α-Synuclein O-GlcNAcylation alters aggregation and toxicity, revealing certain residues as potential inhibitors of Parkinson’s disease

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A compelling link is emerging between the posttranslational modification O-GlcNAc and protein aggregation. A prime example is α-synuclein, which forms toxic aggregates that are associated with neurodegeneration in Parkinson’s and related diseases. α-Synuclein has been shown to be O-GlcNAcylated at nine different positions in vivo. This raises the possibility that O-GlcNAc may alter the aggregation of this protein and could be an important biological mediator of neurodegeneration and a therapeutic target. Here, we expand upon our previous research in this area through the chemical synthesis of six site-specifically O-GlcNAcylated variants of α-synuclein. We use a variety of biochemical experiments to show that O-GlcNAc in general inhibits the aggregation of α-synuclein but can also alter the structure of α-synuclein aggregates in site-specific ways. Additionally, an α-synuclein protein bearing three O-GlcNAc modifications can inhibit the aggregation of unmodified protein. Primary cell culture experiments also show that several of the O-GlcNAc sites inhibit the toxicity of extracellular α-synuclein fibers that are likely culprits in the spread of Parkinson’s disease. We also demonstrate that O-GlcNAcylation can inhibit the aggregation of an aggressive mutant of α-synuclein, indicating that therapies currently in development that increase this modification might be applied in animal models that rely on this mutant. Finally, we also show that the pan-selective antibody for O-GlcNAc does not generally recognize this modification on α-synuclein, potentially explaining why it remains understudied. These results support further development of O-GlcNAcylating agents and therapeutic strategies in neurodegenerative diseases.

O-GlcNAc | synuclein | aggregation | amyloid | Parkinson’s disease

O-GlcNAcylation (Fig. L4) is a widespread intracellular posttranslational modification of serine and threonine residues. In contrast to most other forms of protein glycosylation, O-GlcNAcylase involves the sole addition of the monosaccharide N-acetylglucosamine and can be a dynamic modification through addition by O-GlcNAc transferase (OGT) and subsequent removal by the enzyme O-GlcNAcase (OGA). While the physiological roles of O-GlcNAcylation are diverse, a variety of in vivo and biochemical experiments support an important role for this modification in neurodegenerative diseases (1, 2). For example, tissue-specific knockout of OGT in neurons or even specifically in the forebrain of mice results in neurodegeneration and neuron death (3, 4). Measurement of O-GlcNAc levels in human brains has shown decreased modification in Alzheimer’s disease compared with healthy controls (5, 6). In mice, increasing the amounts of O-GlcNAcylation with a small-molecule inhibitor of OGA (7) slows neurodegeneration and the formation of tau aggregates in a model of Alzheimer’s disease (8). Additionally, enzymatic O-GlcNAcylation of recombinant tau by OGT inhibits the aggregation of this protein in vitro (8, 9). Finally, we have previously contributed to this area by using a semisynthetic strategy to site-specifically O-GlcNAcylate the Parkinson’s disease causing protein α-synuclein at residues 72 or 87 and demonstrated that these modifications display site-specific differences in their ability to inhibit protein aggregation (10, 11). Together, these results support a hypothesis where proper O-GlcNAcylation of certain proteins prevents their aggregation, and loss of this modification is a contributing factor in the development of neurodegenerative diseases. This has encouraged the application of OGA inhibitors that raise the brain O-GlcNAcylations levels as potential treatments for Alzheimer’s and Parkinson’s diseases (7). However, significant fundamental questions remain. For those proteins that contain multiple O-GlcNAcylated sites, like tau and α-synuclein, which of those sites is most inhibitory to aggregation and/or toxicity and should receive the largest attention? Does O-GlcNAc simply stabilize the monomeric state of proteins or can it change the structure of the aggregate, potentially in ways that are less toxic? What are the levels of O-GlcNAcylation of these proteins and do they change during the progression of disease? And finally, how can OGA inhibitors best be tested to explore their potential for the treatment of neurodegenerative diseases? Here, we present results that make progress toward answering all of these questions in the context of α-synuclein and Parkinson’s disease.

Significance

Preventing the aggregation of toxic proteins in neurodegenerative diseases is both an important biological function and a potential therapeutic strategy. Here, we examine the consequences of O-GlcNAcylation on the aggregation and toxicity of α-synuclein, the aggregating protein in Parkinson’s disease. α-Synuclein is modified by O-GlcNAc at least nine different positions in vivo, but the consequences of most of these modifications are unknown. Here, we use synthetic protein chemistry to prepare six different O-GlcNAcylated forms of α-synuclein and show that they have largely inhibitory, but site-specific, effects on the aggregation and cellular toxicity of this protein. These results suggest that O-GlcNAc may be a cellular strategy to prevent protein aggregation, which could potentially be exploited for treatment.


