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Chemical Biology Approaches to Understanding Neuronal O–GlcNAcylation

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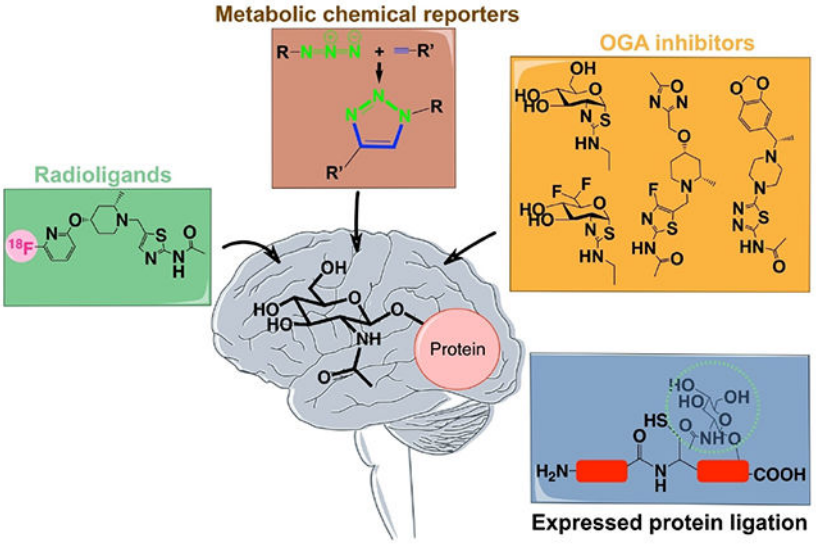
Abstract

O-linked β-*N*-acetylglucosamine (O–GlcNAc) is a ubiquitous post-translational modification in mammals, decorating thousands of intracellular proteins. O–GlcNAc cycling is an essential regulator of myriad aspects of cell physiology and is dysregulated in numerous human diseases. Notably, O–GlcNAcylation is abundant in the brain and numerous studies have linked aberrant O–GlcNAc signaling to various neurological conditions. However, the complexity of the nervous system and the dynamic nature of protein O–GlcNAcylation have presented challenges for studying of neuronal O–GlcNAcylation. In this context, chemical approaches have been a particularly valuable complement to conventional cellular, biochemical, and genetic methods to understand O–GlcNAc signaling and to develop future therapeutics. Here we review selected recent examples of how chemical tools have empowered efforts to understand and rationally manipulate O–GlcNAcylation in mammalian neurobiology.

Graphical Abstract



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1. Introduction

O-linked β-*N*-acetylglucosamine (O–GlcNAc) is a major intracellular form of glycosylation, reversibly decorating serine and threonine side-chains on thousands of nuclear, cytoplasmic, and mitochondrial proteins.[1–7] In several ways, O–GlcNAcylation is conceptually analogous to phosphorylation: In both cases, dedicated enzymes respond to physiological cues by dynamically adding or removing a small covalent moiety to alter target protein functions, often on a relatively short time-scale (minutes).[1–7] In mammals, O–GlcNAc is added by O–GlcNAc transferase (OGT) and removed by O–GlcNAcase (OGA), both ubiquitous nucleocytoplasmic proteins (Figure 1).[1–7] O–GlcNAc controls a wide range of cellular processes[2–4] and is essential in mammals, as deletion of OGT or OGA is lethal in mice.[8–10] O–GlcNAc also influences all tissue types,[1–7] including the brain, where OGT is highly expressed throughout and O–GlcNAc is especially abundant in post-synaptic densities and synapses.[11–14] Mouse models ablating the *ogt* gene in specific populations of dopaminergic[15] or hypothalamic[16,17] neurons revealed functional and behavioral defects, underlining the importance of O–GlcNAcylation in the normal adult brain. Moreover, O–GlcNAcylation of disease-relevant substrates is dysregulated in many clinically important neurological disorders.[18–20] Despite its broad pathophysiological significance, key aspects of O–GlcNAc signaling (e. g., its biochemical effects and the most functionally important OGT substrates) are often unknown.[3,4,21,22]

As these facts illustrate, O–GlcNAcylation in the mammalian nervous system is central to health and disease, motivating efforts to understand it at the biochemical, cellular, and physiological levels. However, because O–GlcNAc is a transient, sub-stoichiometric post-translational modification (PTM), it can be difficult to study with traditional molecular biology or genetic methods alone.[1,2,23,3–6] To address this challenge, many groups have developed chemical tools to answer pressing questions on the cell biology of O–GlcNAc. Here, we highlight selected recent examples and advances in using chemical approaches to understand O–GlcNAc signaling in the mammalian nervous system.

2. Physiological Roles of O–GlcNAc in the Nervous System

2.1 Strategies for Substrate Identification

Because O–GlcNAcylation is generally sub-stoichiometric,[24] substrate identification often requires considerable enrichment of O–GlcNAc-modified proteins or peptides for detection and analysis. Chemical methods for O–GlcNAc enrichment have proven to be a powerful complement to protein-based approaches, such as lectin weak affinity chromatography[25,26] and affinity-capture via catalytic-dead OGA homologs,[27] which are valuable but material- and labor-intensive and do not discriminate between older and newer O–GlcNAc moieties. To overcome this challenge, the Bertozzi laboratory utilized endogenous enzymes in live cells or tissues to convert cell-permeable, non-natural sugars bearing functional groups, such as azides[28] and alkynes, into intermediates that can be used by OGT.[29] This metabolic labeling approach results in the installation of azido- or alkynyl O–GlcNAc analogs onto native substrates.[30] Then, O–GlcNAc substrates can be covalently labeled with a useful probe (e. g., an affinity handle for enrichment or a fluorophore for imaging) by a bioorthogonal “click” reaction, such as copper-catalyzed [3 + 2] azide-alkyne

cycloaddition[31] or strain-promoted azide-cyclooctyne cycloaddition (Figure 2A).[32,33] Researchers have since improved the specificity of metabolic labeling with unnatural monosaccharides[34–37] and harnessed it to characterize O–GlcNAc substrates in brain homogenates[38,39] (see below) and primary cultured neurons.[40,41] Given its success in studying other glycan classes in living fish and mice,[42–44] metabolic labeling may be a valuable strategy for identifying and characterizing neuronal O–GlcNAc substrates *in vivo* in future work.

Chemoenzymatic approaches have also enabled O–GlcNAc substrate identification in neuronal systems. These strategies exploit a Tyr289Leu mutant of β-1,4-galactosyltransferase ($GalT^{Y289L}$), an enzyme that ordinarily transfers galactose to the nonreducing end of GlcNAc residues.[45] The expanded binding pocket of $GalT^{Y289L}$ allows it to transfer GalNAc or C2-modified unnatural sugars from their corresponding UDP nucleotide-sugars.[45] Several groups have used this approach to chemoenzymatically label O–GlcNAc substrates *in vitro* with recombinant-purified $GalT^{Y289L}$ and appropriate cofactors, such as ketone-functionalized galactose or *N*-azidoacetylgalactosamine (GalNAz) (Figure 2B).[46] For instance, the Hsieh-Wilson group used this method to identify 25 OGT substrates in rat brain lysates, including synaptic proteins such as synaptopodin and bassoon.[47] In subsequent work, the authors combined the chemoenzymatic strategy with differential isotopic labeling, which allowed them to quantitatively assess O–GlcNAc dynamics in rat cortical neurons in response to induced excitatory stimulation.[48] In another example, the Hart and Smith laboratories used $GalT^{Y289L}$-mediated GalNAz labeling of O–GlcNAc substrates and an alkyne-functionalized, photocleavable biotin probe to allow affinity purification, photochemical release, and analysis of OGT substrates (Figure 2B), identifying over 500 unique O–GlcNAcylated peptides in healthy mouse brain samples[49–51] and frozen human Alzheimer's disease brain tissues.[52] These and related studies have advanced our knowledge of neuronal proteins and signaling pathways that are influenced by O–GlcNAc and potentially altered in neurodegenerative disorders.

2.2 Discoveries with OGT and OGA Inhibitors

Cell-active small molecule inhibitors of OGT and OGA (Figure 3) have been valuable aids in understanding various aspects of neuronal O–GlcNAc function. We refer the reader to excellent recent articles that review the development of these compounds.[53,54] Here we discuss selected examples of new insights in neurobiology that were made possible by these chemical tools.

