In Vivo Modulation of O-GlcNAc Levels Regulates Hippocampal Synaptic Plasticity through Interplay with Phosphorylation∗

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Melanie K. Tallent‡, Neal Varghis‡, Yuliya Skorobogatko§, Lisa Hernandez-Cuebas§, Kelly Whelan‡, David J. Vocadlo¶, and Keith Vosseller††

From the §Department of Biochemistry and Molecular Biology and ¶Department of Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, Pennsylvania 19102 and the ‡Department of Chemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada

O-Linked N-acetylgalcosamine (O-GlcNAc) is a cytosolic and nuclear carbohydrate post-translational modification most abundant in brain. We recently reported uniquely extensive O-GlcNAc modification of proteins that function in synaptic vesicle release and post-synaptic signal transduction. Here we examined potential roles for O-GlcNAc in mouse hippocampal synaptic transmission and plasticity. O-GlcNAc modifications and the enzyme catalyzing their addition (O-GlcNAc transferase) were enriched in hippocampal synaptosomes. Pharmacological elevation or reduction of O-GlcNAc levels had no effect on Schaffer collateral CA1 basal hippocampal synaptic transmission. However, in vivo elevation of O-GlcNAc levels enhanced long term potentiation (LTP), an electrophysiological correlate to some forms of learning/memory. Reciprocally, pharmacological reduction of O-GlcNAc levels blocked LTP. Additionally, elevated O-GlcNAc led to reduced paired-pulse facilitation, a form of short term plasticity attributed to presynaptic mechanisms. Synapsin I and II are presynaptic proteins that increase synaptic vesicle availability for release when phosphorylated, thus contributing to hippocampal synaptic plasticity. Synapsins are among the most extensively O-GlcNAc-modified proteins known. Elevating O-GlcNAc levels increased phosphorylation of Synapsin I/II at serine 9 (cAMP-dependent protein kinase substrate site), serine 62/67 (Erk 1/2 (MAPK 1/2) substrate site), and serine 603 (calmodulin kinase II site). Activation-specific phosphorylation events on Erk 1/2 and calmodulin kinase II, two proteins required for CA1 hippocampal LTP establishment, were increased in response to elevation of O-GlcNAc levels. Thus, O-GlcNAc is a novel regulatory signaling component of excitatory synapses, with specific roles in synaptic plasticity that involve interplay with phosphorylation.

In contrast to luminal and extracellular complex glycosylation, O-GlcNAc is a single N-acetylgalcosamine O-linked to serines and threonines of cytosolic and nuclear proteins in multicellular eukaryotes. Analogous to the way that kinases and phosphatases regulate phosphorylation, cytosolic and nuclear enzymes dynamically catalyze addition (OGT)2 and removal (O-GlcNAcase) of O-GlcNAc (1, 2). Mechanistic examples of site-specific O-GlcNAc regulatory function are limited but include influences on protein-protein interactions, subcellular protein localization, protein half-life, and interplay with phosphorylation (3–8).

O-GlcNAc, OGT, and O-GlcNAcase are enriched in brain (1, 2, 9), and OGT levels are especially high at neuronal synapses (10, 11). Mouse brain-specific deletion of OGT results in perinatal lethality associated with severe motor defects (12). KCl-induced depolarization of NG-108-15 neuroblastoma cells in culture rapidly activates OGT and increases O-GlcNAc levels, suggesting regulatory potential for dynamic O-GlcNAc modifications in neuronal signaling (13). Our recent proteomic observations revealed strikingly extensive O-GlcNAc modifications of several proteins that function in regulating vesicle (SV) availability for release. For example, the presynaptic proteins synapsin and Bassoon, critical in regulating SV pools, are O-GlcNAc modified at 7 and 19 unique sites, respectively (14, 15). These observations are consistent with immunoelectron microscopy showing 5-fold more intensity of O-GlcNAc localization presynaptically versus postsynaptically in rat cerebellar cortex (11), with staining being most intense surrounding SVs. The evidence strongly suggests evolutionarily conserved functional roles for O-GlcNAc in synaptic transmission, but this has not been examined.

