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O-GlcNAc and neurodegeneration: biochemical mechanisms and potential roles in Alzheimer's disease and beyond.

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Abstract

Alzheimer disease (AD) is a growing problem for aging populations worldwide. Despite significant efforts, no therapeutics are available that stop or slow progression of AD, which has driven interest in the basic causes of AD and search for new therapeutic strategies. Longitudinal studies have clarified that defects in glucose metabolism occur in patients exhibiting Mild Cognitive Impairment (MCI) and glucose hypometabolism is an early pathological change within AD brain. Further, type 2 diabetes mellitus (T2DM) is a strong risk factor for the development of AD. These findings have stimulated interest in the possibility that disrupted glucose regulated signaling within the brain could contribute to the progression of AD. One such process of interest is the addition of *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) residues onto nuclear and cytoplasmic proteins within mammals. *O*-GlcNAc is notably abundant within brain and its presence on hundreds of proteins including several, such as tau and the amyloid precursor protein, which are involved in the pathophysiology AD. The cellular levels of *O*-GlcNAc are coupled to nutrient availability through the action of just two enzymes. *O*-GlcNAc transferase (OGT) is the glycosyltransferase that acts to install *O*-GlcNAc onto proteins and *O*-GlcNAcase (OGA) is the glycosylhydrolase that acts to remove *O*-GlcNAc from proteins. Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) is the substrate for OGT and its levels vary with cellular glucose availability because it is generated from glucose through the hexosamine biosynthetic pathway (HBSP). Within the brains of AD patients *O*-GlcNAc levels have been found to be decreased and aggregates of tau appear to lack *O*-GlcNAc entirely. Accordingly, glucose hypometabolism within the brain may result in disruption of the normal functions of *O*-GlcNAc within the brain and thereby contribute to downstream neurodegeneration. While this hypothesis remains largely speculative, recent studies using different mouse models of AD have demonstrated the protective benefit of pharmacologically increased brain *O*-GlcNAc levels. In this review we summarize the state of knowledge in the area of *O*-GlcNAc as it pertains to AD while also addressing some of the basic biochemical roles of *O*-GlcNAc and how these might contribute to protecting against AD and other neurodegenerative diseases.

Introduction

Alzheimer Disease (AD) is the most common neurodegenerative disease. This progressive disease leads initially to cognitive impairment and ultimately to death. The incidence of AD within populations worldwide is steadily increasing, in large part because age is the largest risk factor. Approximately 26 million people suffer from AD and this number is projected to quadruple by 2050. Given the absence of any disease modifying therapeutics, the disease poses a major challenge to societies who must be prepared to cope with the growing amount of intensive patient care required by the increasing number of AD patients. These facts have stimulated great interest in the causes and mechanisms driving AD as well as an allied search for potential strategies that might slow or halt progression of the disease.

The microtubule-associated protein tau (tau) and the amyloid precursor protein (APP) are the two proteins that give rise to the chief pathological hallmarks of AD; extracellular neuritic (senile) plaques composed of amyloid-β peptide, which are derived from the amyloid precursor protein (APP), and intracellular neurofibrillary tangles (NFTs), which are formed from the microtubule associated protein tau (tau). The post-translational modification of these proteins has emerged as a topic of considerable interest since various modifications have been found to alter the toxicity of these proteins. Consequently, the ability to manipulate the modification state of these proteins and other upstream factors may offer routes to decrease their toxicity in AD. Accordingly, an increasing number of enzymatic and non-enzymatic post-translational modifications have been found on these two proteins and several of these are well implicated in AD pathology (for review see[1, 2]). Among these post-translational modifications, it has emerged that both APP and tau are enzymatically modified with *N*-acetyl-D-glucosamine (GlcNAc) residues *O*-linked to the hydroxyl groups of serine and threonine residues (*O*-GlcNAc). The *O*-GlcNAc modification and its potential involvement in AD will be the chief focus of this review.

O-GlcNAc is a non-canonical form of protein glycosylation that occurs within the nucleus and cytoplasm of multicellular eukaryotes. The *O*-GlcNAc modification, as it is commonly referred to, has been found on diverse families of proteins ranging from low abundance transcription factors through to common cytoskeletal proteins[3]. This modification has emerged as the focus of a field of increasingly broad scope in the last number of years, not only because of the growing number of proteins on which it is found, but also because of the number of important

cellular processes in which these proteins participate. The fact that both tau and APP bear the *O*-GlcNAc modification and are critically involved in the development of AD has raised the possibility that *O*-GlcNAc may play a role in the pathogenesis of this disease. Using a similar rationale, the presence of *O*-GlcNAc on other proteins such as α-synuclein[4], which is a component of Lewy bodies found most notably in Parkinson's disease[5] (PD), as well as superoxide dismutase (SOD)[6] and neurofilament proteins[7], which are involved in ALS, suggests *O*-GlcNAc may play roles in a variety of neurodegenerative diseases. Over recent years, a number of studies have taken the first steps beyond identifying sites of *O*-GlcNAc on proteins involved in neurodegeneration and have moved to unravel the ways in which this enigmatic carbohydrate modification alters the functions of these proteins in both homeostasis and disease. The purpose of this review is to broadly highlight the state of knowledge regarding the biochemical properties of *O*-GlcNAc, with specific emphasis on the presence and potential functions of *O*-GlcNAc on both tau and APP, as well as to discuss the potential contributions of misregulated *O*-GlcNAc in the pathogenesis of AD. Further, based on the presence of *O*-GlcNAc on other proteins involved in neurodegenerative diseases, we speculate on more general roles for *O*-GlcNAc in neurodegeneration and the therapeutic potential of altering protein *O*-GlcNAc levels.

1. The *O*-GlcNAc modification

The discovery, in 1984 by Torres and Hart, that *O*-linked *N*-acetyl-D-glucosamine (GlcNAc) could be found within the inside of lymphocytes marked a paradigm shift in the study of carbohydrates and their biology[8]. This surprising observation was the first, and still the only, demonstration of nucleocytoplasmic protein glycosylation within metazoans. This modification later became known as the *O*-GlcNAc post-translational modification. *O*-GlcNAc stands in contrast to more widely recognized forms of glycosylation, such as *N*-linked glycosylation which is found exclusively within the secretory compartments, the outside of the cell, and in the extracellular milieu[9]. *O*-GlcNAc has now been identified on a diverse array of proteins which localize within the cytoplasm, nucleus, and mitochondria[8]. Proteins which bear the *O*-GlcNAc posttranslational modification are involved in such diverse processes as regulation of gene expression[10], translation[11], and metabolism[12]. The addition of *O*-GlcNAc to protein substrates is carried out by just one glycosyltransferase that is referred to as uridine diphosphate-*N*-acetyl-D-glucosamine: polypeptidyl transferase (OGT). This enzyme uses the donor sugar, uridine 5'-diphospho-*N*-acetylglucosamine (UDP-GlcNAc), to transfer GlcNAc to serine or threonine residues[13, 14]. *O*-GlcNAc can be removed from proteins by a glycoside hydrolase called *O*-GlcNAcase (OGA), which catalyses the hydrolytic cleavage of GlcNAc off from modified proteins[15, 16] (Figure 1).

The enzymatic addition and removal of *O*-GlcNAc from proteins can occur several times during the lifetime of a protein and, though the turnover rates have only been described on a small number of proteins, the half-life of *O*-GlcNAc on studied protein appears to be several hours[17, 18]. *O*-GlcNAc appears to be completely conserved within multicellular eukaryotes and OGA and OGT are highly conserved nucleocytoplasmically localized enzymes, suggesting *O*-GlcNAc plays ancient and fundamental roles in cells.

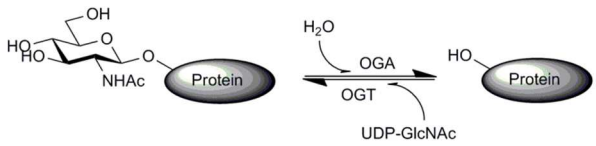

Figure 1. O-GlcNAc is added by OGT and removed by OGA. A glycosyltransferase, uridine diphospho-***N***-acetylglucosamine: polypeptidyl-β-***N***-acetylglucosaminyltransferase, OGT transfers GlcNAc from the donor sugar UDP-GlcNAc to target proteins. Conversely, a glycoside hydrolase, O-GlcNAcase or OGA catalyzes the hydrolytic cleavage of GlcNAc off of modified proteins.

1.1. Tissue and sub-cellular localization

To date no detailed comparative analysis of the abundance of *O*-GlcNAc at the tissue level has been published. However, the mRNA expression levels of the enzymes which install and remove *O*-GlcNAc, OGT and OGA, have been extensively studied within mammals at the tissue level. OGT is universally expressed in all tissues examined, with the highest level of expression in pancreas and brain[13, 14]. Additionally, one study has shown that the level of OGT activity is ten-fold higher in brain than peripheral tissues[19]. OGA mRNA expression has also been detected in all tissues studied, where the expression level was by far the highest in the brain[16]. Brain regions showing particularly high levels of OGT expression and *O*-GlcNAc include the cerebellar cortex and hippocampus[20, 21]. Interestingly, *O*-GlcNAc is also present at all stages of development of the embryonic brain and its levels do not appear to change between the embryonic, post-natal, and adult stages in mice[22]. Collectively, these studies suggest that the *O*-GlcNAc modification may play a particularly important role within mammalian brain. This possibility is further supported by an elegant tissue-specific gene knock-out study performed by Marth and co-workers. These investigators bred mice containing an OGT gene flanked by two lox-p sites (OGT^F). Crossing these OGT^F mice with mice expressing a cre-recombinanse under control of the synapsin-1 promoter (Syn1-CRE), led to both central and peripheral neuron-specific expression of the cre-recombinase and corresponding loss of OGT. These OGT^F/Syn1-CRE offspring were found with lower frequency, were considerably smaller, had abnormal locomotor ability, and none survived longer than 10 days. No histological examination of the OGT^F/Syn1-CRE mice were performed in this study so it is unknown whether these effects were due specifically to the death of neurons. Biochemical analyses, however, revealed that tau is abnormally hyperphosphorylated within both the brain and spinal cord, suggesting *O*-GlcNAcylation may play a role in the regulation of tau phosphorylation. The deleterious effect of losing OGT and *O*-GlcNAc within these mice is also consistent with the high levels of *O*-GlcNAc and OGT within the brain playing essential roles in homeostasis. At the cellular level within brain, particular attention has been directed toward *O*-GlcNAc and OGT levels in neurons. *O*-GlcNAc has been shown to be highly abundant at the nodes of Ranvier,[23] and especially high at the the termini of

neurons[24]. This abundance at nerve termini has stimulated interest from analytical biochemists, who have gone on to identify hundreds of *O*-GlcNAc modified proteins in synaptosomes and identify hundreds of sites on these proteins[25-27]. In terms of brain region-specific *O*-GlcNAc levels, it has been noted that *O*-GlcNAc is particularly abundant in the Purkinje neurons of the cerebellar cortex[20] within mice as well as in radial glia of developing chicken optic tecta[28]. Little however, is known about how the roles of *O*-GlcNAc might differ between the different cell types of the brain and this remains a fertile area for investigation.