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α-Synuclein (Fig. 1B) is a short, 140-amino-acid protein (12) that exists at relatively high concentrations (~50 μM) at neuronal synapses (13). This protein exists as an unstructured monomer in solution but will form an extended α-helix in the presence of cellular membranes or artificial lipids (14–16), where it can carry out its roles in vesicle trafficking and second-messenger release (17). During neurodegeneration in Parkinson’s disease and other synucleinopathies, the protein will form β-sheet-rich amyloid aggregates that are toxic to cells (18). This aggregation process is likely somewhat heterogeneous but broadly consists of two concentration-dependent steps: nucleation and extension (Fig. 1C) (19). Nucleation is the initiation of aggregate formation from α-synuclein monomers to oligomeric structures and small fibers while extension involves the rapid “seeding” of further monomer aggregation by these initial small fibers. Notably, we have shown that seeding capacity plays a key role in cellular toxicity (20, 21), and this process is increasingly accepted as an important component of the progressive spread of aggregates throughout the brain in vivo (22, 23).

α-Synuclein can be modified by a variety of posttranslational modifications, including O-GlcNAc, that have the potential to significantly impact its stability, aggregation propensity, and toxicity in vivo (24, 25). A variety of in vivo proteomics studies from mice and humans have identified up to nine different sites of O-GlcNAc modification on α-synuclein, including several located within the region of the protein required for aggregation that spans approximately residues 61–95 (Fig. 1B) (26–30). Unlike some other posttranslational modifications (e.g., phosphorylation and acetylation), the only way to directly investigate the site-specific effects of O-GlcNAcylation on protein biophysics and biochemistry is the application of synthetic protein chemistry to build the protein from the “ground up.” Previously, we have applied a powerful version of synthetic protein chemistry, expressed protein ligation (EPL) (31), which enables the use of recombinant and synthetic protein fragments, to investigate the consequences of α-synuclein O-GlcNAcylation at residues 72 or 87 (10, 11). We found that modification of threonine 72 (T72) is more inhibitory than O-GlcNAcylation of serine 87 (S87) but also that neither modification completely prevents the aggregation of α-synuclein.

Here, we dramatically expand the characterization of α-synuclein O-GlcNAcylation. First, we synthesized five different modified protein variants bearing O-GlcNAc at threonines T72, T75, or T81 or serine S87, as well as a triply O-GlcNAcylated protein at T72, T75, and T81. The choice of these sites was motivated by a variety of proteomic, structural, and biological studies. For example, the recent solid-state NMR and cryo-EM structures of the α-synuclein fiber place all four of these modifications in the core of the aggregate (32–34). O-GlcNAc modifications of T72 and T81 have been found in at least two independent proteomics experiments (27–30), including one from human brain tissue, and T75 and S87 O-GlcNAcylation has been found in mice and human red blood cells, respectively (26, 29). We have also shown that S87 is subjected to reciprocal phosphorylation (35), and this position is asparagin in rodent α-synuclein, a mutation that has been shown to alter protein aggregation (36, 37).

Using a variety of biophysical and biochemical techniques we show that individual modification sites uniquely affect the two steps of α-synuclein aggregation, while having very little effect on membrane binding, with O-GlcNAcylation of T75 or T81 having the greatest overall inhibitory potential in vitro. Additionally, multiple O-GlcNAc modifications on α-synuclein endowed it with the ability to inhibit the aggregation of unmodified protein. We also use our previously established fibril-growth assay in neuronal cell culture (21), which we demonstrated plays a key role in neuron apoptosis, to demonstrate that modification at T75 could be the most important O-GlcNAcylation site for slowing progression of Parkinson’s disease. These results support the application of OGA inhibitors, although the specific sites of dynamic O-GlcNAcylation still need to be characterized in vivo. However, robust animal models often rely on the expression of an aggregate formation mutant (A53T) of α-synuclein that causes a familial early-onset version of Parkinson’s disease. Therefore, we also synthesized the triply O-GlcNAcylated analog of this mutant and found that these modifications also blocked both steps of aggregation of this protein, indicating that OGA inhibitors could be tested in a range of preclinical models. Finally, we demonstrate that the most widely used pan-antibodies for O-GlcNAc detection can at best only visualize modification at T72 but at none of the other positions and that additional modification prevents even this limited recognition of T72 O-GlcNAcylation. This result could explain why these modifications have been difficult to detect in the past. Unfortunately, it also demonstrates that novel approaches will be needed to determine the levels and dynamics of α-synuclein O-GlcNAcylation not only in cells, but also in healthy and Parkinson’s disease patients. However, our results further support an important role for O-GlcNAcylation in preventing protein aggregation in neurodegenerative diseases.

