in **Fig. 1c**) abolished the glycosylation of CREB. Our studies indicate that CREB is highly monoglycosylated in neurons under basal conditions and is rarely glycosylated simultaneously at multiple sites. Additionally, we have identified all major glycosylation sites on CREB and have established Ser40 as the predominant site of *O*-GlcNAc glycosylation in neurons.

Neuronal activity induces CREB glycosylation at Ser40

We next investigated whether CREB glycosylation is dynamically induced by neuronal stimuli. Although *O*-GlcNAc glycosylation levels are modulated by glucose concentrations and cellular stress[12,14], the signals and signaling pathways that regulate the modification in neurons are largely unknown. Two well-established methods for inducing neuronal activity include membrane depolarization using KCl and activation of *N*-methyl-D-aspartic acid (NMDA) receptors[27]. Notably, treatment of neurons with KCl or NMDA stimulated glycosylation of CREB (**Fig. 1d** and **Supplementary Fig. 2**). Upon membrane depolarization with KCl, the amount of CREB glycosylation increased steadily by 42.0 ± 4.8% over the course of 6 h (**Fig. 1d**). The kinetics of glycosylation was slower and more sustained than that of phosphorylation at Ser133, with glycosylation continuing to increase as phosphorylation declined. Mutation of Ser40 to alanine blocked depolarization-induced CREB glycosylation, whereas mutation of the other glycosylation sites had no effect (**Fig. 1c**). Treatment with the protein synthesis inhibitor cycloheximide did not block the increase in glycosylation (**Supplementary Fig. 3**), suggesting that glycosylation is activated directly by signal transduction pathways without requiring new protein synthesis. Inhibition of L-type calcium channels with nimodipine abolished the depolarization-induced glycosylation of CREB, indicating a requirement for voltage-sensitive calcium influx (**Fig. 1e**). Moreover, inhibition of Ca^{2+} or calmodulin-dependent protein kinases (CaMKs) or mitogen-activated protein kinase (MAPK) blocked the increase in CREB glycosylation, whereas inhibitors of protein kinase C or protein phosphatases PP-2B or PP-1/2A had no effect (**Fig. 1e**). To our knowledge, these results provide the first demonstration that neuronal activity triggers *O*-GlcNAc glycosylation in neurons and specifically leads to the calcium- and kinase-dependent glycosylation of CREB at Ser40.

Induction of Ser40 glycosylation requires phosphorylation

A variety of kinase pathways converge to phosphorylate CREB at Ser133 and activate CREB-mediated transcription in neurons, including the cAMP-induced protein kinase A (PKA), MAPK and CaMKIV pathways[6–8,10,28]. Previously, we found that CREB phosphorylation at Ser133 by PKA occurs independently of the glycosylation status of CREB in 293T cells[24]. However, the involvement of multiple different kinases in CREB Ser133 phosphorylation warrants the investigation of pathway-specific interactions between CREB glycosylation and phosphorylation. CaMKs and MAPK are known to phosphorylate CREB at Ser133 following neuronal depolarization[28]. As these kinases are also necessary for calcium-induced, activity-dependent glycosylation of CREB, we determined the interplay between Ser133 phosphorylation and CREB glycosylation. Notably, S133A mutation blocked the depolarization-induced increase in CREB glycosylation (**Fig. 2a**). However, forskolin-mediated stimulation of Ser133 phosphorylation via the cAMP pathway had no effect on the amount of CREB glycosylation (**Supplementary Fig. 4**). These results provide evidence that phosphorylation is required but may not be sufficient by itself to activate CREB glycosylation.

We next examined the interdependence of Ser133 phosphorylation and Ser40 glycosylation on CREB. *O*-GlcNAc sites on cortical neuronal proteins were labeled with a 2,000-Da mass tag and immunoblotted with a phospho-Ser133 CREB–specific or total CREB–specific antibody to enable visualization of four distinct subpopulations (**Fig. 2b**)[24]. We found that both *O*-GlcNAc glycosylation and Ser133 phosphorylation can occur concomitantly on the same protein molecule (**Fig. 2b**). To further assess the role of glycosylation specifically at Ser40, we constructed a mutant CREB (A5) in which all of the glycosylation sites except Ser40 were mutated to alanines. Both the Ser40-glycosylated and nonglycosylated subpopulations of the A5 mutant were phosphorylated at Ser133 after 10 min of neuronal depolarization (**Fig. 2c**). Furthermore, the amount of phospho-Ser133 was similar across all glycosylated and nonglycosylated subpopulations of wild-type and A5 CREB. Thus, the same molecule of CREB can be modified simultaneously by *O*-GlcNAc at Ser40 and by *O*-phosphate at Ser133, and glycosylation at Ser40 does not affect the amount of Ser133 phosphorylation that occurs in response to neuronal depolarization. To determine the kinetics of glycosylation and phosphorylation within each post-translationally modified subpopulation, we monitored the time course of induction. The kinetics of Ser133 phosphorylation, which include both a rapid and slow phase as reported previously[28], were similar for both the glycosylated and nonglycosylated subpopulations of endogenous CREB upon KCl depolarization (**Fig. 2b**,**d**), confirming further that Ser133 phosphorylation occurs independently of the glycosylation state of CREB. Notably, glycosylation was more rapidly induced in the Ser133-phosphorylated subpopulation than in the total CREB population (**Fig. 2b**,**e**). Together, the results strongly suggest that phosphorylation and glycosylation work cooperatively to regulate CREB activity, with activity-dependent glycosylation induced preferentially on the phosphorylated subpopulation. The close coupling of these two post-translational modifications may allow for graded suppression of CREB following its activation.

Glycosylation represses CREB-dependent transcription

We next determined whether glycosylation modulates the transcriptional activity of CREB. As Ser40 is the major glycosylation site and the only site responsive to neuronal activity, we compared the abilities of wild-type and S40A mutant CREB to regulate CRE-dependent gene expression. A short hairpin RNA (shRNA) was used to knock down endogenous CREB in Neuro2a cells, and shRNA-resistant wild-type CREB or S40A mutant CREB was overexpressed (**Supplementary Fig. 5**). Replacement of endogenous CREB with the S40A mutant resulted in increased luciferase activity under the cAMP response element promoter (CRE-luciferase activity), suggesting that glycosylation functions to repress CREB activity (**Fig. 3a**). The S40A substitution also upregulated the expression of endogenous CREB target genes involved in cell cycle arrest, cell