Cerebellum Development: The cerebellum is a morphologically unique brain region comprising a complex pattern of folia (narrow, leaflike gyri[55]) that coordinates balance, movement, and motor skills.[56] During development, the neurogenesis of granule cells, the most abundant cerebellar neuron type, begins with granule cell precursors (GNPs).[57] The postnatal proliferation of GNPs depends on the Sonic Hedgehog (Shh) pathway,[58] wherein the ligand Shh binds to the transmembrane receptor Patched (Ptch) to relieve the inhibition of the G-protein coupled receptor Smoothened (Smo) (Figure 4A).[59,60] This in turn triggers a signaling cascade in which GLI family transcription factors translocate to the nucleus and activate developmental gene expression programs.[59] Prior studies detected OGT

protein and O–GlcNAcylated substrates throughout the rat cerebellar cortex,[11] hinting at a potential functional connection between O–GlcNAc and cerebellum formation. In a recent study, the Wu group closely examined this hypothesis.[61] The authors observed an increase in global O–GlcNAc levels throughout cerebellar development in mice and discovered that OGT modifies GLI family zinc finger 2 protein (Gli2).[61] Inhibiting OGT or OGA with the small molecules OSMI-1[62] or Thiamet-G[63] (Figure 3) significantly suppressed or elevated Gli2 expression in GNPs, respectively,[61] demonstrating an impact of O–GlcNAc on Shh signaling. Further experiments with these inhibitors revealed that Gli2 O–GlcNAcylation promotes GNP proliferation by preventing Gli2 interaction with histone acetyltransferases (HATs) and Gli2 acetylation.[61] Interestingly, these observations may have relevance to disease as well. Shh signaling is upregulated in a variety of highly malignant brain tumors, including medulloblastoma.[64] Wu and colleagues used a medulloblastoma mouse model to show that inhibition of OGT by OSMI-1 dramatically impaired tumor progression and improved survival by suppressing Gli2 transcriptional activity (Table 1).[61] Notably, the authors also observed elevated O–GlcNAc levels in human samples of Shh-subtype medulloblastoma (Table 1).[61] Together, these results suggest that O–GlcNAc may regulate GNP proliferation and that reducing Gli2 O–GlcNAcylation may be a useful therapeutic strategy in Shh-subgroup brain tumors. In the future, these and similar studies will also benefit from related next-generation OGT inhibitors developed by the Walker laboratory with improved specificity and lower toxicity, such as OSMI-4 (Figure 3),[65] which is now commercially available.

Autophagy: Autophagy is a catabolic process that sequesters and destroys intracellular contents, including cytoplasm, organelles, protein aggregates, and even pathogens.[66] Proteins encoded by autophagy-related genes (ATG) initiate the formation of the phagophore, a double-membrane vesicle that engulfs targeted cargoes and matures into an autophagosome and then autolysosome via fusion to the lysosome (Figure 4B).[66] The ATG4-like proteases mediate two important roles in autophagy. First, they cleave the small ubiquitin-like protein LC3 into LC3-I, which is then lipidated and recruited to the phagophore outer membrane as LC3-II to promote elongation and autophagosome formation.[67] Second, at a later stage, ATG4 proteins hydrolyze membrane-anchored LC3-II to release LC3-I for recycling (Figure 4B).[67] Numerous studies have revealed essential roles for autophagy in various cell types,[68–72] including neurons[73–76] and astrocytes,[77] and emerging evidence has implicated O–GlcNAc in regulating autophagy in several ways.[78–82] For example, the Cho laboratory observed that OGA inhibition by PUGNAc (Figure 3)[83] activated autophagy in neuroblastoma cells and found that glucose starvation increased ATG4B O–GlcNAcylation.[84] PUGNAc treatment elevated ATG4B proteolytic activity, promoting autophagosome processing (Figure 4B),[84] but it remains to be determined which ATG4B glycosites mediate these effects and whether manipulating O–GlcNAc levels could modulate autophagosome maturation in primary neurons via this mechanism.

In another study, the Zhang laboratory exposed rat primary cortical neurons to Thiamet-G for one or seven days.[75] In response, autophagosome-anchored LC3-II was reduced, indicating decreased autophagic flux, and the mechanistic target of rapamycin (mTOR), a serine-threonine protein kinase that regulates various crucial metabolic processes,[67] was

activated.[75] These findings suggest that excessive neuronal O–GlcNAcylation may inhibit autophagy and activate mTOR, resulting in the accumulation of disease-associated proteins, such as α-synuclein (see below).[75]

Interestingly, other studies have revealed that O–GlcNAc can also impact neuronal autophagy without mTOR activation. In a mouse model of Alzheimer's disease (AD), the most common neurodegenerative disorder, autophagic clearance of neuronal substates, such as tau or amyloid-β peptide, is impaired.[85] Reverting this autophagic defect[85] or promoting autophagy via pharmacological enhancers[86,87] reduces two central pathological features of AD: memory deficits and amyloid deposition.[86,87,85] Since OGA inhibitors ameliorate AD phenotypes[88,89,38,90,91,63,92,93] and O–GlcNAc is linked to autophagy,[78–80,75,81,82] the Vocadlo group hypothesized an O–GlcNAc-influenced mechanism that stimulates autophagy to mitigate AD symptoms. Indeed, in multiple cell line and primary neuron experiments and two different mouse models of AD, OGA inhibition stimulated autophagy.[76] In these systems, mTOR signaling remained unchanged.[76] Future studies will be needed to determine the mechanism by which increased O–GlcNAc augments autophagy in these models.

Excitatory Synaptic Transmission: Prior studies revealed that OGT localizes to synapses[11,13] and nerve terminals,[12] prompting researchers to explore possible regulatory roles of O–GlcNAc in synaptic functions. Excitatory synapses are major mediators of neuronal cell-cell communication, learning, and memory.[94] These processes depend on long-term potentiation and long-term depression (LTD), which are changes in synaptic strength due to dynamic insertion and removal, respectively, of ion-channel receptors in the synaptic density.[95,96] The McMahon laboratory discovered a novel form of O–GlcNAc-influenced LTD[97] at hippocampal CA3-CA1 synapses involved in learning (Figure 4C).[98] This discovery allowed the authors to examine the functional effects of manipulating O–GlcNAcylation on behavior *in vivo*, demonstrating that rats injected with Thiamet-G exhibited memory and learning deficits.[97] In another study by the Vocadlo group, elevating O–GlcNAc in male mice with NButGT, an OGA inhibitor (Figure 3),[99] resulted in increased presynaptic function and elevated phosphorylation of synapsin I/II, a presynaptic protein, in hippocampal slices.[100] All together, these findings connect O–GlcNAc modification to synaptic excitability and plasticity.

Mitochondrial Properties: Mitochondria are highly dynamic organelles, undergoing frequent fission and fusion, and are key to neuronal homeostasis.[101] Mitochondrial dynamics and functions are dysregulated in many diseases, including neurodegeneration.[102–105] Prior studies connected O–GlcNAcylation to mitochondrial biology via glucose, an important biosynthetic precursor of UDP-GlcNAc.[106–108] Specific mitochondrial proteins are O–GlcNAc-modified, which impacts organelle motility.[109,110] For instance, the Cho laboratory reported the O–GlcNAcylation of dynamin-related protein 1 (Drp1),[109] a GTPase involved in mitochondrial fission.[101] Culturing neuroblastoma cells or primary neurons either with PUGNAc (Figure 3) or amyloid-β peptide induced aggregation, mitochondrial fragmentation, and increased Drp1 O–GlcNAcylation.[109] Interestingly, in the brains of AD mouse models, Drp1 O–GlcNAcylation was increased compared to

controls,[109] hinting that therapeutic suppression of Drp1 O–GlcNAcylation could perhaps be used to correct mitochondrial dysfunction. Future studies on the impacts of Drp1 *O*–*G*lcNAcylation in neuronal mitochondria will be required to test these possibilities.

Neural Stem Ccell (NSC) Differentiation: NSC differentiation determines mature cell fates and functions.[111,112] In examining human embryonic stem cell differentiation to neural progenitor cells, the Bertozzi laboratory observed a fluctuating profile of global O–GlcNAcylation.[113] At least some of these changes are likely functionally important because treating these cells with Ac_4-5SGlcNAc, an OGT inhibitor developed by the Vocadlo laboratory (Figure 3),[114] potentiated differentiation.[113] Other studies have investigated the impact of O–GlcNAc on NSC fate-switching. In the adult hippocampus, neurogenesis begins with quiescent NSCs, which are stem cells poised in a reversible cell cycle arrest.[115] The Villeda laboratory showed that in aging mice, NSC O–GlcNAc levels declined over time (Table 1).[116] This reduction correlated with decreased neurogenesis and increased gliogenesis (the differentiation of NSCs to glia) in the mature hippocampus (Table 1).[116] Inhibiting OGT in NSCs via OSMI-1 phenocopied these effects of aging,[116] implying diminished NSC O–GlcNAcylation during the NSC neuron-to–glia transition. Consistent with this hypothesis, genetic deletion of OGT in the NSCs of young mice produced similar phenotypes.[116] Collectively, these results demonstrate how pharmacologically manipulating O–GlcNAc can elucidate its role in neuronal differentiation and physiology.

