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Chemical approaches to understanding *O*-GlcNAc glycosylation in the brain

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***O*-GlcNAc glycosylation is a unique, dynamic form of glycosylation found on intracellular proteins of all multicellular organisms. Studies suggest that *O*-GlcNAc represents a key regulatory modification in the brain, contributing to transcriptional regulation, neuronal communication and neurodegenerative disease. Recently, several new chemical tools have been developed to detect and study the modification, including chemoenzymatic tagging methods, quantitative proteomics strategies and small-molecule inhibitors of *O*-GlcNAc enzymes. Here we highlight some of the emerging roles for *O*-GlcNAc in the nervous system and describe how chemical tools have significantly advanced our understanding of the scope, functional significance and cellular dynamics of this modification.**

O-GlcNAc glycosylation, the covalent attachment of β-*N*-acetylglucosamine to serine or threonine residues of proteins, is an unusual form of protein glycosylation[1] (**Fig. 1**). Unlike other types of glycosylation, this single-sugar modification occurs on intracellular proteins and is not elaborated further into complex glycans. The *O*-GlcNAc transferase (OGT) enzyme is a soluble protein that is found in the cytosol, nucleus and mitochondria[2], rather than in the endoplasmic reticulum or Golgi. The dynamics of *O*-GlcNAc are also unique among sugar modifications, being cycled on a shorter time scale than protein turnover[3]. Thus, in many respects *O*-GlcNAc is more akin to phosphorylation than to conventional forms of glycosylation.

Several reviews have described the roles of *O*-GlcNAc in cellular processes, such as transcription[2,4], the stress response[5,6], apoptosis[7,8], signal transduction[2,9], glucose sensing[5,10] and proteasomal degradation[5]. Only a few reviews have highlighted the importance of *O*-GlcNAc glycosylation in the nervous system, and those reports have focused on its potential impact on neurodegenerative diseases[11,12]. However, several lines of evidence suggest that *O*-GlcNAc has crucial roles in both neuronal function and dysfunction. The enzymes responsible for the modification are most highly expressed in the brain[13,14] and are enriched at neuronal synapses[15,16]. Neuron-specific deletion of the OGT gene in mice leads to abnormal development and locomotor defects, resulting in neonatal death[17]. The *O*-GlcNAc modification is abundant in the brain and present on many proteins important for transcription, neuronal signaling and synaptic plasticity, such as cAMP-responsive element binding protein (CREB)[18], synaptic Ras GTPase-activating protein (synGAP)[19] and β-amyloid precursor protein (APP)[20]. An intriguing interplay between *O*-GlcNAc glycosylation and phosphorylation has been observed in cerebellar neurons, wherein activation of certain kinase pathways reduces *O*-GlcNAc levels on cytoskeleton-associated proteins[21]. Finally, recent studies suggest that *O*-GlcNAc is dynamically regulated by excitatory stimulation of the brain *in vivo*[19].

Over the past 5 years, new chemical approaches have been developed to meet the specific challenges associated with studying *O*-GlcNAc glycosylation in the brain. Methods for chemically tagging and identifying *O*-GlcNAc–glycosylated proteins have vastly expanded the number of neuronal proteins known to be *O*-GlcNAc modified. New pharmacological inhibitors of the *O*-GlcNAc enzymes have been discovered that may overcome some of the limitations of genetic approaches. Quantitative proteomics strategies designed to accommodate postmitotic neurons and brain tissue have been used to monitor changes in glycosylation levels in response to neuronal stimuli. Combined with recent advances in mass spectrometry, these powerful tools have provided an unprecedented opportunity to explore the *O*-GlcNAc proteome, manipulate glycosylation levels and study the dynamics of this modification *in vivo*.

In this Review, we describe emerging functions for *O*-GlcNAc glycosylation in the nervous system. We then consider the modification in the context of chemical biology, describing chemical approaches that have accelerated an understanding of *O*-GlcNAc and chemical tools needed to further advance the field.

The enzymes OGT and OGA

OGT and β-*N*-acetylglucosaminidase (OGA or *O*-GlcNAcase) catalyze the reversible addition and removal of *O*-GlcNAc, respectively. Both enzymes are most highly expressed in the brain and exist as several different isoforms[2,22]. Three distinct isoforms of OGT have been identified, including a 110-kDa and a 78-kDa isoform, which can assemble into multimers[23,24], and a smaller mitochondrial isoform. Each variant contains a C-terminal catalytic domain, but differs in the number of tetratricopeptide repeats (TPRs) within its N-terminal domain. The TPRs serve as protein-protein interaction modules that appear to target OGT to accessory proteins and potential substrates, such as the $GABA_A$ receptor interacting factor-1 (GRIF-1)[25] and the related *O*-GlcNAc transferase interacting protein (OIP106)[25], which have been implicated in mitochondrial trafficking to synapses[26,27], and the transcriptional repressor complex mSin3A–histone deacetylase 1 (HDAC1)[28]. In addition,

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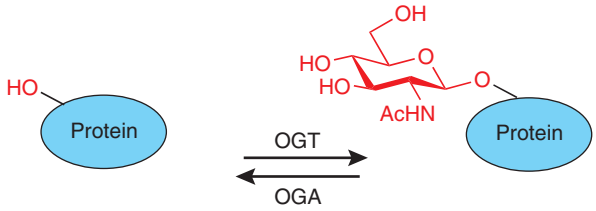

Figure 1 *O*-GlcNAc glycosylation is the addition of β-*N*-acetylglucosamine to serine or threonine residues of proteins. OGT, *O*-GlcNAc transferase; OGA, β-*N*-acetylglucosaminidase.

OGT forms a complex with protein phosphatase-1 (PP1) in the brain[29]. The association between OGT and PP1 is particularly intriguing, as it may provide a direct mechanism to couple *O*-GlcNAc glycosylation to dephosphorylation of specific substrates. Although OGT is found in the nucleus, cytosol and mitochondria, it is particularly enriched in the nucleus[15] and the soluble synaptic compartment[16].

Like OGT, OGA seems to be highly active at neuronal synapses[16], and it is also found in the nucleus and cytosol[30]. OGA contains an N-terminal glycosidase domain and a putative C-terminal histone acetyltransferase (HAT) domain[31]. Two distinct isoforms of OGA exist, a 130-kDa and a 75-kDa variant, which share the same catalytic domain but differ in their C terminus[32]. The potential HAT activity of OGA may provide an interesting mechanism for coupling deglycosylation of nuclear proteins to transcriptional activation. As with OGT, OGA has been shown to interact with specific proteins, including calcineurin (also known as protein phosphatase-2B), amphiphysin and dihydropyrimidinase-related protein 2 (DRP2, also known as CRMP1)[30].