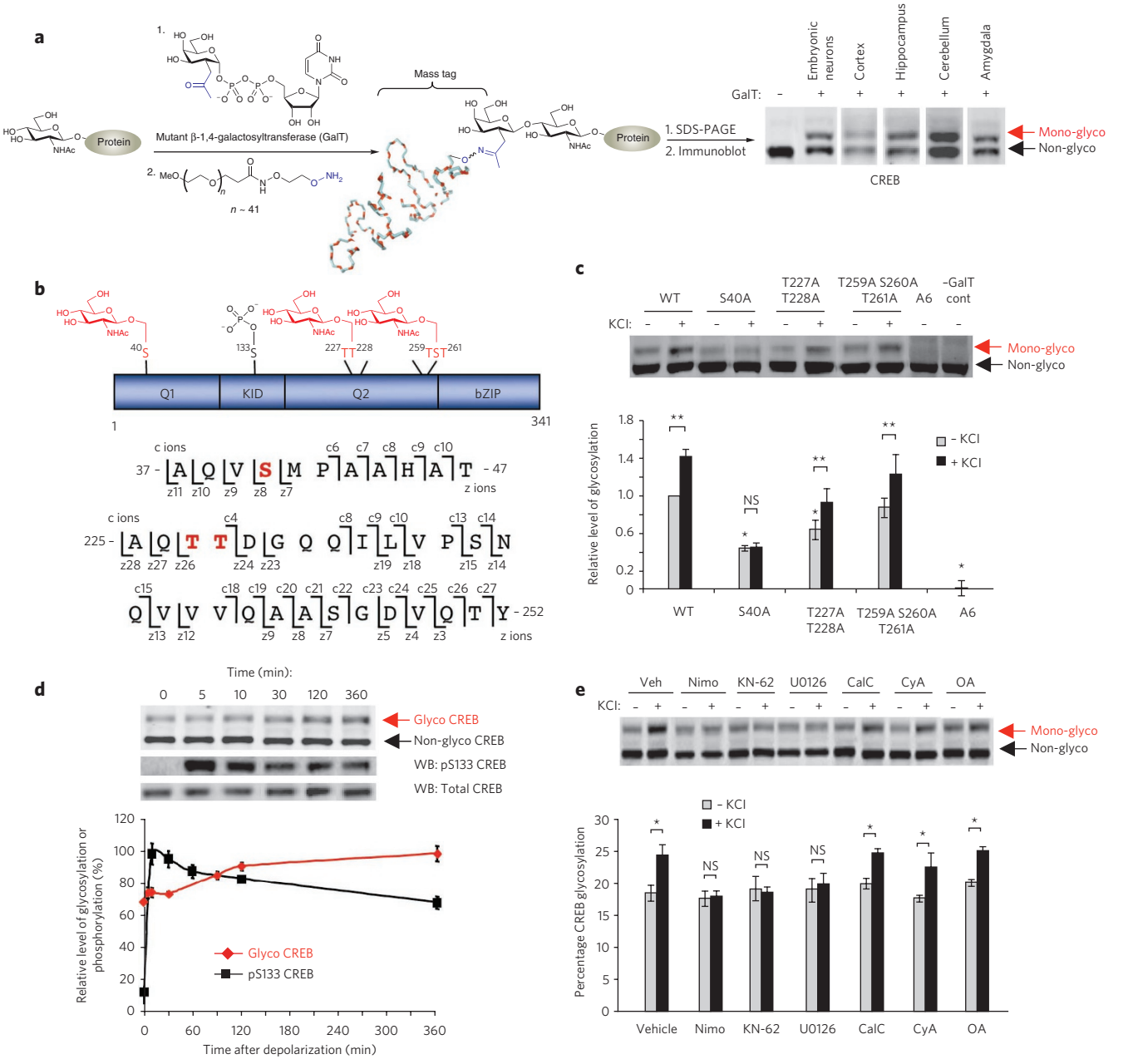

Figure 1 | CREB is *O*-GlcNAc glycosylated at Ser40 in response to neuronal activity. (**a**) Detection of *O*-GlcNAc–glycosylated CREB in neurons by chemoenzymatic labeling with a 2,000-Da mass tag and immunoblotting with a CREB-specific antibody. Mono-glyco, monoglycosylated; Non-glyco, nonglycosylated. (**b**) Glycosylation sites on CREB mapped by ETD-MS. (**c**) Amount of glycosylation of Flag-tagged wild-type (WT) CREB and various alanine mutants after expression in cultured cortical neurons, as detected by chemoenzymatic labeling and immunoblotting with a Flag-specific antibody. All levels were normalized to Flag-tagged WT CREB in untreated cells. Neurons were depolarized with KCl where indicated. $n = 7$ for WT and S40A CREB; $n = 3$–5 for other mutants; $^{*}P < 0.01$ compared to WT unstimulated; $^{**}P < 0.05$; NS, not significant; Veh, vehicle; –GalT cont, control reaction in the absence of GalT. (**d**) Kinetics of endogenous CREB glycosylation and Ser133 phosphorylation upon depolarization of cortical neurons with KCl. Amount of glycosylation or phosphorylation is plotted relative to the maximum signal for each modification. ($n = 4$–6). WB, western blot. (**e**) Level of glycosylation of endogenous CREB in unstimulated or KCl-stimulated cortical neurons upon treatment with inhibitors of L-type calcium channels (nimodipine, Nimo), CaMKs (KN-62), MAPK (U0126), protein kinase C (calphostin C, CalC), PP-2B (cyclosporin A, CyA), or PP-1/2A (okadaic acid, OA). $n = 3$–6, $^{*}P < 0.02$. Error bars, mean ± s.e.m. Full-length blots are presented in **Supplementary Figure 25**.