2.3 Discoveries via Expressed Protein Ligation (EPL)

Emerging evidence has demonstrated that O–GlcNAcylation can reduce the aggregation of several key neuronal proteins, such as tau,[117] TAK-1 binding protein,[93] and the Polycomb group repressor Polyhomeotic in *Drosophila*.[118] A powerful tool to study this and other phenomena at the biochemical level is EPL, which combines protein synthesis, native chemical ligation, and recombinant protein expression to semi-synthesize homogeneous protein samples[119,120] with desired characteristics, such as defined PTMs. The method exploits the chemistry of inteins, protein segments originally from prokaryotes or yeasts that are capable of self-splicing via excision followed by the rejoining of the flanking (extein) sequences (Figure 5A).[121] The Pratt group and others have used EPL to study O–GlcNAc, ligating a peptide or expressed protein thioester with a synthetic peptide bearing both an N-terminal cysteine and an O–GlcNAc-modified residue into a full-length, site-specifically O–GlcNAcylated protein (Figure 5B).[122–126] EPL has greatly empowered the study of O–GlcNAc as a regulator of several important substrates and processes in neurobiology, most prominently α-synuclein, an aggregation-prone protein implicated in several neurodegenerative “synucleinopathies,” such as Parkinson’s disease (PD) and Lewy body dementia.[123–126] We refer the reader to excellent recent reviews on the role of O–GlcNAc in α-synuclein pathophysiology.[18,127, 128] Here, we highlight the emerging usefulness of EPL in studying the roles of O–GlcNAc in chaperone function and liquid-liquid phase separation in the nervous system.

Chaperone Function.—Protein aggregation is a hallmark of numerous neurodegenerative disorders, implying that a loss of protein folding and proteostasis underlies these important

human diseases.[129] To prevent protein misfolding, cells employ small heat shock proteins (sHSP), molecular chaperones of ~12-43 kDa comprising a core α-crystallin domain (ACD) flanked by N-terminal and C-terminal domains.[130] A structurally important cleft in the ACD binds various amyloid-forming proteins, such as amyloid-β(1-42),[131,132] tau,[133] or α-synuclein,[134] all implicated in major neurodegenerative disorders, and sHSPs can reduce amyloid formation by these and other clients. sHSPs themselves can be O–GlcNAcylated, but the functional consequences of this modification are incompletely understood. The Pratt laboratory made use of EPL to discover that O–GlcNAcylation of HSP27 at Ser176 or Thr184 potentiated its ability to reduce amyloid formation by amyloid-β(1-42).[122] Thr184 of HSP27 lies near the IXI motif, a tripeptide sequence that binds the ACD to influence sHSP client binding and oligomerization.[135,136] Interestingly, Thr184-O–GlcNAcylated HSP27 reduced the intramolecular IXI-ACD interaction and increased HSP27 oligomeric size (Figure 5C).[122] Several studies have observed decreased global O–GlcNAc levels in AD brains,[137,52] but O–GlcNAcylation of sHSPs was barely affected,[122] suggesting a potential need to maintain sHSP function through glycosylation in the face of neuronal proteotoxic stress.

Liquid-Liquid Phase Separation (LLPS): In recent years, it has become clear that some membraneless organelles (e. g., the nucleolus or stress granules[138]) and membrane-semi-enclosed compartments (e. g., the postsynaptic density[139]) are biomolecular condensates that exhibit LLPS relative to the surrounding cytoplasm or nucleoplasm.[140] LLPS influences many cellular functions, including transcription, genome organization, and neuronal synaptic signaling.[138,140, 139] Work on ribonucleoprotein granules established PTMs as a possible mode of regulating their LLPS properties.[141] A new study by the Chen laboratory used EPL to characterize the role of site-specific O–GlcNAcylation in the LLPS of SynGAP and PSD-95.[142] SynGAP is a synaptic GTPase-activating protein highly abundant in the dendritic spines of excitatory neurons.[143] SynGAP forms a parallel trimer to bind many copies of PSD-95, a central scaffolding protein that coordinates signaling cascades and shapes the basic architecture of the post-synaptic density (PSD).[144] Using *in vitro* and cell-based assays, the Chen team showed that SynGAP is O–GlcNAc-modified at Thr1306, which abrogates SynGAP/PSD-95 interactions and the size of the condensates they form.[142] These results suggest that O–GlcNAc modification may serve as a crucial mode of LLPS regulation in other membraneless organelles as well. Moreover, this notion may also have broad implications for neurodegenerative diseases in light of the abovementioned observations that O–GlcNAcylation can reduce the aggregation of several clinically significant proteins[118,117, 93] and the known impact of LLPS on disease-associated proteins, such as TDP-43 (TAR DNA-binding protein 43), tau, and α-synuclein.[145]

3. O–GlcNAcylation in Neurological Diseases: Dysregulation and Potential Therapeutic Approaches

In addition to their power to elucidate normal neurobiological processes, chemical methods show great promise in manipulating O–GlcNAc for future clinical benefit in nervous system injuries and pathologies. Recent studies have employed these tools to probe therapeutic

potential in numerous disease models and human clinical trials.[18,146–151] In particular, there has been significant progress in the pharmacological targeting of O–GlcNAcylation on tau[88,89,38,90,91,63,92,93] and α-synuclein[123–126] for AD, PD and related disorders. This literature has been extensively reviewed elsewhere.[18,127,128,19,151] Beyond these relatively well-studied targets, O–GlcNAc is dysregulated on myriad neuronal proteins in a range of other pathologies. Here we feature emerging efforts to target O–GlcNAc in other neurological disorders.

Amyotrophic Lateral Sclerosis (ALS):

ALS is the most common human motor neuron disease, affecting both upper and lower limbs and eventually resulting in respiratory failure with shortened lifespan.[152] Genetic predisposition, environment, and aging in combination influence the majority of ALS cases.[153] Studies have identified ALS-causative mutations in only a handful of proteins, including TDP-43 and the detoxifying enzyme superoxide dismutase 1 (SOD1).[152] Prior work reported reduced O–GlcNAcylation in the spinal cords of an ALS mouse model overexpressing mutant human SOD1 (Table 1).[154] Intriguingly, as noted above, TDP-43 itself is O–GlcNAc-modified, and its O–GlcNAcylation reduces ALS-associated TDP-43 hyperphosphorylation and protein aggregation while enhancing TDP-43's mRNA splicing function.[155] These results hint that directly manipulating TDP-43 O–GlcNAcylation may ameliorate pathological ALS phenotypes, analogous to prior findings with tau and α-synuclein in other models.

No cure for ALS currently exists in part because of the multiple factors contributing to disease, such as stress from reactive oxygen species (ROS) and aging.[156] Neurons deploy various strategies to manage these insults, such as the ROS-detoxifying enzyme non-selenocysteine-containing phospholipid hydroperoxide glutathione peroxidase (NPGPx), a member of the GPx family.[157] The Lee laboratory uncovered an NPGPx-mediated mechanism involving O–GlcNAcylation to cope with motor neuron degeneration triggered by oxidative stress during aging.[158] Wild type mice displayed increased O–GlcNAc levels in their spinal cords with aging, whereas NPGPx-knockout (KO) mice exhibited no changes in O–GlcNAc levels (Table 1).[158] Oral administration of Thiamet-G to KO mice for three months improved locomotor activity, reduced oxidative stress levels, and attenuated motor neuron degeneration.[158] Importantly, the authors observed lower levels of NPGPx in ALS patients as well.[158] It will be interesting to determine in future studies how NPGPx signaling leads to increased O–GlcNAc levels during oxidative stress and which glycoprotein targets are most important for ensuring neuronal survival and function in this context.

