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Authors

Marotta, Nicholas P
Lin, Yu Hsuan
Lewis, Yuka E
et al.

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O-GlcNAc modification blocks the aggregation and toxicity of the Parkinson's disease associated protein α-synuclein

Nicholas P. Marotta[1], **Yu Hsuan Lin**[1], **Yuka E. Lewis**[1], **Mark R. Ambroso**[2], **Balyn W. Zaro**[1], **Maxwell T. Roth**[3], **Don B. Arnold**[3], **Ralf Langen**[2], and **Matthew R. Pratt**[1,3,4]

[1]Department of Chemistry, University of Southern California, Los Angeles, CA 90089, United States

[2]Department of Biochemistry and Molecular Biology and the Zilka Neurogenetic Institute, University of Southern California, Los Angeles, CA 90089, United States

[3]Department of Molecular and Computational Biology, University of Southern California, Los Angeles, CA 90089, United States

Abstract

Several aggregation-prone proteins associated with neurodegenerative diseases can be modified by O-linked *N*-acetyl-glucosamine (O-GlcNAc) *in vivo*. One of these proteins, α-synuclein, is a toxic aggregating-protein associated with synucleinopathies, including Parkinson's disease. However, the effect of O-GlcNAcylation on α-synuclein is not clear. Here, we use synthetic protein chemistry to generate both unmodified α-synuclein and α-synuclein bearing a site-specific O-GlcNAc modification at the physiologically-relevant threonine residue 72. We show that this single modification has a notable and substoichiometric inhibitory-effect on α-synuclein aggregation, whilst not affecting the membrane binding or bending properties of α-synuclein. O-GlcNAcylation is also shown to affect the phosphorylation of α-synuclein *in vitro* and block the toxicity of α-synuclein that was exogenously added to cells in culture. These results suggest that increasing O-GlcNAcylation may slow the progression of synucleinopathies and further support a general function for O-GlcNAc in preventing protein aggregation.

INTRODUCTION

O-GlcNAc modification (Fig. 1a) is the enzymatic addition of the single monosaccharide *N*-acetyl-glucosamine to proteins in the cytosol, nucleus and mitochondria[1,2]. This

[4]Correspondence should be addressed to matthew.pratt@usc.edu.

AUTHOR CONTRIBUTIONS

N.P.M, YH. L., Y.E.L., M.R.A, B.W.Z, M.R., D.B.A., R.L, and M.R.P. designed experiments and interpreted data. N.P.M. carried out the synthesis of the synuclein proteins, performed aggregation reactions and collected CD and DLS spectroscopy, ThT measurements, and TEM images. YH. L. performed cell-death assays. Y.E.L. performed mutant synuclein experiments. M.R.A. performed membrane binding experiments. B.W.Z. performed SDS-PAGE analysis. M.R. collected primary neurons. N.P.M, YH. L., Y.E.L., and M.R.P. prepared the manuscript.

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modification, which occurs on serine and threonine side-chains, is a uniquely dynamic form of glycosylation through the addition by the enzyme O-GlcNAc transferase (OGT) and removal by the enzyme O-GlcNAcase (OGA). Several lines of evidence link O-GlcNAcylation to neurodegenerative diseases[3,4]. Neuron-specific knockout of OGT in mice results in locomotor defects, increased hyperphosphorylated tau, and death by 10 days after birth[5]. Several aggregation-prone proteins that directly contribute to neurodegeneration are themselves modified by O-GlcNAc, including tau[6,7] and α-synuclein[8,9]. Increasing O-GlcNAcylation *in vivo* using small-molecule inhibitors of OGA has been shown to reduce hyperphosphorylation of tau in healthy rats[10] and slow neurodegeneration in a mouse model of Alzheimer's disease[11]. Additionally, experiments where recombinant tau was enzymatically O-GlcNAcylated demonstrated that this modification can directly block that aggregation of tau *in vitro*[11]. Therefore, the maintenance of "normal" O-GlcNAcylation levels may be a protective mechanism that inhibits protein aggregation and is potentially lost in neurodegenerative diseases.

We are interested in examining the consequences of O-GlcNAcylation on α-synuclein, the aggregating protein in Parkinson's disease and other synucleinopathies[12]. α-Synuclein is a 140 amino-acid protein that is prevalent in pre-synaptic neurons of the central nervous system[13]. In the cytosol, the protein is an unfolded monomer, while it adopts an extended α-helical conformation when associated with cellular membranes, where it performs its likely physiological roles in vesicle trafficking[14]. In contrast, in diseased cells, α-synuclein consists of β-sheet rich aggregates that are amyloid in structure (Fig. 1b)[15]. A range of aggregate-species that form *in vitro* closely resemble the aggregates in Parkinson's disease brain-samples, biochemically supporting their importance in disease. Furthermore, recent experiments in human patients, animal models, and cell culture have shown that extracellular α-synuclein aggregates are toxic and that the protein is likely transferred from neuron-to-neuron where it can propagate disease pathology[16–18]. O-GlcNAc has been identified as an *in vivo*, endogenous modification of α-synuclein at threonine 64 and 72 in mice and serine 87 in humans, residues that are conserved between the species[8,9,19], raising the possibility that O-GlcNAcylation may affect α-synuclein aggregation (Fig. 1c). In support of this hypothesis, we previously demonstrated that addition of synthetic peptides corresponding to fragments of the aggregation-prone region of α-synuclein (residues 61–95) will accelerate the *in vitro* aggregation of full-length protein[20]; however, the O-GlcNAcylated peptides at threonine 72 (T72) do not have any effect on the kinetics of aggregation. Unfortunately, in the same study we also found that although recombinant tau is O-GlcNAcylated by OGT[11,21], α-synuclein is not[20]. This is not necessarily surprising as OGT appears to require accessory proteins in order to modify some of its substrates[22], but it does prevent the use of this method to study α-synuclein O-GlcNAcylation.