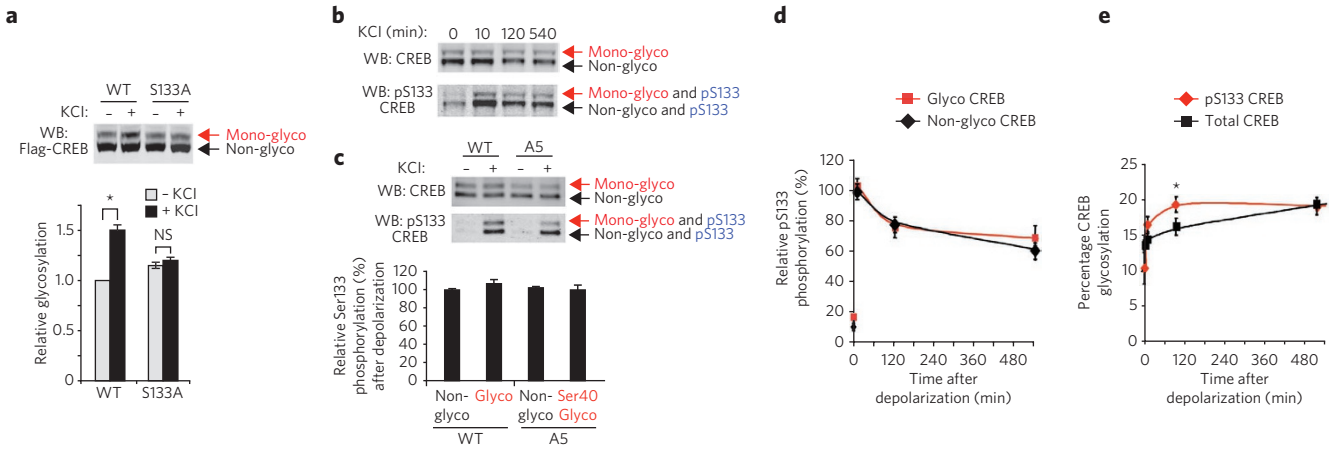

Figure 2 | Neuronal activity induces CREB glycosylation preferentially on the Ser133-phosphorylated subpopulation. (**a**) Glycosylation is not induced on S133A CREB. The amount of glycosylation was analyzed on Flag-tagged wild-type (WT) CREB or CREB^{S133A} expressed in cortical neurons. $n = 22$, $^*P < 0.001$. Mono-glyco, monoglycosylated; Non-glyco, nonglycosylated. WB, western blot. (**b**) Chemoenzymatic labeling of endogenous CREB for visualizing phosphorylation and glycosylation within the same protein molecule and for quantifying the each modification within distinct post-translationally modified subpopulations. (**c**) Quantification of phospho-Ser133 (pS133) on the nonglycosylated and glycosylated subpopulations of Flag-tagged WT or A5 mutant CREB following 10-min depolarization of neurons. All values were normalized relative to nonglycosylated, Flag-tagged WT CREB. $n = 3$; Glyco, glycosylated. (**d**,**e**) Kinetics of Ser133 phosphorylation (**d**) and glycosylation (**e**) for specific post-translationally modified subpopulations of endogenous CREB. Relative amounts of S133 phosphorylation and glycosylation were calculated as described in Methods. $n = 4$, $^*P < 0.03$; error bars, means ± s.e.m. Full-length blots are presented in **Supplementary Figure 25**.

survival and mitochondrial function, including *Cdkn1a*, *Nr4a2* and *Opa3* (**Supplementary Fig. 6**). To confirm that the effects of mutating Ser40 are likely due to loss of an *O*-GlcNAc moiety at Ser40, we overexpressed the β-N-acetylglucosaminidase enzyme (*O*-GlcNAcase or OGA) to decrease *O*-GlcNAc glycosylation in cells, and we assessed the activity of wild-type and S40A CREB. As expected, OGA overexpression enhanced CRE-luciferase activity in cells expressing wild-type CREB, mimicking the effects of the S40A

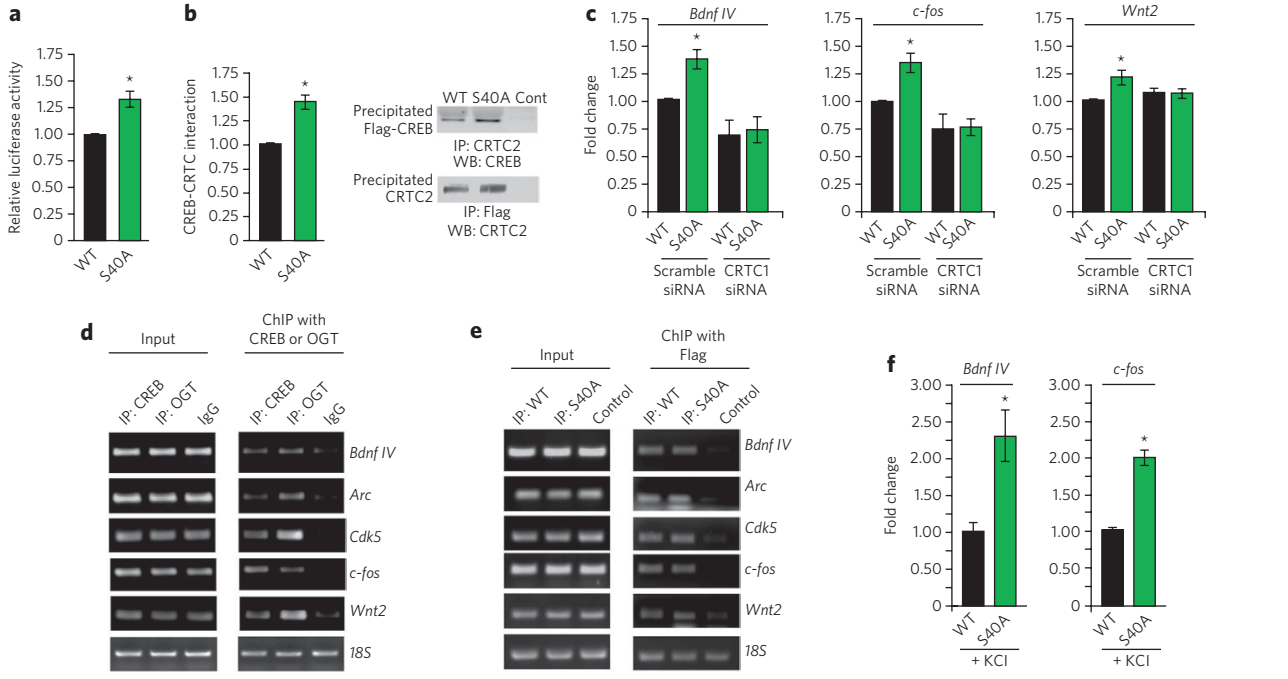

Figure 3 | Glycosylation at Ser40 represses CREB activity via a CRTC-dependent mechanism. (**a**) CRE-luciferase activity in Neuro2a cells expressing wild-type (WT) or S40A CREB. $n = 11$, $^*P < 0.01$. (**b**) Reciprocal coimmunoprecipitation of the CREB–CRTC2 complex from Neuro2a cells expressing WT or S40A CREB. The bar graph represents the average of all coimmunoprecipitation experiments ($n = 8$; $^*P < 0.01$. Cont, control. (**c**) qPCR analysis of *Bdnf* exon IV, *c-fos* and *Wnt2* expression in cultured cortical neurons electroporated with the indicated siRNAs or expression vectors using *RPL3* as an internal control. Fold change is plotted relative to neurons electroporated with wild-type CREB and scramble siRNA. $n = 4$–9; $^*P < 0.01$. (**d**) ChIP with a CREB-, OGT- or IgG–specific antibody was followed by PCR for the indicated promoters. $n = 3$. (**e**) ChIP with a Flag-specific antibody after electroporation of neurons with Flag-tagged WT CREB, Flag-tagged S40A CREB or no vector as a control. PCR was performed for the indicated promoters. $n = 3$. (**f**) qPCR analysis of *Bdnf* exon IV and *c-fos* expression after membrane depolarization of cultured cortical neurons expressing WT or S40A CREB. $n = 10$, $^*P < 0.01$; error bars, mean ± s.e.m. Full-length blots are presented in **Supplementary Figure 25**.

mutation (**Supplementary Fig. 7**). Notably, cells expressing S40A CREB did not undergo any further increase in CRE-dependent transcription upon OGA overexpression, suggesting that OGA overexpression enhances CREB activity by decreasing glycosylation at Ser40. Together, these data suggest that Ser40 glycosylation represses the transcriptional activity of CREB.

