

Investigation of All Disease-Relevant Lysine Acetylation Sites in α -Synuclein Enabled by Non-canonical Amino Acid Mutagenesis

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eLife Assessment

This **valuable** work provides new insights into the role of lysine acetylation of alpha-synuclein, the protein involved in Parkinson's Disease. The evidence is mostly **solid**, but the claims around the potential disease relevance based on seeding assays and structural work need to be toned down, or else supported by additional experimental evidence. Overall, the work will be of interest to researchers in the fields of protein biophysics and post-translational modifications, as well as Parkinson's Disease.

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Abstract

Aggregates of α -synuclein (α S) are hallmarks of synucleinopathies, including Parkinson's Disease (PD) and Multiple System Atrophy (MSA). We have recently shown that α S lysine acetylation in the soluble monomer pool varies between healthy controls, PD, and MSA patients. To study the effects of lysine acetylation at all disease-relevant sites of α S, we first compared production of acetylated α S through either native chemical ligation or non-canonical amino acid (ncAA) mutagenesis. Since yields were comparable, ncAA mutagenesis was deemed superior for scanning many acetylation sites. We expressed and purified 12 disease-relevant variants and studied their binding to membranes as well as their aggregation propensities, and found that acetylation of lysine 12, 43, and 80 had particularly strong effects. To understand the implications for acetylation of monomeric α S found in

healthy cells, we performed NMR experiments to study protein conformation and fluorescence correlation spectroscopy experiments to quantify lipid binding. We also investigated the effects of acetylation at lysine 12, 43, and 80 on fibril seeding in neurons. Collectively, our biochemical and cell biological investigations indicated that acetylation of K₈₀ could inhibit aggregation without conferring negative effects on monomer function in healthy cells. Therefore, we studied the structures of fibrils with K₈₀ acetylation through cryo-electron microscopy to uncover the structural basis for these effects. Finally, we identified inhibition of HDAC8 as a way of potentially increasing acetylation at K₈₀ and other inhibitory sites for therapeutic benefit.

Introduction

α -Synuclein (α S) is a 14 kDa protein that typically exists at presynaptic terminals in healthy neurons, where its primary function is believed to be in synaptic vesicle trafficking and regulating neurotransmission (Burré et al., 2010 [DOI](#); Cabin et al., 2002 [DOI](#)). Aggregates of α S commonly characterize several neurodegenerative diseases such as Parkinson's Disease (PD), Dementia with Lewy Bodies (DLB) and Multiple System Atrophy (MSA), which are referred to as synucleinopathies. Evidence indicates that distinct pathology is caused by α S fibrils formed in different disease environments, or α S “strains.” Aggregation seeding experiments showed that α S strains have distinct abilities to propagate pathology, where α S fibrils from MSA patients are much more potent in seeding aggregation than those from DLB (Peng et al., 2018 [DOI](#)). In addition to this, recent cryo-electron microscopy (cryo-EM) experiments showed that structures of α S fibrils vary between different pathological contexts in PD/DLB (Yang et al., 2022 [DOI](#)) and MSA (Schweighauser et al., 2020 [DOI](#)). Despite these findings, the mechanism underlying these differences remains to be understood. It has been suggested that post-translational modifications (PTMs) may contribute to these differences (Baskakov, 2021 [DOI](#)). Among the PTMs that have been studied on α S thus far are *N*-terminal acetylation, phosphorylation, O-GlcNAcylation, lysine acetylation, lysine ubiquitination, tyrosine nitration and glutamate arginylation (Baskakov, 2021 [DOI](#); Chen et al., 2019 [DOI](#); Pancoe et al., 2022 [DOI](#); Schaffert and Carter, 2020 [DOI](#)).

We have recently published a comprehensive study of the relative levels of PTMs in the soluble α S monomer pool between MSA, PD, and DLB patients vs. healthy controls (Zhang et al., 2023 [DOI](#)). While many of the PTMs identified have been previously studied in chemical detail by our laboratory and others, (Moon et al., 2021 [DOI](#); Pancoe et al., 2022 [DOI](#)) lysine acetylation stood out as a PTM that is very common and highly physiologically relevant in other proteins, but had received relatively little attention to date in the context of α S. Given that other α S PTMs have found great significance as biomarkers (e.g. pS₁₂₉ – a hallmark of PD (Fujiwara et al., 2002 [DOI](#))) and drug targets (e.g. kinase inhibitors (Pagan et al., 2019 [DOI](#))), we wished to investigate these acetyl lysine (^{Ac}K) sites more thoroughly.