**Results**

**Synthesis of Five O-GlcNAcylated Variants of α-Synuclein.** To investigate the effect of site-specific O-GlcNAcylation on α-synuclein, we used EPL to prepare these proteins semisynthetically.
from synthetic and recombinant fragments. An EPL-based strategy requires cysteines residues at any ligation sites between these fragments, but α-synuclein contains no native cysteine residues. This apparent limitation is actually a feature, however, as it allows us to introduce cysteine residues in place of any alanine in α-synuclein and then chemically desulfurize these mutations back to the native sequence at the end of our synthetic route. With these considerations in mind, we deconstructed α-synuclein into three fragments (Fig. 2); an N-terminal protein-histioye synthet that can be obtained from recombinant expression as an in-frame fusion to an intein from Anaabaena variabilis, a synthetic peptide containing a chemically placed O-GlcNAc modification, and a recombinant C-terminal fragment from Escherichia coli. Using this general strategy, we generated five differentially O-GlcNAcylated α-synuclein proteins with glycosylation at either threonine 72 (gT72), threonine 75 (gT75), threonine 81 (gT81), serine 87 (gS87), or a triply modified protein at all three threonine residues (gT72,75,81) (Fig. 2). At each step of the synthesis, the peptides/proteins were purified by RP-HPLC and their identity was confirmed by electrospray ionization mass spectrometry, including the final semisynthetic products (SI Appendix, Fig. S1). Critically, we previously demonstrated that semisynthetic α-synuclein with no modifications prepared by the same route behaves identically to recombinant full-length protein prepared in E. coli (10, 11).

O-GlcNAc Modification Has Little-To-No Effect on Monomeric α-Synuclein or Its Micelle-Bound Structure. With these semisynthetic proteins in hand, we first set out to determine if any of the O-GlcNAc modification events changed the native monomeric and unfolded state of α-synuclein in solution. Comparison of the five O-GlcNAcylated proteins with recombinant, unmodified α-synuclein using dynamic light scattering gave very similar Stokes radii for all of the proteins and confirmed their monomeric states (SI Appendix, Fig. S2). Furthermore, circular dichroism revealed that none of the O-GlcNAc modifications resulted in the formation of any significant secondary structure (SI Appendix, Fig. S3), indicating that these modifications would have no effect on soluble α-synuclein in the cytosol. As mentioned above, the ability of α-synuclein to bind and bend membranes is likely a major physiological function of this protein in neurons by helping to regulate second messenger release. Accordingly, we tested whether O-GlcNAc affected the interaction of α-synuclein with the negatively charged vesicles by incubating the different proteins with a large excess (1:100) of the negatively charged lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1-rac-glycerol) for 20 min. Subsequent analysis by circular dichroism readily shows the formation of the expected α-helix (minima around 208 and 222 nm) by unmodified α-synuclein in each of the experiments (SI Appendix, Fig. S4). Individual O-GlcNAc modifications at all four sites had essentially no effect on α-helix formation. The triply modified protein [α-synuclein (gT72,75,81)] displayed a slight decrease in secondary structure formation, which may not be surprising as it would represent a cumulative effect of the modifications. This indicates that O-GlcNAcylation may not have a large effect on the normal biology of α-synuclein in contrast to phosphorylation that has been shown to more strongly alter the micelle-bound structure (35). However, these in vitro systems are artificial and need to be corroborated with cellular assays before any definitive conclusions can be drawn.

O-GlcNAc Inhibits Monomeric α-Synuclein Aggregation (Nucleation) with Site-Specific Differences. We next moved to test if O-GlcNAcylation can block the initiation of α-synuclein aggregation by inhibiting the nucleation of protein monomers. To perform this analysis, we simultaneously subjected either unmodified α-synuclein or an O-GlcNAcylated variant to aggregation conditions (50 μM protein concentration, agitation at 1,000 rpm, and 37 °C) for 7 d. Notably, this concentration is within the measured physiological range of α-synuclein concentrations in vivo (13). Reaction aliquots were removed after different lengths of time (0, 48, 96, and 168 h) and then added to a solution of the dye thioflavin T (ThT), which displays increased fluorescence upon binding of amyloid aggregates. In all of the experiments, unmodified α-synuclein showed robust aggregation with a 20- to 30-fold increase in ThT fluorescent signal (Fig. 3A). However, the O-GlcNAcylated proteins showed interesting differences in their inhibitory capacity (Fig. 3A). Consistent with our previous results, α-synuclein(gT72) was a very poor aggre gator while α-synuclein(gS87) did form amyloid fibers but with slightly slower kinetics. α-Synuclein(gT75) displayed a similar inhibitor capacity compared with α-synuclein(gT72), while α-synuclein(gT81) was the most inhibitory of all of the individual O-GlcNAc modifications. Notably, α-synuclein(gT72,75,81) was completely refractory to any aggregation. To examine the structure of any aggregates that formed in these reactions, we
analyzed the 168-h timepoint using transmission electron microscopy (TEM) and visualized structures that were highly consistent with the ThT data (Fig. 3B and SI Appendix, Fig. S5). Unmodified α-synuclein formed long regular fibers, as expected, while α-synuclein(gS87) formed fibers that were shorter and of a grossly different shape. This was followed by α-synuclein(gT72) that formed very small and broken fiber segments and α-synuclein(gT75) and (gT81) that formed rare and highly irregular aggregates. Finally, we did not observe any aggregates from α-synuclein(gT72,75,81) despite scanning significant areas of the TEM grid. Importantly, these results are largely consistent with our previously published analysis of α-synuclein(gT72) and (gS87) but highlight the potentially greater importance of O-GlcNAcylation at T75 or T81.