Here, we investigate the consequences of T72 O-GlcNAcylation on full-length α-synuclein. We selected T72 as our first target for O-GlcNAc modification since it has been identified in multiple proteomics experiments[8,9] and because it lies within the region of α-synuclein that is required for aggregation *in vitro* (residues 71–82). First, we demonstrate that mutation of α-synuclein residue 72 to alanine (T72A), which would prevent O-GlcNAcylation at this position, dramatically reduces the aggregation of the protein. This would make any loss-of-

function studies in cell culture or animals extremely difficult to interpret. To overcome this roadblock, we then used synthetic protein chemistry to generate site-specifically O-GlcNAcylated α-synuclein at T72 for subsequent gain-of-function experiments. Comparing this protein to either synthetic, unmodified material or completely recombinant material, a well-accepted standard in the field of α-synuclein biochemistry, we show that the single O-GlcNAc modification at T72 completely blocks the formation of both fiber and oligomer aggregates but has no effect on membrane binding or bending. We then demonstrate that O-GlcNAcylation inhibits the toxicity of α-synuclein when it is exogenously added to neurons in culture. Since O-GlcNAcylation can affect subsequent phosphorylation, we also show that O-GlcNAcylation at T72 alters physiologically-relevant phosphorylation events on α-synuclein by three different kinases. We further demonstrate that O-GlcNAcylation can act in a sub-stoichiometric fashion to slow α-synuclein aggregation. Finally, to explore the mechanism behind these observations, we find that O-GlcNAcylation primarily blocks aggregation by preventing the incorporation of α-synuclein into aggregates and thereby lowering the effective concentration of aggregation-prone material. These studies support an important role for O-GlcNAcylation in potentially inhibiting the progression of not only Alzheimer's disease but Parkinson's disease as well.

RESULTS

An α-Synuclein loss-of-function O-GlcNAcylation mutant has compromised aggregation

One common method to investigate the effects of posttranslational modifications in living cells or animal models is the expression of a loss-of-function point mutant of the protein of interest that cannot be endogenously modified. For example, the consequences of phosphorylation of α-synuclein serine 129 have been studied using a serine to alanine (S129A) mutation[23]. Therefore, one possibility to understand the role of O-GlcNAcylation would be overexpression of either wild-type or a T72A mutant protein and compare their effects in living cells. However, several studies have found that mutations in the region of α-synuclein that is responsible for aggregation, including T72 to proline or glutamic acid, can themselves have dramatic effects on the aggregation of the protein[24]. Therefore, we recombinantly expressed both wild-type α-synuclein and the mutant protein α-synuclein(T72A) (Supplemental Fig. S1) and subjected them to aggregation conditions (50 μM protein concentration at 37 °C with constant agitation) for 4 days. To determine the extent of aggregation, a combination of thioflavin T (ThT) fluorescence and and transmission electron microscopy (TEM) were employed. Notably, we chose to use an end-point-type assay, as the presence of ThT in continuous assays was recently shown to accelerate aggregation[25]. Analysis by fluorescence showed that wild-type protein aggregated with the expected kinetics beginning around 24 h, while α-synuclein(T72A) only displayed a small amount of ThT signal at 72 and 96 h (Supplementary Fig. S2). Analysis by TEM showed that both proteins form fiber structures of the expected diameter of ~10 nm (Supplementary Fig. S2). These data demonstrate that mutation of α-synuclein at T72 to prevent O-GlcNAcylation has a direct inhibitory effect on the aggregation of the protein, rendering any loss-of-function experiments by expression living cells impossible to accurately interpret.

Synthesis of O-GlcNAcylated α-synuclein

Since the loss-of-function mutation (T72A) in α-synuclein itself inhibits aggregation, we chose to directly test the effects of O-GlcNAcylation at this residue by preparing the protein semi-synthetically by expressed protein ligation (EPL). The only absolute requirement for the semisynthesis of proteins using traditional EPL is a cysteine residue at any ligation sites. α-Synuclein contains no native cysteine residues, so we chose to introduce cysteines at residues 69 and 76, which are normally alanine residues in the native primary sequence. Introduction of these cysteine residues enables the retrosynthesis of α-synuclein (Fig. 2a) into a synthetic peptide (**1**, residues 69–75), a recombinant protein with an N-terminal cysteine residue (**2**, residues 76–140) and a recombinant protein thioester (**3**, residues 1–68). We reasoned that the introduced cysteines could be chemically desulfurized to alanine residues, yielding semisynthetic α-synuclein with no amino-acid mutations. As schematized in Fig. 2b, peptide **1** was prepared using standard Fmoc-based solid-phase peptide synthesis on the Dawson aminobenzyol resin that enables the generation of C-terminal peptide thioesters (Supplementary Fig. S3)[26]. Importantly, the N-terminal cysteine residue remained protected as a thioproline to prevent auto-ligation. Protein **2** was heterologously expressed in *E. coli* (Supplementary Fig. S4). Notably, we found that the initiating methionine-residue was conveniently removed during expression by an endogenous methionine aminopeptidase. Incubation of peptide **1** and protein **2** resulted in formation of the ligation product **4** in high yield (Supplementary Fig. S5). The new N-terminal cysteine residue of **4** was then deprotected by treatment with methoxylamine to give protein **5** (Supplemental Fig. S6). To prepare protein thioester **3**, the appropriate α-synuclein fragment (residues 1–68) was recombinantly expressed as an N-terminal fusion with an engineered DnaE intein from *Anabaena variabilis* (Supplemental Fig. S7). Subsequent incubation of thioester **3** with protein **5** gave full-length, unmodified α-synuclein (Supplemental Fig. S8). Finally, radical-based desulfurization was used to convert the two cysteine residues required for the ligations to the native alanine residues (synthetic α-synuclein, Fig. 2c and Supplemental Fig. S9).