1.2. *O*-GlcNAc transferase (OGT)

OGT in mammals is encoded by a single gene located on the X chromosome[29] and it has been shown that deletion of the *ogt* gene is embryonic lethal in mice[29]. This glycosyltransferase was first cloned by the Hanover and Hart groups[13, 14]. These authors demonstrated that the *ogt* gene is highly conserved across a number of eukaryotes from *C. elegans* to humans[13, 14]. The primary product of the *ogt* gene is a 1036 amino acid protein of ~110 kDa[13, 14] that is found in both the nucleus and the cytosol (referred to as ncOGT). This variant is the best studied and it has been shown to use an ordered bi-bi catalytic mechanism and display considerable variability in its K_m values for protein substrates. Notably, the K_m for UDP-GlcNAc for different protein substrates varies from single digit micromolar to high double digit micromolar, as seen for tau which is poorly modified in vitro[30-32]. These observations suggest that some protein substrates are likely constitutively modified at physiological UDP-GlcNAc concentrations whereas modification of others, such as tau, varies depending on the cellular UDP-GlcNAc concentration[30]. Two other isoforms of OGT have been shown to arise by alternative splicing: a shorter mitochondrial isoform (mOGT) as well as the shortest form of OGT (sOGT)[33, 34]. The exact significance of these two shorter forms of OGT remains little explored, however, these enzymes lack some of the tetratricopeptide repeat (TPR) sequences that are situated to the C-terminal region of the enzyme relative to the catalytic units. The structure of OGT has been solved[35] and previous proposals[36-38] that the TPR domain would serve to bind and direct some substrates into the glycosyltransferase active site have recently received clear structural support for one protein substrate[39]. Given recent proteomic data, and in light of the structure of OGT, it appears that this enzyme predominantly modifies disordered regions of proteins. Quite remarkably, this enzyme has been shown to cleave one binding partner that is a substrate for glycosylation through a mechanism dependent on glycosylation. Given that the TPRs appear critical for binding proteins OGT likely participates in protein complexes and binds partners through this domain. Much remains to be learned about how OGT substrate specificity might be defined at the cellular and biochemical levels.

1.3. *O*-GlcNAcase (OGA)

Like the *ogt* gene, the human *O*-GlcNAcase (OGA) gene, originally named *MGEA5,* has also been cloned and is located on chromosome 10[16, 40]. The *MGEA5* gene gives rise to an enzyme that is 916 amino acids in length. Both the optimal pH for this enzyme and the substrate specificity were deduced and indicate that OGA is a β-*N*-acetylglucosaminidase with a neutral pH optimum[16], which is the same as the enzyme that was termed HexC and partly characterized much earlier after biochemically purification[41]. Two different isoforms of OGA have been described; a smaller isoform referred to as the nuclear variant of OGA (nvOGA or OGA-S), which arises by alternative splicing and contains only 662 amino acids[42]. The nuclear variant of OGA bears this name due to the nuclear localization of this isoform, whereas the full length protein (OGA-L) shows both nuclear and cytosolic localization[42, 43]. Both OGA-L and the OGA-S contain the core β-*N*-acetylglucosaminidase domain but differ in that OGA-S does not contain the inactive acetyltransferase-like domain found within the full length protein[42]. The catalytic mechanism of OGA is established and it is known that OGA recognizes predominantly the GlcNAc moiety of its substrates[30]. Enzymatic characterization of both OGA-L and OGA-S reveals that OGA-S is significantly less active than OGA-L with respect to the hydrolysis of *O*-GlcNAc[44]. Within brain tissue, the short isoform appears to only be expressed during development of the embryo[45]. Earlier biochemical studies reported several binding partners but these observations have not been pursued.

1.4 Inhibitors

To study the roles that *O*-GlcNAc plays within cells and organisms two general approaches have been used: genetic approaches and chemical approaches. Overexpression as well as knock-down of both OGA and OGT has been performed in a number of different systems[46-51]. Notably, as mentioned above, the conditional knock-out of OGT within neurons of mice leads to neuronal death and tau hyperphosphorylation[29]. Dramatically altering the levels of these proteins, however, may have effects independent of *O*-GlcNAc since both of these large multi-domain proteins have been shown to interact with several protein partners including those involved in regulating gene expression. For this reason, chemical approaches offer some benefit in that they do not directly modify the amount of the target protein present in cells, yet they are able to significantly reduce the activity of the target in cells and *in vivo*. Interestingly, it has been shown the inhibitors of OGA and OGT lead to modest changes in the expression levels of these proteins, highlighting the presence of a cellular homeostatic system that is likely working to try to maintain balanced *O*-GlcNAc levels. The other major advantage of chemical approaches is that the chemical agent can be withdrawn and the system allowed to return to an unperturbed state.

A small number of OGT inhibitors having some activity in cells have been described[52-54]. Among these are some, discovered through high-throughput screening, that are able to inactivate OGT within cells. These show modest efficiency at decreasing *O*-GlcNAc levels and likely hit other cellular targets. A different approach has been to use an acetylated GlcNAc analogue, 2-acetamido-2-deoxy-5-thio-D-glucosamine, which can be converted by cells to form UDP-5SGlcNAc, which then inhibits OGT and also modestly lowers the cellular concentration of UDP-GlcNAc[55]. This compound shows a K_i value of about 8 μM for human OGT and an EC_{50} value of between 0.5 and 5 μM for decreasing *O*-GlcNAc levels in cultured cells[55]. Further work in the area of OGT inhibitors is sorely needed.

In contrast to the case for OGT, a number of good OGA inhibitors have been uncovered. The first inhibitors of OGA to be

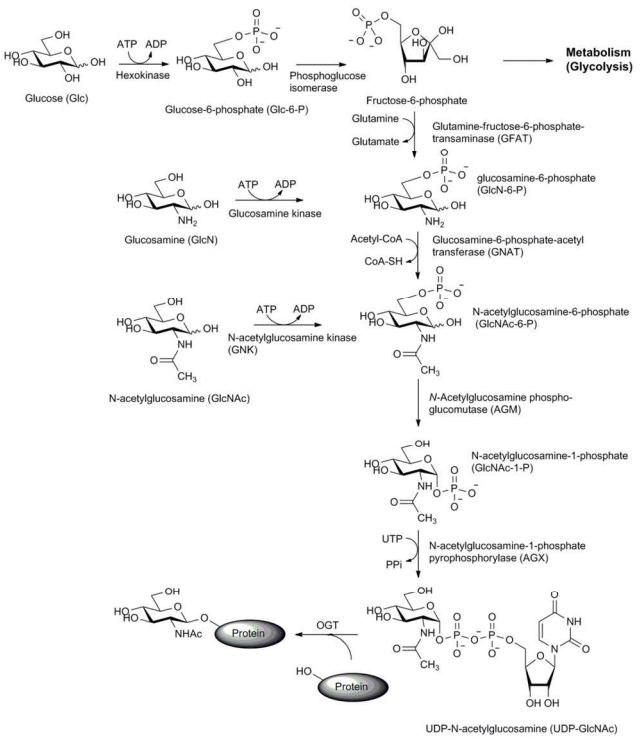

Figure 2. The Hexosamine biosynthetic pathway. UDP-GlcNAc is produced by the hexosamine biosynthetic pathway (HBSP) from cellular glucose via the action of two enzymes from central metabolism (hexokinase and phosphoglucose isomerase) and the four enzymes which make up the HBSP (GFAT, GNAT, AGM and AGX).

described were PUGNAc and LOGNAc, which were shown to be inhibitors of a different β-*N*-acetylhexosaminidase[56]. PUGNAc was later found to also potently inhibit OGA (K_I = 20 nM)[15, 57, 58] but was later shown to equally well inhibit human lysosomal hexosaminidase B[58]. Another early tool was streptozotocin, a GlcNAc analogue, which is a weak OGA inhibitor (K_I = 2 mM)[59], which, when coupled to its well characterized toxicity and the availability of better reagents, makes it an inappropriate tool to study *O*-GlcNAc[60-62]. A known compound[63], NAG-thiazoline, resembles the enzyme intermediate found in the reaction mechanism of OGA and is a potent inhibitor but one that also inhibits the functionally related β-hexosaminidases[58]. Adding a pendant alkyl chain to the thiazoline ring resulted in inhibitors having good selectivity for human OGA over functionally related lysosomal β-hexosaminidases. These compounds exploit an extended pocket within the active site of OGA and one of the compounds derived from this study is NButGT, which is relatively potent (K_i = 600 nM) and 800-fold selective for OGA over the lysosomal β-hexosaminidases. This selectivity is manifested both *in vitro* but also in *vivo*, where it acts to increase *O*-GlcNAc but does not affect ganglioside levels. The same approach to selectively was pursued by Van Aalten and co-workers to take a known compound to generate the potent and selective inhibitor GlcNAcstatin[64]. These GlcNAcstatins have not yet been used *in vivo*, perhaps owing to the difficulty in synthesizing large amounts. Rational modification of NButGT gave rise to the compound Thiamet-G, which differs from NButGT only by replacement of a methylene unit with an amine functionality. Thiamet-G is highly potent (K_i = 20 nM), highly selective (37,000-fold) for human OGA, and is remarkably stable in solution. In addition, thiamet-G is also able to cross the blood-brain barrier. All of these properties thus make thiamet-G useful for *in vivo* experiments. Indeed, Thiamet-G is now commercially available from several vendors and has been used in over a dozen different studies since its first description including several in mice[65-71].