OGT and O-GlcNAc are most abundant in hippocampus (16), a brain region implicated in learning and memory through forms of synaptic plasticity such as long term potentiation (LTP). LTP is an activity-dependent strengthening of synaptic responses thought to be an electrophysiological correlate to some forms of learning/memory. Phosphorylation is a critical mechanism regulating synaptic processes. For example, hippocampal LTP involves phosphorylation/activation of kinases such as CaM kinase II, p42/44 Erk (MAPK 1/2), and PKA (17, 18). Although postsynaptic mechanisms are critical to LTP-based learning and memory (19), presynaptic mechanisms, including synapsin phosphorylation, have been implicated in

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To whom correspondence should be addressed: 245 N. 15th St., Philadelphia, PA 19102. Tel.: 215-762-8789; Fax: 215-762-4452; E-mail: keith.vosseller@drexelmed.edu.

The abbreviations used are: OGT, O-GlcNAc transferase; LTP, long term potentiation; FEPSP, field excitatory postsynaptic potentials; HFS, high frequency stimulus; PPF, paired-pulse facilitation; STP, short term potentiation; ACSF, artificial cerebrospinal fluid; PKA, cAMP-dependent protein kinase; CaM, calmodulin; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase.
hippocampal LTP (20–23). Synapsins I/II tether SVs to the actin cytoskeleton, limiting their availability for release. During synaptic activity, Synapsins I/II are phosphorylated, dissociate from vesicles, and thus promote vesicle availability for release (24–26). Synapsin I and II have been linked to activity-dependent increased SV release probability in hippocampal synaptic plasticity (27, 28), and synapsin phosphorylation by PKA at serine 9 has been linked to enhanced neurotransmitter release (29, 30).

O-GlcNAc may compete directly with phosphorylation at specific serines and threonines (31–33). Alternatively, O-GlcNAc may contribute to up-regulation of phosphorylation events (34). Given the potential for interplay between O-GlcNAc and phosphorylation (35), O-GlcNAc may modulate phosphorylation-based signaling in synaptic processes. Here we utilize acute hippocampal slice electrophysiology to examine the influence of modulating O-GlcNAc levels on synaptic transmission/plasticity and functionally related phosphorylation events.

**EXPERIMENTAL PROCEDURES**

Reagents—Antibodies used in Western blotting were as follows: anti-synaptophysin mouse monoclonal IgG (Chemicon, Temecula, CA); anti-O-GlcNAc transferase (OGT) rabbit polyclonal (kind gift of Gerald Hart, The Johns Hopkins University, Baltimore); anti-O-GlcNAc (110.6) IgM monoclonal (36); anti-active CaM kinase II (Thr(P)286) (Promega, Madison, WI); rabbit polyclonal IgG anti-synapsin (Cell Signaling, Danvers, MA); rabbit polyclonal IgG anti-phospho-synapsin (Ser9) (Cell Signaling); rabbit polyclonal IgG anti-phospho-Ser62/67 (37) synapsin (PhosphoLutions, Aurora, CO); rabbit polyclonal IgG anti-ERK 1/2 (Promega, Madison, WI); rabbit polyclonal IgG and anti-ERK 1/2 (pTPY185/187) rabbit polyclonal IgG (BIOSOURCE). The pharmacological inhibitor of O-GlcNAc case 9d was synthesized as described (38).