To investigate the mechanism, we evaluated whether glycosylation affects the ability of CREB to associate with DNA or transcriptional coactivators. Binding of CREB to the CRE promoter was unaffected by the S40A mutation in an electrophoretic mobility shift assay (**Supplementary Fig. 8**). CREB interacts with two coactivators, the Ser133 phosphorylation–dependent CBP and the Ser133 phosphorylation–independent CRTC (refs. 9,11). As the S40A mutant increased CREB activity when Ser133 phosphorylation was not activated (**Fig. 3a** and **Supplementary Fig. 6**), we examined whether glycosylation affects the CREB-CRTC interaction. Binding of CREB to CRTC was significantly enhanced by the S40A mutation in reciprocal coimmunoprecipitation assays ($P < 0.01$; **Fig. 3b** and **Supplementary Fig. 9**). Furthermore, knockdown of CRTC2 expression in Neuro2a cells abolished the increases in *Cdkn1a*, *Nr4a2* and *Opa3* transcript levels observed in cells expressing S40A CREB relative to cells expressing wild-type CREB (**Supplementary Figs. 6** and **10**). Together, these findings indicate that glycosylation impairs the ability of CREB to activate transcription by disrupting the CREB-CRTC interaction.

We next determined whether glycosylation at Ser40 regulates gene expression in neurons. In particular, we focused on well-characterized neuronal CREB target genes that are important for brain development and memory consolidation, including *Bdnf* exon IV, *Arc*, *Cdk5*, *c-fos* (also known as *Fos*) and *Wnt2* (refs. 29–33). Expression of S40A CREB in cortical neurons increased the transcript levels of *Bdnf* exon IV, *Arc*, *Cdk5*, *c-fos* and *Wnt2* relative to those in wild-type CREB (**Fig. 3c** and **Supplementary Fig. 11**). The observed increases most likely underestimate the contribution of *O*-GlcNAc glycosylation to CREB activity given the moderate transfection efficiency of primary neurons (~30–40%) and the contribution of other transcription factors to the regulation of those genes. Therefore, we used CREB small interfering RNA (siRNA) to obtain a rough approximation of the contribution of CREB to the expression of each gene. Assuming equal transfection efficiency of the cDNA plasmids and siRNA, the observed increases correspond to approximately 2.5- to 3.6-fold inductions in CREB activity (**Supplementary Fig. 12**). Consistent with a mechanism involving direct regulation of these genes, both CREB and OGT were bound to the promoters of each gene (**Fig. 3d**), and wild-type and S40A CREB showed comparable promoter occupancy in chromatin immunoprecipitation (ChIP) assays (**Fig. 3e**). Moreover, siRNA-mediated