1.5. The HBSP: Biosynthesis of UDP-GlcNAc

As mentioned above, OGT is a bisubstrate enzyme requiring two different substrates: a protein substrate and the donor sugar UDP-GlcNAc. UDP-GlcNAc is involved in central metabolism and is also used as a substrate by a number of other GlcNAc transferases. UDP-GlcNAc can be synthesized from glucose by the serial action of five different enzymes within a biosynthetic pathway that is referred to as the hexosamine biosynthetic pathway[72] (HBSP) (See Figure 2). Glucose, upon entry into the cell, is phosphorylated by hexokinase at the 6-hydroxyl and is then isomerized, by phosphoglucose isomerise, to fructose-6-phosphate[73, 74]. The action of glutamine fructose-6-phosphate transaminase (GFAT) then converts fructose-6-phosphate to glucosamine-6-phosphate in what is the rate limiting step for the HBSP[75]. This transformation involves the transamination of the amine functionality of glutamine to the carbonyl group of fructose-6-phosphate, followed by isomerisation to generate glucosamine-6-phosphate[72]. The acetyl group is then installed by glucosamine-6-phosphate acetyltransferase[76] (GNAT) to form *N*-acetylglucosamine-6-phophate (GlcNAc-6-phosphate) followed by another isomerisation involving phosphorylation of the anomeric hydroxyl group and dephosphorylation of the 6-hydroxyl group the enzyme by N-acetylglucosamine phosphoglucomutase[77] (AGM) to produce *N*-acetylglucosamine-1-phosphate (GlcNAc-1-phosphate). Finally, in the last step, *N*-acetylglucosamine-1-phosphate pyrophosphorylase (AGX) catalyzes the transfer of uridine-5'-diphosphate from UTP to *N*-acetylglucosamine 1-phosphate to form UDP-GlcNAc[78]. Additionally, both glucosamine and *N*-acetylglucosamine can also enter into the HBSP via so-called 'salvage' pathways. In these cases, glucosamine or *N*-acetyglucosamine are phosphorylated by their respective kinases, to yield glucosamine-1-phosphate or *N*-acetylglucosamine-1-phosphate, which are both HBSP intermediates[79, 80] (Figure 2).

1.6 Metabolic control of *O*-GlcNAc levels

As shown above in Figure 2, between 2-3% of all glucose that enters the cell is shunted down the HBSP[72]. Because UDP-GlcNAc is made from dietary glucose, the HBSP has been proposed as a nutrient sensing pathway and indeed, studies have shown that UDP-GlcNAc levels vary with glucose availability[81, 82]. Though UDP-GlcNAc itself is a feedback inhibitor of the HBSP, flux through this pathway can be increased by providing cells exogenous nutrients including lipids, uridine, or glucosamine[81], as well as by inducing various cellular stresses[83], which all lead to increased *O*-GlcNAc levels.

Multiple papers have shown in adipocytes that increased flux through the HBSP can lead to the development of insulin

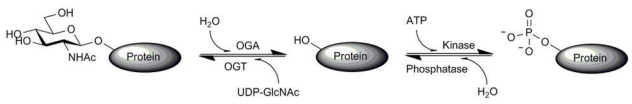

Figure 3. *O*-GlcNAc can be reciprocal to phosphorylation. The existence of serine or threonine residues that are known to be both phosphorylated and *O*-GlcNAc modified dictates that such residues can exist in one of three different states; phosphorylated, glycosylated, or free hydroxyl. The formation of these states is regulated by the appropriate enzymes. Direct competition for the same serine or threonine residues or sites nearby could result in a dynamic equilibrium between these three states.

resistance[84, 85]. Based on these results it was proposed that *O*-GlcNAc may act as a nutrient sensor because OGT catalyzed *O*-GlcNAcylation of some proteins depends on the concentration of UDP-GlcNAc[86]. In times of hyperglycemia, UDP-GlcNAc levels should be increased and thus lead to increased *O*-GlcNAc levels, which has been reproducibly shown[87, 88]. Thus increased *O*-GlcNAc levels could act to sense nutrient availability and studies using both chemical and genetic approaches have supported this view. First, using a chemical approach, treatment of 3T3-L1 adipocytes with the OGA inhibitor, PUGNAc, was shown to result in increased *O*-GlcNAc levels and result in impaired glucose uptake and insulin resistance in these cells[89]. Other data using different inhibitors, however, has not replicated these findings regarding glucose uptake or insulin resistance neither in 3T3-L1 adipocytes, nor in rats or diabetic mice[90-92]. The mechanism by which PUGNAc acts in this regard therefore remains unclear, though it is known to have some off-target effects[93, 94]. Overexpression of OGT in muscle and fat cells[51] as well as in the liver[50] resulted in insulin resistance and perturbed glucose homeostasis, respectively. Finally, with respect to the brain, starvation of mice, and thus decreased glucose availability, results in dramatically lower levels of *O*-GlcNAc in the brain[95]. Collectively, these studies show that *O*-GlcNAc fluctuates in response to glucose availability and suggest that such fluctuations may act to sense glucose availability. The discrepancy in results between the use of genetic methods and inhibitors suggests there may be differences in these approaches, as suggested in an earlier section of this review. Whether these changes in *O*-GlcNAc levels, seen under hypo- or hyperglycemic conditions, serve a functional role in adaptation to nutrient excess or deprivation or, instead, whether they might have deleterious effects such as inducing insulin resistance, remains a question of interest. Reconciling the differences observed when using genetic and chemical methods could yield valuable insights into the mechanisms by which the *O*-GlcNAc system may modulate cellular signalling.

1.6 Biochemical effects of *O*-GlcNAc on proteins

Study into the biophysical effects of glycosylation on proteins has been a longstanding topic, though the majority of studies have focused on cell surface glycosylation. Studies into *N*-glycans, which have at their core a GlcNAc residue β-linked to the amide nitrogen side chain of asparagines, have proposed that N-glycosylation assists proteins in achieving their specific folds[96], increases the structural rigiditity of proteins[97] and increases resistance to thermal unfolding and aggregation[98, 99]. The size and complexity of *N*-glycans, which comprise more than eight carbohydrate residues, is significantly greater than for the *O*-GlcNAc modification. However, it has been established that simply the presence of the first *N*-linked GlcNAc residue accounts for the resistance to thermal unfolding that is seen for RNAseB. Indeed, elongation to a more complex glycan does not further enhance the protection afforded by just one residue against thermal unfolding[98]. More recent evidence suggest that the single GlcNAc in an *N*-glycan might be able to stabilize proteins through carbohydrate-π interactions with the nearby protein sidechains[100].

By analogy to *N*-glycans, it is possible that a basic biochemical role for *O*-GlcNAc may be to stabilize proteins to prevent them from unfolding or aggregating. Notably, *O*-GlcNAc is found most abundantly on disordered regions of proteins, which is consistent with a potential protective role since it is fairly well established that proteins containing large intrinsically disordered regions are particularly susceptible to misfolding to form aggregates. A few studies have provided some suggestive evidence supporting the idea that *O*-GlcNAc may offer protection against protein aggregation. First, overexpression of OGT in Chinese hamster ovary (CHO) cells was suggested to result in less protein aggregation in cells following incubation at 45 °C[101]. Second, overexpression of OGA to reduce *O*-GlcNAc levels appeared to reduce the thermal stability of Sp1 in cells[102]. Finally, *O*-GlcNAc has been shown to increase the solubility of keratins[103]. In addition to these suggestive findings, *O*-GlcNAc has been shown using nuclear magnetic resonance (NMR) studies to alter the conformation of the N-terminus of the murine estrogen receptor[104]. More direct biochemical data has shown that *O*-GlcNAc modification of TAB1 decreases its thermally induced aggregation and *O*-GlcNAc has also been shown to influence tau aggregation as will be further discussed below[71].

1.7 Effects of *O*-GlcNAc on other PTMs

The *O*-GlcNAc modification has drawn parallels to serine / threonine (Ser/Thr) phosphorylation because both of these modifications are dynamic and can be added or removed multiple times during the lifespan of a particular protein[18]. In a few cases *O*-GlcNAc has been found to be reciprocal to Ser/Thr phosphorylation[105, 106]. *O*-GlcNAc site-mapping studies on proteins which are both *O*-GlcNAc modified and phoshorylated indicates that *O*-GlcNAc sites sometimes occur near phosphorylation sites, although this is not universally the case. Proteomics studies using OGA inhibitors have shown that pharmacologically increased *O*-GlcNAc levels lead to changes in protein phosphorylation. For these reasons it has been hypothesized that *O*-GlcNAc and phosphorylation can compete for the same Ser/Thr residues and can thus exist in a dynamic equilibrium as shown in Figure 3.

However, recent large scale proteomic analysis showed that *O*-GlcNAc and Ser/Thr phosphorylation were not coincident more than would be expected by chance[27]. On the basis of these observations, it appears unlikely that *O*-GlcNAc serves in a widespread manner to antagonize protein phosphorylation for regulatory purposes. However, such direct or proximal competition is likely to occur in specific instances, as seen for CK2[107] and CamKIV[108]. Given these more recent large scale site

mapping studies, the possible relationship between *O*-GlcNAc and phosphorylation might be more likely to stem from an indirect mechanism where *O*-GlcNAc modification of kinases or phosphatases could alter the activity of these enzymes. Notably in this regard, it has been observed, through large scale proteomics studies as well as targeted studies on specific proteins, that decreases in protein phosphorylation can be induced by acute administration of OGA inhibitors[70, 109-111] but that longer term treatment with inhibitors does not influence phosphorylation on proteins such as tau[67, 69, 70]. This transient effect suggests *O*-GlcNAc may influence the activity of kinases and phosphatases in the short term but that such changes diminish over time, perhaps because the cell gradually adapts to sustained changes in global *O*-GlcNAc levels.