Lysine acetylation is a reversible PTM that can be introduced at specific sites by lysine acetyltransferases (KATs) or non-enzymatically added by reaction with abundant cytosolic acetyl coenzyme A. Lysine deacetylation is catalyzed by lysine deacetylases (KDACs), which include Zn²⁺-dependent histone deacetylases (HDACs) and NAD⁺-dependent sirtuins (Ruijter et al., 2003 [DOI](#); Sauve et al., 2006 [DOI](#)). In addition to our comprehensive PTM study in patient samples, there has been some previous evidence for the role of lysine acetylation in synucleinopathies. It has been suggested that activity imbalances between KATs and KDACs on histone or non-histone proteins are pathologically relevant to PD. In fact, activators of some sirtuins and inhibitors of specific KDACs/KATs have shown potential as therapeutics (Wang et al., 2020 [DOI](#)). Identified as a substrate of these enzymes, α S was found acetylated on Lys6 and Lys10 in mouse brain. Sirtuin-2 was found to deacetylate those sites and enhance the toxicity of α S (de Oliveira et al., 2017 [DOI](#)). It is notable that in this work, semi-synthetic, acetylated α S was used for the deacetylation assay, however for other

experiments glutamine was used to mimic lysine acetylation, which is a common strategy of choice in the field of biochemistry or biophysics, due to easier access to the site-specifically, homogeneously modified construct.

Recently, many more disease-relevant lysine acetylation sites have been identified in patient tissue. Eight ^{Ac}K sites were identified by Goedert and Scheres in mass spectrometry (MS) studies accompanying a cryo-EM structure of αS fibrils from MSA patients (Lys21/23/32/45/58/60/80/96, **Figure 1A**) (Schweighauser *et al.*, 2020). Ten ^{Ac}K sites, many overlapping those found by Goedert and Scheres, were identified by MS in our previously noted studies of soluble αS from patients (Lys12/21/23/34/43/45/58/60/96/102, **Figure 1A**) (Zhang *et al.*, 2023). In our accompanying mechanistic studies of the PTMs, authentic constructs of phosphorylated αS were produced through semi-synthesis because phosphorylation occurred at a few key sites with established semi-synthetic routes, but lysine acetylation was investigated only through glutamine mimics due to challenges in systematically investigating a large number of PTM sites where there was less literature to identify key targets. (Zhang *et al.*, 2023) Thus, there have not been studies of the effect of authentic lysine acetylation in αS at the sites identified from patient tissue.

In this work, we set out to study lysine acetylation at all 12 disease-relevant sites of αS (**Figure 1A**). We began by comparing the efficiency of producing acetylated αS through either native chemical ligation (NCL) or non-canonical amino acid mutagenesis (ncAA mutagenesis). We found that ncAA mutagenesis provided comparable yields, and was therefore superior for scanning many acetylation sites due to the ease of generating new constructs. Once the 12 αS ^{Ac}K variants were expressed and purified, we studied their binding to membranes as well as their aggregation propensities. We performed NMR, fluorescence correlation spectroscopy (FCS), and transmission electron microscopy (TEM) experiments on acetylated variants that showed perturbed membrane binding or aggregation. NMR and FCS experiments were enabled by our ncAA mutagenesis approach which made it facile to produce isotopically or fluorescently labeled αS. We went on to characterize the seeding ability of select ^{Ac}K constructs in neurons, determine a cryo-EM structure of fibrils with a particular ^{Ac}K site of interest, and to test HDAC selectivity in deacetylating these sites. The combination of the site-specific incorporation approach and a variety of biological characterization methods provides a systematic understanding of lysine acetylation, identifying a few key ^{Ac}K sites as significant for further investigation and potential therapeutic intervention.