We next embarked on the synthesis of α-synuclein bearing an O-GlcNAc modification at T72, which we termed α-synuclein(gT72). The synthesis followed the same route as that for unmodified α-synuclein with peptide **1** replaced by glycopeptide **7** (Fig. 3a). The selectively protected O-GlcNAcylated threonine residue was first synthesized using high-yielding thioglycoside chemistry[20], followed by Fmoc-based solid phase peptide synthesis. On-resin deprotection of the O-GlcNAc moiety was accomplished using hydrazine before cleavage and purification of the glycopeptide by RP-HPLC (Supplementary Fig. S10). Notably, hydrazine-treatment did not result in any deprotection of the N-terminal cysteine residue. Following the same reaction scheme as in Fig. 2b, ~10 mg of α-synuclein(gT72) was readily prepared (Fig. 3a and Supplementary Fig. S11 – S14).

O-GlcNAcylation blocks α-synuclein aggregation but has no effect on membrane binding

Comparison of unmodified α-synuclein to α-synuclein(gT72) using circular dichroism revealed that O-GlcNAcylation does not result in the formation of any significant secondary structure (Supplementary Fig. S15), and dynamic light scattering showed no formation of oligomeric or aggregate structures during synthesis or purification (Supplemental Fig. S15). Recombinant α-synuclein, synthetic α-synuclein or α-synuclein(gT72) at a concentration of

50 μM were then simultaneously subjected to aggregation conditions for 7 days. After 72, 120 and 168 h, reaction aliquots were removed and added to a solution of ThT. Analysis by fluorescence showed that recombinant and synthetic α-synuclein formed fibril aggregates; however, α-synuclein(gT72) resulted in no ThT fluorescence over the entire assay (Fig. 3b). To visualize the structure of any aggregates that formed, TEM was performed after 7 days. Both of the unmodified α-synuclein preparations formed protein-fibers characteristic of amyloidogenic proteins (Fig. 3c and Supplemental Fig. S16). In stark contrast, α-synuclein(gT72) did not form any fibrous aggregates (Fig. 3c), as the only protein aggregates that we visualized by TEM were rare and amorphous in nature (Supplemental Fig. S17). To determine the relative amounts of protein that remained in solution after 7 days of aggregation, we separated the insoluble and soluble fractions by centrifugation. The soluble fraction was then concentrated by lyophilization and both fractions were resuspended in 8M urea to break-up any aggregates. Analysis by SDS-PAGE showed that essentially all of the unmodified α-synuclein was incorporated into insoluble structures, while a large majority of α-synuclein(gT72) remained in solution (Fig. 3d). Next, we tested whether O-GlcNAcylation inhibits the formation of oligomeric structures that are also toxic. Protein samples that had been subjected to 7 days of aggregation were analyzed using SEC-MALS (size exclusion chromatography with inline multi-angle light scattering), which enables the size determination of protein complexes (Fig. 3e). As expected, recombinant α-synuclein formed oligomers that are very consistent in size with previous SEC-MALS experiments[27,28]. In contrast, α-synuclein(gT72) formed essentially no oligomeric species. Taken together, these data demonstrate that O-GlcNAcylation blocks the formation of both fibers and oligomers and promotes the solubility of α-synuclein under aggregation conditions.

α-Synuclein binds strongly to cellular membranes and negatively charged vesicles where it forms an extended α-helix[29], and we have previously shown that α-synuclein can independently induce membrane curvature and convert vesicles into tubular structures *in vitro*[30,31]. Notably, the formation of small vesicles and tubes by α-synuclein has also been reported *in vivo* and in neuron culture[32], and recent studies on the role of α-synuclein in synaptic vesicle endocytosis demonstrated that α-synuclein acts at the earliest stages of this process in neurons[33]. Together, these data establish membrane binding and bending as a relevant readout on the endogenous function of α-synuclein. To test whether O-GlcNAcylation had any effect on these properties, recombinant α-synuclein or α-synuclein(gT72) where incubated with a large excess (1:100) of membrane vesicles for 20 min, and the extent of α-helix formation was measured using circular dichroism (Fig. 4a). As expected, recombinant α-synuclein formed an α-helix in the presence of negatively charged lipid vesicles (POPG or POPS) that could be competed by the addition of the zwitterionic lipid (POPC). Notably, we observed essentially no differences for α-synuclein(gT72), which demonstrates that there are no significant changes in the affinity and mode of binding upon O-GlcNAcylation. Next, we incubated either recombinant α-synuclein or α-synuclein(gT72) with POPG (1:20 ratio) for 20 min and found that both proteins induced the formation of tubular structures with a diameter of ~30 nm as visualized by TEM (Fig. 4b and Supplemental Fig. S18) Together, these data indicate that O-

GlcNAcylation will likely have little to no effect on α-synuclein's ability to bind or remodel membranes during its endogenous functions.

O-GlcNAcylation affects subsequent α-synuclein phosphorylation

In addition to its direct effects, O-GlcNAcylation has been demonstrated to affect phosphorylation of several substrate proteins. α-Synuclein is phosphorylated at multiple residues, including serine 87 (S87) and 129 (S129)[23]. Both of these phosphorylation sites have been shown to be closely associated with protein aggregates *in vivo*, indicating that they may have detrimental effects in PD. However, another model has emerged from a series of *in vitro*, cellular, and *in vivo* experiments that suggests a protective role for these phosphorylation events[34–37]. Briefly, these experiments showed that simultaneous phosphorylation at S87 and S129 by casein kinase 1 (CK1) can inhibit aggregation, and they demonstrated that polo-like kinases (PLKs) and G protein-coupled receptor kinase 5 (GRK5) can phosphorylate α-synuclein aggregates an promote their degradation. Although the exact consequences of α-synuclein phosphorylation still need to be completely elucidated, we chose to test if O-GlcNAcylation affects these phosphorylation events. Recombinant α-synuclein or α-synuclein(gT72) were incubated with CK1, polo-like kinase 3 (PLK3), or GRK5. The proteins were then separated by SDS-PAGE and analyzed by Western blotting (Supplementary Fig. S19). O-GlcNAcylation may have a small effect of increasing phosphorylation at S87 by CK1, while inhibiting the modification at S129 by all three kinases. It is therefore possible that O-GlcNAcylation of α-synuclein at T72 may not only directly block aggregation but also effect phosphorylation events that may play protective or detrimental roles in PD.