Transcriptional regulation

Early studies revealed that the *O*-GlcNAc modification is enriched on chromatin[33] and is found on RNA polymerase II and many of its transcription factors[34]. As described in several reviews[2,4,35], *O*-GlcNAc glycosylation has been shown to both enhance and suppress the activity of transcription factors. *O*-GlcNAc can disrupt protein-protein interactions, as in the case of Sp1, whose glycosylation represses transcription at Sp1-driven promoters[36,37]. In other cases, it can promote protein-protein interactions, as in the case of signal transducer and activator of transcription 5A (STAT5A), whose glycosylation enhances its activity by recruiting the transcriptional coactivator CREB-binding protein (CBP)[38]. *O*-GlcNAc may also play a more general role in transcriptional repression through a mechanism involving the targeting of OGT to an HDAC1 complex by the corepressor mSin3A[28]. In addition to altering protein-protein interactions, *O*-GlcNAc can affect post-translational modifications. For instance, glycosylation stabilized the tumor-suppressor protein p53 by decreasing its phosphorylation and subsequent degradation by the proteasome[39].

Much less is known about the transcriptional roles of *O*-GlcNAc in the brain. However, CREB, a transcription factor important for neuronal survival, long-term memory storage and drug addiction[40,41], was shown to be *O*-GlcNAc glycosylated in the rodent brain[18]. Glycosylation occurred at two major sites within the Q2 transactivation domain of CREB and disrupted binding of CREB to $TAF_{II}130$, a component of the basal transcriptional machinery. As a result, glycosylation repressed the transcription of CRE-mediated genes both *in vitro* and in cells[18]. It will be interesting to investigate whether glycosylation of CREB is dynamically regulated in neurons, and whether it downregulates specific genes associated with memory storage and cell survival.

Early growth response-1 (EGR1), a transcription factor important for neuronal survival and long-term memory storage[42,43], was also recently shown to be *O*-GlcNAc modified in the brain[19]. Notably, glycosylation of EGR1 was found to be dynamically modulated by excitatory stimulation of the brain *in vivo*. Stimulation of rats with the glutamate receptor agonist kainic acid led to a ten-fold increase in glycosylation of EGR1 within its transactivation domain[19]. Although the functional implications of this response will require further investigation, these studies raise the exciting possibility that neuronal activity regulates the transcriptional activity of EGR1 by modulating EGR1 glycosylation levels.

Proteomic studies of *O*-GlcNAc–modified proteins from the brain have also underscored the importance of *O*-GlcNAc in regulating transcription. Approximately one-quarter of the neuronal *O*-GlcNAc–modified proteins known to date are transcriptional regulatory proteins[19,44] (**Fig. 2**). This includes numerous transcription factors (for example, SRY box–containing gene 2 (SOX2), activating transcription factor 2 (ATF2) and EGR1), as well as transcriptional coactivators (steroid receptor coactivator-1 (SRC1)), repressors (p66β and BRAF-HDAC complex 80 (BHC80)) and corepressors (transducin-like enhancer of split 4 (TLE4), carbon catabolite repression 4-negative on TATA-less (CCR4–NOT)). For instance, SOX2 is a member of the high-mobility group box (HMG) superfamily of minor groove DNA binding proteins, and it functions to regulate transcription on different promoters depending on its interactions with specific protein partners[45]. SOX2 interacts with proteins through its highly conserved HMG DNA binding domain, which also contains its *O*-GlcNAc modification site[44]. One of the well-established functions of SOX2 is its crucial role in the maintenance of embryonic stem cell pluripotency in partnership with OCT4 (also known as POU5F1)[46]. In the adult rat brain, SOX2 expression has been reported to occur in actively dividing adult neuronal precursor cells and in neurogenic astroglia[47]. Another example of the expanding role of *O*-GlcNAc in transcription is the modification of two proteins (including a ubiquitin ligase) in CCR4–NOT[44], a large protein complex involved in mRNA metabolism and in the global control of gene expression[48]. Together with earlier studies demonstrating glycosylation of RNA polymerase II, these findings suggest that *O*-GlcNAc participates in regulating several aspects of transcription.

Synaptic proteins and neuronal communication

Consistent with the observation that OGT and OGA are highly active at synapses, proteomic studies have uncovered a number of synaptic proteins in the *O*-GlcNAc proteome[19,44,49,50] (**Fig. 2**). Many of these proteins are enriched in the postsynaptic density, where they participate in the regulation of dendritic spine morphology and associate with the cytoskeleton. For instance, synaptopodin[44], synGAP[19], and SH3 and multiple ankyrin repeat domains protein 2 (SHANK2)[49] are crucial for the normal formation of dendritic spine apparatuses[51–53], and synaptopodin and δ-catenin have important roles in learning and memory[51,54].

O-GlcNAc modifications are also highly abundant in presynaptic terminals. Several proteins involved in neurotransmitter release or synaptic vesicle endocytosis, such as bassoon[44], piccolo[49], synapsin[49] and clathrin assembly protein (AP180)[55], are *O*-GlcNAc glycosylated. The *O*-GlcNAc–modified collapsin response mediator protein 2 (CRMP2, also known as DPYSL2)[19] has key roles in axon formation, elongation and branching[56]. Moreover, many cytoskeletal proteins themselves are known to be glycosylated, including tau[57], the neurofilament proteins NF-H[58], NF-L[49] and NF-M[49], and the microtubule-associated proteins MAP1B[44] and MAP2B[44].

Additional functional studies are needed to define the mechanisms by which *O*-GlcNAc regulates these proteins. Nonetheless, its prevalence on proteins intricately involved in neurotransmitter release and cytoskeletal rearrangements underlying synaptic plasticity suggests roles

for *O*-GlcNAc in regulating key neuronal functions. As described below, emerging evidence indicates that *O*-GlcNAc levels can be dynamically modulated in response to neuronal stimuli. Moreover, the potential interplay between *O*-GlcNAc and kinase pathways in neurons may provide a powerful means to control protein function and modulate neuronal communication processes.

Neurodegenerative disease

O-GlcNAc glycosylation has been implicated in several neurodegenerative diseases, such as Alzheimer's disease[57,59,60] and amyotrophic lateral sclerosis (ALS)[61]. The genes encoding OGA and OGT map to chromosomal regions associated with late-onset Alzheimer's disease[62] and dystonia-Parkinsonism syndrome[63], respectively. Moreover, *O*-GlcNAc levels are abnormally altered in the brains of individuals with Alzheimer's disease, although the magnitude and direction of the change seems to depend on the subcellular protein fraction[59,60].