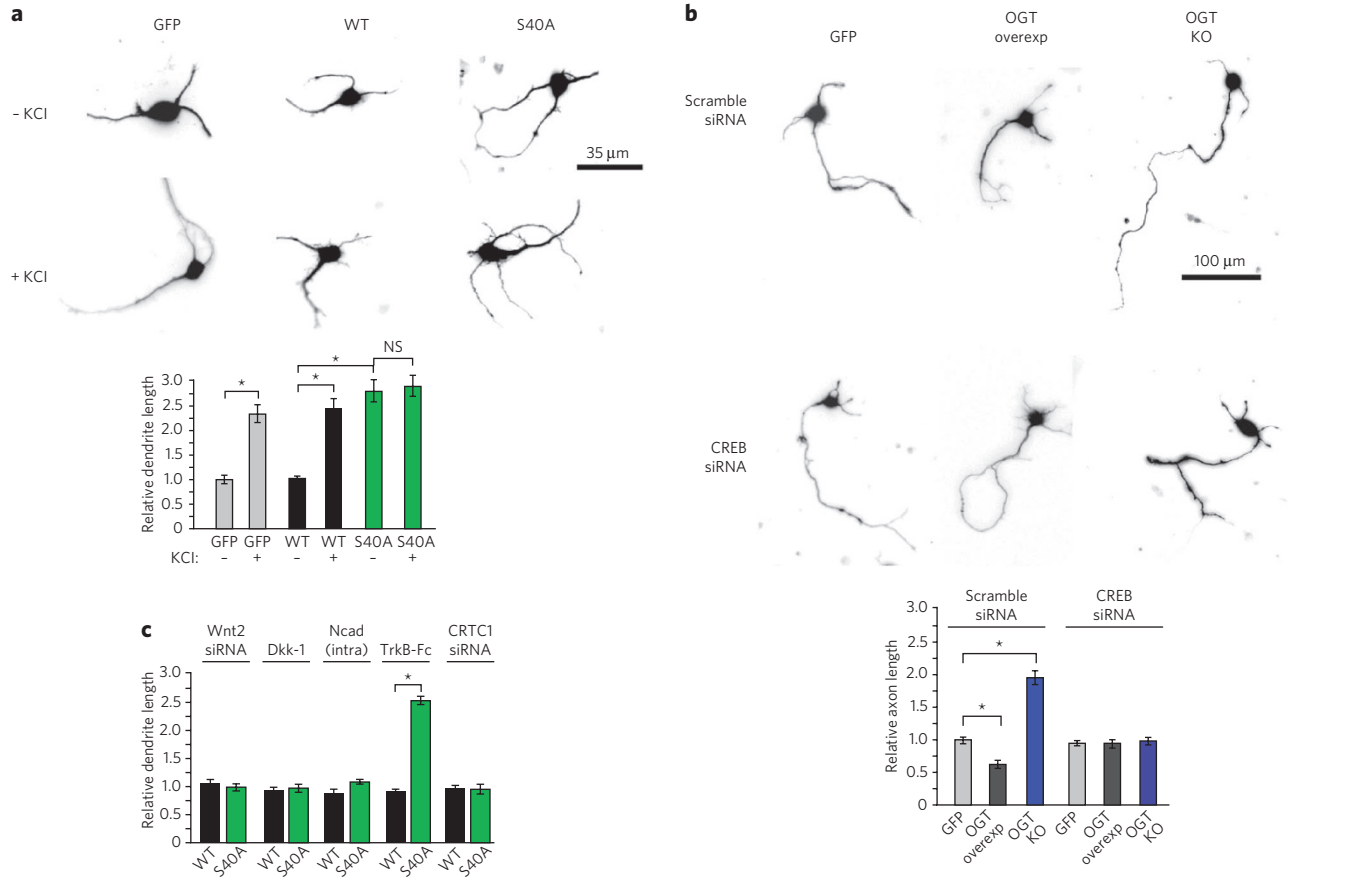

Figure 4 | CREB glycosylation at Ser40 represses dendritic and axonal growth. (**a**) Relative total dendrite lengths of cortical neurons expressing GFP, wild-type (WT) CREB or S40A CREB. For each condition, 30 dendrites were measured in each of three independent experiments; *$P < 0.001$; NS, not significant. (**b**) Relative axon lengths of *Ogt*-floxed cortical neurons expressing GFP, Flag-tagged OGT (OGT overexp) or Cre recombinase (OGT KO). Neurons were also transfected with scramble or CREB siRNA as indicated. For each condition, 30 axons were measured in each of three independent experiments; *$P < 0.001$. (**c**) Relative dendrite lengths of unstimulated neurons transfected with WT or S40A CREB, Wnt2 signaling inhibitors (Wnt2 siRNA, Dickkopf-1 (Dkk-1), Ncad(intra)) or CRTC1 siRNA as indicated. The BDNF signaling inhibitor TrkB-Fc was added in solution to neurons. For each condition, 30 dendrites were measured in each of three independent experiments; *$P < 0.001$. Lengths are shown relative to unstimulated GFP-expressing neurons in **a**. Error bars, mean ± s.e.m.

knockdown of CRTC1 reversed the effects of S40A CREB on neuronal gene expression (**Fig. 3c** and **Supplementary Figs. 11** and **13**). Thus, glycosylation of CREB at Ser40 modulates the constitutive expression of genes that are important for neuronal development, survival and synaptic plasticity via a CRTC-dependent mechanism.

To investigate whether glycosylation also contributes to activity-induced gene expression, we depolarized neurons expressing wild-type or S40A CREB with KCl. Blocking glycosylation of CREB at Ser40 increased the levels of *Bdnf* exon IV and *c-fos* transcripts to a greater extent in membrane-depolarized neurons than in unstimulated neurons (**Fig. 3f**). These results, together with the observation that neuronal activity enhances glycosylation of the Ser133-phosphorylated CREB subpopulation, suggest dual functions for CREB glycosylation: to repress basal transcript levels and to attenuate activity-dependent CREB-induced gene expression.

CREB glycosylation at Ser40 regulates neuronal growth

As CREB has critical roles in axon growth, activity-dependent dendrite development and synaptogenesis[1,31,34], the ability of O-glycosylation to regulate CREB-mediated gene expression could have important consequences for neuronal development. To assess the functional consequences of Ser40 glycosylation on neuronal growth, we assayed axonal and dendritic extension in cortical neurons expressing wild-type or S40A mutant CREB (**Supplementary Fig. 14**). Dendrites of neurons expressing wild-type CREB or a GFP control had similar lengths, and their growth was stimulated by membrane depolarization, as expected (**Fig. 4a**). In contrast, neurons expressing S40A CREB had significantly longer dendrites than wild-type CREB-expressing neurons (2.77-fold increase)—with lengths comparable to those of depolarized neurons—and their dendrites showed no further elongation upon membrane depolarization ($P < 0.001$; **Fig. 4a**). Additionally, neurons expressing S40A CREB had significantly longer axons than wild-type CREB– and GFP-expressing controls ($P < 0.001$; **Supplementary Fig. 15**). Thus, blocking glycosylation of CREB accelerates the rate of both dendrite and axon elongation and leads to dysregulation of basal and activity-induced dendritic growth.

To confirm that these effects result from direct O-glycosylation of CREB, we performed OGT gain- and loss-of-function experiments. *Ogt*-null neurons were generated from *Ogt*-floxed mice[23] by expression of Cre recombinase in cultured cortical neurons (**Supplementary Fig. 16**). *Ogt* knockout stimulated axonal growth, whereas its overexpression (**Supplementary Fig. 17**) attenuated axonal growth (**Fig. 4b**). In both cases, siRNA-mediated knockdown of endogenous CREB reversed the effects of *Ogt* knockout or overexpression and restored axon lengths to those of GFP-expressing neurons (**Fig. 4b**), indicating that *O*-GlcNAc glycosylation regulates axonal growth through a CREB-dependent mechanism.

To further investigate the underlying molecular mechanisms, we considered known mediators of dendrite and axon elongation. Activation of CREB drives the expression of the secreted mitogen Wnt2 to regulate activity-dependent dendritic growth, whereas application of the neurotrophin brain-derived neurotrophic factor (BDNF) leads to axon elongation[31,35]. Earlier, we showed that both *Wnt2* and *Bdnf* transcript levels were significantly higher in cortical neurons expressing S40A CREB compared to those expressing wild-type CREB ($P < 0.01$; **Fig. 3c**). To examine whether CREB glycosylation modulates dendritic growth via the Wnt2 pathway, we knocked down *Wnt2*, overexpressed the Wnt2 antagonist Dickkopf-1 or overexpressed the β-catenin sequestrant Ncad(intra). All three treatments blocked the enhancement of dendritic growth following neuronal depolarization (**Supplementary Fig. 18**), a result that demonstrates the efficacy of the Wnt2 blockers and is consistent with the importance of Wnt2 in dendritic growth[31]. Most notably, the same treatments reversed the stimulatory effects of S40A CREB on dendritic growth (**Fig. 4c** and **Supplementary Fig. 19**).

To probe the mechanism of glycosylation-mediated axonal growth, we treated neurons with the BDNF and NT-4/5 scavenger TrkB-Fc. Addition of TrkB-Fc to the cells blocked the effects of S40A CREB specifically on axonal, but not dendritic, growth (**Fig. 4c** and **Supplementary Figs. 19** and **20**). Furthermore, knockdown of CRTC1 abolished the S40A CREB–dependent increases in gene expression of both *Wnt2* and *Bdnf* (**Fig. 3c**), dendritic growth (**Fig. 4c** and **Supplementary Fig. 19**) and axonal growth (**Supplementary Fig. 20**). Together, the results demonstrate that CREB glycosylation modulates dendrite and axon elongation via the CRTC-dependent downregulation of Wnt2 and BDNF signaling, respectively. These findings provide strong evidence that O-glycosylation of CREB has a large, chronic, repressive effect on multiple developmental pathways and functions as a key regulator of neuronal growth.