O-GlcNAcylation inhibits α-synuclein toxicity

Although O-GlcNAcylation blocks the formation of α-synuclein fibers and oligomers, it is possible that it does not inhibit the genesis of other insoluble or soluble species that are still toxic. α-Synuclein aggregates are found intracellularly in PD; however, many α-synuclein overexpression studies in neurons or cell-lines did not observe toxicity without additional cellular stress[38–41]. Additionally, we showed here that α-synuclein(T72A) has a intrinsic aggregation deficiency (Supplementary Fig. S2), which prevents the direct effects of O-GlcNAcylation at this site to be meaningfully tested in an overexpression model. Notably, a growing body of evidence supports the extracellular, cell-to-cell transmission of α-synuclein aggregates to previously healthy neurons[16,42]. In particular, the exogenous addition of toxic α-synuclein-species to culture media results in the death of neurons and neuronal culture-models, enabling us to directly test the effects of O-GlcNAcylation with our synthetic material. Accordingly, we initiated aggregation reactions containing recombinant α-synuclein or α-synuclein(gT72). After 7 days, aggregates were first collected by centrifugation and then resuspended in culture media using sonication. To control for the presence of any species that remained soluble, the supernatant was concentrated by lyophilization and also resuspended in culture media. These samples (25 μM concentration based on monomer) were analyzed by TEM (Supplementary Fig. S20) and then added to either primary rat cortical neurons or SH-SY5Y cells, a neuroblastoma cell-line that expresses endogenous α-synuclein. After 60 h, cell toxicity was measured using the small-molecule ethidium homodimer. Ethidium homodimer is an environmentally sensitive

fluorescent dye that excluded from healthy cells but gains access through the disrupted membranes of dying cells, where it intercalates into DNA. Recombinant α-synuclein aggregates caused a substantial increase in ethidium homodimer signal in both cell types (Fig. 5). In contrast, treatment with α-synuclein(gT72) resulted in no significant change in toxicity in neurons and less death in SH-SY5Y cells compared to unmodified protein (Fig. 5). Notably, treatment of cells with either of the supernatants resulted in no significant changes in toxicity (Fig. 5). Because SH-SY5Y cells divide in culture, we also measured the effect of α-synuclein on their proliferation (Fig. 5). Consistent with the ethidium homodimer signal, unmodified α-synuclein aggregates inhibited proliferation, while α-synuclein(gT72) had no effect. Again, the soluble material from both proteins had no significant effect on proliferation (Fig 5). Together, these data indicate that α-synuclein O-GlcNAcylation prevents the formation of toxic species, and that it might slow the cell-to-cell spread of toxicity in the brain.

O-GlcNAcylation largely prevents incorporation of α-synuclein monomers into aggregates

To investigate how O-GlcNAcylation inhibits α-synuclein aggregation, we first asked if O-GlcNAcylation has a substoichiometric effect on fiber formation. Aggregation reactions were prepared that contained either recombinant or synthetic α-synuclein, α-synuclein(gT72) or different ratios of α-synuclein(gT72) and unmodified α-synuclein. Analysis by ThT fluorescence (Fig. 6a) demonstrated that as little as 10% O-GlcNAcylated α-synuclein resulted in delayed aggregation kinetics. Larger percentages (25 & 50%) increased this delay and yielded overall lower-levels of ThT fluorescence. Examination by TEM, showed that increasing percentages of O-GlcNAcylated did not have a noticeable effect on the structure of the fibers that were formed (Fig. 6b and Supplementary Fig. S21). The aggregation of α-synuclein is concentration dependent. Since, O-GlcNAcylation does not change the structure of the fibers formed, its sub-stoichiometric effect could be explained by the exclusion of α-synuclein(gT72) from the fibers, which would in effect simply lower the concentration of aggregation-competent protein. To test this possibility, aggregation reactions were initiated containing either unmodified α-synuclein at 50 μM, the same protein at 25 μM, or a 1:1 mixture of unmodified α-synuclein (25 μM) and α-synuclein(gT72) (25 μM). Analysis of the aggregation kinetics by ThT fluorescence showed that aggregation was less efficient at 25 μM and in the 1:1 mixture compared to 50 μM α-synuclein, with the 1:1 mixture showing slightly more aggregation at later time points (Fig. 6c). Visualization of the aggregates by TEM again showed no major changes to the fiber structure (Fig. 6d and Supplementary Fig. S21). At the 168 h time point, aggregates from the 25 μM and 1:1 reactions were isolated by centrifugation and the supernatant was concentrated by lyophilization. Both samples were dissolved in an 8M urea solution to break up any aggregates and then separated by SDS-PAGE. Western blotting showed that the vast majority of α-synuclein(gT72) remained in solution during the aggregation reaction (Supplementary Fig. S22), suggesting that a small fraction of α-synuclein(gT72) is incorporated into aggregates but the majority remains in solution. Finally, we tested whether O-GlcNAcylation inhibits the incorporation of α-synuclein monomers into a large amount of pre-formed fibers. Specifically, fibers were formed by subjecting unmodified α-synuclein to aggregation conditions for 7 days. At this time the fibers were sonicated to break the fibers and increase the available “ends” for elongation. They were then added to either buffer or

unmodified α-synuclein or α-synuclein(gT72) to give a mixture of 25 μM fibers and 25 μM monomeric protein. Aggregation conditions were then reinitiated and the extent of fiber formation was measured using ThT fluorescence (Supplementary Fig. S23). Fibers that were added to buffer gave relatively stable ThT fluorescence signal over the course of the experiment. In contrast, fibers mixed with unmodified α-synuclein yielded an immediate increase in ThT signal that plateaued within 24 h. Fibers mixed with α-synuclein(gT72) showed a similar increase in ThT signal with a small but statistically-significant decrease in the aggregation kinetics. Analysis of the aggregates that formed in these reactions by TEM (Supplementary Fig. S23) showed that addition to unmodified α-synuclein formed long, straight fibers of ~10 nm in diameter, while those formed by α-synuclein(gT72) are somewhat more irregular in shape, suggesting that O-GlcNAcylation may have an effect on the structure of the fibers when it is incorporated into them. Together, these three experiments indicate that O-GlcNAcylated α-synuclein remains more soluble during the aggregation reaction and is incorporated at a reduced efficiency compared to unmodified α-synuclein; however, this equilibrium between soluble and aggregated α-synuclein(gT72) can be driven by the presence of a large amount of pre-formed, unmodified fibers. (Supplementary Fig. S24).