In the pathology of Alzheimer's disease, the microtubule protein tau becomes hyperphosphorylated, which in turn favors its aggregation to form neurofibrillary tangles, which are hallmarks of the disease[64]. Tau is extensively *O*-GlcNAc glycosylated in the adult rat brain, although the estimated 12 or more modification sites have yet to be mapped[57]. Importantly, several studies suggest that *O*-GlcNAc glycosylation of tau negatively regulates its ability to be phosphorylated. For instance, inducing tau glycosylation with OGA inhibitors or by overexpression of OGT decreases tau phosphorylation at specific sites[60,65]. Conversely, stimulation of hyperphosphorylated tau using the phosphatase inhibitor okadaic acid leads to hypoglycosylated tau in human neuroblastoma cells[66]. Neuron-specific deletion of the OGT gene in mice induces hyperphosphorylated tau similar to that found in Alzheimer's disease[17]. As impaired glucose uptake and metabolism have been linked to Alzheimer's disease and seem to worsen as the disease progresses[67], one theory is that tau glycosylation becomes reduced in affected individuals, leading to hyperphosphorylated tau. Consistent with this view, mouse models of starvation that mimic this impaired glucose metabolism show reduced tau glycosylation and a corresponding increase in tau phosphorylation at specific sites[60,68].

Abnormal *O*-GlcNAc glycosylation may also contribute to neurodegenerative diseases in more diverse ways. The amyloid precursor protein (APP), which forms the β-amyloid plaques characteristic of Alzheimer's disease, is both *O*-GlcNAc glycosylated and phosphorylated[20]. In an animal model of ALS, the *O*-GlcNAc levels of neurofilament protein M are decreased at the same time as its phosphorylation levels are increased[61]. Finally, *O*-GlcNAc glycosylation has been demonstrated to inhibit the proteasome[69], thus providing a mechanism to couple ubiquitin-mediated protein degradation to the general metabolic state of the cell. Blocking the removal of *O*-GlcNAc from the proteasome leads to increased protein ubiquitination[69] and possibly neuronal apoptosis[70]. Proteasomal dysfunction and ubiquitinated inclusion bodies are found in the diseased tissue of individuals with ALS, Parkinson's disease, Huntington's disease and Alzheimer's disease[71]. Thus, aberrations in glucose metabolism and the *O*-GlcNAc glycosylation of specific proteins have been associated with several neurodegenerative disorders. It will be important to determine the extent to which such changes are crucial to the development and progression of these diseases.

O-GlcNAc dynamics and cycling

A unique feature of *O*-GlcNAc glycosylation is its ability to undergo dynamic cycling, in contrast to other, more static forms of protein glycosylation. Studies have shown that *O*-GlcNAc levels are altered by extracellular stimuli on a similar time scale to phosphorylation. For instance, a transient increase in glycosylation of the transcription factor nuclear factor activated T cells (NFAT) was observed within 5 min after T- or B-cell activation[72].

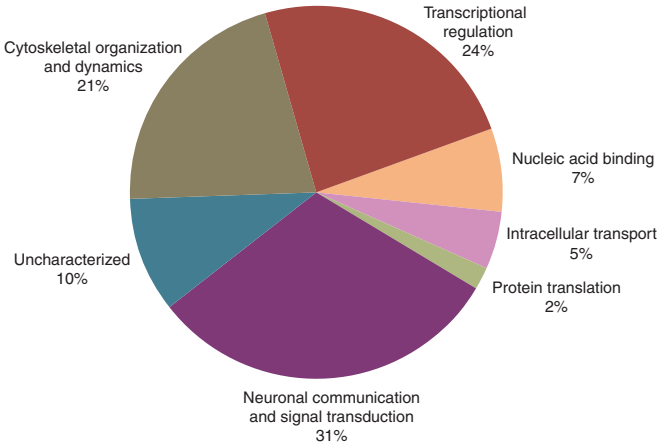

Figure 2 *O*-GlcNAc proteome from rodent brain tissue. Approximately 31% of the known *O*-GlcNAc proteins are involved in neuronal communication and signaling, 24% participate in transcriptional regulation, and 21% are associated with cytoskeletal structures. Proteins were classified according to categories described by Schoof *et al.*[111]

O-GlcNAc levels are highly responsive to glucose concentrations and influx through the hexosamine biosynthesis pathway (HBP) in neurons and other cell types[73,74]. Approximately 2–5% of all cellular glucose is metabolized through the HBP pathway to generate UDP-GlcNAc[75]. As OGT activity is exquisitely sensitive to UDP-GlcNAc concentrations[76], *O*-GlcNAc glycosylation may act as a sensor for the general metabolic state of the cell. Consistent with this idea, *O*-GlcNAc has been intricately linked to cell survival[17] and is induced by many forms of cell stress[77].

In the brain, phosphorylation serves as a central mechanism for neuronal communication by regulating ion channels, neurotransmitter receptors, gene transcription and synaptic vesicle release[78,79]. Protein kinases and phosphatases work together to coordinate different forms of synaptic plasticity, and they are necessary for the induction and maintenance of postsynaptic long-term potentiation and long-term depression[80]. Thus, the potential interplay between *O*-GlcNAc glycosylation and phosphorylation has exciting implications for many neuronal functions. Early studies showed that activation of protein kinase C (PKC) or cAMP-dependent protein kinase (PKA) significantly decreased overall *O*-GlcNAc glycosylation in the cytoskeletal protein fraction of cultured cerebellar neurons[21]. Conversely, inhibition of PKC, PKA, cyclin-dependent protein kinases or S6 kinase increased overall *O*-GlcNAc levels in these fractions. A more complex relationship was observed with tyrosine kinases and phosphatases: inhibition of tyrosine phosphatases led to a decrease in overall *O*-GlcNAc levels, whereas inhibition of tyrosine kinases induced both increases and decreases in *O*-GlcNAc, depending on the protein fraction.

O-GlcNAc also seems to have important signaling functions that are independent of its relationship to phosphorylation. Recent quantitative proteomics studies have shown that *O*-GlcNAc glycosylation is dynamic and is induced by excitatory neuronal stimuli *in vivo*[19]. Dynamic changes in *O*-GlcNAc glycosylation of specific proteins were observed when rats were administered kainic acid, which activates multiple excitatory signaling pathways involved in the stress response and synaptic plasticity. The increases in glycosylation reached a maximum after 6 hours and then returned to basal levels, demonstrating the reversible nature of *O*-GlcNAc in the brain. Interestingly, not all of the *O*-GlcNAc–modified proteins