Beyond its interaction with phosphorylation, *O*-GlcNAcylation has also been shown to have cross-talk with other post-translational modifications. One such example of this is the interaction of *O*-GlcNAcylation and ubiqutinylation. For example, the *O*-GlcNAcylation of histone H2B appears to facilitate its monoubiquitination[112] and decreased global ubiquitination was observed upon either glucosamine or PUGNAc treatment[113]. More recently, it has been suggested that *O*-GlcNAc impairs ubiquitination of other proteins such as β-catenin, perhaps through direct competition. Further research is needed to better understand the relationships between *O*-GlcNAc and ubiquitin as well as other post-translational modifications.

2. Alzheimer's Disease (AD)

Alzheimer's Disease (AD) was first described in 1906 by a German physician, Alois Alzheimer, after he evaluated the clinical symptoms and the subsequent autopsy results of one of his patients, Auguste D.[114, 115]. In the years since its first description, AD has been revealed as a progressive neurodegenerative disorder that affects memory and learning. The most important known risk factor for the development of AD is age[116], however, type II diabetes is also a major risk factor as will be discussed further below. AD can be subdivided into two different subtypes categorized by the age at onset: early onset AD (EOAD) and late onset or sporadic AD (LOAD)[117]. EOAD accounts for about 1-6% of all cases of AD and typically develops between 30-60 years of age[117]. LOAD, however, is the most common form of the disease and is defined by an average age of onset of 60-65 years age or later[117]. A family history of AD can occur in people who are diagnosed with either EOAD or LOAD. However, a far higher fraction, 50-60%, of all EOAD patients have disease that can be attributed to a familial origin[118, 119]. Due to the increasing incidence of LOAD (hereafter referred to as AD), and the associated societal burden, extensive research in the AD field is being directed at identifying potential disease modifying therapeutic strategies. A critical step in the process of identifying disease modifying therapeutics for AD is understanding the pathological features of the disease, how they arise, and how they progress.

When Alzheimer first presented the case of Auguste D. he described the presence of evenly distributed atrophy in the brain[114, 115], which is the most obvious late stage feature of AD. Upon silver staining of brain sections from Auguste D. Alzheimer described two distinct pathologies. The first of these was the presence of 'tangles' of neurofibrills, which he correctly identified as being intracellular. In the cerebral cortex, Alzhiemer described the second pathology as being "minute miliary foci which are caused by the deposition of a special substance in the cortex"[114, 115]. Today, we know that these 'tangles' of neurofibrills are indeed intracellular tangles made up of a microtubule-associated protein called tau and that the "minute miliary foci" are neuritic plaques caused by the deposition of Alzheimer's "special substance" which has been revealed to be peptides cleaved from the amyloid precursor protein.

2.1 *O*-GlcNAc in AD – The link between AD-related pathology and impaired glucose uptake/metabolism.

Major strides have been made in understanding the temporal relationship between the two main pathologies in AD. Among the most notable recent achievements comes from the Alzheimer disease neuroimaging initiative (ADNI), which is a private-public consortium of basic scientists and clinicians aiming to find better ways to detect AD as well as evaluate the progression of AD over time using imaging modalities and biomarkers. This work involves longitudinally following a cohort of patients over years, who display dementia characteristic of AD, those having early dementia referred to as mild-cognitive impairment (MCI), those who suffer from subtle memory complaints, and those who are cognitively normal and healthy who function as a control group[120, 121]. Based on this longitudinal data changes in AD clinical parameters and biomarkers can be tracked within individual patients. Already evident from these ongoing studies is the fact that the earliest changes in AD are the presence of amyloid-β peptides in the cerebrospinal fluid that parallel the formation of amyloid plaques[120, 121]. Subsequently, impairments in glucose metabolism are seen and this is followed by the appearance of tau within the CSF. Finally, changes in cognitive performance appear after these changes in CSF biomarkers[120, 121]. Two points are particularly salient to this review. First, ADNI data indicates aberrant glucose metabolism follows amyloid pathology and precedes tau pathology. Positron emission tomography studies using 2-fluoro-2-deoxy-glucopyranose support these ADNI data, showing that cerebral glucose metabolism declines progressively with normal aging and becomes further impaired in AD[122-124]. Conversion from MCI to AD also tracks significantly with the degree of impairments in glucose metabolism[125-127] . Biochemical evidence suggests that insulin and insulin receptor levels decrease within normal aging brain while levels of insulin receptors remain higher in concentration within AD brain as compared to age-matched controls[128] which may be a compensatory mechanism working to counteract impairments in glucose utilization. The major neuronal glucose transporter, GLUT3, has been shown to be decreased in AD brain[129] and its translocation to the plasma membrane is reported to result from the action of insulin[130] as well as by depolarization of the membrane[131]. Therefore, the loss of synaptic connections within AD brain associated with neuronal death[132] may thus explain the decreased expression or translocation of the GLUT3 and resulting impairments in glucose utilization. Second, glucose metabolism becomes impaired prior to tau becoming detectable in the CSF. Although this data is correlative, it is consistent with a scenario in which impaired glucose metabolism

may be a factor contributing to tau pathology.

In addition to human data focused on AD pathology, studies of various transgenic mouse models of AD have also shown impairments in glucose metabolism occur within the brain[133-138]. This work is important because it suggests that impaired glucose metabolism can arise from toxic gain of function of these proteins and is not simply a confounding symptom in AD patients. Notably, Type II diabetes mellitus (T2DM) is a major risk factor for AD[139, 140] and various mechanisms have been proposed to account for this observation (see for example[141-143]). However, because insulin resistance leads to decreased cellular glucose uptake and can influence levels of cell surface glucose transporters, T2DM can lead to impaired brain glucose utilization. Indeed, high blood glucose levels have been found in humans to correlate with glucose hypometabolism in regions susceptible to neurodegeneration in AD[144, 145]. Consistent with these observations in humans, it has also been found that diet-induced insulin resistance in animal models[146], including transgenic AD models[147-149], exacerbates AD pathologies. These findings suggest that impaired brain glucose metabolism is both driven by and exacerbated AD pathologies. These data also suggest that early maintenance of glucohomeostasis by lifestyle changes or therapeutic intervention may protect against AD.

On balance, the data described above point to impairments in glucose metabolism and utilization being a central feature of AD. The deleterious effects of T2DM as well as toxic tau and Aβ species on brain glucose utilization likely contributes to severity of AD pathologies. Mechanistically, however, the processes by which impaired glucose utilization drives these pathologies in the brain remains unclear. We believe the current data is pointing to *O*-GlcNAc being one factor linking impairments in glucose metabolism with these hallmark pathologies in AD. Because UDP-GlcNAc is derived from glucose via the hexosamine biosynthetic pathway, these early impairments in glucose utilization in the AD brain should lead to lower levels of UDP-GlcNAc and thus lower levels of *O*-GlcNAc modified proteins. This appealing hypothesis has been supported through studies in which brain tissue from AD patients were found to have lower *O*-GlcNAc levels when considering post-mortem delay[110] and more recently in a different patient population where decreased overall cytosolic *O*-GlcNAc levels were observed in frontal cortex but not cerebellum[150]. An earlier study using different analytic tools also observed no change in *O*-GlcNAc levels within the cytosolic fraction but did see increased *O*-GlcNAc levels within the detergent insoluble fraction of AD brain tissue from several regions other than cerebellum[151].

If the *O*-GlcNAc modification plays a protective role by limiting Aβ and tau toxicity in the brain then decreased glucose utilization and consequent lower *O*-GlcNAc levels could represent a failure of this protective mechanism. Such a molecular link could offer one mechanism by which T2DM and impaired brain glucose utilization drives AD pathologies.

3. Alzheimer's Disease (AD): *O*-GlcNAc and amyloid-β

The "special substance" contained within the "minute miliary foci" that Alzheimer described was first isolated in 1984[152]. Glenner and Wong purified a 4500 Da peptide from neuritic plaques, which they speculated was a product arising from cleavage of a larger serum protein[152]. This peptide, initially referred to as the A4 peptide, was later purified from neuritic plaques and was shown to form higher order aggregates that assemble into and are the major constituent of plaques[153]. Not long after, the gene encoding the A4 peptide (found on chromosome 21[154]) was cloned and shown to encode a protein of 695 amino acids resembling a cell surface receptor[155]. This protein was named the amyloid precursor protein (APP) and the A4 peptide derived from it was renamed the amyloid-β peptide. APP is a member of the conserved type I transmembrane proteins that are found across a number of species and orthologs are found in *Caenorhabditis elegans*[156], *Drosophila Melanogaster*[157], *Danio Rerio* (zebrafish)[158] and *Xenopus Laevis*[159]. APP has a large extracellular domain and can exist as one of three different isoforms that are 695, 751, or 770 amino acids in length[160]. These species are generated by alternative splicing and the 695 amino acid form of APP is primarily expressed in the brain whereas the 751 and 770 amino acid isoforms are expressed in other tissues[161].

Since the discovery of the amyloid-β peptide and the realization that it is produced from the larger APP, many studies have aimed to address how the amyloid-β peptide is generated from the precursor protein. It has emerged that APP is processed by three different proteases: α[162], β[163], and a γ [164, 165] secretase complex, for which the complete set of protein components are still being identified (see Figure 4). The cleavage of APP by α-secretase within the extracellular domain of APP gives rise to what is referred to as either the non-amyloidogenic[166], whereas cleavage within this domain by β-secretase results in processing within what is termed the amyloidogenic pathway[167, 168]. Cleavage by α-secretase within the extracellular domain liberates most of the extracellular domain, which is referred to as sAPPα, a soluble fragment, and the C-terminal fragment, APP-CTFα, which remains bound in the membrane[162]. Further cleavage of APP-CTFα within the transmembrane region by the γ-secretase complex results in the formation of the non-amyloidogenic product, p3, and the APP intracellular domain, AICD[166]. Conversely, cleavage by β-secretase within the extracellular domain gives rise to the soluble sAPPβ fragment and the membrane bound portion, APP-CTFβ[163]. Cleavage of the APP-CTFβ fragment within the transmembrane region by the γ-secretase complex gives rise to the amyloid-β peptide (either 40 or 42 amino acids, Aβ40 or Aβ42) and the AICD[167, 168].