DISCUSSION

Here, we used synthetic protein chemistry to show that site-specific O-GlcNAcylation of α-synuclein at T72 inhibits protein aggregation by most-likely shifting the equilibrium of the aggregation reaction towards soluble material. Although there is currently no atomic-scale structural information about α-synuclein aggregates, our data is consistent with experiments using electronic spin resonance (EPR)[43] and solid-state NMR[44] spectroscopies, which demonstrate that T72 lies in the core of a fiber. O-GlcNAcylation also inhibits the toxicity of α-synuclein in a well-established extracellular assay when added to primary neurons or a neuronal cell-line. We believe that this difference probably results from both a reduction in the direct toxicity of aggregates in culture and reduced uptake of the protein, as aggregates have been shown to be endocytosed more efficiently than monomeric protein[16]. In contrast to the inhibition of aggregation by O-GlcNAcylation, the modification does not interfere with membrane binding or bending by α-synuclein, indicating that it would not disrupt the normal biological functions of the protein. This has important implications for neurodegenerative diseases. OGA inhibitors that raise O-GlcNAcylation levels should be pursued as possible therapeutics in both Alzheimer's disease and synucleinopathies, particularly in cases where genetic screening can inform early treatment decisions to potentially slow the onset of protein aggregation. Coupled with previous data on the aggregation prone proteins tau and TAB1[11], as well as recent evidence that O-GlcNAc prevents the aggregation of the Polycomb protein Polyhomeotic (Ph) in *Drosophila*[45], these data add further support to a model where O-GlcNAcylation prevents indiscriminate protein aggregation in general. Finally, we also found that a T72A mutant of α-synuclein that would prevent O-GlcNAcylation has a large effect on the aggregation of α-synuclein, preventing the straight-forward interpretation of any loss-of-function experiments [e.g., over-expression of wild-type α-synuclein and α-synuclein(T72A) in cell culture or animal models]. This highlights the unique utility of synthetic protein chemistry to investigate the direct effects of

posttranslational modifications on proteins, including modification of α-synuclein by O-GlcNAcylation (this study), ubiquitination[46,47], phosphorylation,[48], acetylation[49], and nitration[50].

METHODS

Methods and any associated references are available in the online version of the paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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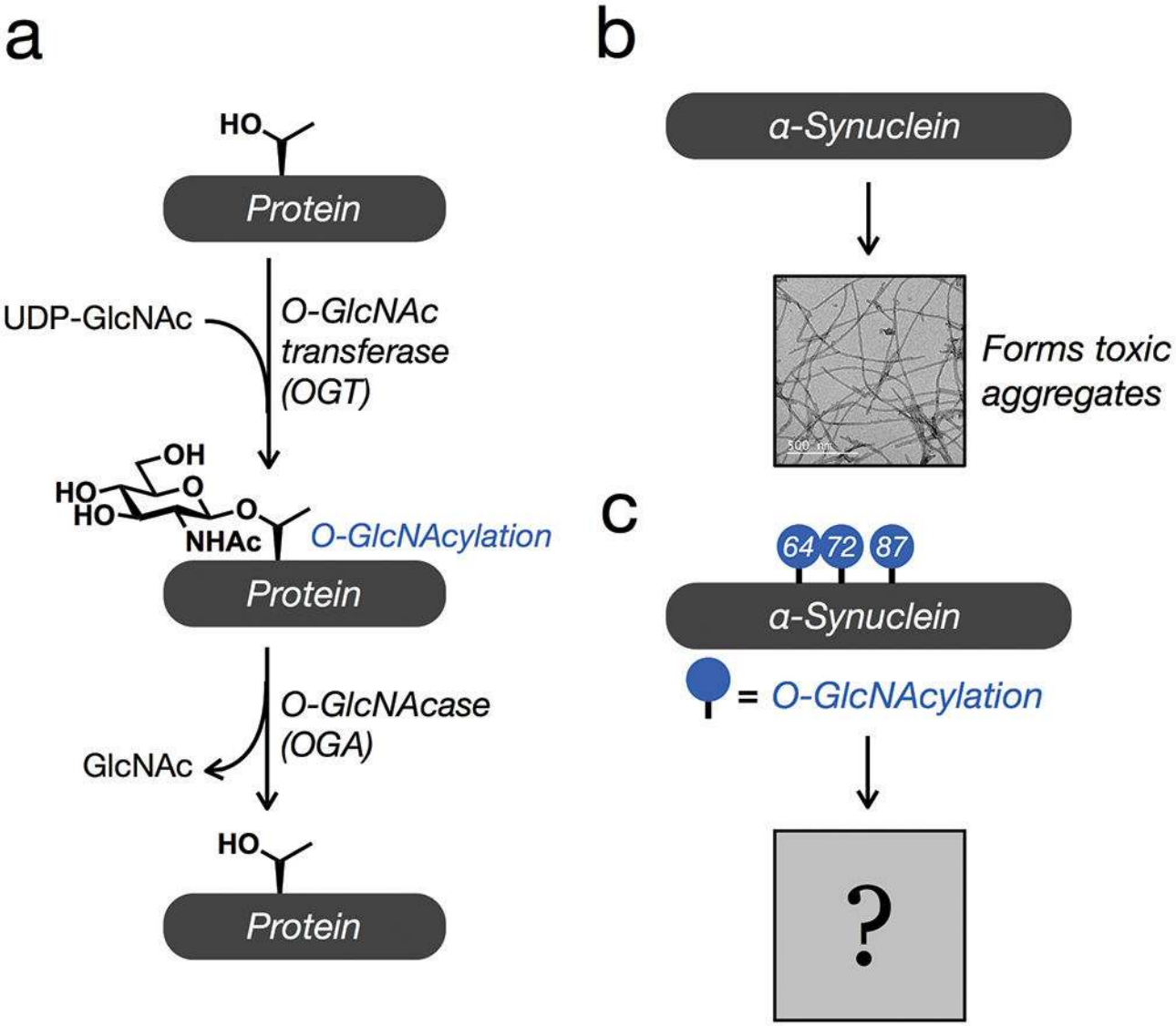