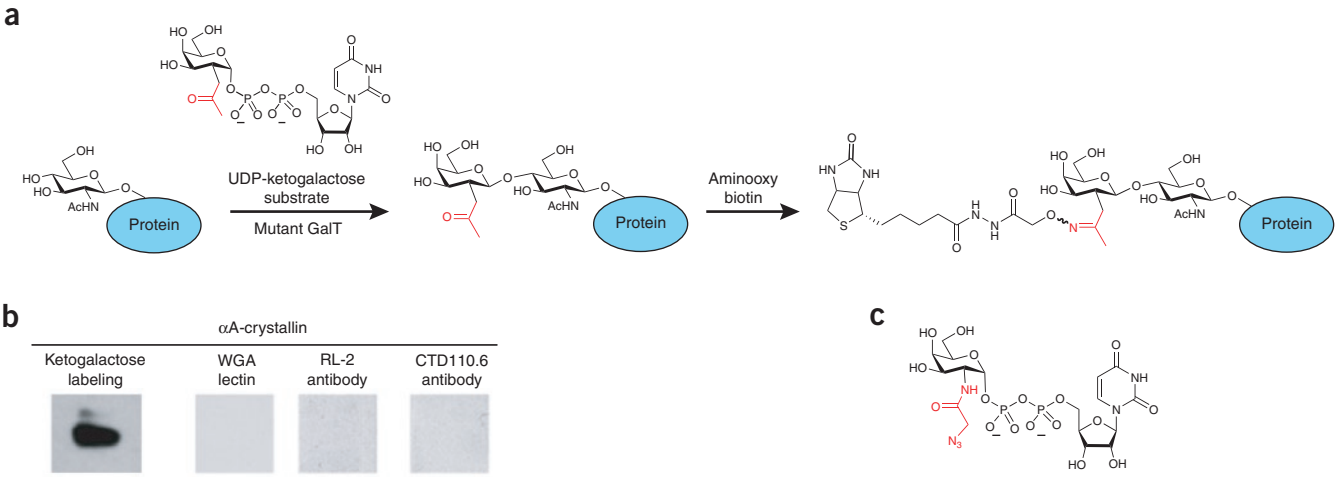

Figure 3 Strategy for chemically tagging *O*-GlcNAc proteins. (**a**) An engineered mutant GalT enzyme transfers the UDP-ketogalactose substrate onto *O*-GlcNAc proteins. The ketone functionality is then reacted with an aminooxy biotin derivative. Tagged *O*-GlcNAc glycoproteins are then detected by chemiluminescence or isolated by streptavidin affinity chromatography. Adapted from ref. 19. (**b**) Detection of αA-crystallin using the ketogalactose-biotin tagging approach and comparison with other methods. The chemical tagging approach provides significantly improved sensitivity relative to antibodies and lectins. 0.75 μg of αA-crystallin was used for the ketogalactose labeling method; 5 μg of protein was used for the lectins and antibodies. (**c**) The UDP-ketogalactose substrate can be replaced with the UDP-azidogalactose substrate shown, and an azide-alkyne [3+2] cycloaddition reaction can be used to attach biotin or fluorescent dyes.

showed site-specific increases in glycosylation, suggesting that *O*-GlcNAc is subject to complex regulation within neurons.

Altogether, the emerging evidence suggests that *O*-GlcNAc represents a key regulatory modification in the brain. Not only is it present on many functionally important proteins, it seems to be reversible, differentially regulated and responsive to neuronal activity. Further studies are needed to elucidate the molecular mechanisms involved and how activation of specific signaling pathways leads to the regulation of OGT and OGA. Moreover, with the exception of the last example, changes in *O*-GlcNAc glycosylation have been monitored only on a global level, and the specific proteins undergoing dynamic changes in glycosylation remain largely unknown. As described below, new chemical approaches are being developed that should enable such questions to be addressed and provide new insights into the complex cellular dynamics of *O*-GlcNAc.

Chemical approaches to address the challenges of *O*-GlcNAc

Recent advances in our understanding of the cellular functions of *O*-GlcNAc have been accelerated by the development of chemical tools for studying the modification. Although *O*-GlcNAc glycosylation was discovered more than 20 years ago, its extent and functional significance are only beginning to be understood. A major challenge has been the difficulty of detecting and studying the modification *in vivo*. Like other post-translational modifications, *O*-GlcNAc is often dynamic, substoichiometric, targeted to specific subcellular compartments and prevalent on low-abundance regulatory proteins. The sugar is also enzymatically and chemically labile, being subject to reversal by cellular glycosidases and cleavage on a mass spectrometer. As with many protein kinases, the lack of a well-defined consensus sequence for OGT has precluded the determination of *in vivo* modification sites on the basis of the primary sequence. Here we highlight some chemical approaches designed to overcome these challenges and advance a fundamental understanding of *O*-GlcNAc.

Rapid, sensitive detection. Traditional methods for detecting *O*-GlcNAc glycosylation include the use of wheat germ agglutinin (WGA) lectin[81], pan-specific *O*-GlcNAc antibodies[73,77] or radiolabeling using β-1,4-galactosyltransferase (GalT)[82], which transfers [^{3}H]Gal from UDP-[^{3}H]galactose to terminal GlcNAc groups. Although these approaches have greatly facilitated studies of the modification, many *O*-GlcNAc proteins still elude detection. For instance, tritium labeling suffers from low sensitivity, often necessitating exposure times of several days to months. *O*-GlcNAc antibodies and lectins have limited binding affinity and specificity, and therefore are best suited for highly glycosylated proteins with several modification sites.

Recently, chemical approaches have been developed to tag *O*-GlcNAc proteins with reporter groups such as biotin to enable more rapid, sensitive detection. Khidekel and co-workers designed an unnatural substrate for GalT containing a ketone moiety at the C2 position of UDP-galactose[83] (**Fig. 3a**). An engineered enzyme with a single Y289L mutation that enlarges the binding pocket[84] was used to transfer the ketogalactose sugar onto *O*-GlcNAc proteins of interest. Once transferred, the ketone functionality was reacted with an aminooxy biotin derivative and detected by chemiluminescence using streptavidin conjugated to horseradish peroxidase. This approach provides a significant improvement in sensitivity, enabling detection of proteins beyond the reach of traditional methods (**Fig. 3b**). The biotin handle also facilitates isolation of the glycoproteins from cell or tissue extracts, thus circumventing the need to develop purification procedures for each protein of interest. As such, virtually any protein can be readily interrogated for the modification, and comparisons can be made across specific functional classes. For instance, Tai and co-workers have applied the approach to demonstrate that numerous transcriptional regulatory proteins are *O*-GlcNAc glycosylated *in vivo*, including transcription factors, transcriptional coactivators and corepressors[85]. A variant of this method is now commercially available, in which a UDP azidogalactose substrate is used in conjunction with 'click' chemistry to transfer alkyne-containing reporter groups[86] (**Fig. 3c**).