In 1991 a breakthrough occurred when it was discovered that a mutation in the APP gene can give rise to EOAD, which appears nearly identical to LOAD in terms of clinical onset and pathophysiology[169]. This advance was considered important because it allowed the formulation of what has become known as the 'amyloid hypothesis' or the 'amyloid cascade hypothesis'[170]. The central idea behind the amyloid hypothesis is that the production of the amyloid-β peptide is sufficient to commence the pathological cascade which ultimately results in all of the pathological hallmarks of AD, including the amyloid plaques and the neurofibrillary tangles composed of tau. Implicit in this hypothesis is that the development of neurofibrillary tangles are downstream of plaque formation and this idea is now quite well supported by the accumulating ADNI data[120, 121].

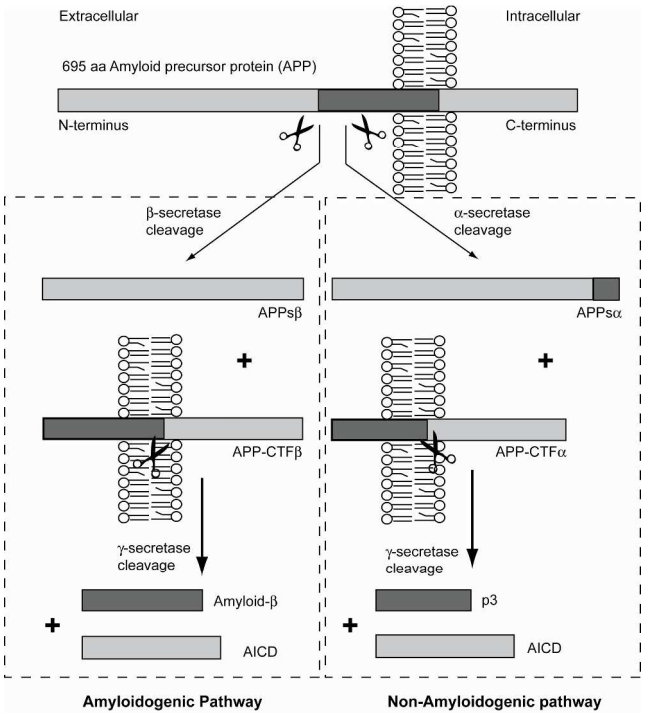

Figure 4. Proteolytic processing of the amyloid precursor protein (APP). Proteolytic processing of APP can proceed along one of two pathways depending on the action of three different proteases, α, β and γ-secretase. These two pathways generate either non-amyloidogenic products or amyloidogenic products. Upon β-secretase cleavage, the soluble APPsβ and the membrane bound APP-CTFβ fragments are generated. The APP-CTFβ can then be cut by γ-secretase to liberate the amyloidogenic amyloid-β peptide and the APP intracellular domain (AICD). Upon α-secretase cleavage, the soluble APPsα and the membrane bound APP-CTFα fragments are generated. The APP-CTFα can then be cut by γ-secretase to liberate the non-amyloidogenic p3 peptide and the AICD.

A great deal of attention has been focused on the post-translational modification of APP and proteins that process APP (for review see[1, 2]). With regard to *O*-GlcNAc, APP was first suggested to be modified by Griffith *et al*., who used both antibody detection and ^{3}H-galactosyltransferase labelling to indentify the presence of an *O*-linked GlcNAc residue[171]. Further to this, Jacobsen *et al.* have also used antibody binding to suggest that APP is *O*-GlcNAc modified but they further showed that increased *O*-GlcNAc leads to decreases in the Aβ40 amyloid-β peptide being released from cultured SH-SY5Y cells[172]. This work, however, made use of the non-selective OGA inhibitor PUGNAc, and thus should be interpreted with some caution. Very recently, however, Kim and co-workers described the long-term treatment of an AD mouse model of amyloid-β deposition (5xFAD mice) with the selective OGA inhibitor NButGT[173]. These investigators saw significant reductions in the number of amyloid plaques, the amount of the amyloidogenic Aβ40 and Aβ42 peptides, and a significant prevention of cognitive impairment[173]. Additionally, these investigators attempted to explain these *in vivo* effects of NBuGT treatment by treating Chinese hamster ovary (CHO) cells expressing the Swedish mutation in APP, which results in substantially increased production of Aβ40 and Aβ42. In a dose dependent manner, it was shown that NButGT decreases the amount of the C-terminal fragment of APP (APP-CTF). These effects were suggested to result from decreases in γ-secretase activity and, more specifically, due to *O*-GlcNAc modification of the nicastrin component of the γ-secretase complex[173]. It should be noted, however, that levels of Aβ40 and Aβ42 were not evaluated in this work and thus it is difficult to conclude if the in vitro effect on APP-CTF can explain the *in vivo* effects of NButGT. With the exception of mapping the *O*-GlcNAc of nicastrin to Serine-708, none of the above mentioned studies have described the unambiguous identification of *O*-GlcNAc modification sites on APP nor any other functionally involved proteins. Given the current data, it is difficult to speculate what roles, if any, *O*-GlcNAc present on APP might serve in its normal biological function or in Alzheimer-type neurodegeneration. This area therefore merits closer attention from investigators to verify and extend these findings by identifying sites of *O*-GlcNAc modification, clarifying the effects of cellular *O*-GlcNAc on APP processing, and addressing the biological roles of *O*-GlcNAc on these proteins.

4. Alzheimer's Disease (AD): *O*-GlcNAc and tau

4.1 Discovery, Gene Structure, and Biochemical Function

In 1975, the Kirschner group co-purified and characterized a protein from repeated cycles of polymerization of porcine brain tubulin[174]. This protein, which was capable of greatly accelerating tubulin polymerization *in vitro* was given the name "tau factor". At that time one remarkable property of tau factor was immediately evident; tau could be boiled for 5 minutes and still maintain its tubulin polymerization activity *in vitro*. The human gene encoding microtubule associated protein tau (*MAPT*), as tau factor is now called, is located on the long arm of chromosome 17[175]. Disruption of the *MAPT* gene results in mice that develop normally and are able to reproduce normally[176]. Recently, however, tau deficiency has been shown to cause Parkonsinism in aged mice[177]. The absence of a clear phenotypic effects until greater than 12 months of age is speculated to stem from the presence of various other functionally redundant microtubule associated proteins. Alternative splicing of tau in the human brain gives rise to six different isoforms which range in size from 352 amino acids to 441 amino acids[178]. The isoforms differ in their inclusion or exclusion of three different exons; exon 2, exon 3 and exon 10. Inclusion of exon 10 results in tau isoforms that contain four imperfect microtubule binding repeats (referred to as 0N4R tau), whereas exclusion of exon 10 results in isoforms having three repeats (referred to as 0N3R tau)[179]. Inclusion of exon 2 results in one N-terminal insert to generate 1N3R and additional inclusion of exon 10 leads to 1N4R tau. Inclusion of exons 2 and 3 results in two N-terminal inserts in 2N3R and the presence of exon 10 leads to 2N4R tau[179] (see Figure 5 for the depiction of the longest isoform of human tau (2N4R)). Within human brain roughly equal proportions of the 3R and 4R tau isoforms are expressed[178] whereas rodent brain expresses primarily the 3R tau isoforms[180]. Interestingly, expression of tau protein in the human brain is developmentally regulated, with the

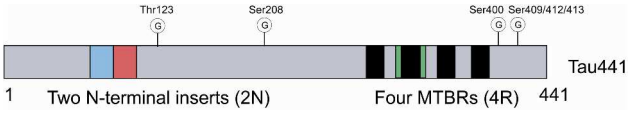

Figure 5: The longest human isoform of tau and the locations of serine/threonines which have been shown to be *O*-GlcNAc modified.

3R isoforms predominating in the fetal human brain. Altered levels of tau isoform expression within the human brain is thought to play a role in dementia[181] and the ability to alter isoform ratios is a topic of current interest.

4.2 Tau Structural Characteristics

As expected given the extreme stability of tau in solution, tau contains many hydrophilic amino acids and a paucity of hydrophobic amino acids. For example, the longest human isoform of tau (441 amino acids) contains 80 serine and threonine amino acids, no tryptophan residues, and only five tyrosine residues[182]. Given these features, it came as no major surprise that tau is a predominately unstructured and elastic protein[183]. Circular dichroism indicates that tau contains little secondary structure and behaves like a random coil[184]. Recent biophysical studies have supported the idea that tau may assume a general global conformation in solution that is more compact than a random coil[185] and also suggested that a precursor to tau aggregation is its adoption of further compressed conformation that has been termed the "paperclip" conformation[186, 187].

4.3 Role in Alzheimer's Disease

Tau has been revealed to exist within the bovine brain in a non-phosphorylated form as well as a phosphorylated form[188]. Phosphorylation of tau was shown to impair its ability to bind to and enhance microtubule polymerization[189-192]. This loss of function upon phosphorylation has deleterious consequences as tau is normally found almost exclusively within the axon of neurons[193] where it stabilizes microtubules to allow efficient movement of cargo over long distances[194]. Indeed, some investigators believe that failures in axonal transport play a significant and early role in neurological disease[195]. The fact that phosphorylation impairs the ability of tau to bind microtubules implies that the normal regulatory function of tau phosphorylation may be to tune the affinity of this protein for microtubules and regulate its behaviour within the axon.

Tau shot to the forefront of AD research in the mid-1980's when it was discovered to be the major component of neurofibrillary tangles by Grundke-Iqbal and coauthors[196, 197]. Neurofibrillary tangles had been previously shown to consist of so called paired-helical filaments (PHFs) because of the twisted pair appearance of these filaments seen by electron microscopy[198-200]. These PHFs can be conveniently enriched by exploiting their unique insolubility in a common detergent, *N*-lauroylsacrosinate (sarkosyl)[201]. Antibodies raised against PHFs from AD brains were found to react with purified tau from bovine brain. Tau protein in the mammalian brain contains roughly 1.9 moles of phosphate per mole of tau protein spread across ~10 different sites[202]. During the course of Alzheimer disease, tau becomes hyperphosphorylated leading to an increase in the stoichiometry of phosphorylation to 2.6 and 6-8 moles of phosphate per mole of soluble AD brain tau and paired-helical filament tau, respectively, across ~45 different sites[202]. A general hypothesis for tau dysfunction is that tau hyperphosphorylation leads to detachment from microtubules thereby increasing the amount of unbound heavily phosphorylated tau which then aggregates to form paired-helical filaments, which ultimately give rise to the neurofibrillary tangles that are characteristic of AD.