Pharmacological Treatments and Hippocampal Slice Preparation—Male mice (6–8 weeks old) were intravenously injected in the tail vein with 9d (75 mg/kg) in saline or with saline alone for controls for the time indicated. Mice were anesthetized with isoflurane and decapitated, and the brains were rapidly removed into ice-cold glycerol/artificial cerebrospinal fluid (glycerol/ACSF) (130 mM NaCl, 3.5 mM KCl, 1.25 mM NaH2PO4, 1.5 mM MgSO4·7H2O, 2.0 mM CaCl2, 24 mM NaHCO3, and 10 mM glucose, 260 mM glycerol) gassed with 95% O2, 5% CO2. The hippocampus was homogenized in 0.32 M sucrose containing a glass Teflon tissue grinder and centrifuged at 2,600 × g for 2 min. Supernatants were collected and spun at 14,500 × g for 10 min to obtain pellet P2. P2 was processed by layering over a Percoll gradient comprising 23, 10, and 3% Percoll solutions, and the synaptosome-enriched fraction was recovered at the 23 to 10% interface. The preparations were washed into oxygenated HEPES-buffered saline (142 mM NaCl, 2.8 mM KCl, 1.2 mM K2HPO4, 1 mM MgCl2, 5 mM d-glucose, and 10 mM HEPES, 1 mM CaCl2, pH 7.4) and collected by centrifugation.

**Tissue Lysis and Western Blotting**—Samples for Western blotting of hippocampal slices and fractions from synaptosome preparation were obtained by lysis in 6 M urea buffer containing 200 mM Tris, pH 7.8, and 5 mM EDTA added to two slices and subjected to sonication with a microtip on ice using a Branson Sonifier 250. (Duty cycle %: 20, output control: 2, 10 pulses per sample.) Samples were clarified at 14,000 rpm for 10 min. Protein levels in the supernatant were determined by a protein assay (Bio-Rad). For O-GlcNAc Western blotting, 40 μg of sample was loaded on precast 10% SDS-polyacrylamide gels (Bio-Rad), whereas for all other blotting, 10 μg of sample was loaded per lane. Protein was transferred to polyvinylidene difluoride for Western blotting. Densitometry of bands on Western blots was performed on the Alpha Innotech-Alphalmager™ 3400 (San Leandro, CA).

**Immunoprecipitation**—Whole hippocampus was placed in 100 μl of RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 25 mM Tris-HCl containing phosphatase inhibitor mixture I and II (Sigma) and protease inhibitor mixture (Sigma)). Samples were sonicated on ice as described for hippocampal slice lysis, left on ice for 10 min, and clarified at 14,000 rpm for 10 min. The supernatants were pre-cleared with agarose-bound protein A/G (Santa Cruz Biotechnology, Santa Cruz, CA), and immunoprecipitations were per-
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formed using 3 μg of anti-synapsin (Cell Signaling, Danvers, MA) and 30 μl of a 50% slurry of agarose-bound protein A/G. Immunoprecipitates were washed three times in ice-cold RIPA buffer and eluted by boiling in 2× SDS sample buffer.

RESULTS

OGT and O-GlcNAc Enrichment at Hippocampal Synapses—Previous O-GlcNAc proteomic analysis of whole brain post-synaptic density preparations (14), O-GlcNAc immunohistochemistry of cerebellar neurons (11), and biochemical fractionation (10) suggest enrichment of O-GlcNAc at brain synapses. We examined this in mouse hippocampus by Western blotting of increasingly enriched synaptosomal fractions from 6- to 8-week-old male C57B6/J mice. Equal amounts of whole hippocampal initial homogenate, a crude endosomal pellet (P2), a synaptosomal fraction, or supernatant from the P2 preparation (S2) were separated by SDS-PAGE for Western blotting. As expected, the synaptosomal marker synaptophysin was strongly enriched in synaptosomes, indicating successful fractionation (Fig. 1). Additionally, enrichment of OGT in synaptosomes was also observed (Fig. 1), indicating concentration of OGT at hippocampal nerve terminals. Consistent with this observation, increased O-GlcNAc reactivity patterns (particularly at high molecular weights) are observed in the synaptosome fraction, with several O-GlcNAc reactive bands being detected exclusively in synaptosomes (Fig. 1).