A complementary strategy involves tagging *O*-GlcNAc–modified proteins through metabolic labeling of living cells with *N*-azido-

acetylglucosamine (GlcNAz)[87]. Vocadlo and co-workers showed that the azido sugar is processed by enzymes in the hexosamine salvage pathway and forms *O*-GlcNAz–modified proteins. The bioorthogonal azide moiety can then be derivatized with a FLAG peptide or a biotin tag using the Staudinger ligation. Treatment of Jurkat cells with peracetylated *N*-azidoacetylglucosamine led to labeling of the highly glycosylated nuclear pore protein p62, as detected by immunoprecipitation of p62 and western blotting. Although the labeling efficiency and sensitivity of this approach could not be evaluated and may depend on cell type, the ability to incorporate bioorthogonal groups selectively into *O*-GlcNAc proteins represents a powerful strategy for the detection of *O*-GlcNAc proteins in living cells.

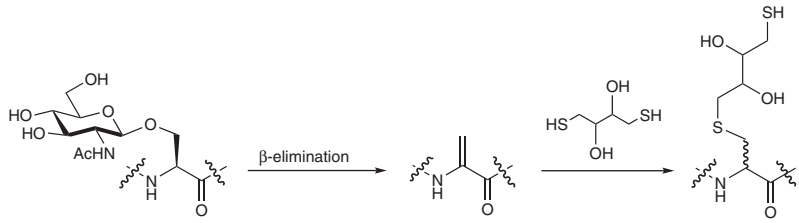

Figure 4 The BEMAD strategy for mapping glycosylation sites. In this approach, the GlcNAc sugar undergoes a β-elimination reaction. Michael addition with dithiothreitol produces a sulfide adduct that is stable to MS/MS analysis.

Mapping *O*-GlcNAc glycosylation sites. Mapping the sites of *O*-GlcNAc glycosylation within proteins is essential for elucidating the functional roles of *O*-GlcNAc in specific biological contexts. Unfortunately, direct observation of the *O*-GlcNAc moiety by mass spectrometry during collision-induced dissociation (CID) is difficult, as the glycosidic linkage is labile and readily cleaved, providing little peptide fragmentation[44]. Moreover, although OGT seems to favor sequences rich in proline, serine and threonine residues[44], there is no apparent consensus sequence that directs the action of OGT.

The development of chemical tools coupled to mass spectrometry has greatly facilitated the localization of *O*-GlcNAc to short peptide sequences within proteins, and this combination can be used to determine exact glycosylation sites. One approach, which relies on β-elimination followed by Michael addition with dithiothreitol (BEMAD), results in replacement of the labile GlcNAc moiety with a more stable sulfide adduct[50] (**Fig. 4**). As this adduct is not cleaved upon CID, sites of glycosylation can be more readily determined. However, selectivity controls must be carried out to distinguish *O*-GlcNAc from *O*-phosphate and other *O*-linked carbohydrates. Extension of BEMAD to proteomic studies for the high-throughput mapping of *O*-GlcNAc sites is an important future goal.

A second approach capitalizes on the ability to selectively biotinylate *O*-GlcNAc proteins using ketone- or azide-containing UDP-galactose sugars as described above (**Fig. 3**). In addition to isolating intact proteins, the biotin handle can be used to enrich *O*-GlcNAc peptides following proteolytic digestion[44]. This enrichment step is essential for mass spectrometric detection because the *O*-GlcNAc–modified species represents only a small fraction of the total peptides. Unlike BEMAD, the approach enables direct detection of the *O*-GlcNAc moiety by mass spectrometry, with the GlcNAc-ketogalactose-biotin tag providing a unique fragmentation pattern for unambiguous identification of *O*-GlcNAc peptides[44]. Notably, the strategy has been applied to both individual proteins and complex mixtures to localize the modification to short sequences in more than 50 different proteins. However, mapping exact glycosylation sites remains challenging in most cases owing to the lability of the *O*-glycosidic linkage. As a potential solution to this problem, Khidekel and co-workers demonstrated that this ketogalactose-biotin tagging approach could be combined with BEMAD to identify specific glycosylation sites on the HIV-1 Rev binding protein[44]. Similarly, Wang and co-workers recently used azidogalactose-biotin tagging in conjunction with BEMAD to map glycosylation sites on vimentin[88].

Newer mass spectrometry approaches such as electron transfer dissociation (ETD) and electron capture dissociation (ECD) promise to greatly accelerate our ability to identify *O*-GlcNAc modification sites. ETD and ECD produce sequence-specific fragmentation of peptides without the loss of labile post-translational modifications such as phosphorylation and glycosylation[89]. As such, these approaches are ideal for mapping *O*-GlcNAc glycosylation sites. Vosseller and co-workers demonstrated that ECD can be used to identify modification sites on several neuronal proteins following enrichment of the glycopeptides by lectin weak-affinity chromatography[49]. More recently, Khidekel and co-workers applied the ketogalactose-biotin tagging approach in conjunction with ETD to map glycosylation sites on proteins from rat brain lysates[19]. In contrast to ECD, which requires the use of Fourier transform instrumentation, ETD can be carried out directly in appropriately modified ion trap mass spectrometers. Thus, the speed, sensitivity and accessibility of ETD to most laboratories make it an ideal emerging technology. With further methodological refinements and advances in database search algorithms for fragment ions, we anticipate that ETD and ECD will become increasingly powerful tools for the study of *O*-GlcNAc glycosylation.

Proteome-wide analyses. As described earlier, the analysis of *O*-GlcNAc on a proteome level has begun to reveal exciting functional roles for *O*-GlcNAc in the brain. Chemical strategies to tag, enrich and detect *O*-GlcNAc peptides and proteins have been instrumental in this regard, enabling the first proteome-wide studies of *O*-GlcNAc. Specifically, the ketogalactose-biotin tagging approach (**Fig. 3**) has been applied in combination with high-throughput LC-MS/MS analysis to identify more than 45 *O*-GlcNAc–glycosylated proteins from the mammalian brain[19,44]. Direct detection of the modified species was observed in each case, which allowed mapping of the glycosylation sites to short peptide sequences. Notably, many of the proteins identified participate in the regulation of gene expression (for example, CCR4–NOT, SOX2, SRC1, HCF and TLE4), neuronal signaling (WNK1, bassoon, PDZ-GEF) and synaptic plasticity (synGAP, synaptopodin), suggesting that *O*-GlcNAc may contribute to neuronal communication processes. In another approach, Nandi and co-workers metabolically labeled HeLa cells with *N*-(2-azidoacetyl)glucosamine[90]. Tryptic digestion of the captured proteins led to the identification of 199 putative *O*-GlcNAc–modified proteins. As the presence of the GlcNAc moiety was inferred rather than detected directly, independent confirmation of the modification by immunoprecipitation was required and demonstrated for 23 of the proteins.