Neurofilament (NF) Dysfunction and Aggregation:

NFs are neuronal intermediate filaments comprising light, medium and heavy subunits that structurally support neurons.[159] NF protein aggregation is a common feature of multiple neurodegenerative diseases including ALS[160] and is likely causative in at least some subtypes[161] of Charcot-Marie-Tooth disease (CMT), one of the most common inherited neurological disorders.[162] NF proteins are composed of an N-terminal head domain, a central coiled-coil rod domain, and a C-terminal tail domain.[159] The medium and heavy

NF proteins (NF–M and NF–H) contain various lysine-serine-proline (KSP) epitopes in their tails that are subject to phosphorylation[163] and O–GlcNAcylation.[164,165] NFs influence axon radial growth an axonal transport, likely regulated in part by these tail-domain PTMs.[159] Early evidence detected functionally important NF O–GlcNAcylation in rodent brains.[164,165, 25, 26] For example, the Brandt laboratory generated the NL6 antibody that recognizes the KSP repeats in the tail domain of NF–M.[166] Using this tool, the authors observed a downregulation of NF–M O–GlcNAcylation in the spinal cords of transgenic rats expressing the ALS-causative human SOD^{G93A} mutant (Table 1).[166] A later study by Shan and colleagues employed an OGA inhibitor, NButGT (Figure 3), and the NL6 antibody to demonstrate that NF–M O–GlcNAcylation was significantly reduced in the SOD^{G93A} transgenic strain, compared to wild type mice.[154] Notably, OGA blockade significantly increased O–GlcNAcylated NF–M in the spinal cords of wild type mice but not in the SOD^{G93A} transgenic mice.[154]

Despite these rodent studies, little is known about NF O–GlcNAcylation in humans. Recently, we used mass spectrometry to identify several specific O–GlcNAcylation sites in the head and tail domains of human NF light (NF–L), which is required to form filamentous NF networks *in vivo*[159] (Huynh and Boyce, manuscript in preparation). Combining genetic, cell biological, and chemical tools (e. g., Thiamet-G), we found that NF–L O–GlcNAcylation modulates its assembly into full-length filaments. Interestingly, we also observed a loss of O–GlcNAcylation in CMT-causative mutants of NF–L, suggesting that dysregulated PTMs may contribute to neuronal dysfunction in this context. Our findings provide insights into human NF O–GlcNAcylation and will facilitate future efforts to investigate its potential role in other neurodegenerative disorders.

Spinal Cord Injury (SCI):

Several studies suggest that modulating O–GlcNAcylation may hold promise for supporting organ recovery following injuries.[167,168] In recent work, the Zhang laboratory found that Thiamet-G treatment reduced lesion size after experimental SCI in rats, promoting spinal cord, and motor function recovery (Table 1).[169] Further studies will be needed to determine the mechanisms underlying these observations. Interestingly, inhibiting OGT may be a useful approach in developing future cell-based therapies for SCI. As noted above, the Bertozzi laboratory showed that OGT inhibition enhanced NSC differentiation.[113] More recently, bioengineering efforts used OGT inhibitors for experimental stem cell therapies. Prior studies had sought to harness the regenerative power of NSCs to repair SCIs.[170–172] Building on this approach, the Liu laboratory used three-dimensional bioprinting to create a material mimicking spinal cords and deliver NSCs treated with OSMI-4 (Figure 3) to the injury site.[173] The biomimetic scaffolds allowed long-term culturing of NSCs with OSMI-4 to promote their differentiation into mature neurons, in turn promoting axonal regeneration *in vitro* and motor function recovery in rats after experimental SCI.[173] This example illustrates the power of combining chemical tools with bioengineering to pioneer novel approaches to therapeutic intervention in neurological conditions.

Temporal Lobe Epilepsy (TLE):

TLE is the most common type of focal epilepsy. While the underlying mechanisms are complex, some evidence indicates that O–GlcNAc may be involved. For instance, the McMahon laboratory observed a slight reduction in epileptiform activity (i. e., spike waves that correlate with epilepsy) at the CA3-CA1 synapse in cultured hippocampal brain slices from young adult male rodents upon Thiamet-G treatment.[174] Acutely elevating O–GlcNAcylation with Thiamet-G and glucosamine, a metabolic precursor of UDP-GlcNAc, [2] suppressed cortical hyperexcitability, a sign of synaptic dysfunction,[175] in awake mice. [174] These findings link O–GlcNAcylation to synaptic excitability and may suggest an opportunity to manipulate O–GlcNAc for seizure control. In another recent study, the Lubin laboratory observed that global O–GlcNAc and OGT levels declined in the hippocampi of both epileptic rats (using the kainic acid-induced experimental model) and human patients with TLE (Table 1).[176] Interestingly, OGA inhibition via acute Thiamet-G treatment (three days) reduced epileptiform activity in the hippocampus of the rat model (Table 1).[176] However, chronic Thiamet-G treatment (two weeks) neither reverted hippocampal atrophy in rats, as judged by MRI, nor slowed chronic epilepsy progression.[176] Also, culturing human TLE hippocampal or cortical slices with Thiamet-G reduced the spontaneous seizure-like activity but not the epileptiform activity (Table 1).[176] These mixed results underline the need to better understand both the electrophysiological underpinnings of epilepsy and the key downstream targets of O–GlcNAcylation in order to design future therapies.

Stroke: Stroke refers to the brain damage caused by an interruption or cessation of the blood supply. In one study using the well-established middle cerebral artery occlusion (MCAO) model of stroke in rodents,[177] Thiamet-G injection either prophylactically (three days before MCAO) or therapeutically (30 minutes before MCAO and daily for three days thereafter)[178] significantly reduced infarct volume, inhibited the inflammatory response, and modestly decreased the activation of NF-κB p65 signaling, an important transcriptional pathway that regulates immune and inflammatory gene expression programs (Table 1). [179] Whether inhibiting OGA only after stroke might mitigate damage and neurological impairment remains to be explored.

Down Syndrome: Down syndrome is a trisomic genetic disorder wherein abnormal cell division leads to an extra copy of chromosome 21, causing intellectual disability, motor defects, and other conditions.[180] A widely used experimental model of Down Syndrome is Ts2Cje mice, which carry an extra copy of a segment of chromosome 16 (syntenic to human chromosome 21) via a translocation to chromosome 12.[181] One study reported that these mice display reductions of O–GlcNAcylation in the hippocampus during aging (Table 1).[182] The authors observed reduced O–GlcNAcylation and concomitant elevated phosphorylation of amyloid precursor protein (APP) and tau in the hippocampus, which are particularly relevant due to the high prevalence of AD in humans with Down Syndrome.[182] Intranasal injection of Thiamet-G in Ts2Cje mice elevated APP and tau O–GlcNAcylation, decreased their phosphorylation in the hippocampus while boosting autophagy, as indicated by increased LC3-II levels, clearance of the autophagy substrate SQSTM1, and reduced nitrated proteins, such as 3-nitrotyrosine (Table 1).[182] The functionally important substrates

and pathways affected by OGA inhibition in this system will be an important area for future studies.

4. OGA Inhibitors as Drug Candidates for Human Neurodegeneration

As noted, hyperphosphorylation and aggregation of tau characterize AD and other tauopathies.[183] Therefore, reducing tau phosphorylation and aggregation to mitigate AD may be an attractive therapeutic goal. Tau is modified by O–GlcNAc, and O–GlcNAcylation reduces tau phosphorylation in cultured cells[117] and rats.[184] Indeed, several other studies in rodent models of AD have reported benefits of elevating O–GlcNAcylation via Thiamet-G treatment, such as reducing proteotoxicity, cognitive defects and behavioral dysfunction.[88,89,38,90,91,63,92,93] Other work has leveraged GalNAz labeling and click chemistry (Figure 2) to confirm increased tau O–GlcNAcylation in response to Thiamet-G treatment.[38] However, it remains unclear whether tau hyperphosphorylation is always a faithful proxy for AD outcomes in these systems, as increasing O–GlcNAcylation attenuated tau aggregation without changing its phosphorylation in some instances.[88–91,63,92,93] Importantly, these studies differed in how Thiamet-G was delivered (e. g., gavage, water administration, lateral ventricle injection), which mouse model of AD was used, and the biological sex(es) examined (i. e., male,[91,63] female,[88,38] or both[89]). Additional standardized, carefully controlled studies will be required to further characterize the pharmacokinetic properties of Thiamet-G or other OGA inhibitors and apply this knowledge to optimize dosing in AD models.

Building on the promising data on modulating O–GlcNAc in preclinical AD models, at least three OGA inhibitors have entered human clinical trials in recent years (Figure 6).[185] First, MK-8719 (Merck/Alectos)[186] induces strong O–GlcNAcylation in rat brains hours after oral administration.[187] In an AD mouse model expressing human tau^{P301L},[188] eight-week MK-8719 treatment attenuated tau pathology and brain atrophy, and improved behavioral phenotypes, such as locomotor activity.[187] These results led to Phase I human trials, which showed low toxicity in healthy volunteers.[149] Second, LY3372689 (Eli Lilly) has been tested as a potential treatment for AD and other tauopathies, with promising safety and pharmacokinetic parameters after single and multiple oral doses in healthy volunteers.[146,147, 150] Third, ASN90 (Asceneuron S.A.) potently inhibits rodent and human OGA, crosses the blood-brain barrier, and exhibits favorable drug-like properties for the central nervous system.[39] In a methodical effort to characterize its efficacy *in vivo*,[39] A team led by Permanne and colleagues injected ASN90 in multiple mouse models of tauopathy with varying degrees of disease progression[189,190] as well as PD mouse models.[191] Chronic ASN90 treatment (3.5 months for AD models and 3–6 months for PD) ameliorated multiple pathological phenotypes, including (1) tau accumulation and phosphorylation (2) hippocampal α-synuclein phosphorylation at Ser129 (which correlates with aggregation)[192] (3) motor dysfunction (4) cognitive decline, and (5) prolonged survival.[39] To determine whether ASN90 promotes the O–GlcNAcylation of α-synuclein *in vivo*,[39] the team employed a chemoenzymatic mass-tagging method[47] that involves GalT-mediated GalNAz labeling of native O–GlcNAc moieties on α-synuclein, followed by a click reaction with dibenzocyclooctyne-polyethylene glycol (PEG) probes[33] (Figure 2). In brain homogenates from PD mouse models, O–GlcNAcylated α-synuclein tagged with