Figure 1. O-GlcNAc modification and α-synuclein

a, O-GlcNAc modification (O-GlcNAcylation) is a dynamic modification of intracellular proteins by the monosaccharide *N*-acetyl-glucosamine (GlcNAc). **b**, The protein α-synuclein forms toxic amyloid-aggregates that contribute to the progression of neurodegenerative diseases, including Parkinson's disease. α-Synuclein is O-GlcNAcylated at three different sites but the effects on protein aggregation and toxicity were unknown. Here, we determine the consequences of O-GlcNAcylation at threonine residue 72.

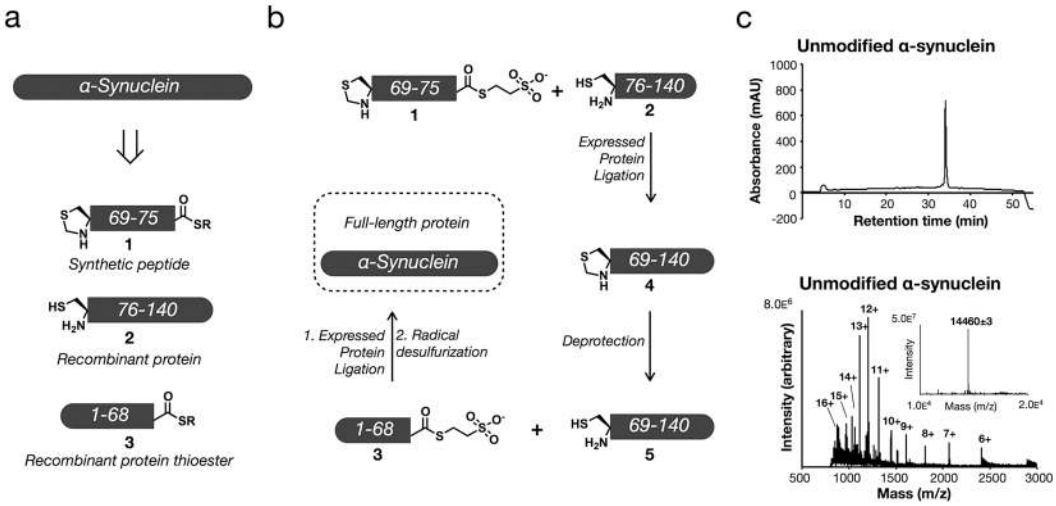

Figure 2. Semisynthesis of α-synuclein

a, α-Synuclein was retrosynthetically deconstructed into a synthetic thioester-peptide (**1**), a recombinant protein (**2**) and a recombinant protein thioester (**3**) obtained using intein chemistry. **b**, Synthetic scheme outlining the preparation of α-synuclein. Synthetic thioester-peptide **1** was first incubated with recombinant protein **2**, resulting in the ligation reaction that yielded protein **4**. The N-terminal thioproline of **4** was then transformed to the corresponding cysteine (**5**) by treatment with methoxylamine. Protein **5** was then ligated to the protein thioester **3** to give full-length α-synuclein. The cysteines required for the ligation reactions were then desulfurized to the native alanine residues to give synthetic α-synuclein with no amino acid mutations. (**c**) Characterization of synthetic α-synuclein using RP-HPLC and ESI-MS. Analysis by RP-HPLC showed that synthetic α-synuclein was pure, as evidenced by the appearance of only one, sharp peak. Characterization by ESI-MS gave a range of charge-states that could be deconvoluted to a molecular mass (14,460 ± 3 Da) in excellent agreement with the predicted weight of 14,460 Da.