Recently, biochemical tools such as antibodies and lectins have been exploited for proteomic analyses of *O*-GlcNAc. Vosseller and co-workers used a uniquely packed, 39-foot lectin affinity column to enrich *O*-GlcNAc peptides and identified 18 proteins from the postsynaptic density fraction of murine brain tissue[49]. Antibody affinity chromatography using a general *O*-GlcNAc antibody identified 45 putative *O*-GlcNAc proteins from COS7 cells[88]. The challenge with both approaches is that the weak binding affinity of antibodies and lectins necessitates gentle washing conditions and can lead to false positives, such as interacting proteins or nonspecific binding proteins. In many cases, further confirmation of the

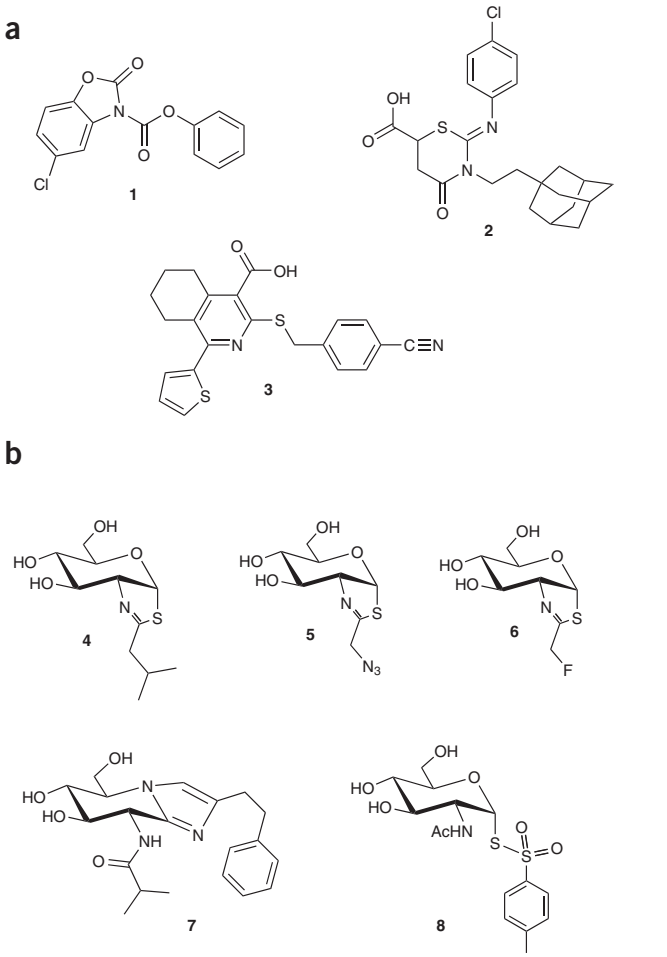

Figure 5 Small-molecule inhibitors of *O*-GlcNAc transferase (OGT) and β-*N*-acetylglucosaminidase (OGA). (**a**) OGT inhibitors (**1**–**3**) identified by screening a 64,416-member library of compounds. The benzoxazolinone compound **1** inhibits OGT activity in oocytes and prevents meiotic progression. (**b**) Representative OGA inhibitors (**4**–**8**) with enhanced selectivity for OGA over β-hexosaminidase.

presence of the modification can be provided by evaluating individually immunoprecipitated proteins or by directly detecting *O*-GlcNAc–modified peptides by mass spectrometry analysis.

Together, proteome-wide studies have revealed that *O*-GlcNAc is a ubiquitous and abundant modification found on proteins with considerable functional significance and breadth. In less than 5 years, the number of known *O*-GlcNAc proteins has expanded from tens to hundreds, with many more proteins yet to be discovered.

Small-molecule inhibitors of OGT and OGA. Historically, genetic manipulation of OGT and OGA activity *in vivo* has proven difficult. Whole-animal and conditional deletions of the OGT gene have revealed that OGT is essential for cell survival and mouse embryogenesis[17,91]. Modulation of OGT using small interfering RNAs is complicated by the long half-life of the protein and often produces only partial knockdown of OGT[92]. Although deletion of OGA in *Caenorhabditis elegans* leads to metabolic changes and increased dauer formation[93], no mammalian knockout of OGA has yet been reported. These features have challenged efforts to study *O*-GlcNAc glycosylation and have limited the use of conventional genetic tools to elucidate its role in cellular processes.

A complementary approach involves the generation of pharmacological agents to inhibit OGT and OGA. A well-established inhibitor of OGT, alloxan, shows multiple nonspecific effects, such as inhibition of OGA[94], glucokinase inhibition[95] and formation of superoxide radicals[96]. By screening a 64,416-member library of compounds using a novel fluorescence-based assay, Gross and co-workers identified several promising new compounds that inhibit OGT activity *in vitro*[97] (**Fig. 5a**, compounds **1**–**3**). These compounds were selective in inhibiting OGT but not another related enzyme, MurG, which also uses UDP-GlcNAc as a substrate. Recently, a benzoxazolinone compound (**Fig. 5a**, compound **1**) was shown to inhibit OGT activity in oocytes, where it prevents meiotic progression[98]. In the future, these numerous, chemically distinct OGT inhibitors might be used in parallel to distinguish the potential nonspecific effects of individual inhibitors *in vivo*.

Many of the early OGA inhibitors such as PUGNAc (*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate) exhibit nonspecific activity toward β-hexosaminidase[99] and only partially inhibit the short isoform of OGA[100]. Recently, several laboratories have designed more selective OGA inhibitors that either are structural variants of early hexosaminidase inhibitors and/or have been rationally designed using information about the enzyme active site. For instance, Macauley *et al.* and Knapp *et al.* functionalized the nonspecific hexosaminidase inhibitor GlcNAc-thiazoline with longer alkyl chains or fluoro or azido groups to generate new OGA inhibitors that show greater than 3,000-fold selective inhibition of OGA over β-hexosaminidase[101,102] (**Fig. 5b**, compounds **4**–**6**). In other studies, extending the *N*-acyl group of PUGNAc led to the creation of new inhibitors with greater than ten-fold selectivity for OGA over β-hexosaminidase[99,103]. A drawback of these compounds is that they inhibit OGA more weakly than PUGNAc. In response to this problem, Dorfmeuller and co-workers designed a nagstatin derivative based on the crystal structure of NagJ, a bacterial homolog of human OGA[104] (**Fig. 5b**, compound **7**). The isobutanamido group at the N8 position (tetrahydroimidazopyridine numbering) provides improved selectivity by fitting into a pocket that is larger than the corresponding pockets in other hexosaminidases, whereas the phenethyl group at the C2 position offers stronger inhibition ($K_i = 4.6 \pm 0.1$ pM) for the bacterial homolog of OGA by interacting with a solvent-exposed tryptophan residue. Nonetheless, a related nagstatin derivative still showed weaker inhibition toward human OGA than PUGNAc[105]. More recently, Kim and co-workers designed a compound that strongly inhibits the short isoform of OGA[100] (**Fig. 5**, compound **8**), an isoform that is only partially inhibited by 1 mM PUGNAc. However, selectivity may be an issue with this compound, as thiosulfonate moieties have been shown to react with exposed cysteine residues of proteins[106]. Notably, compounds **4** and **7** have been tested in cell culture and were shown to increase overall cellular *O*-GlcNAc levels.