the PEG probe resulted in a band (~30 kDa) distinct from that of the unmodified protein (15 kDa).[39] Using this assay, the team observed approximately 20% of total α-synuclein modified by O–GlcNAc in vehicle-treated controls, whereas chronic ASN90 treatment increased overall α-synuclein O–GlcNAcylation by 1.5-fold.[39] A Phase I clinical trial of ASN90 has demonstrated no concerning toxicity and good brain penetration following oral administration.[148] Together, these results emphasize both the promise of OGA inhibitors as therapeutic agents in a range of intractable neurodegenerative diseases and the utility of chemical approaches to dissect neuronal O–GlcNAc signaling and improve next-generation pharmaceuticals.

5. *In vivo* Imaging of OGA Using Radioligands

As previous sections have illustrated, pharmacological manipulation of O–GlcNAcylation holds therapeutic promise for a wide range of neurological disorders. However, the limited access to the brain in human patients makes it difficult to assess the extent of *in vivo* target engagement by OGA (or future OGT) inhibitors after administration. Detailed data on the degree of enzyme engagement in the brain or elsewhere greatly facilitates dose projections and optimization of inhibitor delivery for maximal effects. To this end, collaborative efforts among researchers at Eli Lilly (specializing in radiosynthesis) and Merck and Alectos (specializing in radio-imaging and biophysics) have created and developed OGA inhibitor positron emission tomography (PET) ligands. Specifically, [^{18}F]MK-8553 has been used successfully to measure OGA engagement and dose selection of MK-8719 (see above) in rodent brains.[193] A different OGA radioligand, [^{18}F]LSN3316612, based on LSN3316612, a selective and high-affinity OGA inhibitor (Figure 7) targets OGA rapidly (within minutes) and specifically,[194] allowing the quantification of OGA occupancy by ASN90 in the brains of live mice.[39] [^{18}F]LSN3316612 also labeled OGA in other organs of rhesus monkeys, displaying a whole-body biodistribution.[194] In evaluating the pharmacokinetics of [^{18}F]LSN3316612 in healthy human volunteers, the authors observed similar robust probe uptake in the brain and whole-body biodistribution via PET scans.[195] The development of two additional, related probes, [^{3}H]LSN3316612 and [^{11}C]LSN3316612 (Figure 7), allowed the authors to detect OGA distribution in native tissues and postmortem AD brain regions via autoradiography.[196] These regional radioactivity measurements and summed PET images illuminate OGA distribution in distinct brain regions in rodents, monkeys, and humans, informing future efforts to target OGA in the specific regions affected in various neurological disorders. Analogous OGT PET probes would be a useful complement to these reagents, providing a complete picture of the enzymology of O–GlcNAcylation in a tissue-specific fashion. In the future, such probes may find valuable research uses beyond evaluating target engagement by clinical drug candidates, such as studying enzyme distribution and activity in response to stimuli or in genetic models of disease.

6. Summary and Outlook

As in many realms of glycobiology, chemical approaches have made key contributions to exploring the mechanisms and functions of O–GlcNAc signaling. In a field as complex as human neurobiology, the development of versatile chemical tools has been particularly instrumental in characterizing the role of O–GlcNAcylation. Going forward, we anticipate

that new areas of investigation and potential therapeutic applications will continue to emerge. For example, a very recent study reported promising results with Thiamet-G in ameliorating the phenotypes of intervertebral disc degeneration in a rat model.[197] However, outstanding challenges remain in the field, pointing to the need for further advances in chemical approaches in order to understand the neuronal roles of O–GlcNAc. We highlight some of these challenges here.

- For OGT substrate identification, there is a need to improve metabolic labeling and biorthogonal chemistry to better detect, characterize, and image neuronal O–GlcNAcylation *in vivo*. Next-generation sugar probes and detection reagents with good bioavailability and blood-brain barrier penetration could unlock access to O–GlcNAc substrates in different brain compartments and open doors to new studies.
- OGT and OGA have thousands of substrates but limited intrinsic amino acid sequence preferences, relying on cofactor proteins, subcellular localization, and other mechanisms to achieve substrate specificity. Therefore, using catalytic OGT or OGA inhibitors in experimental models often produces pleiotropic effects on many substrates and processes. To detect and manipulate site- and substrate-specific O–GlcNAcylation, the field needs (1) chemical reagents capable of inducing or inhibiting the O–GlcNAcylation of specific proteins or glycosylation sites and (2) delivery methods that selectively target particular regions of the brain or other organs. This is a challenging problem, to be sure, but small molecules that promote or abrogate the interactions of OGT and OGA with cofactor proteins or PROTAC-style molecules that target OGT/OGA to specific substrates may be paths forward to accomplishing these goals.
- New ways of extending *in vitro* mechanistic insights (e.g., made by EPL) into *in vivo* systems would allow us to test phenotypes observed at the substrate level in cellular and whole-organism experimental models. EPL-like methods that permit the glycoengineering of key substrates in live cells or tissues could address this problem.
- Translating molecular and preclinical knowledge of O–GlcNAcylation into diagnostics and treatments will be a major goal of future work. Recent progress in understanding the role of O–GlcNAc in neural stem cell maintenance and differentiation hints at the potential of manipulating O–GlcNAc in regenerative medicines, such as stem cell therapies that promote organ recovery. Studies that systematically test this hypothesis in additional experimental systems and injury types are still needed. As noted above, PET probes for OGT and OGA might be deployed for new experimental purposes, such as measuring enzyme activity or expression in response to varying nutrient conditions or behavioral stimuli or in control versus genetically modified animals (e.g., disease models). From a translational stand-point, these approaches could hold promise for diagnostics or precision medicine.

In the face of these significant challenges, interdisciplinary efforts that combine current and next-generation chemical tools with complementary new approaches, such as increasingly

sophisticated whole-organism genetic models (aided by the CRISPR revolution), intravital imaging, bioengineering, and optogenetic technologies will propel the field forward. Indeed, we expect that chemical methods will continue to play a central and essential role in understanding the neurobiology of O–GlcNAcylation and in manipulating it for therapeutic benefit in a variety of neurological diseases.

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Biographies

Michael Boyce received his Ph.D. in cell biology from Harvard Medical School under the supervision of Professor Junying Yuan and performed postdoctoral research on the chemical biology of O–GlcNAc with Professor Carolyn Bertozzi, then at the University of California, Berkeley. His independent laboratory at Duke University takes an interdisciplinary approach to study cell signaling through protein glycosylation in diverse aspects of mammalian cell biology, such as vesicle trafficking, cytoskeletal dynamics, protein ubiquitination, and the regulation of nucleotide-sugar pools.

Duc Tan Huynh obtained a bachelor's degree in Biochemistry from the University of California, Los Angeles in 2017. For his doctoral degree, he matriculated at Duke University and conducts his thesis research under the supervision of Professor Michael Boyce. He combines cell biological and biochemical approaches to elucidate the regulation of O–GlcNAc modification in the neuronal cytoskeleton. The long-term goal of his work is to gain mechanistic insights into the physiology of O–GlcNAc in the brain and its dysregulation in nervous system disorders.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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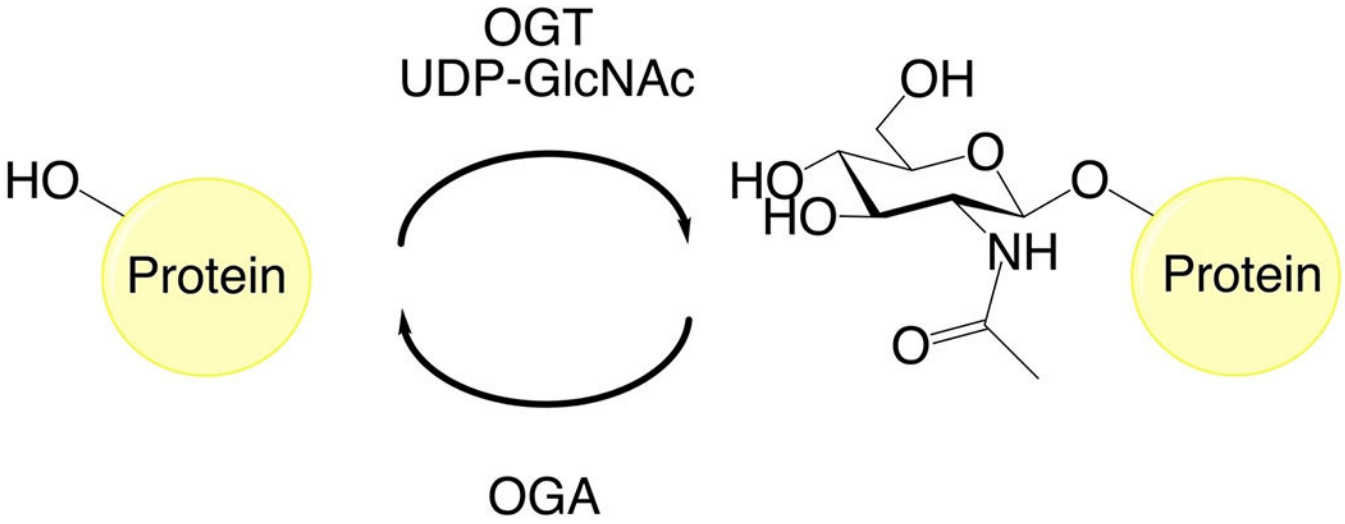