Pharmacological in Vivo Elevation of O-GlcNAc Alters Synaptic Plasticity in Hippocampal Slices—To examine potential functional roles of O-GlcNAc at neuronal synapses, we first examined effects of modulating O-GlcNAc in vivo on electrophysiological responses in acute hippocampal slices. We tested the ability of an O-GlcNAcase inhibitor (9d) (37) to pharmacologically elevate in vivo O-GlcNAc levels. 6–8-Week-old male C57B6/J mice were injected intravenously in the tail vein with 75 mg/kg of 9d or with saline vehicle, and the hippocampus was dissected for determination of O-GlcNAc levels by Western blotting at 3, 5, and 7 h. Maximal O-GlcNAc elevation occurred between 5 and 7 h, whereas levels of the presynaptic marker synaptophysin were unchanged (Fig. 2A). Dose-response studies indicated that 75 mg/kg 9d was the lowest dose for inducing maximal O-GlcNAc levels (data not shown). Following in vivo elevation of O-GlcNAc by 5 h 9d intravenous treatment (Fig. 2B), the slope of fEPSPs evoked by varied stimulus intensities at the CA1 Shaffer collateral pathway (input-output analysis) in acute hippocampal slices was unchanged (n = 7 after 9d; n = 8 for control; Fig. 2C). The range of stimulus intensities used to evoke threshold, half-maximal, and maximal fEPSPs and to generate LTP was also not different between control and 9d slices (p = 0.5).

PPF, a form of short term facilitation of synaptic transmission attributed to presynaptic mechanisms, was significantly decreased at 15- (p = 0.006) and 100-ms (p = 0.043) inter-stimulus intervals in hippocampal slices from 9d-treated mice (Fig. 2E). With an interstimulus interval of 50 ms, PPF was not significantly different between control and 9d slices (p = 0.246). In control slices, the ratio of slope of the 2nd fEPSP to the 1st was 1.9 ± 0.14, 1.9 ± 0.10, and 1.8 ± 0.13 at 15-, 50-, and 100-ms interstimulus intervals, respectively. In slices from 9D-treated mice, the ratio of the 2nd to the 1st fEPSP slope was 1.5 ± 0.11, 1.7 ± 0.10, and 1.5 ± 0.03 at 15-, 50-, and 100-ms interstimulus intervals, respectively. The level of LTP measured as percent increase in base-line slope of fEPSPs induced by a single train of stimuli at 100 Hz was enhanced significantly over 60 min by 9d treatment (Fig. 2D, p = 0.03). 60 min following the train, the initial slope of fEPSPs recorded from control slices was increased by 47 ± 14% (n = 8) relative to the base-line pre-train value. In slices from 9d-treated animals (n = 7), the initial slope of the fEPSP was increased by 77 ± 10% 60 min following the train. Short term potentiation (STP) measured from 1 to 5 min following the train was also significantly increased in 9d-treated mice compared with control mice (p = 0.007). 1 min following the train, fEPSP slope was increased by 215 ± 14% in control mice and 322 ± 24% in 9d-treated mice.
FIGURE 2. In vivo pharmacological inhibition of O-GlcNAcase elevates O-GlcNAc levels and enhances hippocampal LTP. A, C57Bl/6J 6-week-old male mice were tail vein-injected (intravenously [I.V.]) with 75 mg/kg 9d for 0 (vehicle injected for 7 h), 3, 5, or 7 h, and hippocampal lysates were analyzed by Western blotting for levels of O-GlcNAc with anti-O-GlcNAc antibody 110.6 (36) or with anti-synaptophysin. B, O-GlcNAc levels were examined by Western blotting (WB) in whole brain or in hippocampal slices (in duplicate animals) in response to intravenous 5-h 9d treatment. C, eEPSPs were evoked in acute hippocampal slices to determine influence of 9d basal synaptic transmission in response to stimuli generating threshold, half-maximal (half-max), and maximal (max) responses. No differences between control and 9d slices are observed. D, PPF in response pairs of stimuli at 15–100-ms interstimulus intervals. 9d treatment results in significant suppression of PPF at 15 and 100-ms interstimulus intervals. Inset shows representative recordings with a 100-ms interstimulus interval. Scale bars, 0.5 mV, 50 ms. E, LTP in response to single train high frequency stimulation at 100 Hz. Inset shows representative recordings from a control and 9d-treated slices. Scale bars 0.5 mV, 5 ms.