Because NFTs are such large structures found within the cell body of neurons it was initially presumed that NFTs are the toxic species responsible for tau-driven neurodegeneration. However, newer data has lead to revised thinking about this view. Using doxycycline inducible expression of transgenic mutant human tau that aggregates rapidly in the mouse brain the rapid formation of NFTs could be observed[203]. However, when transgene expression was halted, NFTs continued to grow even as neurodegeneration was blocked and memory improved[203]. This data implies that the formation of NFTs may serve to sequester toxic species of tau, which are likely soluble tau oligomers. Work by Le Corre *et al*., using a different transgenic tau mouse has shown that prevention of tau hyperphosphorylation does not block the formation of NFTs although it does block the onset of motor impairment and presumptive neuron loss[204]. This work is also consistent with the idea that the toxic tau species are low molecular weight oligomers. Interestingly, it also suggests that hyperphosphorylation is required in order to produce the toxic tau species but is not essential for NFT formation. Further support for the toxic tau species being a smaller oligomer comes from cell-based studies which have shown that tau misfolding and oligomer formation can be propagated from the outside of cells to the inside of cells and can be transferred between cells by low molecular weight species[205, 206]. Further confirmation of this process has now also been achieved *in vivo* where it has been shown that tau pathology is propagated along synaptic circuits from one connected neuron to another in a prion-like manner.[207]

4.4 NFTs and mechanism of their formation

A clear correlation between the number of NFTs and the severity of clinical dementia in AD has been demonstrated and argues strongly for tau dysfunction playing a critical role in AD pathogenesis[208]. This data contrasts to the lack of a strong correlation between the number of amyloid plaques and disease severity in AD[209]. For this reason, it is imperative to understand how NFTs arise in the AD brain since doing so could improve our understanding of the processes that ultimately lead to neuronal cell death and, by extension, enable the development of approaches that might block disease progression. Following the cloning of the *MAPT* gene, recombinant techniques made it possible to produce tau isoforms recombinantly within *E.coli*. Notably, recombinant tau on its own could not aggregate efficiently even at very high concentrations[210]. This observation suggested that hyperphosphorylation of tau is necessary to drive oligomer and PHF formation. Indeed, Alonso *et al.,* have shown that phosphorylated tau from AD brain can easily assemble into authentic PHFs/NFTs whereas enzymatic dephosphorylation of this tau completely blocks its ability to aggregate[211]. Aggregation of recombinant tau, however, has been achieved *in vitro* without the need for tau phosphorylation by the inclusion of polyanions (such as heparin)[212] or fatty acids (such as arachidonic acid)[213] within aggregation reactions. The general rationale for why

phosphorylation is thought to be due to this negatively charged modification decreasing the pI of tau, which leads to it being less positively charged at physiological pH values and therefore better able to self associate[214]. The presence of polyanions presumably acts in a similar manner, but in trans, to screen the positive charges of tau and render the tau-anion complex less positively charged.

4.5 Tau and the *O*-GlcNAc modification

Tau was first shown to be modified through studies by Arnold and Hart, who found bovine tau is extensively modified by *O*-GlcNAc with an average stoichiometry proposed to be greater than four moles of GlcNAc per mole of tau protein[215]. Even at that time, it was noted that the presence of *O*-GlcNAc on tau protein might play a role in regulating the function of tau or perhaps the degree to which it can be phosphorylated[215]. Multiple different studies have lead to the observation that phosphorylation and *O*-GlcNAc on tau show some reciprocity. Several early independent studies have made use of both chemical and genetic approaches to note this reciprocal relationship. Gong and coworkers described a series of elegant studies that suggested that the addition of *O*-GlcNAc regulates the extent to which tau is phosphorylated *in vitro* in tissue culture cells and *ex vivo* in rat brain slices[110]. These authors demonstrated that when PC12 cells over expressing human tau are treated with the OGA inhibitor PUGNAc, which increases *O*-GlcNAc levels,[56] there is a significant reduction in the extent of tau phosphorylation as measured using antibodies for several phosphoepitopes. These investigators also observed a similar phenomenon in rat brain slices studied *ex vivo*. Most notably, they found that cortex from AD brain showed lower levels of global *O*-GlcNAc, as compared to age and post-mortem delay matched brain, whereas *O*-GlcNAc levels in the cerebellum were unchanged. Suggestively, these authors also failed to detect *O*-GlcNAc on neurofibrillary tangles. Based on these collective findings, Gong and coworkers proposed that impaired glucose metabolism, which occurs early in disease progression in AD brain, would result in less UDP-GlcNAc production and consequently lower levels of *O*-GlcNAc on tau[110]. The net consequence of these potentially lower UDP-GlcNAc levels were suggested to be increased tau phosphorylation. Since their first study, Gong and coworkers have shown that short term fasting of mice leads to decreased *O*-GlcNAc levels and increased tau phosphorylation[95], lending support for this proposal regarding reciprocity. This reciprocal relationship was also observed independently by Lefebvre et al. who, using both galactosyltransferase labeling and wheat germ agglutinin, noted that more extensively phosphorylated tau had less *O*-GlcNAc. They also noted that by increasing tau phosphorylation using a protein phosphatase 1/2a inhibitor, okadaic acid, a corresponding decrease in tau *O*-GlcNAc levels could be induced[109, 216]. Finally, mouse genetics studies mentioned above, in which the gene encoding OGT was deleted specifically in neuronal tissue using the Cre-Lox system, revealed significantly increased phosphorylation of tau that was mirrored by a global decrease in *O*-GlcNAc levels[29]. More recently, the OGA inhibitor thiamet-G[70] was shown to increase global *O*-GlcNAc levels within brain. Following acute treatment of mice with this compound, Yuzwa *et al.* found that inhibitor treatment lowered tau phosphorylation at several pathologically relevant sites including Thr-231, Ser-396, and Ser-422[70] in a time dependent manner that was inversely related to the time dependent increases seen in global *O*-GlcNAc levels.

Other than observations regarding the reciprocal relationship between *O*-GlcNAc and phosphorylation on tau, little is known about the functional significance of tau *O*-GlcNAcylation. One reason for this lack of information is that the sites at which *O*-GlcNAc is found on tau were unknown, making site-directed mutagenesis studies of limited utility. Using a bacterial co-expression system Yuzwa *et al*. succeeded in mapping four *O*-GlcNAc sites on tau at positions Thr-123, Ser-208, Ser400 and one of Ser-409, Ser-412, or Ser-413[71, 217] (see Figure 5). One of these sites, Ser-400, was independently observed within both rat brain[4] and the JNPL3 mouse model[71]. The Ser-208 and Ser-400 sites have also been identified *in vitro* by NMR using recombinant OGT and tau peptides[31]. Further, Smet-Nocca, Lippens and coworkers used this site information to show that *O*-GlcNAc at Ser-400 blocks priming phosphorylation by cyclin-dependant kinase 2 (CDK2/cyclinA3) at Ser-400 and subsequent sequential phosphorylation by glycogen synthase kinase 3β (GSK3β) at Ser-400 and Ser-396[31]. These observations provide a molecular rational for how increased *O*-GlcNAc can lead to the decreased levels of phosphorylation at Ser-396 elicited *in vivo* by treatment of mice with thiamet-G[70].

All of the studies described above have collectively shown that tau is *O*-GlcNAc modified at a number of different residues and that reciprocal modification by phosphorylation can occur over shorter times, such as observed upon acute dosing using OGA inhibitors. The potential impact of increased *O*-GlcNAc antagonizing tau phosphorylation has stimulated consideration of what the effect of sustained increases in *O*-GlcNAc levels would be on the formation and/or toxicity of hyperphosphorylated tau, which is of clear relevance in the various tauopathies including AD. Mutations in the *MAPT* gene are known to give rise to a group of diseases collectively referred to as frontotemporal dementia linked to chromosome-17 (FTDP-17); a progressive brain disorder that leads to impairments in behaviour, language, and movement[218]. Capitalizing on this knowledge, researchers have created transgenic mice expressing tau with some of the more common FTDP-17 mutations to produce tauopathy mice such as the JNPL3 mouse model[219]. JNPL3 mice express the 0N4R isoform of P301L tau under the control of the mouse prion promoter. These mice develop robust tau hyperphosphorylation and neurofibrillary pathology in the spinal cord, brainstem, and the hindbrain but and less so in the forebrain. At roughly 6.5 months of age these mice develop motor impairments leading to muscle atrophy and weight loss due to loss of motor neurons. Eventually, these mice loose a significant amount of body weight and become moribund. A number of other tau mouse models have since been produced carrying other FTDP-17 mutations including the Tg4510 mice, which carry the same P301L mutation but expressed under control of the tetracycline operator (*tetO*), which makes it possible to control expression of the mutant gene using doxycycline[203]. Other mouse models will not be described in detail here but readers are pointed to an excellent recent review[220].