Figure 1.
O–GlcNAc modification. O–GlcNAc transferase (OGT) uses the nucleotide-sugar uridine diphosphate (UDP)-GlcNAc to add O–GlcNAc to serine or threonine resides of intracellular proteins, and O–GlcNAcase (OGA) catalyzes its removal.

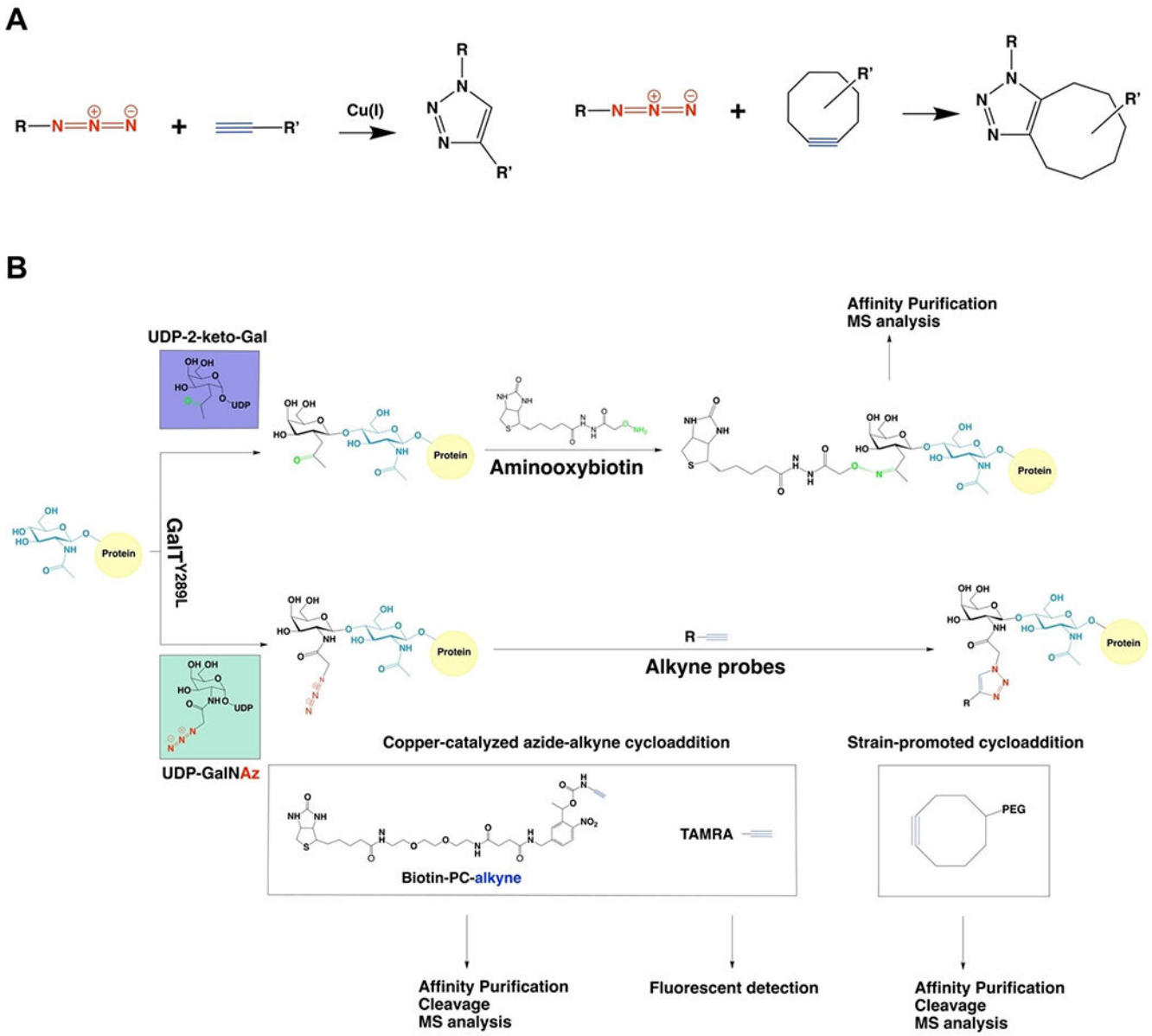

Figure 2.

An overview of metabolic labeling. (**A**) Copper-catalyzed azide-alkyne [3 + 2] cycloaddition[31] and strain-promoted azide-cyclooctyne cycloaddition.[32,33] (**B**) UDP-2-ketO–Galactose and UDP-*N*-azidoacetylgalactosamine (UDP-GalNAz) are used to metabolically label O–GlcNAc-modified proteins via $GalT^{Y289L}$-mediated transfer. Different tags, such as aminooxybiotin or alkyne probes, are then reacted with labeled glycans to tag the O–GlcNAc moieties for affinity purification, fluorescence imaging, or other purposes.

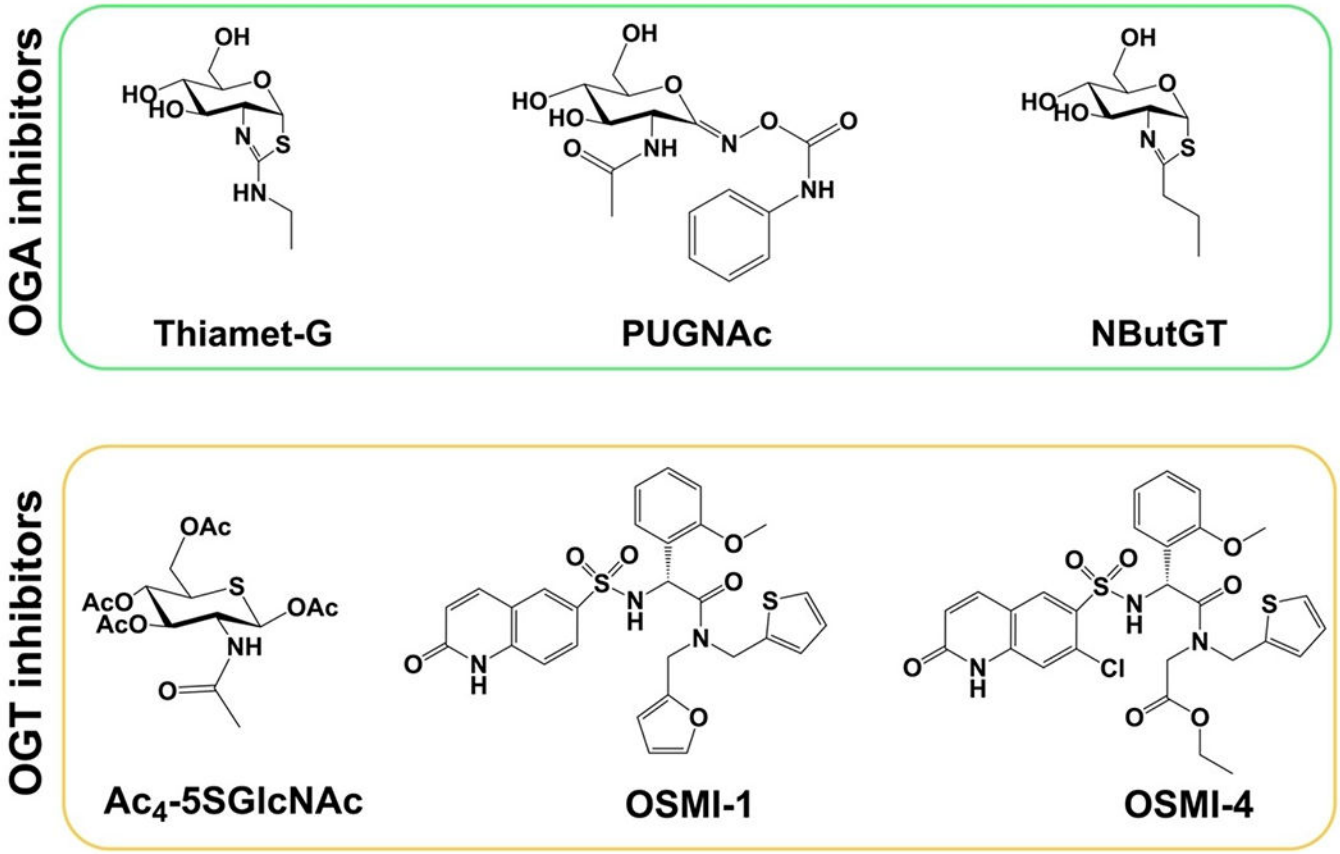

Figure 3.