FIGURE 3. Pharmacological inhibition of OGT lowers O-GlcNAc levels and inhibits LTP. A, acute hippocampal slices from C57Bl/6J 6-week-old male mice were treated 4 h with either the OGT inhibitor alloxan or the inactive analogue uracil at 5 mM in ACSF and lysed for O-GlcNAc Western blotting (WB). The influence of alloxan treatment was examined on input-output basal synaptic transmission in response to various stimulus intensities (B), PPF in response pairs of stimuli at 25–100-ms intervals (C), and LTP in response to single train HFS at 100 Hz (D). Half-max, half-maximal; max, maximal. Because STP is mediated by presynaptic mechanisms (41), these results suggest that 9d treatment results in an increase in presynaptic function.

Pharmacological Reduction of O-GlcNAc Levels Inhibits LTP—Alloxan is an inhibitor of OGT (42) and has been used to reduce levels of O-GlcNAc in cells (43–45). We incubated acute hippocampal slices in ACFS for 4 h with a concentration of alloxan previously shown to maximally reduce O-GlcNAc levels in cells (5 mm) or with 5 mm of the analogue uracil (which lacks OGT inhibitory action) (42) as a control. Alloxan (n = 8) reduced O-GlcNAc levels (Fig. 3A) but had no effect compared with uracil (n = 8) on basal CA1 Shaffer collateral pathway basic synaptic transmission as measured by the input-output in response to different stimulus intensities (Fig. 3B, p = 0.26). The stimulus intensities used to generate the input-output relationship was also not affected by alloxan treatment (p = 0.4). Furthermore, alloxan treatment did not affect PPF using interstimulus intervals from 15 to 100 ms (Fig. 3C, p = 0.614). However, pretreatment with alloxan strongly inhibited LTP measured as percent increase in baseline slope of eEPSPs (Fig. 3D, p = 0.021). In uracil-treated slices, 60 min following LTP induction, the eEPSP slope was increased by 41 ± 12% relative to the base line (n = 8). However, in alloxan-treated slices, only a 9.5 ± 3.7% increase in eEPSP slope remained 60 min following the trains (n = 8). When eEPSP slopes from 51 to 60 min following the train are compared with the base line, no significant enhancement is present in alloxan-treated mice (p = 0.08), whereas uracil-treated slices show significant enhancement (p = 0.005). Thus, blocking O-GlcNAc elevation with alloxan prevents generation of LTP. The results show that SC CA1 hippocampal LTP is enhanced by pharmacological elevation of O-GlcNAc and is reciprocally inhibited by pharmacological reduction of O-GlcNAc levels.

Elevation of O-GlcNAc Levels Increases Synapsin Phosphorylation—The influences of O-GlcNAc elevation on synaptic plasticity could be linked to effects on phosphorylation-based signaling known to function in synaptic transmission/LTP. As synapsins are among the most extensively O-GlcNAc-modified proteins (14, 15), are implicated in synaptic plasticity through known phosphorylation-dependent mechanisms (20–23), and because altered PPF in response to 9d treatment suggested presynaptic influences (Fig. 2E), we examined the effects of in vivo pharmacological elevation of O-GlcNAc on synapsin phosphorylation. Known sites of synapsin phosphorylation and O-GlcNAc modifications, and the enzymes thought to catalyze addition of these modifications, are depicted in Fig. 4. Elevation of O-GlcNAc in acute hippocampal slices was achieved as in electrophysiological studies, except that slices were lysed and examined by Western blotting using antibodies specific for
total Synapsin I/II levels and synapsin phosphorylation events at serine 9 (P-site 1) and serines 62/67 (P-site 3 and 4). Elevation of O-GlcNAc levels in response to 9d treatment was confirmed (Fig. 5A). Additionally, immunoprecipitated Synapsin I from hippocampal lysates of 9d-treated mice was modified by increased levels of O-GlcNAc (Fig. 5B). 9d treatment of mice had no effect on total Synapsin I/II protein levels, but phosphorylation of synapsin at both serine 9 and serines 62/67 was increased in response to elevation of O-GlcNAc (Fig. 5A). Densitometry was used to quantify changes in intensity of Synapsin IA-phosphorylated bands from nine independent experiments (Fig. 5C). Serine 9 and serine 62/67 phosphorylation in 9d-treated samples was increased relative to total synapsin levels by 160 and 104%, respectively.