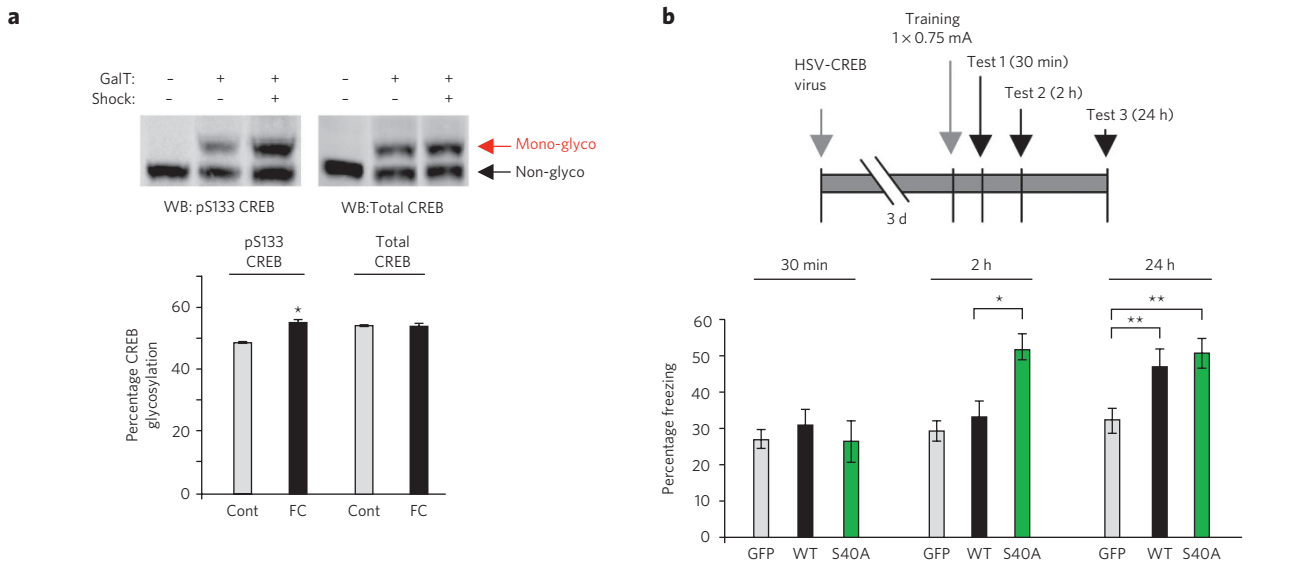

Figure 5 | CREB glycosylation at Ser40 modulates long-term conditioned fear memory. (**a**) The amount of glycosylation of activated Ser133-phosphorylated CREB and total CREB in the amygdala 15 min after auditory fear conditioning (FC). $n = 3$, $^*P < 0.01$; Cont, control. (**b**) Freezing behavior after auditory fear conditioning of mice infused with HSV vectors expressing GFP, WT or S40A CREB. $n = 11$ for GFP, $n = 16$ for WT and $n = 20$ for S40A at 2 h and 24 h, $n = 6$ for all vectors at 30 min. $^*P < 0.05$, $^{**}P < 0.005$. Error bars, means ± s.e.m. Full-length blots are presented in **Supplementary Figure 25**.

As the directed growth of axons and dendrites in response to synaptic activation, depolarization and trophic factors is critical for the formation and maturation of neuronal circuits[36], these results also implicate *O*-GlcNAc in regulating such processes.

Glycosylation at Ser40 modulates long-term memory

Having shown that CREB glycosylation affects important cellular processes, we next examined whether glycosylation has an impact on higher-order brain functions *in vivo*. Extensive studies have shown that CREB is not only a requirement but also a critical driving force for the consolidation of long-term conditioned fear memories[3,37–41]. We first investigated whether glycosylation is induced on endogenous CREB in response to auditory fear conditioning in mice. Specifically, the amount of CREB glycosylation in the lateral amygdala of fear-conditioned mice was compared to that in tone-only trained controls. CREB phosphorylation was induced in the amygdala, consistent with previous studies (**Supplementary Fig. 21**)[39]. An increase in glycosylation (13.6 ± 0.3%) was detected within the activated subpopulation (that is, CREB phosphorylated at Ser133; **Fig. 5a**) but not the total CREB population. Therefore, glycosylation is specifically induced upon physiologic activation of the amygdala *in vivo* to modify Ser133-phosphorylated CREB. This induction of CREB glycosylation was comparable to the induction observed in neuronal cultures after 10-min depolarization with KCl (**Fig. 1d**). The chemoenzymatic mass-tagging approach also revealed that more than half of the phosphorylated CREB subpopulation (54.7 ± 1.4%, 10 min after fear conditioning) was glycosylated, suggesting that CREB glycosylation may contribute substantially to amygdala function.

To determine whether CREB glycosylation affects memory formation *in vivo*, we bilaterally injected replication-defective herpes simplex virus (HSV) vectors expressing wild-type CREB and GFP, S40A CREB and GFP, or GFP alone into the lateral amygdala of mice before fear conditioning (**Supplementary Fig. 22**) and assessed memory 30 min, 2 h and 24 h after training. Similarly to what has been seen in previous experiments[39,41], mice infused with wild-type CREB vector had enhanced memory compared to GFP vector-infused mice after 24 h but not after 30 min or 2 h (**Fig. 5b**; $F_{1,25} = 4.34$, $P = 0.048$), indicating that CREB overexpression increases long-term memory. Notably, mice infused with S40A CREB vector showed significant memory enhancement by 2 h after training compared to mice infused with wild-type CREB or GFP ($F_{2,45} = 9.70$, $P = 0.0003$), and the enhanced memory persisted at 24 h (**Fig. 5b**). The mice showed the same short-term memory at 30 min independent of the genotype, suggesting that the enhancements at 2 h and 24 h most likely represent changes in long-term memory and are not due to other nonspecific events such as differences in pain sensation or cell death. Taken together, the data suggest that expression of S40A CREB in the amygdala may promote more rapid long-term memory consolidation than expression of wild-type CREB.

We next tested further whether the effect of S40A CREB represents enhanced long-term memory formation, a CREB-dependent process that requires *de novo* mRNA and protein synthesis[3,37]. Mice were injected with anisomycin at various points after training, and then memory was assessed. Anisomycin is a commonly used inhibitor of protein synthesis[3,37,42], although it may also have secondary effects such as the activation of p38 MAPK[43]. Previous reports have shown that anisomycin injection disrupts protein synthesis–dependent long-term memory consolidation but not protein synthesis–independent short-term memory[42]. Anisomycin injection immediately after training blocked the memory enhancement of S40A CREB at 2 h (**Supplementary Fig. 23**; $F_{1,12} = 24.57$, $P = 0.0003$), whereas anisomycin injection 2 h after training failed to block the memory enhancement at 24 h (**Supplementary Fig. 24**; $F_{1,13} = 0.23$, $P = 0.64$). These results provide further evidence that mice expressing S40A CREB have enhanced, consolidated long-term memory at 2 h. Collectively, our studies show that disinhibition of CREB activity through a single glycosylation site mutation can modulate the formation of long-term memory, and they demonstrate a new role for *O*-GlcNAc glycosylation in memory processing.