The chemical structures of OGT/OGA inhibitors discussed here: Thiamet-G,[63] PUGNAc,[83] NButGT,[99] Ac_4-5SGlcNAc,[114] OSMI-1,[62] or OSMI-4.[65]

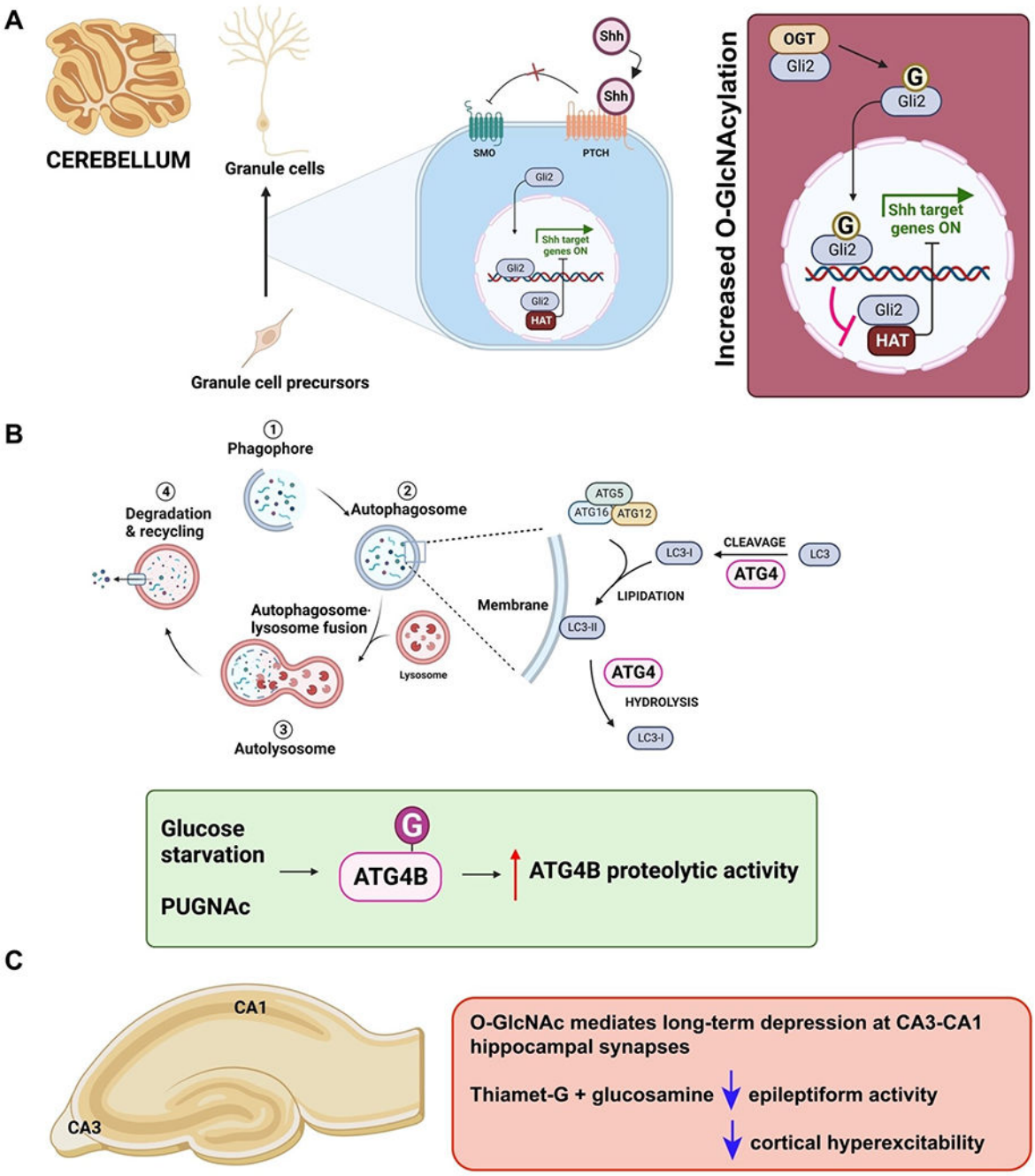

Figure 4.

Examples of O–GlcNAc function in normal neuronal physiology. (**A**) Cerebellar development depends on the maturation of GNPs to granule cells. In this process, Shh binds to Ptch to relieve Smo inhibition.[59] Transcription factor Gli2 then translocates to the nucleus, where its interaction with HATs inhibits gene transcription.[198] O–GlcNAcylation of Gli2 reduces Gli2 acetylation, promotes Shh signaling and GNP proliferation.[61] (**B**) An overview of autophagy. Glucose deprivation or OGA blockade by PUGNAc increases

ATG4B proteolytic activity in autophagosome maturation.[84] (**C**) Summary of O–GlcNAc's impacts on CA3-CA1 hippocampal synapses and electrophysiology.[97]

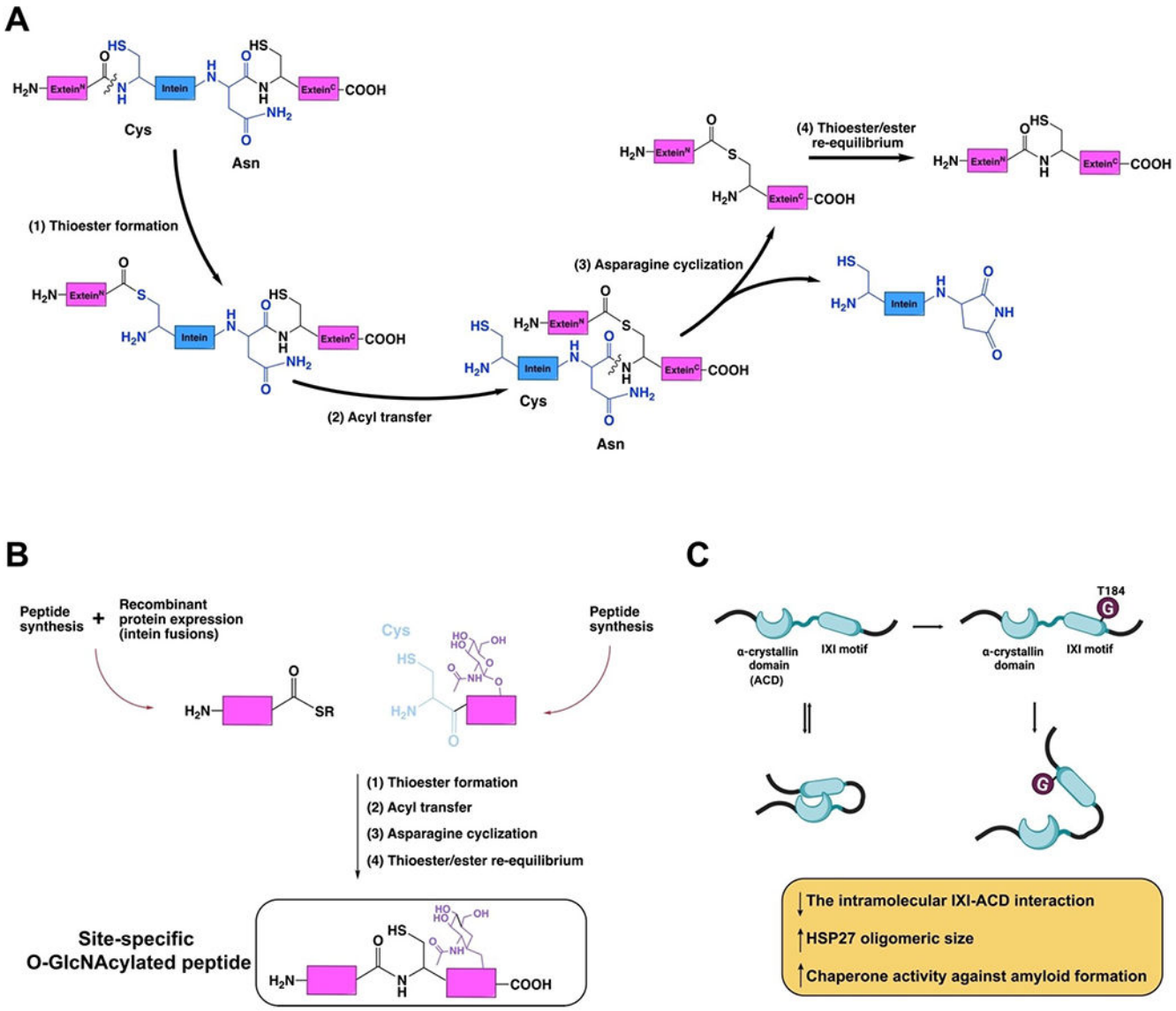

Figure 5.