**Activation-specific Phosphorylation of Erk 1/2 in Response to Elevated O-GlcNAc**—Serines 62/67 of Synapsin I are thought to be Erk 1/2 kinase substrate sites that when phosphorylated promote movement of SVs from a “reserve” to a “readily releasable” pool (46). It was possible that increased serine 62/67 phosphorylation in response to elevated O-GlcNAc was because of increased activity of Erk 1/2. Erk 1/2 plays important roles in hippocampal LTP and hippocampus-dependent learning (47). The influence of Erk 1/2 on LTP appears to be complex but likely involves post-synaptic effects on increased K⁺ channel-dependent depolarization (48) as well as presynaptic effects through synapsin phosphorylation (20). Increased O-GlcNAc levels have previously been linked to Erk 1/2 activation in neutrophils (34). Erk 1/2 is activated through phosphorylation by MEKs at a conserved threonine (202 in human Erk 1) and tyrosine (204 in human Erk 1). Erk is phosphorylated at these sites rapidly in response to high frequency stimulation that induces LTP in the CA1 region of hippocampal slices (17, 49). Intravenous 9d treatment pharmacologically elevated O-GlcNAc (data not shown) and caused increased hippocampal slice Erk 1/2 activation state-specific phosphorylation, whereas total levels of Erk 1/2 were unchanged (Fig. 6A).

**Activation-specific Phosphorylation of CaM Kinase II in Response to Elevated O-GlcNAc**—CaM Kinase II activation through Ca²⁺ and calmodulin-dependent autophosphorylation at threonine 286/287 is critical for establishment of hippocampal LTP (50). Post-synaptic phosphorylation of CaM kinase II substrates such as α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor is implicated in LTP. Additionally, presynaptic synapsin is also phosphorylated at two distinct CaM kinase II sites at residues 603 and 567 (rat numbering, P-sites 2 and 3), which has been implicated in synapsin function in regulating availability of SVs for release (51). Thus, we examined CaM kinase II activation-specific phosphorylation, and phosphorylation of Synapsin I at serine 603. In vivo intravenous injection of 9d caused pharmacological elevation of O-GlcNAc (data not shown) and led to a modest increase in hippocampal slice CaM kinase II activation-specific phosphorylation, whereas expression levels were unaltered (Fig. 6A). We used densitometry to quantitate the 9d-induced increase in CaM kinase II phosphorylation from eight replicate experiments, which shows an average increase of 33.2% normalized for total CaM kinase II α protein levels (Fig. 6B). Additionally, a

**FIGURE 4.** Post-translational modifications of mouse Synapsin Iα N and C terminus and the enzymes thought responsible for catalyzing their addition.

**FIGURE 5.** In vivo pharmacological elevation of O-GlcNAc levels increases hippocampal synapsin phosphorylation at serine 9 (P-site 1) and serines 62/67 (P-site 3/4). Lysates of hippocampal slices from triplicate control saline versus 9d (5 h) intravenously treated mice were Western-blotted (WB) for O-GlcNAc, total synapsin, synapsin phosphorylated at serine 9, and synapsin immunoprecipitated (IP) from hippocampal lysates. C, densitometry performed on Synapsin Iα isoform in nine replicate Western blots were plotted as average percent increase of either phosphorylated serine 9 or phosphorylated serine 62/67 in 9d-treated samples normalized for total O-GlcNAc levels. Densitometry values from control versus 9d-treated Western blot bands of synapsin phosphorylated serine 9 and serines 62/67 were compared using Student’s t test. *, p < 0.05; ***, p < 0.001.
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FIGURE 6. In vivo pharmacological elevation of O-GlcNAc increases activation-specific phosphorylation of Erk 1/2 and CaM kinase II and leads to increased synapsin phosphorylation at CaM kinase II substrate site. A, Western blotting (WB) of hippocampal slice lysates from duplicate control saline versus 9d (5 h) intravenously treated mice were performed for total Erk 1/2, Erk 1/2 phosphorylated at activation-specific threonine/tyrosine 202/204, total CaM kinase II, phospho-286/287 CaM kinase II, and phospho-603 synapsin. B, densitometry performed on eight replicate Western blots was plotted as 9d-induced average percent change in total CaM kinase II levels or activation-specific CaM kinase II phosphorylation (residue 286/287) normalized for total CaM kinase levels. Densitometry values from control versus 9d-treated Western blot bands of phosphorylated CaM kinase II were compared using Student’s t test. *, p < 0.05; **, p < 0.01.