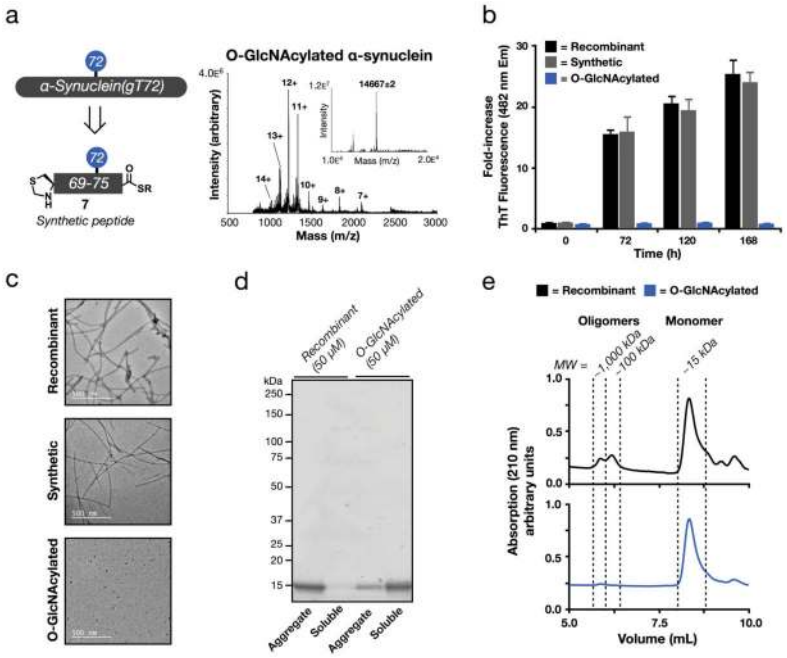

Figure 3. O-GlcNAcylation blocks α-synuclein aggregation

a, α-Synuclein bearing an O-GlcNAc modification at threonine 72 [α-synuclein(gT72)] was prepared using the same semisynthetic route outlined in Fig. 2b by replacing peptide **1** with glycopeptide **7**. Analysis of purified α-synuclein(gT72) by RP-HPLC showed only one, sharp peak. Characterization by ESI-MS gave a range of charge-states that could be deconvoluted to a molecular mass (14,667 ± 2 Da) in good agreement with the predicted weight of 14,663 Da. **b**, O-GlcNAcylation blocks α-synuclein fiber formation. Recombinant α-synuclein or synthetic α-synuclein or α-synuclein(gT72) (50 μM) were subjected to aggregation conditions (agitation at 37 °C) before analysis by ThT fluorescence (λ_{ex} = 450 nm, λ_{ex} = 482 nm) at the indicated time points. y-Axis is fold-change in fluorescence compared to recombinant α-synuclein at t = 0 h. Results are the mean ±s.e.m. of three separate experiments. Both recombinant and synthetic α-synuclein give strong ThT fluorescence signals over the course of the aggregation assay, while α-synuclein(gT72) results in no detectable increase in the signal. **c**, The same reactions were analyzed by TEM after 7 days; scale bar: 500 nm. Mature, rigid fibers with an approximate diameter of 10 nm, consistent with amyloid structures, were readily visualized in the recombinant and synthetic α-synuclein aggregation reactions, but were completely lacking in the α-synuclein(gT72) reaction. **d**, The majority of α-synuclein(gT72) remains soluble during aggregation. After 7 days, aggregation reactions were also separated by centrifugation, resuspended in 8M urea to disassociate any aggregates, and analyzed by SDS-PAGE and coomassie staining. Essentially all of the recombinant α-synuclein formed insoluble aggregates, while the majority of α-synuclein(gT72) was found in the soluble fraction. **e**, O-GlcNAcylation blocks α-synuclein oligomer formation. Aggregation reactions were analyzed by SEC-MALS after 7 days. Faster eluting peaks corresponding to molecular weights of approximately 1,000 and 100 kDa were detectable in the recombinant α-synuclein aggregation reaction, but were essentially absent in the α-synuclein(gT72) reaction.

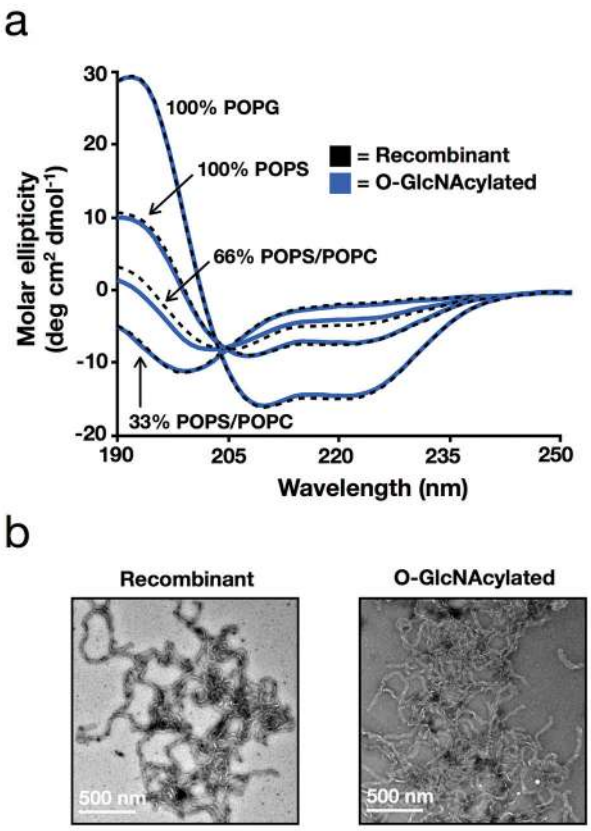

Figure 4. O-GlcNAcylation has no effect on α-synuclein membrane binding or bending

a, O-GlcNAcylation has no effect on α-synuclein α-helix formation upon membrane binding. Recombinant α-synuclein or α-synuclein(gT72) were incubated with an 100-fold excess of the indicated, preformed vesicles and analyzed using circular dichroism (CD). In the presence of negatively charged vesicles (POPG or POPS), both recombinant α-synuclein amd α-synuclein(gT72) gave indistinguishable CD spectra consistent with the formation of an extended α-helix. The introduction of a zwitterionic lipid (POPC) reduced the α-helix formation equally for both proteins. **b**, Unmodified α-synuclein and α-synuclein(gT72) bend membranes into tubules equally. Recombinant α-synuclein or α-synuclein(gT72) were incubated with a 20-fold excess of POPG vesicles and analyzed using TEM. Both proteins remodeled the vesicles into tube structures of approximately 30 nm in diameter. POPG = 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-RAC-(1-glycerol)]; POPS = 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; POPC = 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