The discovery of potent, selective inhibitors of OGT and OGA provides powerful tools for perturbing *O*-GlcNAc glycosylation in cells and *in vivo*. Application of these inhibitors in specific contexts should reveal new insights into the functional roles of *O*-GlcNAc and cellular mechanisms for the regulation of OGT and OGA.

Monitoring *O*-GlcNAc dynamics. The dynamic nature of *O*-GlcNAc is a unique characteristic that distinguishes it from other forms of glycosylation. As described earlier, this feature has important implications for the regulation of protein structure and function and the interplay with other post-translational modifications. An exciting challenge in the future will be to understand the cellular dynamics of the modification, as well as the signaling pathways and mechanisms by which

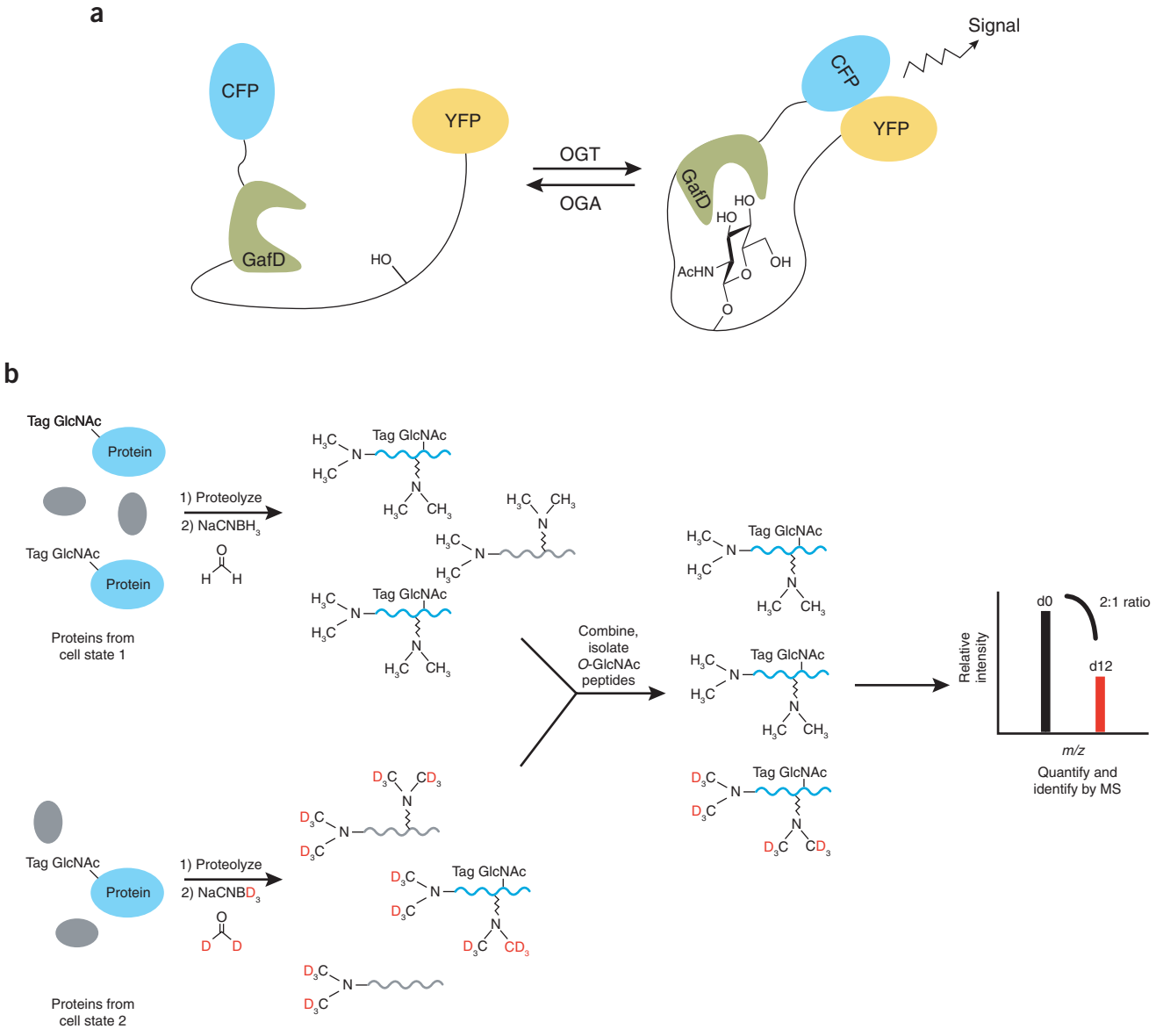

Figure 6 Chemical tools for monitoring *O*-GlcNAc dynamics. (**a**) A fluorescence resonance energy transfer (FRET)-based sensor designed to detect the dynamics of *O*-GlcNAc glycosylation in living cells. Upon glycosylation, binding of the GafD lectin to the *O*-GlcNAc moiety induces a conformational change and produces a stronger FRET signal. CFP, enhanced cyan fluorescent protein; YFP, yellow fluorescent protein. (**b**) The quantitative isotopic and chemoenzymatic tagging (QUIC-Tag) approach for quantitative proteomics. Proteins from two different cell states are tagged with a ketogalactose-biotin group as shown in **Figure 3a**. After proteolytic digestion, the peptides are isotopically labeled and combined. The biotinylated *O*-GlcNAc peptides are captured using avidin chromatography, and quantified and sequenced by mass spectrometry. This approach enables the identification of *O*-GlcNAc proteins undergoing changes in glycosylation in response to cellular stimuli and allows for those changes to be monitored at specific specific sites within proteins. Adapted from ref. 19.

O-GlcNAc is regulated on specific proteins. Toward this end, several groups have developed chemical approaches to monitor changes in *O*-GlcNAc glycosylation levels in response to cellular stimuli.

Carrillo and co-workers designed a fluorescence resonance energy transfer (FRET)-based sensor for the detection of *O*-GlcNAc dynamics in living cells[107]. Their sensor consisted of two fluorophores (enhanced cyan and yellow fluorescent protein) separated by a known peptide substrate for OGT and an *O*-GlcNAc lectin (GafD). Upon *O*-GlcNAc glycosylation, the lectin was expected to bind the glycosylated peptide substrate and bring the two fluorophores in close proximity, producing a stronger FRET signal (**Fig. 6a**). As anticipated, an increase in FRET (7–30%) was observed upon stimulation of transfected HeLa cells with PUGNAc and glucosamine. In the future, it will be interesting to examine changes in OGT activity in response to a variety of cellular stimuli.