strong increase in Synapsin I phosphorylation at the CaM kinase II substrate site serine 603 was observed in response to elevation of O-GlcNAc (Fig. 6A), whereas total levels of synapsin were unchanged (data not shown).

DISCUSSION

The enrichment of OGT and O-GlcNAc we observe in synaptosomes indicates unique targeting of O-GlcNAc modifications to neuronal synapses and suggests evolutionarily conserved functions in regulating synaptic transmission/plasticity. To test this hypothesis, we examined the influence of modulation of O-GlcNAc levels on synaptic responses. We made use in vivo of an O-GlcNAcase inhibitor (9d) (37), lacking nonspecific effects associated with previous O-GlcNAcase inhibitors, such as O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc) (37) and streptozotocin (52), to pharmacologically elevate O-GlcNAc. Alloxin was used to reciprocally inhibit OGT and lower O-GlcNAc levels (42, 43). We find that modulation of O-GlcNAc specifically alters electrophysiological read outs of synaptic plasticity, while not affecting basal synaptic transmission. Activity-dependent alterations in synaptic molecular states underlie the phenomenon of synaptic plasticity. In particular, activity-dependent long lasting strengthening of synaptic responses (LTP) is thought to encode some forms of learning/memory in the hippocampus. Thus, it appears that the post-translational regulatory potential of O-GlcNAc is specifically utilized in modulating plasticity at hippocampal synapses.

The 9d-induced decrease in PPF and increase in STP indicated potential presynaptic contributions to increased LTP in response to elevated O-GlcNAc. Consistent with this observation, the most extensively O-GlcNAc-modified proteins appear to be presynaptic and to be involved in regulating SV pools. Although the study of hippocampal CA1 LTP has largely focused on N-methyl-D-aspartic acid receptor-dependent postsynaptic mechanisms (17, 53), presynaptic mechanisms also likely contribute. For example, Erk 1/2 (MAPK 1/2) acting upstream of synapsin phosphorylation has been linked to hippocampal LTP. Activation of Erk by transgenic expression of constitutively active Ras leads to increased Synapsin I serine 62/67 phosphorylation associated with enhanced hippocampal CA1 LTP. This effect was synapsin-dependent, as this increased LTP was lost in Synapsin I/II double knock-out mice (20).

Hippocampus-dependent learning (e.g. contextual fear conditioning) also leads to phosphorylation of Synapsin I at serine 62/67 (P-sites 4/5) (Erk substrate sites), which is Erk-dependent (20). Additional evidence links synapsin with signals for enhanced neurotransmission and suggests potential roles in plasticity. For example, brain-derived neurotrophic factor and nerve growth factor of neurotrophin enhances glutamate release from rat brain synaptosomes and cultured neurons through Erk-dependent phosphorylation of synapsins (54, 55), and this effect may be through altered phosphorylation-dependent interaction of synapsin with actin (54).

Additionally, Synapsins I and II have been linked to increased SV release probability in hippocampal synaptic transmission enhancement/plasticity at glutamatergic synapses in response to prolonged stimulation (27). Phosphorylation of synapsin is linked to its dissociation from SVs and redistribution out of nerve terminals, which is thought to increase the availability of SVs for release. Thus, our observation of increased synapsin phosphorylation in response to elevation of O-GlcNAc is likely associated with increased presynaptic release probability of neurotransmitter.