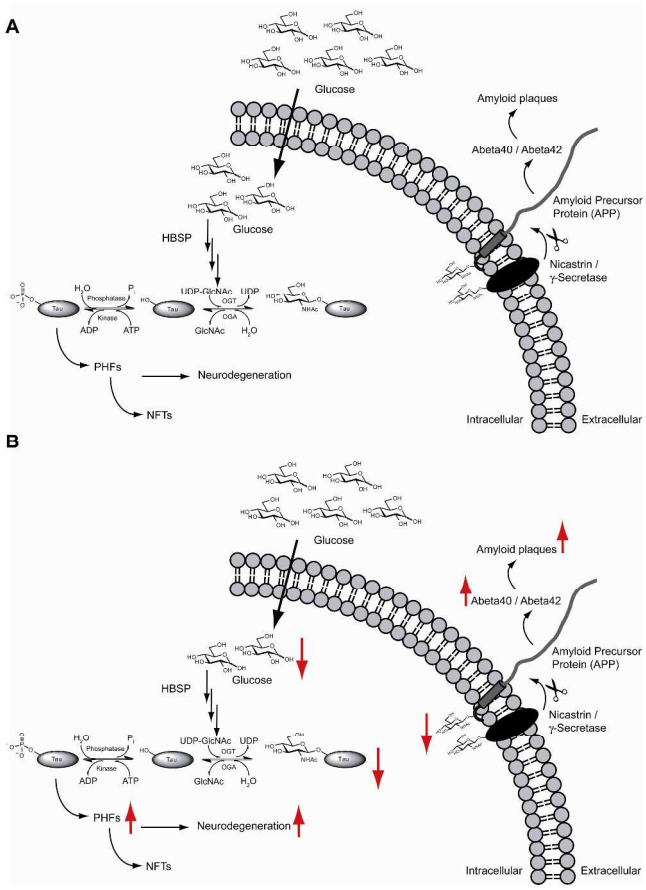

Figure 6. *O*-GlcNAcylation may play a protective role within the brain (Panel A) which becomes deficient in AD (Panel B).

Recently, it was shown by Yuzwa *et al.* that long-term treatment of JNPL3 mice with thiamet-G[71] leads to a prevention of neuron loss and a reduction in the number of NFTs in the brains of these animals[71]. Using a rabbit polyclonal antibody against *O*-GlcNAc at Ser-400 of tau in combination with mass spectrometry analysis of tau isolated from the brains of these mice, these authors showed that Ser-400 is indeed modified and that OGA inhibition resulted in increase tau *O*-GlcNAcylation at this residue. Within this study, it was found that long term inhibition did not lead to a reduction in tau phosphorylation nor a decrease in tau hyperphosphorylation, suggesting that decreased tau aggregation was hindered directly by *O*-GlcNAc modification[71]. These observations were further supported by *in vitro* studies showing that *O*-GlcNAc on recombinant aggregation-prone tau fragments inhibited their aggregation *in vitro*[71]. More recent data has found that increased *O*-GlcNAc modification of two other tau constructs, including the full length isoform, leads to decreased aggregation propensity while not affecting either the local or global conformation of tau[32]. This data suggests that *O*-GlcNAc on tau may play a protective role and thus the impairments in glucose utilization seen in AD brain may result in a failure of this protective function (Figure 6). Independent findings using thiamet-G in Tg4510 mice have just been reported and these closely support the findings of Yuzwa *et al.*[71]. A new monoclonal antibody directed toward *O*-GlcNAc at Ser-400 was used to show in Tg4510 mice that thiamet-G administration resulted in a 7-fold increase in tau *O*-GlcNAcylation at this residue. OGA inhibition within the Tg4510 model resulted in a similar decrease in NFT burden but it was also noted in this study that the levels of pathological hyperphosphorylated tau species were decreased but that the phosphorylation of non-pathological tau was unaffected. These observations are consistent with studies from the Gong group in which it was noted that tau aggregates were not *O*-GlcNAc modified[110]. The absence of an effect on tau phosphorylation upon chronic dosing with an OGA inhibitor is consistent with other reports in which OGA inhibitors have been used in mouse models for weeks to months[67, 69, 71]. In our opinion the Tg4510 mouse model, which displays more uniform penetrance of tau pathology as compared to jnPL3 mice, is well suited to examine the detailed role of *O*-GlcNAc in the formation of tau pathology. This collective data indicates that chronic OGA inhibition leads to increases in tau *O*-GlcNAc modification at Ser-400, but also likely more widely at multiple modification sites. The data is now clear that these increases in tau *O*-GlcNAcylation lead to a lower aggregation propensity for tau, perhaps by a combined effect mediated by increasing the solubility of tau, destabilizing growing filaments, and antagonizing its pathological hyperphosphorylation.

The somewhat enigmatic observation that OGA inhibition affects tau phosphorylation when mice are acutely treated with inhibitors, yet not when animals are treated for weeks, may stem from multiple scenarios. One situation could be that these different post-translationally modified tau species are in relatively low abundance. Phosphorylation and *O*-GlcNAcylation may therefore be present on different pools of tau. Acute administration could then lead to effects on the activity of kinases and phosphatases, which the cell could adapt to over time. Alternatively, normal phosphorylation and pathological hyperphosphorylation may be of sufficiently high abundance such that phosphorylation could be antagonized by *O*-GlcNAcylation, as suggested by Lippens and coworkers[31]. However, such a decrease in phosphorylation efficiency may be overcome *in vivo* through the sustained action of kinases that, overtime, enable normal levels of phosphorylation. In both cases, it appears likely that pathological hyperphosphorylation remains blocked. Blockade of pathological hyperphosphorylation may either be direct, or indirect and mediated by increased *O*-GlcNAc influencing other cellular processes. We believe it is indeed likely that the protective effects of *O*-GlcNAc are mediated through multiple mechanisms. To address these mechanisms new tools will be required to specifically detect *O*-GlcNAc on tau at various modification sites. Site specific monoclonal and polyclonal antibodies that have been generated against tau *O*-GlcNAc modified tau, such as those developed against Ser-400[66, 217], will be useful tools. The recent development of multiple reaction monitoring (MRM) strategies for the sensitive detection of *O*-GlcNAc at specific sites of proteins from tissues[71, 221] should similarly prove useful, particularly when used in conjunction with approaches for monitoring tau phosphorylation. Further mechanistic studies on the effects of *O*-GlcNAc in cells and in vivo are likely to shed light on these questions and in vitro studies on the effects of *O*-GlcNAc modification should also help clarify

the processes at play.

5. Potential role of *O*-GlcNAc in other neurodegenerative disorders

In addition to the roles of *O*-GlcNAc on tau and APP in AD, a few studies have provided suggestive hints that *O*-GlcNAc may play a more general role in neurodegenerative disorders. First, Corbo *et al.* demonstrated that a pathological feature of amyotrophic lateral sclerosis (ALS) is the accumulation of axonal spheroids which are composed of neurofilament proteins (a type of intermediate filament)[222]. The light and medium chains of neurofilament proteins (NF-L and NF-M) are both *O*-GlcNAc modified as well as phosphorylated[7, 223]. Additionally, these two modifications appear, once again, to be reciprocal to one another[224]. Given that neurofilament phosphorylation has been suggested to precede their accumulation in axons[225], these studies suggest that *O*-GlcNAc on neurofilament proteins may play a role similar to *O*-GlcNAc on tau as described above. In the context of ALS, Shan *et al*., have shown that *O*-GlcNAc levels are globally decreased in the spinal cord of mice carrying a mutation in super-oxide dismutase 1 (SOD1)[226]. Familial mutations in SOD1 give rise to 20% of human cases of familial ALS[227] . If *O*-GlcNAc provides a protective benefit, decreased *O*-GlcNAc in SOD mutation carriers might be a participating factor in ALS pathology. Interestingly, SOD1[6] and neurofilament proteins[7] are all *O*-GlcNAc modified and all are found within protein aggregates in ALS. Accordingly, increased *O*-GlcNAc levels generated using OGA inhibitors could be a novel therapeutic strategy for ALS. Considering another aggregation prone protein implicated in neurodegenerative disease, *O*-GlcNAcylation sites have been identified on all of α, β and γ-synuclein[4, 228]. α-synuclein is the principle component of Lewy Bodies in PD[5] and mutations in this protein are linked to PD[229]. Recently, Marotta *et al*., have shown that an *O*-GlcNAc modified α-synuclein peptide does not aggregate in the presence of unmodified peptide[230]. This work suggests that *O*-GlcNAc may inhibit the aggregation of α-synuclein in PD and a deficiency in *O*-GlcNAc, perhaps associated with aging, might contribute to this process. It is interesting in this regard that mutations in glucose transporter 1 (Glut-1) have been linked to AD[231], perhaps impaired glucose metabolism is a general features in neurodgenerateive diseases. Given the observations made for tau in AD models, perhaps increasing *O*-GlcNAc levels in PD thus might lead in an analogous manner to protective benefits in this disease by preventing the formation of toxic oligomers of α-synuclein. In a distinct vein, recent genetic studies in *C. elegans* showed that OGA and OGT null alleles affected the toxicity of two different length poly-glutamine (polyQ) expansions of the Huntingtin protein (Htn)[232].

Collectively, these studies, when viewed in light of the role of *O*-GlcNAc in AD, suggests that *O*-GlcNAc may play a role in various neurodegenerative disorders involving protein aggregation. Indeed, Yuzwa *et al*[71]. have speculated that *O*-GlcNAc modification may serve a general protective function by stabilizing proteins against aggregation. This concept is supported by observations regarding tau[32, 71], α-syuclein[230], and also for proteins that are not known to be involved in protein aggregation diseases such as TAB1, where it was found *O*-GlcNAc modification stabilized the protein against thermal aggregation[71]. Studies performed in cells have also suggested that increase *O*-GlcNAc may decrease the aggregation propensity of proteins. Thus, therapeutic strategies aimed at modulating *O*-GlcNAc levels may hold significant promise for the treatment of these disorders.

6. Perspectives and Conclusions

O-GlcNAc has attracted significant attention in recent years owing, in part, to the; (i) development of new tools that have enabled detection and manipulation of this modification within cells and *in vivo*[233-235], (ii) the potential for interplay with protein phosphorylation was uncovered[104, 106, 110, 236-238], (iii) clear connection to stress response emerged[239] and, (iv) observation that OGT is an epigenetic regulator of gene expression[240, 241]. Knowledge regarding the functional roles of *O*-GlcNAc, however, remains in its infancy. As new tools, large datasets, and confirmatory studies become more common within the field, the principle functions of *O*-GlcNAc will doubtless become more clear. One notable study in this regard has been the large scale proteomic site mapping of *O*-GlcNAc and phosphorylation, which suggested that *O*-GlcNAc does not influence protein phosphorylation anymore than would be expected by chance[27]. This unexpected finding supports the idea that the effects on phosphorylation observed on altering *O*-GlcNAc levels in the short term may be a consequence of altered activity of kinases and phosphatases, which are themselves *O*-GlcNAc modified. The short term effects of increased *O*-GlcNAc on tau phosphorylation associated with OGA inhibition that sustained in mice treated for longer periods of time, suggests that cells are able to adapt to increased cellular *O*-GlcNAc levels to maintain homeostasis. This data, coupled with the absence of obvious toxic effects associated with treating rodents with OGA inhibitors for extended periods of time, suggests that *O*-GlcNAc may play a predominantly protective role within cells, a hypothesis that is consistent with early studies showing increased *O*-GlcNAc offers protection against a wide array of cellular stresses[239].