DISCUSSION

Since the discovery of *O*-GlcNAc, the *O*-GlcNAc modification has been shown to have key roles in many cellular processes, ranging from glucose homeostasis and the stress response to insulin signaling and transcription[12–14,44,45]. Despite suggestive evidence linking *O*-GlcNAc to neuronal signaling and neurodegeneration[13,15,16,18–21], a mechanistic understanding of how this modification contributes to important neuronal functions has been lacking. In this work, we demonstrate that *O*-GlcNAc glycosylation regulates CREB, a central transcription factor in the brain. Our studies show that *O*-GlcNAc glycosylation of CREB at a specific site, Ser40, has important effects on neuronal gene expression, axonal and dendritic growth and long-term memory.

In contrast with previous studies linking changes in *O*-GlcNAc to cell stress and metabolism[12,44,45], we found that *O*-GlcNAc glycosylation was dynamically induced by neuronal activity, both *in vitro* and *in vivo*, and upon activation of the MAP and CaM kinase pathways. As these same pathways regulate the phosphorylation of many proteins, our results suggest a strong coupling of glycosylation to phosphorylation in neurons. Moreover, the sustained levels of both basal and induced CREB glycosylation suggest that glycosylation may affect CREB activity over longer time periods compared to phosphorylation and may be important for longer-lasting alterations in cellular function.

Our chemoenzymatic strategy combined with site-directed mutagenesis provided a powerful method to study the complex interplay between *O*-GlcNAc and phosphorylation and to dissect the function of the modification at individual sites. The complex relationship between the two modifications on CREB expands the model of *O*-GlcNAc glycosylation and phosphorylation as simply opposed to, independent of or synergistic with one another. Furthermore, we found that specific sites of *O*-GlcNAc glycosylation can be activated independently and uncoupled from one another, depending on the stimulus. We showed previously that CREB is glycosylated within Thr259-Ser260-Thr261, in the binding domain of $TAF_{II}130$, a component of the TFIID transcriptional complex[25]. Hyperglycosylation of CREB disrupts the interaction of CREB with $TAF_{II}130$ *in vitro*. Here we demonstrate a distinct function for Ser40 glycosylation, and together our results highlight the capacity for *O*-GlcNAc glycosylation to exert multiple site-specific functions in a protein. As CREB has been shown to be SUMOylated, acetylated and ubiquitinated[46,47], these studies raise the possibility that CREB activity may be controlled through the complex, combinatorial effects of various post-translational modifications. The continued development of new chemical methods to dissect such intricate interrelationships will be essential for understanding the regulation of CREB and numerous other proteins.

The ability of *O*-GlcNAc to hinder the binding of transcription factors to transcriptional coactivators such as CRTC represents a previously uncharacterized function for the *O*-GlcNAc modification. Overall nuclear CRTC abundance has been proposed to limit the pool of active CREB[11]. Thus, glycosylation adds the capacity to control a defined subpopulation of CREB and may provide an elegant mechanism for the regulation of specific subsets of genes. Supporting this notion, both CREB and OGT were localized to the promoters of glycosylation-sensitive genes. As OGT has been shown to associate with several transcriptional regulatory complexes, including the mSin3a-HDAC complex and the Polycomb repressor complex[48,49], it may have the potential to glycosylate CREB locally while it is bound to specific gene promoters. This would enable even a small cellular pool of glycosylated CREB to effect substantial changes in gene expression and allow for the differential regulation of a subset of CREB-mediated genes. Future studies will examine the effects of CREB glycosylation on a genome-wide scale.

Our results suggest that *O*-GlcNAc glycosylation functions as a constant repressor of CREB activity, thereby controlling the amount of basal expression of CREB-mediated genes such as *Bdnf*, *Wnt2* and *c-fos*. By keeping basal transcript levels low, *O*-GlcNAc glycosylation would provide a larger dynamic range for gene induction to enable neurons to respond properly upon activation. Consistent with this notion, blocking glycosylation of CREB led to dysregulation of KCl-induced dendritic growth. As both CREB repression (via glycosylation) and activation (via phosphorylation) regulate a common *Wnt2*-dependent pathway, the increase in *Wnt2* transcripts observed upon expression of S40A CREB may saturate the Wnt2–β-catenin pathway and desensitize the cell to further increases in *Wnt2* expression following depolarization. Thus, *O*-GlcNAc glycosylation of CREB may contribute to the proper establishment of neural connections through its ability to repress the expression of trophic factors in the absence of developmental cues.

In addition to modulating constitutive transcription, CREB glycosylation also limited activity-dependent transcription. These results suggest that the *O*-GlcNAc modification may regulate the kinetics of CREB deactivation in response to neuronal depolarization and other stimuli. The close coupling of CREB's activation to its inhibition could be an important strategy for balancing the expression of inducible genes and restoring neuronal homeostasis.

Consistent with its roles in regulating gene expression and dendritic and axonal growth, glycosylation of CREB has a considerable impact on long-term memory consolidation. One hypothesis is that expression of S40A CREB enhances the levels of plasticity-related transcripts before and after associative learning. Such changes in gene expression would be expected to facilitate the more rapid accumulation of transcripts necessary for synaptic remodeling and memory consolidation. Although further studies are needed to address the underlying molecular mechanisms of long-term memory, to our knowledge our results provide the first demonstration that the addition of a GlcNAc sugar to a single site within a protein—a seemingly minor chemical perturbation—has important functional consequences in neurons and can influence long-term memory formation. These findings also point to the potential for *O*-GlcNAc glycosylation to contribute to complex, higher-order brain functions.

Our study expands the scope of cellular regulation by *O*-GlcNAc glycosylation to the brain and demonstrates that it has important functions in the nervous system. We provide mechanistic insights into how protein O-glycosylation adds a new layer of regulation to phosphorylation-dependent neural processes. Furthermore, our studies identify a previously unknown mechanism for balancing basal gene expression with activity-induced gene expression in neurons. These results, combined with the observation that many transcription factors and synaptic proteins are *O*-GlcNAc modified[13,15,16], demonstrate the functional breadth and potential of *O*-GlcNAc glycosylation as a critical regulator of neuronal function.