O-GlcNAc may compete with phosphorylation at identical or nearby sites (31, 35, 56–58). Reciprocity between O-GlcNAc and phosphorylation has been demonstrated in neuronal culture globally (59) and specifically on the protein Tau (60, 61). However, contrary to this model of reciprocity, we observe several increased phosphorylation events in response to elevation of O-GlcNAc. Increased synapsin serine 9 phosphorylation indicates that the activity of enzymes thought to target this site (PKA and/or CaM kinase IV) may be up-regulated, but this has not yet been examined. Alternatively, synapsin modification by O-GlcNAc may more directly up-regulate serine 9 phosphorylation through mechanisms such as increased substrate availability or increased recruitment of PKA. PKA itself, independ-
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ently of synapsin, has been linked to LTP through effects on post-synaptic α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor trafficking, GluR1 receptor phosphorylation, and cAMP-response element-binding protein phosphorylation (late LTP-specific) (62). Thus, it will be of interest to examine the relationship between O-GlcNAc and PKA activity in hippocampal plasticity.

Erk 1/2 is a well known component of proliferative signaling pathways, but in terminally differentiated neurons it is involved in synaptic plasticity and memory processes (18, 63). We have not discriminated between pre- and post-synaptic pools of activated Erk 1/2 in this study. In addition to the presynaptic substrate synapsin, Erk 1/2 is known to play important roles postsynaptically in expression of early LTP in the CA1 SC pathway in acute hippocampal slices (18, 63). The mechanism through which elevated O-GlcNAc is linked to Erk 1/2 activation is not known but may be occurring through modification of upstream Erk signaling components such as MEKs and Raf. Elevation of O-GlcNAc has been previously linked to Erk 1/2 activation in neutrophils (34), and thus a common mechanism may underlie O-GlcNAc regulation of Erk 1/2 activation in various cell types. It is of interest that Erk 1/2 activation appears not to be involved in basal synaptic transmission but is essential for HFS-induced LTP in the CA1 region of hippocampal slices (49, 63). This parallels our observations with regard to elevated O-GlcNAc and increased LTP, in that basal synaptic transmission is unaffected, supporting a model in which activation of Erk is a key candidate for mediating LTP enhancement by O-GlcNAc elevation.

CaM kinase inhibitors block OGT activation in response to KCl depolarization of a neuroblastoma cell line (13), suggesting potential signaling connections between CaM kinase and dynamic O-GlcNAc modifications in the context of neuronal plasticity. CaM kinase II activation and function in LTP is dependent on Ca2+ influx and Ca2+ binding by autophosphorylation at threonine 286 and association with calmodulin (50). Autophosphorylation of CaM kinase II converts it to a Ca2+-independent constitutively active form. As we observe a modest increase in CaM kinase II phosphorylation at threonine 286 in response to elevated O-GlcNAc, it will be of interest to examine if this effect involves influences on Ca2+ flux. O-GlcNAc has been implicated in regulation of Ca2+ levels in other systems, including cardiomyocytes (64), and through specific modification of the inositol 1,4,5-trisphosphate receptor (65). However, direct elevation of Ca2+ levels by increased O-GlcNAc may be expected to influence basal synaptic transmission directly, which we do not observe. O-GlcNAc regulatory influences acting downstream of Ca2+ flux might allow O-GlcNAc to act as a modulatory factor specifically in neuronal plasticity. Our observations of increased Erk 1/2 and CaM kinase II activation-specific phosphorylation along with increased synapsin phosphorylation in response to elevation of O-GlcNAc indicate that these up-regulated signaling events likely mediate, at least in part, the enhancement of LTP observed in response to 9d pharmacological treatment. In conclusion, this is the first evidence for O-GlcNAc modifications playing specific regulatory roles in neuronal plasticity, which involves interplay with phosphorylation events.

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