In addition to monitoring OGT activity, identifying the intracellular signaling pathways and dynamics of *O*-GlcNAc glycosylation on specific protein substrates represents an important, challenging goal. General *O*-GlcNAc antibodies (CTD110.6 and RL-2) have been extremely valuable for measuring global changes in glycosylation in response to cellular stimuli[73,77]. However, a limitation of these antibodies is that they detect only a small subset of the *O*-GlcNAc–modified proteins[19,83]. Moreover, it remains difficult to identify the specific proteins that undergo changes in glycosylation. In response to these challenges, Khidekel and co-workers have developed a method to probe the dynamics of *O*-GlcNAc

glycosylation *in vivo* using quantitative proteomics[19] (**Fig. 6b**). The quantitative isotopic and chemoenzymatic tagging (QUIC-Tag) approach involves chemoenzymatically labeling proteins from two different cell states (for example, stimulated versus unstimulated) with a ketogalactose-biotin reporter group as described above. Following proteolytic digestion, the peptides are isotopically labeled using reductive amination chemistry to distinguish the two populations and subsequently combined. The biotinylated *O*-GlcNAc peptides are enriched using avidin chromatography, then quantified and sequenced by mass spectrometry. Using this approach, proteins undergoing dynamic changes in glycosylation can be identified, and glycosylation changes at specific sites within proteins can be monitored in response to cellular stimulation. Khidekel and co-workers found that *O*-GlcNAc glycosylation is induced on certain proteins upon excitatory stimulation of the rodent brain *in vivo*. Whereas some sites of glycosylation underwent robust changes in occupancy, other sites remained unchanged, suggesting that *O*-GlcNAc is subject to complex regulation within neurons. The reversible and highly regulated nature of *O*-GlcNAc, along with the observation that excitatory stimulation induces glycosylation, supports the idea that *O*-GlcNAc is an important, regulatory modification in the brain.

More recently, Wang and co-workers used immunoaffinity chromatography in conjunction with SILAC (stable isotope labeling with amino acids in cell culture), a well-established method for quantitative proteomics[108], to study the dynamic interplay between *O*-GlcNAc and phosphorylation by glycogen synthase kinase-3 (GSK3)[88]. Heavy and light isotope-labeled cells were treated with lithium chloride to inhibit GSK3, and *O*-GlcNAc proteins of interest were isolated by affinity chromatography using a general *O*-GlcNAc antibody. Forty-five putative *O*-GlcNAc–glycosylated proteins were identified by mass spectrometry, ten of which showed enrichment after lithium chloride treatment, suggesting that these proteins underwent increases in *O*-GlcNAc glycosylation. In four of the cases, the glycosylation increases that were observed indirectly by mass spectrometry were confirmed by immunoprecipitation. Interestingly, other proteins showed either no change or decreased glycosylation, suggesting that a complex interplay is likely to exist between phosphorylation and *O*-GlcNAc glycosylation within signaling networks.

With these new tools, in-depth studies of the dynamics of *O*-GlcNAc within cells are now within reach. Understanding the molecular mechanisms by which this dynamic signaling comes about and regulates specific proteins is a future challenge that promises to propel the field in exciting new directions.

Conclusion and future challenges

Over the past decade, a surge of discoveries in *O*-GlcNAc glycosylation has revealed new roles for this modification in the nervous system. *O*-GlcNAc is abundant in the brain and present on many diverse proteins involved in transcription, neuronal signaling and synaptic plasticity. Indeed, recent studies have begun to uncover the functional roles of *O*-GlcNAc, its complex dynamics in the brain and the interplay between *O*-GlcNAc and phosphorylation. Many of these discoveries have been accelerated by the development of new chemical tools, such as those for detecting the *O*-GlcNAc modification in cells and for inhibiting OGT and OGA. Combined with advances in mass spectrometry, these new technologies have provided an unprecedented opportunity to define the *O*-GlcNAc proteome, manipulate *O*-GlcNAc enzymes and explore the dynamics of this modification *in vivo*.

Although the pace and scope of understanding *O*-GlcNAc has expanded considerably, much remains to be discovered. Owing to the challenge of studying the modification, evidence linking *O*-GlcNAc to specific biological functions has often been indirect or correlative. This is particularly true in the brain, where the complexity of the nervous system and its unique technical challenges (for example, postmitotic cells, the complex organization of the brain, the presence of multiple cell types and the blood-brain barrier) render *O*-GlcNAc more difficult to investigate. Nonetheless, in-depth functional studies on proteins will be essential in the future to determine the roles of *O*-GlcNAc in neuronal-specific contexts.

The development of new chemical tools to produce homogeneously glycosylated proteins will represent an important step toward this goal. Unlike phosphorylation, *O*-GlcNAc glycosylation cannot be readily mimicked by any naturally occurring amino acid. At best, alanine mutagenesis of specific glycosylation sites within proteins can be used to effect changes in function. Chemical methods such as nonsense suppression[109] and native chemical ligation[110] may allow for the construction of *O*-GlcNAc–glycosylated proteins *in vitro* and in cells. In the future, these methods may provide new insights into whether and how *O*-GlcNAc glycosylation affects protein structure, modulates protein-protein interactions and influences other post-translational modifications.

One of the central challenges of neuroscience is to understand the unique molecular and cellular heterogeneity of the brain as it relates to systems-level phenomena such as learning and memory. Sensitive methods to detect the modification on small subpopulations of cells or proteins will be required to dissect the role of *O*-GlcNAc in fear, addiction and other complex learning and memory models. Despite significant progress, faster, higher-throughput methods are still needed to identify *O*-GlcNAc proteins and study *O*-GlcNAc dynamics *in vivo*. For instance, the ability to directly monitor the glycosylation status of specific proteins using chemical tagging approaches or site-specific *O*-GlcNAc antibodies will be essential. To facilitate the production of *O*-GlcNAc antibodies, facile synthetic routes to access *O*-GlcNAc–modified peptides are needed. Moreover, the continued development of methods to precisely map glycosylation sites, particularly on small quantities of material and on selected proteins of interest, will be crucial for any functional studies.

New sensitive and selective OGT and OGA inhibitors will be important tools for finely dissecting the role of each enzyme in neuronal function and dysfunction. Given the diversity of OGT and OGA substrates, and the lethality of deleting the OGT gene in mice, creative new genetic or chemical approaches are needed to more selectively target functional subsets of OGT and OGA by interfering, for instance, with the enzymes in certain subcellular compartments.

From the time of its discovery, the appeal of *O*-GlcNAc has been both the intrigue of understanding its unique biology and the great technical challenges associated with its study. Over the past 5 years, we have seen a surge of new chemistry designed to meet these obstacles. Strengthened by an arsenal of chemical tools, the future of *O*-GlcNAc is primed for new and exciting discoveries.

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