With regard to the roles of *O*-GlcNAc in AD, the field is similarly at an early stage. The longstanding observations that diabetes is a risk factor for AD and contributes to impaired brain glucose metabolism, coupled with longitudinal studies showing glucose hypometabolism follows amyloid pathology but precedes tau pathology has stimulated interest in nutrient responsive changes within diseased brains. The link between AD and decreased *O*-GlcNAc levels described by Gong *et al*. opened the possibility that *O*-GlcNAc could be a contributing factor in AD. The demonstrated protective effects of inhibiting OGA in both tau and amyloid models, now replicated, have raised considerable interest in this approach as a therapeutic strategy to slow disease progression. Worthwhile to strengthen the protective benefits against tau-induced toxicity would be studies in tau mouse models harbouring different FTDP-17 mutations such as the R406W mutation. Likewise, it would also be of use to known if, when NFTs are allowed to form and the transgene is then repressed, does thiamet-G block further growth of NFTs.

While such studies are eventually likely to be published, the data has spurred interest in the basic mechanisms by which *O*-GlcNAc acts to protect against these two pathologies since

greater clarity in this area could lead to the development of useful targeted therapeutics as well as useful biomarkers for downstream clinical studies. The significant reduction in amyloidogenic Aβ40 and Aβ42 observed by Kim et al[173] suggests *O*-GlcNAc plays a significant role in APP processing. Even if *O*-GlcNAc does not act directly on APP, the study of how such large reductions in plaque load and Aβ40 and Aβ42 arise in this animal model will likely provide new insights into the pathogenesis of AD. *O*-GlcNAc could, in theory, be involved in either the production of amyloid-β peptides or the clearance of amyloid-β or amyloid plaques. In order to distinguish between these two possibilities, investigators will need to thoroughly characterize amyloid-β production using cell models and approaches that allow the monitoring of clearance of proteins and peptides such as pulse-chase experiments. In addition, to assess whether OGA inhibition leads to plaque clearance investigators could treat APP mutant mice with an OGA inhibitor after the appearance of plaques in the brain. Temporal control of mutant APP transgene expression could also be used to asses whether *O*-GlcNAc influences plaque clearance as OGA inhibitor treatment should yield more marked differences when mutant APP expression is shut off. Similarly, the roles of specific *O*-GlcNAc modified residues on APP or APP processing proteins could be evaluated using site-directed mutagenesis. Much remains to be uncovered in this area of research.

While the role of *O*-GlcNAc on tau is somewhat clearer, many questions also remain. We have identified several *O*-GlcNAc modification sites on tau but perhaps more remain to be uncovered. We have also found that *O*-GlcNAc can antagonize tau phosphorylation in the short term but sustained increases in *O*-GlcNAc do not block normal tau phosphorylation. However, increased *O*-GlcNAc does appear to prevent the formation of pathologically hyperphosphorylated tau species and also blocks the formation of NFTs in a mouse model. Finally, *O*-GlcNAc modification of tau decreases its aggregation propensity *in vitro*. We do not understand, however, the stoichiometry of *O*-GlcNAc on tau in both healthy and diseased brain or whether it even matters if *O*-GlcNAc on tau is substoichiometric or stoichiometric. This depends in part on how *O*-GlcNAc modification of tau affects the pathological processes associated with tau toxicity. Nor do we have a good sense of how levels of *O*-GlcNAc change during normal aging and whether this exacerbated in AD. Similarly, we lack the tools to monitor *O*-GlcNAc at all the sites known on tau and we lack knowledge of how dynamic these sites are within human brain. Finally, are there other mechanisms by which *O*-GlcNAc acts to protect against tau toxicity, as these authors believe, or is the effect mediated solely through modification of tau. Answers to these and other questions will gradually unravel the roles played by *O*-GlcNAc in neurodegenerative diseases.

In our opinion, with respect to the role of *O*-GlcNAc on tau in the AD brain, it would be of substantial value to the field to be able to monitor the longitudinal changes in the *O*-GlcNAc modification of tau with both advancing age and with advancing AD pathology. Because of the availability of both polyclonal and monoclonal *O*-GlcNAc tau antibodies we believe that these sorts of studies are now becoming a possibility. Investigators could use human brain tissue of differing ages as well as pathologically staged AD brain tissue to conduct this work. In addition, perhaps *O*-GlcNAc on tau could be detected in the cerebrospinal fluid of healthy controls and AD patients and used to monitor longitudinal change in these patients to see if there was a correlation with disease progression.

The review of papers presented above presents a generally harmonious, yet admittedly incomplete, view of the literature. There are of course papers that do not readily fit within the proposed models. Specifically, studies making use of *C. elegans* as a model system for various neurodegenerative diseases indicates that suppression of OGT, with accordingly lower levels of *O*-GlcNAc, rescued the toxic phenotype associated with tau containing the V337M FTDP-17 mutation[232] as well as Q40 and Q150 polyglutamine expansions of the Huntingtin protein. This observation is notable, since loss of OGT leads to decreased *O*-GlcNAc whereas inhibition of OGA would lead to increased *O*-GlcNAc. These results therefore argue against a protective role for *O*-GlcNAc as has been shown in other systems described above. The models examined are, however, quite different and loss of OGT is tolerated in *C. elegans* whereas in mammals it is essential for stem cell viability[232]. There may also be as yet unappreciated differences between using OGA inhibitors and genetic approaches involving knockout animal models. Very recently, van Leuven *et al.* have also used thiamet-G in a transgenic tauopathy model[242]. The Tau.P301L mice, in which the mutant tau transgene is driven by the *thy1* gene promoter, were treated for approximately 10 weeks with this inhibitor in drinking water. The results are generally consistent with those observed by Yuzwa *et al*. insofar as treatment prevented motor defects as well as a loss in body weight. These authors also noted that no changes in phosphorylation of tau were observed, consistent with previous findings, but were unable to detect *O*-GlcNAc on tau isolated from these mice. This surprising observation departs from earlier studies and is notable since *O*-GlcNAc modification of tau from rodent tissue has been observed by several different groups using various methods including site specific antibodies[66, 67, 71, 217] and mass spectrometry[4, 71, 217]. Nevertheless, these authors also observed that administration of OGA inhibitors resulted in a marked improvement in respiratory tract defects even over just a few days of administration. These observations are consistent with the idea mentioned earlier that OGA inhibition may well have other protective effects against tau toxicity independent of direct modification of tau. Finally, among the earliest papers on *O*-GlcNAc in AD is a report from Griffith and Schmidtz[151] which indicates that *O*-GlcNAc levels in the cerebellum of AD brain tissues are the same as in control tissues, consistent with reports from the Gong group[110, 150], but the study indicates that the cytoskeletal fraction within most other brain regions have increased levels of *O*-GlcNAc. These authors used different antibodies from those typically used but also used an enzymatic labeling method to detect *O*-GlcNAc to obtain similar results. The discrepancies between this study and other reports are not simple to reconcile since different analytical methods were used and post-mortem delay was not uniformly accounted for. Nevertheless, this data points to a clear need for further studies in the area of *O*-GlcNAc levels within AD brain tissues. Mostly useful would be studies aiming to correlate *O*-GlcNAc levels with disease progression within different brain regions

affected by hypometabolism with consideration of the post-mortem delay.

In summary, the influence of *O*-GlcNAc in AD is now of clear interest to both academic and pharmaceutical communities. The potential protective effects of *O*-GlcNAc in other human neurodegenerative disorders such as ALS, PD, and Huntington's disease are only just beginning to emerge and the next number of years will undoubtedly provide novel insight in this area. The fact that *O*-GlcNAc may play important roles in AD and these other neurodegenerative disorders suggests that it may play a generally protective role in the brain, which would be consistent with its high abundance in this organ. This protective role could become compromised during aging due to decreased glucose metabolism within the aging brain, and this could contribute, in part, to the etiology or progression of neurodegeneration. For this reason we speculate that pharmacological intervention, perhaps OGA inhibition, could have significant impact on various neurodegenerative disorders. Despite limited biochemical knowledge in this area, on the basis of the collective literature, we believe *O*-GlcNAc likely plays a critical role in regulating protein stability. We find it notable that *O*-GlcNAc is predominantly found on regions of proteins that are disordered since it well etablished that proteins with extensive regions of intrinsic disorder are often implicated in protein aggregation disorders including neurodegenerative diseases such as AD, PD, ALS, among others[243].

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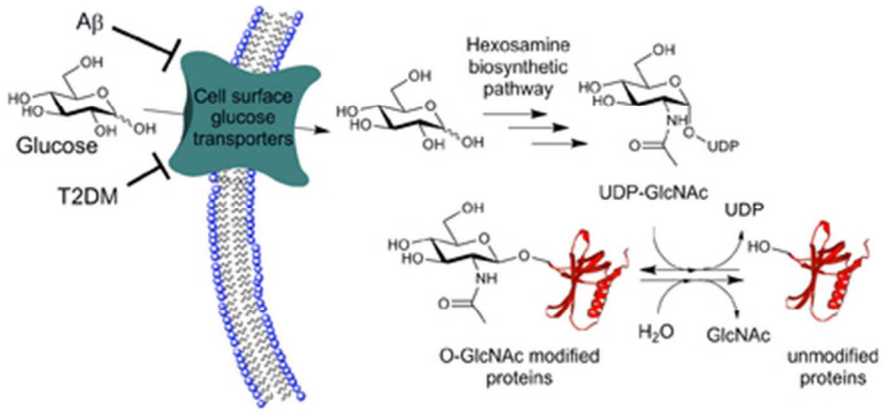

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