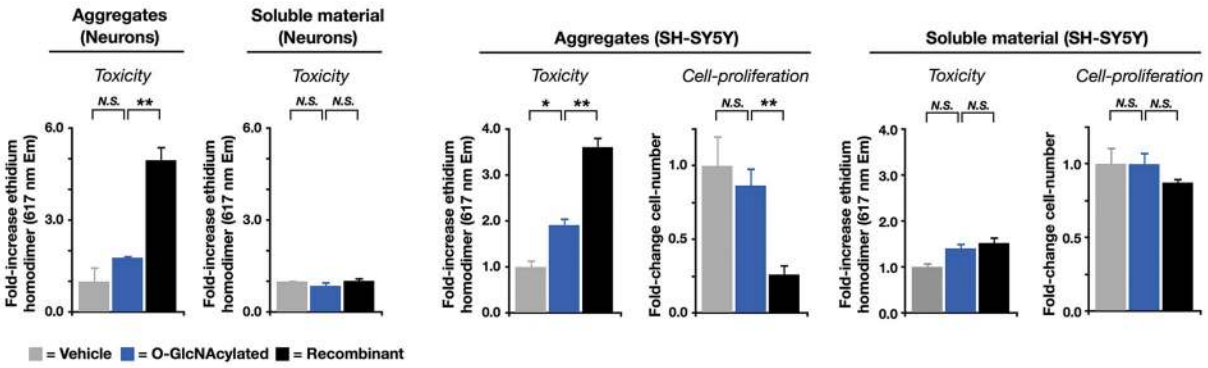

Figure 5. O-GlcNAcylation blocks α-synuclein toxicity

Primary rat cortical neurons in culture or SH-SY5Y cells were treated for 60 h with vehicle or insoluble material or remaining soluble material (25 μM based on monomer concentration) collected from aggregation reactions initiated with either α-synuclein(gT72) or recombinant α-synuclein. In both cell types, toxicity was measured with ethidium homodimer fluorescence (λ_{ex} = 528 nm, λ_{ex} = 617 nm). Treatment with recombinant α-synuclein resulted in significantly more toxicity than α-synuclein(gT72) in both cells types tested. Cellular proliferation was measured in SH-SY5Y cells by cell counting. Again, treatment with recombinant α-synuclein slowed cellular proliferation to a significantly larger extent than α-synuclein(gT72). Results are the mean ±s.e.m. of three separate experiments. Statistical significance (two-tailed, t-test): *P < 0.05, **P = < 0.01.

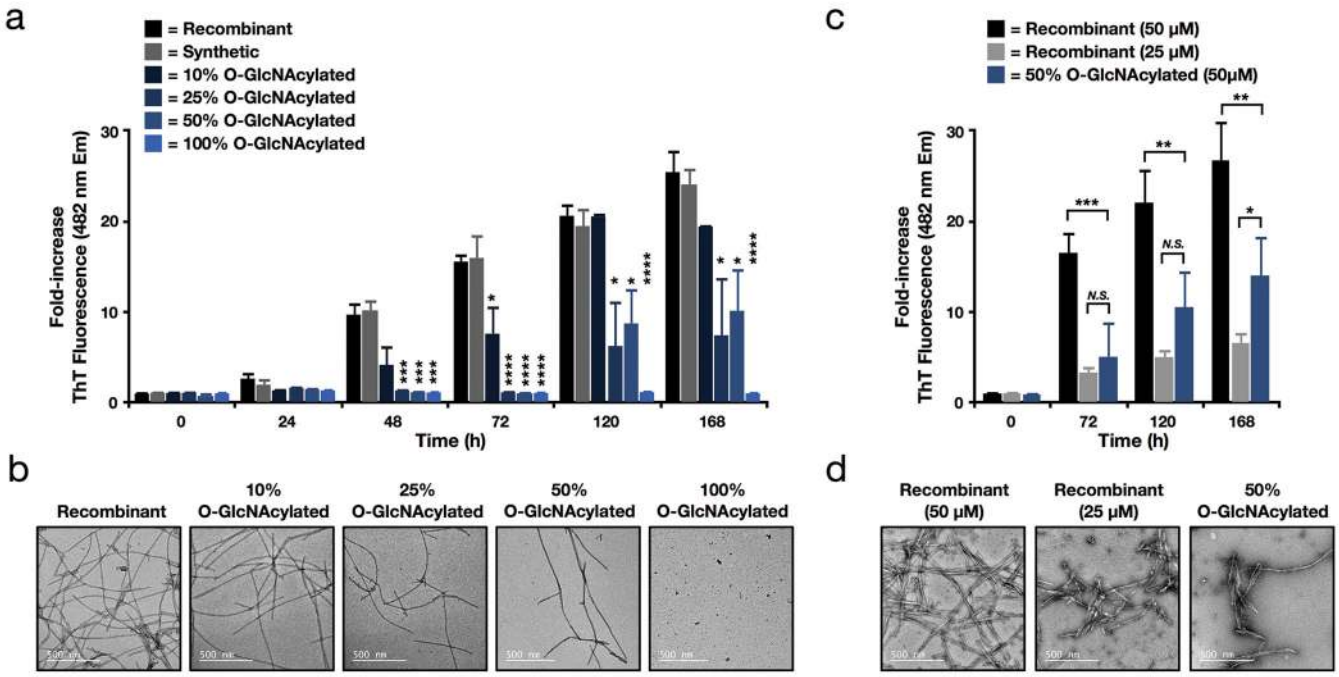

Figure 6. O-GlcNAcylated α-synuclein is largely excluded from the protein aggregates

a, Recombinant α-synuclein or synthetic α-synuclein (50 μM) or the indicated mixtures of α-synuclein(gT72) and recombinant α-synuclein (50 μM total concentration) were subjected to aggregation conditions (agitation at 37 °C) before analysis by ThT fluorescence (λ_{ex} = 450 nm, λ_{ex} = 482 nm) at the indicated time points. y-Axis is fold-change in fluorescence compared to recombinant α-synuclein at t = 0 h. Increasing percentages of α-synuclein(gT72) compared to recombinant α-synuclein resulted in slower aggregation kinetics and lower overall ThT fluorescence levels. **b**, The same reactions were analyzed by TEM after 7 days; scale bar: 500 nm. Increasing percentages of α-synuclein(gT72) compared to recombinant α-synuclein resulted in the formation of fewer overall fiber structures but did not change the gross structures of the fibers formed. **c**, Recombinant α-synuclein at 50 μM or 25 μM or a 1:1 mixture of recombinant α-synuclein (25 μM) and α-synuclein(gT72) (25 μM) were were subjected to aggregation conditions (agitation at 37 °C) before analysis by ThT fluorescence (λ_{ex} = 450 nm, λ_{ex} = 482 nm) at the indicated time points. y-Axis is fold-change in fluorescence compared to recombinant α-synuclein at t = 0 h. The 1:1 mixture of recombinant α-synuclein and α-synuclein(gT72) (50 μM concentration) gave less aggregation than α-synuclein alone but more than recombinant α-synuclein at a concentration of 25 μM. **d**, The same reactions were analyzed by TEM after 7 days; scale bar: 500 nm. All three aggregation reactions formed fibers that were similar in structure and size. All results are the mean ±s.e.m. of at least three separate experiments. Statistical significance (two-tailed, t-test): *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.