METHODS

Quantification of *O*-GlcNAc glycosylation and Ser133 phosphorylation on CREB. Cultured neurons or dissected brain tissues were lysed and chemoenzymatically labeled with a PEG mass tag as described previously[24], with the modifications noted in the **Supplementary Methods**. The lysates were subjected to 4–12% SDS-PAGE and immunoblotted. CREB-specific (Chemicon) and phospho-Ser133 CREB–specific (Affinity BioReagents) antibodies were used to quantify the percentage of glycosylation on endogenous CREB (**Figs. 1a**,**d**,**e** and **2b**,**d**,**e** and **Supplementary Figs. 3** and **4**). An antibody to Flag (M2, Sigma) was used to quantify the percentage of glycosylation on exogenously expressed CREB mutants (**Figs. 1c** and **2a**,**c**). A phospho-Ser133 CREB–specific antibody was also used to measure the amount of phospho-Ser133 relative to total CREB. All western blots were visualized and quantified using an Odyssey Infrared Imaging System and software (Li-Cor, Version 2.1).

To quantify *O*-GlcNAc stoichiometries, the intensities of the PEG-shifted band (glycosylated protein fraction) and the unshifted band (nonglycosylated protein fraction) were measured. The resulting values were corrected for local background, and the PEG-shifted bands were further corrected against the region of corresponding molecular weight in the unlabeled negative control lane. The percentage of glycosylation was calculated from the signal intensities as the percentage of the glycosylated protein fraction over the total protein fraction (the sum of the glycosylated and nonglycosylated fractions). For **Figures 1c** and **2a**, this value was further normalized to the percentage of glycosylation for Flag-tagged wild-type CREB in untreated neurons and averaged across multiple independent sample sets. For kinetic studies, the percentage of glycosylation was further normalized relative to the glycosylation stoichiometry of the basal sample for each sample set and averaged across multiple independent sample sets.

The data in **Figure 2d** were calculated from those in **Figure 2b** by measuring the relative levels of S133 phosphorylation in the nonglycosylated or monoglycosylated CREB population. The pS133 signals were corrected for the amount of total CREB in each fraction and then normalized with respect to the amount of basal pS133 phosphorylation in each case. The data in **Figure 2e** were calculated from those in **Figure 2b** by measuring the glycosylation stoichiometries from the CREB and pS133 CREB immunoblots as described above.

Cell treatments. For experiments using exogenously expressed CREB mutants (**Figs. 1c** and **2a**,**c**), wild-type or mutant pLenti CREB constructs were electroporated into neurons. Neurons were treated with KCl (55 mM, Sigma; 2 h for **Figs. 1c**,**e** and **2a** and **Supplementary Fig. 3**; 5 min for **Fig. 2c**; 5 min–9 h for **Figs. 1d** and **2b**,**d**,**e**) or forskolin (10 μM, 2 h) after 4–6 days in vitro (DIV) or NMDA (25 μM, 5 min and 10 min) after 13 DIV. Prior to KCl treatments, both treated and control neurons were silenced overnight with tetrodotoxin (1 μM; Tocris Biosciences). For NMDA treatments, cells were transferred to a 37 °C warming plate, and the medium was exchanged with warm HEPES-buffered control salt solution (20 mM HEPES, 55 mM glucose, 5 mM KCl, 0.8 mM $MgCl{\cdot}6H_2O$, 120 mM NaCl, 16.2 mM $CaCl_2$, pH 7.4). Where indicated in the individual figures, cells were treated with the following inhibitors for 30 min before the addition of KCl: nimodipine (5 μM), KN-62 (5 μM), U0126 (10 μM), calphostin C (2.5 μM), cyclosporin A (5 μM), okadaic acid (50 nM), cycloheximide (0.3 mg ml^{-1}) or vehicle (water, EtOH or DMSO). All drugs except KCl and tetrodotoxin were from Axxora Alexis.

Neurite outgrowth. Neurons were electroporated with wild-type or S40A pLEMPRA, pMaxGFP, pcDNA3-Dkk-1-Flag or Ncad(intra) vectors and Wnt-2 (Santa Cruz), CRTC1 or scramble (Invitrogen) siRNA as indicated in the individual figures and then plated at a density of 25,000 neurons cm^{-2}. Neurons from B6.129-*Ogt*^{tm1Gwh}/J mice (Jackson Laboratories) were electroporated with pMaxGFP, the CRE recombinase pBOB-CAG-iCRE-s.d. (Addgene) or pA2UCOE-OGT vector, along with scramble or CREB siRNA as indicated. One day before imaging for dendrites, neurons were depolarized with KCl (50 mM) where indicated in the individual figures. After 1 DIV, neurons were treated with TrkB-Fc (R&D Biosystems; 0.7 μg ml^{-1}) where indicated. After 4–5 DIV, all neurons were fixed with 4% paraformaldehyde for 20 min at 23 °C, washed twice with PBS and once with H_2O and mounted onto glass slides. Transfected GFP-expressing cells were imaged using a Nikon Eclipse TE2000-S inverted microscope equipped with Metamorph software. Neurite lengths were quantified with NeuronStudio (Version 0.9.92)[50]. The longest neurite was assigned as the axon, and the remaining neurites were assigned as dendrites. Staining of representative cultures with the dendrite-specific marker MAP2 confirmed the results. Lysates from neurons electroporated with wild-type or S40A CREB were immunoblotted for Flag-CREB to confirm equal amounts of CREB expression. Neurons from B6.129-*Ogt*^{tm1Gwh}/J or C57BL/6 mice were immunostained with the OGT-specific antibody DM17 (Sigma) to confirm the effects of OGT knockdown. Overexpression of Flag-tagged OGT was confirmed by immunoprecipitation using Flag-specific M2 affinity resin (Sigma) per the manufacturer's protocol, followed by western blotting for OGT using the OGT-specific antibody DM17.

Statistics *P* values were calculated from Student's paired *t*-test when comparing within groups and from Student's unpaired *t*-test when comparing between groups. ANOVA was used to analyze *in vivo* data. All calculations were performed using the program Excel.

Additional methods. Information on DNA plasmids and virus construction, cell culture and transfection, glycosylation site mapping and quantification, glycosylation-phosphorylation interaction studies, luciferase reporter assays, electrophoretic mobility shift assay, quantitative PCR with reverse transcription, auditory fear conditioning and amygdala biochemistry, coimmunoprecipitation and ChIP experiments can be found in the **Supplementary Methods**.

All mouse experiments were approved by the Institutional Animal Care and Use Committee at Caltech.

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Author contributions

L.C.H.-W. designed, directed and coordinated the project. P.M.C. and J.E.R. designed and performed the experiments except where otherwise noted. D.E.M. and E.C.P. performed the MS analyses; R.L.N. prepared the HSV. P.M.C., J.E.R. and L.C.H.-W. wrote the manuscript, and all authors participated in editing it.

Competing financial interests

The authors declare no competing financial interests.

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