To evaluate qualitative structural differences between different α-synuclein aggregates proteinase-K (PK) digestion has regularly been employed. PK displays broad selectivity in the α-synuclein primary sequence and will essentially completely degrade the unfolded protein. However, when aggregates (either fibers or oligomers) are formed, they will inhibit the accessibility of the aggregated region to PK, resulting in stabilized fragments that can be visualized by SDS/PAGE and staining. This type of analysis, demonstrating that this protein does not form any aggregates. An additional prominent band at a slightly higher molecular weight is clearly visible from α-synuclein(gT72) and (gS87) all display a band of ~12 kDa that likely corresponds closely to the prominent degradation product of unmodified protein. An additional prominent band at a slightly higher molecular weight is clearly visible from α-synuclein(gS87) aggregates. PK digestion of α-synuclein(gT81) results in a different pattern of bands with a notable band at a uniquely low molecular weight. Finally, triply O-GlcNAcylated protein formed only one band that was essentially totally destroyed upon increased PK treatment, further demonstrating that this protein does not form any aggregates. Again, these PK results are very consistent with our ThT and TEM analysis.

Together, our ThT, TEM, and PK results demonstrate that α-synuclein O-GlcNAcylates itself has a significantly inhibitory effect on the aggregation of α-synuclein monomers in solution, the nucleation step of the process. Additionally, they demonstrate that when aggregates do form, the site-specific nature of the O-GlcNAc modification can “force” the protein into aggregate structures that differ from unmodified protein. Next, we tested the possibility that O-GlcNAcylated α-synuclein would inhibit the aggregation of unmodified protein when they are both present in the same reaction. We previously found
that this is not the case with the single modification of α-synuclein (gT72) (10). Combined with the fact that the triply modified protein completely prevents any fiber formation, we chose to focus on α-synuclein(gT72,75,81). Specifically, we subjected unmodified α-synuclein at either 25 or 50 μM concentrations or a one-to-one mixture of α-synuclein and α-synuclein(gT72,75,81) to a total concentration of 50 μM to aggregation conditions. As expected, analysis by ThT fluorescence and TEM shows that unmodified α-synuclein at 25 μM forms fibers more slowly than the corresponding 50 μM reaction conditions (SI Appendix, Fig. S6). Intriguingly, the one-to-one mixture of modified and unmodified α-synuclein yielded the least aggregation, indicating that higher levels of O-GlcNAcylation on individual α-synuclein proteins could act substoichiometrically to slow fiber formation.

The Majority of O-GlcNAcylation Events Block the Extension of α-Synuclein Fiber “Seeds” into Larger Aggregates. We then examined whether O-GlcNAcylation can inhibit the extension step of protein aggregation that appears to play an important role in the spread of Parkinson’s disease pathology and neurodegeneration in the brain. First, we generated unmodified α-synuclein fibers by subjecting the corresponding monomeric protein (50 μM concentration) to aggregation conditions for 7 d. After this time, the mature aggregates were separated from any remaining monomer by centrifugation and then subjected to tip sonication to generate a high concentration of seeds, or preformed fibers (PFFs), that have the potential to be extended by additional monomeric α-synuclein. These seeds were then added to either buffer, additional unmodified protein, or the different O-GlcNAcylated α-synuclein variants (25 μM monomeric protein concentration, 20% PFFs by weight based on monomeric protein) and the resulting mixtures agitated for 48 h. After different lengths of time, aliquots were removed and analyzed by ThT fluorescence (Fig. 4A). As expected, the PFFs added to buffer showed no increase in ThT signal, while their addition to unmodified α-synuclein resulted in a 30- to 50-fold increase in fluorescence by the termination of the assay, resulting from the extension of the seeds. When the PFFs were aggregated with the triply modified protein, α-synuclein(gT72,75,81), the extension into larger aggregates was completely blocked. Each of the individually O-GlcNAcylated proteins also inhibited the extension reaction to different extents. Notably, however, the inhibitory capacity differed from the trend seen in the nucleation reactions above. Consistent with our previous results (10), α-synuclein (gT72) increases the lag phase of aggregation over 48 h, but not the plateau of ThT fluorescence. The next least inhibitory modification at S87 more dramatically increased the lag phase of aggregation and resulted in a plateau ∼50% of the ThT fluorescence compared with unmodified monomer at the conclusion of the assay. Finally, α-synuclein(gT75) and (gT81) were more potent and very similar in both the increase in aggregation lag time and lower plateau levels. Gratifyingly, visualization of the aggregates formed after 48 h by TEM confirmed our ThT results (Fig. 4B and SI Appendix, Fig. S7). As expected, long fibers were easily visible from PFFs extended with either unmodified protein or α-synuclein(gT72), while the fibers formed after α-synuclein(gS87) treatment are a combination of long fibers and short “plate-like” oligomers (SI Appendix, Fig. S8). α-Synuclein(gT81) formed a combination of irregular fiber-like structures as well as the structures similar to the oligomers formed by α-synuclein(gS87) (SI Appendix, Fig. S8). Finally, the structures formed from α-synuclein(gT75) or (gT72,75,81) appear to be at best PFFs surrounded by amorphous protein aggregates (gT75) or no material at all (gT72,75,81). These findings suggest that this triple-modification pattern alters the structures that monomeric α-synuclein can adopt and makes it incompatible or incapable of adopting a confirmation that is aligned with that of α-synuclein at the ends of the growing fibrils. To evaluate the structure and stability of these aggregates, we again employed PK digestion (SI Appendix, Fig. S9). Interestingly, all of the proteins showed a similar digestion pattern, indicating that unmodified PFFs template and control the structure of the fibers in the extension reaction. However, the different O-GlcNAcylation sites again showed different amounts of stable fibers, highly consistent with our other data. These results show that O-GlcNAcylation sites again showed different amounts of stable fibers, highly consistent with our other data. These results show that O-GlcNAcylation at certain sites on α-synuclein is capable of inhibiting the extension of unmodified PFFs. Notably, the PK digestion suggests that any fibers formed during this extension reaction are determined by the confirmation of the unmodified PFFs. For certain O-GlcNAcylation sites, gT81 and gS87, this inhibition potentially promotes the formation of oligomers, and unmodified oligomers have previously been shown to be toxic (38).