Principles of EPL: (**A**) Intein-mediated protein splicing involves (1) Extein^{N}-Intein cleavage of the peptide backbone and thioester formation between Extein^{N} and Intein at its cysteine (Cys) side chain (2) acyl transfer to connect Extein^{N} with Extein^{C} (3) asparagine (Asn) cyclization to release the intein (4) Extein^{N}-Extein^{C} thioester/ester equilibrium to form a native amide bond.[121] (**B**) Semi-synthesis of an O–GlcNAcylated protein by EPL. **(C)** The effects of Thr184-O–GlcNAcylation of HSP27.[122]

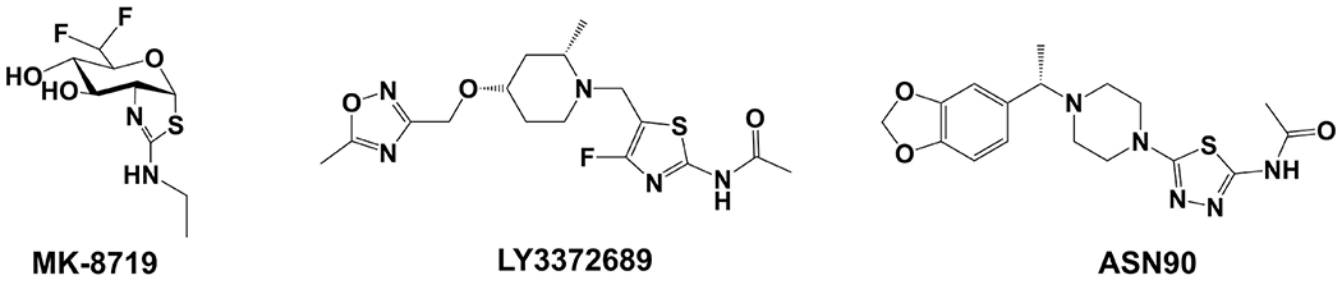

Figure 6.
Chemical structures of OGA inhibitors in clinical development: MK-8719,[186] LY3372689,[146,147, 150] and ASN90.[39]

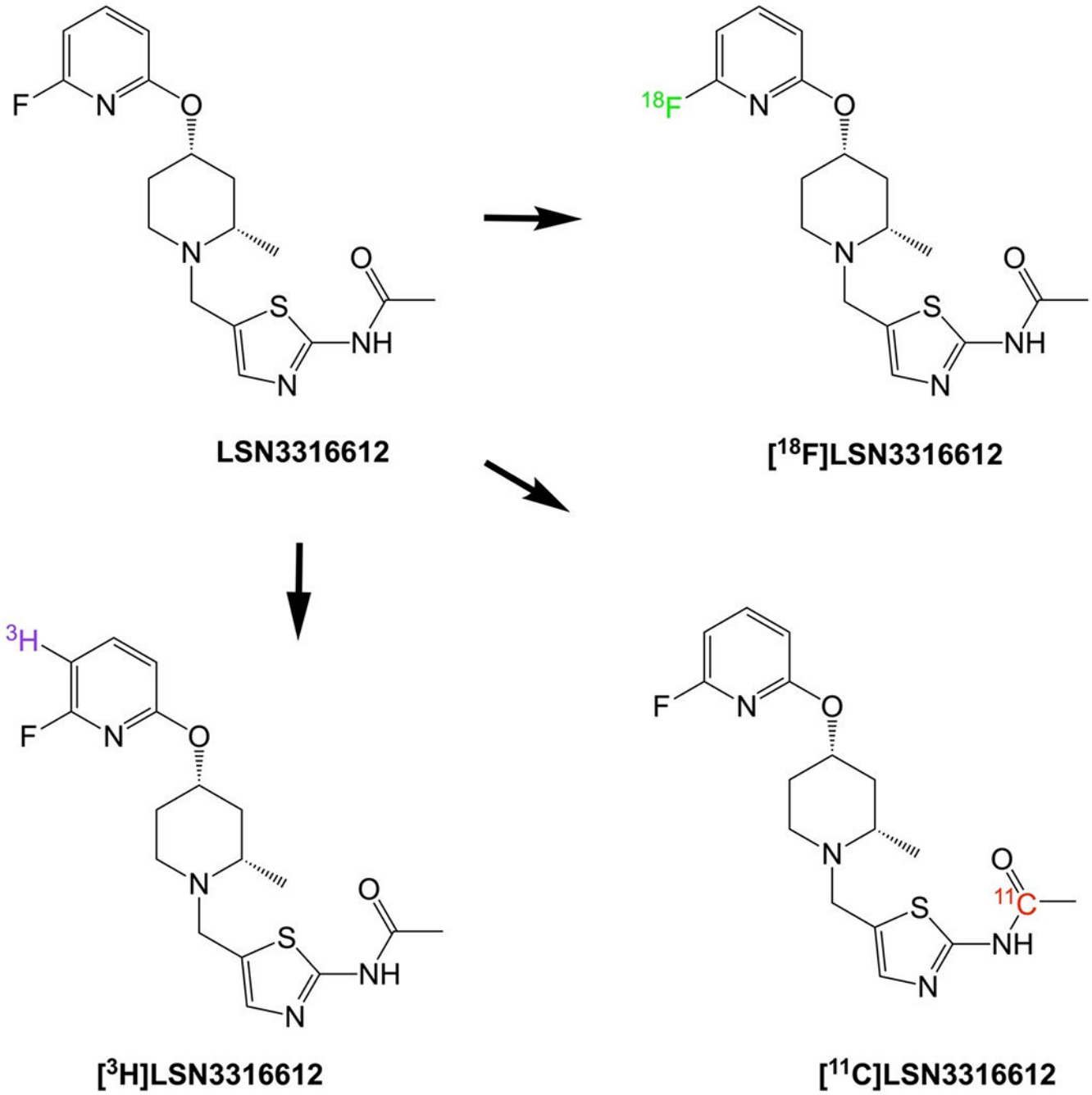

Figure 7.
Chemical structures of the OGA inhibitor LSN3316612 and OGA PET radioligands based on it.[196]

Table 1.

Selected contributions of chemical approaches to understanding the neurobiology of O–GlcNAc.

HIPPOCAMPUS	AGING[116] • O-GlcNAcylation in hippocampal NSCs and neuron-to-glia transition decline over time TLE[176] • O-GlcNAc is reduced in the hippocampus of epileptic rodents and human TLE patients • Acute Thiamet-G injection in epileptic rats decreases epileptiform activity • *Ex vivo* culture of TLE patient brain slices with Thiamet-G reduces the spontaneous seizure-like activity DOWN SYNDROME[182] • O-GlcNAc levels are reduced in a Down syndrome mouse model during aging • Thiamet-G treatment: ○ Elevates O-GlcNAcylation and reduces phosphorylation of amyloid protein precursor and tau ○ Boosts autophagic clearance and reduces oxidative damage
CEREBRAL CORTEX	STROKE[178] • In an MCAO model of stroke, Thiamet-G treatment: ○ Reduces infarct volume ○ Inhibits the inflammatory responses via modest activation of NF-κB p65 sigaling
CEREBELLUM	SHH-SUBTYPE MEDULLOBLASTOMA[61] • O-GlcNAcylation and Shh signaling are upregulated in human tumors • OSMI-1 suppresses Gli2 transcriptional activity in a mouse model of medulloblastoma: ○ Inhibits tumor progression ○ Improves survival
SPINAL CORD	ALS[154] • Global O-GlcNAc levels and NF-M O-GlcNAcylation are reduced AGING[158] • Mounting oxidative stress contributes to motor neuron degeneration • NPGPx ameliorates oxidative stress by increasing O-GlcNAcylation in aging mice SCI[169] • In a rat model of SCI, Thiamet-G treatment reduces lesion size and promote motor function recovery