Fig. 4. Most, but not all, O-GlcNAcylation sites inhibit the extension step of α-synuclein aggregation. (A) PFFs were formed by aggregation unmodified α-synuclein (50 μM) for 168 h, followed by sonication. PFFs were then added to either buffer, additional unmodified α-synuclein, or the indicated O-GlcNAc modified proteins (25 μM monomeric protein concentration, 20% PFFs). These mixtures were subjected to aggregation conditions (agitation at 37 °C) and aliquots were removed at the indicated times for analysis by ThT fluorescence (λem = 450 nm, λex = 482 nm). The y axis shows fold change in fluorescence compared with PFFs only at t = 0 h. Results are mean ± SEM of three experimental replicates. Aggregation of α-synuclein(gT72) and (gS87) were performed simultaneously and compared with the same unmodified protein control. Likewise, α-synuclein(gT75) and (gT871) aggregation reactions were performed simultaneously. (B) The same reactions were analyzed by TEM after 168 h.
O-GlcNAcylation Inhibits α-Synuclein Toxicity. Next, we explored whether O-GlcNAc modifications reduce the toxicity of exogenous α-synuclein administered to neurons in culture. While the initial steps of α-synuclein aggregation are thought to be largely intracellular, a causative role for extracellular α-synuclein in the progression of Parkinson’s disease has been established in cell culture and in vivo (39–41). For example, intracerebral injection of PFFs from recombinant α-synuclein causes progressive neurodegeneration and seeds additional aggregation in mice (40). Critically, these results also allow the toxicity of different recombinant and synthetic variants to be tested in a physiologically relevant fashion. Recently, we established an assay in neuronal culture that uses a combination of PFFs and α-synuclein monomer to monitor the toxicity associated with overall amyloid formation (21). Importantly, we showed that this process at the neuronal membranes results in increased apoptosis of neurons compared with treatment with PFFs alone. We chose to employ this assay here, as it would more likely reflect the endogenous biology where combinations of unmodified and O-GlcNAcylated α-synuclein are likely to coexist. Additionally, it tests the potential for O-GlcNAcylated protein to slow the spread of aggregates and the progression of disease. Accordingly, hippocampal neurons were isolated from the brains of P0 mouse pups and cultured for 14 d. At this time, the neurons were treated with monomeric, unmodified α-synuclein or the different glycosylated variants for six additional days. The integrity of the cellular membranes was then determined using both lactate dehydrogenase (LDH) release and the SYTOX Green fluorescence, and neuron apoptosis was measured by visualizing caspase-3 activity (SI Appendix, Fig. S10). As expected from our previous experiments, the unmodified monomer induced no detectable toxicity, which was replicated by all of the O-GlcNAcylated variants.

Simultaneously, we also treated neurons with either unmodified PFFs alone or in combination with the different monomeric proteins (11% PFFs and 89% monomer by weight based on monomeric protein) and assayed cellular toxicity using the same assays (Fig. 5A). As expected, PFFs exhibited some toxicity that was increased upon the cotreatment of PFFs and unmodified monomer.

Fig. 5. O-GlcNAcylation site-specifically inhibits neuronal toxicity in a membrane-based extension model. (A) Hippocampal neurons were plated in 96-well plates and treated with either Tris buffer, unmodified α-synuclein PFFs, or these PFFs plus the indicated monomeric proteins. After 6 d, compromised cellular membranes were measured using SYTOX Green or LDH release, and cell death was measured using caspase-3 activation. In the case of SYTOX Green and LDH release, cell fixation (paraformaldehyde (PFA)) and cell lysate were used as positive controls. Data shown represent the mean ± SEM of three independent biological experiments, each performed in triplicate. Statistical significance was determined using a one-way ANOVA test followed by Dunnett test (PFFs plus unmodified α-synuclein versus PFFs plus O-GlcNAcylated proteins). N.S., not significant. (B) Hippocampal primary neurons were plated on coverslips and treated with PFFs in the absence or in the presence of the indicated monomeric proteins. At the indicated time, the neurons were washed three times before fixation and stained using a total α-synuclein antibody (epitope 1–20). Neurons were counterstained by MAP2, a specific neuronal marker (green), and the nucleus were stained by DAPI. White arrows show the deposition of the PFFs at the outer plasma membrane. Asterisks indicate a growing aggregate at the cell plasma membrane. The data are consistent between two biological replicates.
Again, we observed interesting site-specific differences for the different O-GlcNAcylated proteins. In agreement with our in vitro aggregation reactions (Fig. 4), cotreatment of PFFs with α-synuclein(gT75) or (gT72,75,81) resulted in significantly less toxicity compared with PFFs plus unmodified monomer and similar to PFFs alone. Additionally, α-synuclein(gT81) and α-synuclein(gS87), which both formed oligomers (Fig. 4 and SI Appendix, Fig. S8) showed increased toxicity compared with PFFs alone (P < 0.05–0.01, one-way ANOVA test followed by Dunnett test). These results are consistent with these oligomers being toxic to neurons. However, it is also possible that α-synuclein aggregation in the presence of membranes is different from the same reaction in solution, as it requires the PFFs and α-synuclein monomers to be accommodated on the membrane in an orientation that enables PFF extension (42). Finally, we observed no increase in toxicity upon cotreatment of PFFs and α-synuclein(gT72), despite its ability to form mature fibers in our in vitro extension assay (Fig. 4). Again, this observation could reflect membrane-associated differences in α-synuclein aggregation.

To better understand the toxicity of α-synuclein(gT72) and (gT81), we next investigated the aggregation of these proteins on the neurons by immunocytochemistry (ICC). Primary neurons were again treated with PFFs alone or in combination with unmodified α-synuclein, α-synuclein(gT72), or (gT81) for up to 6 d (Fig. S8). As previously shown (21), the addition of PFFs alone resulted in the formation of a thick layer of α-synuclein at the cell plasma membrane that is visible in as little as 6 h and relatively consistent over 6 d (Fig. 5B, indicated by the white arrows). Also in line with our published results, the addition of a mixture of PFFs and unmodified α-synuclein monomers resulted in the formation of an entangled network of α-synuclein aggregates that grew over time (Fig. 5B, indicated by the white asterisks). In this model, α-synuclein PFFs that bind to the extracellular face of the plasma membrane serve as seeds for the aggregation of the additional monomeric protein, leading to the formation of formed toxic fibrils that could in turn serve as secondary nucleation sites for the formation of highly toxic oligomeric and fibrillar species (21, 38, 43, 44). Treatment with PFFs and α-synuclein(gT72) monomers also led to the deposition of fibrils at the plasma membrane and promoted the growth of large aggregates over time (Fig. 5B, white asterisks). These data are largely in line with our in vitro seeding assay showing that O-GlcNAcylation at T72 does not prevent the extension of unmodified PFFs (Fig. 4). However, these aggregates are different in shape and appear less “compact” and uniform compared with the analogous treatment with unmodified monomers, potentially explaining their reduced toxicity. Finally, incubation with PFFs and α-synuclein(gT81) was largely indistinguishable from treatment with PFFs alone (Fig. 5B, white arrows). This result corroborates our in vitro finding that gT81 inhibits the extension of PFFs, resulting in the formation of irregular fibril-like structures and oligomers (Fig. 4 and SI Appendix, Fig. S8). Based on our all data, we believe that the mixture of PFFs and α-synuclein(gT81) monomers promotes the formation of toxic oligomers instead of fibers, which could be driving the toxicity in neurons and is an important topic of future study.

O-GlcNAc Inhibits the Aggregation of an Early-Onset Parkinson’s Disease Mutant of α-Synuclein, Opening a Potential Opportunity for Additional in Vivo Studies. All of the above data suggest that increasing the levels of α-synuclein O-GlcNAcylation in the brain with an OGA inhibitor might slow the progression of Parkinson’s disease. Unfortunately, the vast majority of Parkinson’s cases are sporadic and undetected until the presentation of symptoms that are thought to arise from an already significant burden of protein aggregates. Therefore, it is not necessarily surprising that any treatment strategy aimed at inhibiting further α-synuclein aggregation would be minimally efficacious. Additionally, genetic mouse models of Parkinson’s disease built around wild-type α-synuclein have yielded variable results (45). Importantly, there are several mutations in α-synuclein that similarly cause early-onset Parkinson’s disease. We chose to focus on the most well-characterized of these mutations, alanine 53 to threonine (A53T) (46). α-Synuclein(A53T) forms aggregates more rapidly than wild-type protein in vitro and causes Parkinson’s symptoms in patients in their 40s, and has been used to create mouse models that give some clinical phenotypes. Accordingly, we first prepared the triply glycosylated version of this mutant protein, α-synuclein(A53T, gT72,75,81), using the same synthetic scheme that we employed for the wild-type proteins (SI Appendix, Fig. S11). Consistent with our results on α-synuclein(gT72,75,81), O-GlcNAcylation of α-synuclein(A53T) did not affect the unstructured, monomeric state of the protein and only had a small effect on its micelle-bound structure (SI Appendix, Fig. S12). In contrast, these three O-GlcNAc modifications were again able to completely block both the nucleation and extension steps of α-synuclein(A53T) aggregation, as determined by ThT fluorescence, TEM, and PK cleavage (Fig. 6). These results support the application of OGA inhibitors in α-synuclein(A53T) expressing mice to begin to examine the potential for O-GlcNAcylation to slow the progression of Parkinson’s disease symptoms in these models.

Discussion

Here, we used synthetic protein chemistry to prepare a small panel of differentially O-GlcNAcylated α-synuclein proteins, which are otherwise inaccessible via standard protein expression systems, and determined how they affect protein aggregation and toxicity. More specifically, we found that modification of T72, T75, or T81 strongly inhibits the nucleation step of aggregation, but O-GlcNAc at S87 only slightly slows the kinetics of this process (Fig. 3). Additionally, all four modifications, including at S87, significantly alter the structure of the aggregates that do form (Fig. 3C). Interestingly, O-GlcNAcylation of T75, T81, or S87 inhibits the extension of PFFs formed from unmodified α-synuclein, but modification at T72 does not (Fig. 4). However, O-GlcNAcylation of T81 or S87 promotes the formation of plate-like oligomers (SI Appendix, Fig. S8), while modification at T75 does not. These data on T72 and S87 O-GlcNAcylation are largely consistent with our published studies (10, 11). Our NMR analysis of T72 O-GlcNAcylation showed stronger inhibition of aggregation by this modification. However, our current aggregation reaction setup is better temperature controlled, which we believe explains these differences by more efficiently promoting aggregation. The triply glycosylated material was completely refractory to both steps of aggregation in vitro (Figs. 3 and 4). Additionally, this higher level of modification was able to inhibit the aggregation of unmodified protein in a coaggregation experiment (SI Appendix, Fig. S6).

Data from the structural models of the α-synuclein fiber can be used to explain many of our observations (32–34). While all of the modification sites we analyzed here are in the core of the aggregate, T72 faces toward a portion of the fiber (residues 51–67) that was more flexible in the NMR, providing one explanation as to why it can be more easily accommodated into a growing fiber in the extension step of aggregation. S87 lies toward the end of the aggregate core, approximately residues 61–95 as determined by NMR and EPR spectroscopy and cryo-EM (32–34, 47, 48). This potentially explains how O-GlcNAcylation at this site alters the structure of the aggregate core as visualized by PK digestion. Based again on the structural models, we hypothesize that modification of S87 “shrinks” the core of the fiber by preventing the participation of the last 10–15 amino acids, although other scenarios are certainly possible. In either case, it is consistent with experiments from the Lee laboratory showing that mutation of S87 to asparagine, the native residue of mouse
obtained results that are largely consistent with our in vitro aggregation and structural studies (Figs. 4 and 5). Both α-synuclein(gT75) and α-synuclein(gT72,gT75,81) were essentially harmless in the toxicity assays (Fig. 5 A). In contrast, α-synuclein(gT81) and α-synuclein(gS87) that form oligomeric species when added to PFFs (Fig. 4 and SI Appendix, Fig. S8) were toxic to cells (Fig. 5 A). This result is not necessarily surprising, as oligomers are known to be highly toxic. In support of this model, we used ICC to directly observe this extension reaction on neurons in culture, and we did not find any extension of the PFFs by α-synuclein(gT81) into large extracellular aggregates (Fig. 5 B). As noted above, our results with α-synuclein(gT72) are somewhat divergent, as this protein does not inhibit the extension of PFFs in vitro (Fig. 4) but does not induce toxicity in cultured neurons (Fig. 5 A). Analysis by ICC potentially explains this difference. The addition of PFFs and α-synuclein(gT72) results in the formation of large aggregates at the cell membrane, but they appear different in their shape and compactness compared with the unmodified monomer control (Fig. 5 B). This might be explained by the fundamental differences in α-synuclein aggregation in the absence or presence of cellular membranes (42). Notably, T72 has been shown to point directly away from the lipid bilayer when α-synuclein forms its extended α-helix (14). This alignment potentially explains how O-GlcNAc at T72 could alter the interaction of α-synuclein monomers with PFFs and therefore the extension and toxicity at membranes. However, there are other potential toxicity mechanisms at play. For example, it has been consistently observed that upon internalization, extracellular PFFs can serve as seeds and promote the formation and accumulation of toxic, intracellular α-synuclein inclusions over time (39, 40, 49). Therefore, it is possible that the different O-GlcNAc modifications also affect this process. We believe that it is actually likely that multiple aggregation pathways exist that could be exerting their toxic effects through multiple cellular mechanisms, and we plan to test the effects of O-GlcNAc on the internalization and seeding of PFFs in neurons in the future.

Together, these results indicate that O-GlcNAcylation of T75 and potentially T72/T81 are the most important modifications from the perspective of α-synuclein aggregation and potentially neurodegeneration, as the relative importance of the different aggregation steps and conditions (i.e., on-membrane versus solution) has yet to be determined. An obvious next step will be the determination of these modifications in animal models of Parkinson’s disease, and OGA inhibitors of the function in the brain are available (7, 8). However, many Parkinson’s disease animal models that recapitulate α-synuclein aggregation and neurodegeneration rely on familial mutants of α-synuclein (45). We show here that O-GlcNAcylation is also capable of inhibiting the aggregation of one such mutant, α-synuclein(AS3T). We hope that this encourages the targeted testing of OGA inhibitors in these animal models. Finally, it is still unknown how much α-synuclein is O-GlcNAcylated in healthy neurons, whether this modification is lower in Parkinson’s disease patients, or even if the amounts of this modification can be increased in cells or in vivo. To determine if current Western blotting reagents could be used to explore this question, we attempted to visualize the O-GlcNAc modifications on our synthetic proteins using the most widely used global O-GlcNAc antibodies CTD110.6 (50) and RL2 (51) (SI Appendix, Fig. S13). We found that CTD110.6 did not recognize any of the O-GlcNAc modifications, while RL2 only weakly binds to modification at T72 and does not recognize the remainder of the singly O-GlcNAcylated sites. Furthermore, RL2 identification of the T72 modification is blocked by additional O-GlcNAcylation at T75 and T81. While this result can help explain why α-synuclein O-GlcNAcylation remained undiscovered until recently with the advent of modern proteomic ionization techniques, it also means that alternative approaches (i.e., chemoenzymatic detection) will need to be utilized to determine the in vivo relevance of α-synuclein O-GlcNAcylation.
in Parkinson’s disease patients and to evaluate animal models, a problem we are currently exploring.

In summary, our data presented here further support a model where O-GlcNAcylation may inhibit aggregation and play a protective role in Parkinson’s disease and other protein aggregation disorders. The process of synuclein aggregation and toxicity is highly complex in Parkinson’s disease. There are multiple types of interactions that do not all lend itself to contribute to the formation of toxic species, including nucleation of synuclein intracellularly, extension of PFFs both intracellularly and on membranes, secondary nucleation on PFFs, and uptake and seeding of PFFs into previously healthy cells. Here, we show that several O-GlcNAcylation sites inhibit the nucleation of aggregation in vitro and that modification of T75 or multiple O-GlcNAc sites can also block the extension of PFFs in solution. Additionally, these same sites and α-synuclein(gT72) can block toxicity during PFF extension on cellular membranes. It remains to be seen whether O-GlcNAc can also affect the uptake and intracellular seeding of monomeric α-synuclein, a question we are currently exploring.

Materials and Methods
Vertebrate animal experiments were approved by the Ecole Polytechnique Fédérale de Lausanne institutional review board. Experimental procedures for α-synuclein semisynthesis and purification; dynamic light scattering analysis; α-synuclein/membrane interaction assay; CD spectroscopy; α-synuclein aggregation reactions; analysis of protein aggregation by ThT, TEM, and PK digestion; primary neuron toxicity analysis; and ICC analysis are described in SI Appendix, Experimental Methods.

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24. Oueslati A, Fournier M, Lashuel HA (2010) Role of post-translational modifications in α-synuclein associated with Parkinson disease and other protein aggregation disorders. The process of synuclein aggregation and toxicity is highly complex in Parkinson’s disease. There are multiple types of interactions that do not all lend itself to contribute to the formation of toxic species, including nucleation of synuclein intracellularly, extension of PFFs both intracellularly and on membranes, secondary nucleation on PFFs, and uptake and seeding of PFFs into previously healthy cells. Here, we show that several O-GlcNAcylation sites inhibit the nucleation of aggregation in vitro and that modification of T75 or multiple O-GlcNAc sites can also block the extension of PFFs in solution. Additionally, these same sites and α-synuclein(gT72) can block toxicity during PFF extension on cellular membranes. It remains to be seen whether O-GlcNAc can also affect the uptake and intracellular seeding of monomeric α-synuclein, a question we are currently exploring.

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