Results and Discussion

Comparison of ncAA Mutagenesis and NCL

Protein semi-synthesis is a powerful approach to site-specifically incorporate modifications of interest into a protein sequence (Thompson and Muir, 2020) and it has been a method of choice for many αS PTM studies (Moon *et al.*, 2021), including Lys acetylation (de Oliveira *et al.*, 2017). To test this approach to synthesizing acetylated αS, we chose ^{Ac}K₈₀ as an example, and combined solid-phase peptide synthesis (SPPS) (Merrifield, 1963) by which χ -acetyllysine is incorporated, with the expression of protein fragments and a three-part NCL sequence using acyl hydrazides (Zheng *et al.*, 2013) (**Figure 2A**, additional details in SI Scheme S1).

N-terminal thioester fragment αS₁₋₇₆-MES (**1a**) and C-terminal fragment αS₈₅₋₁₄₀-C₈₅ (**4**) were each recombinantly expressed as a fusion with Mxe GyrA intein. The *N*-terminal thioester was generated by adding excess sodium 2-mercaptoethane sulfonate (MESNa) to cleave the intein by *N,S*-acyl shift (Muir *et al.*, 1998) (reported yield 24.1 mg/L (Pan *et al.*, 2020a)). Endogenous methionyl aminopeptidase in *E. coli* processes the *N*-terminus of the 85-140 peptide to expose the *N*-terminal cysteine (Xiao *et al.*, 2010) which further reacts with aldehydes or ketones *in vivo* to form thiazolidine derivatives (Liu *et al.*, 2016).

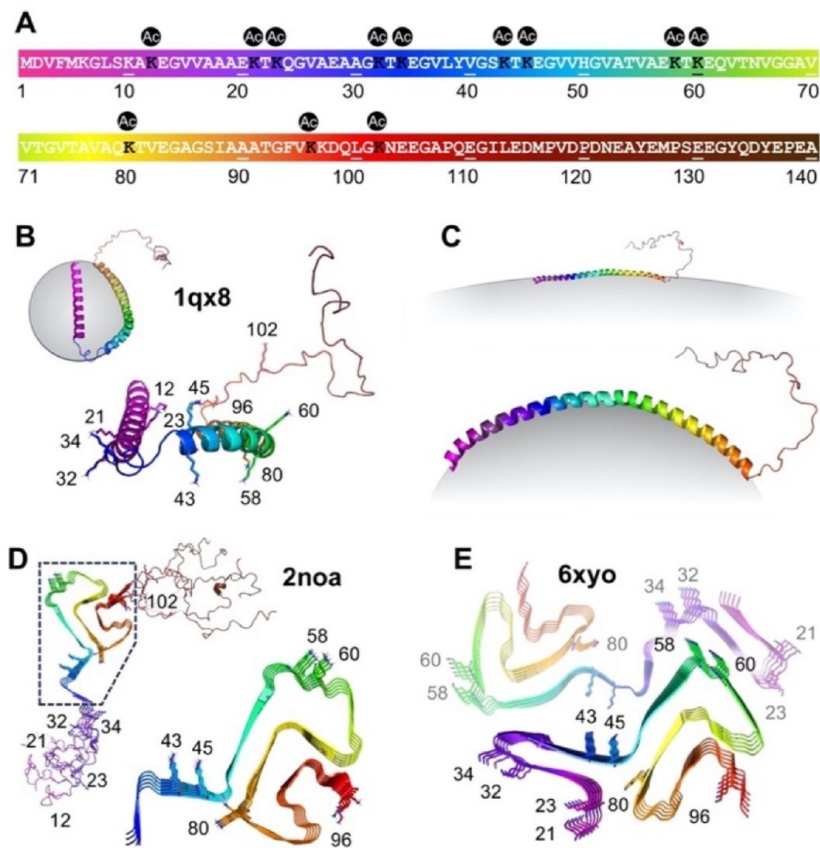


Figure 1.

Neurodegeneration-relevant Lys acetylation sites in α S.

(A) α S sequence with positions 12, 21, 23, 32, 43, 45, 58, 60, 80, 96 and 102 marked. (B) Solution NMR structure of micelle-bound α S (PDB: 1qx8). (C) Proposed structure of vesicle-bound α S. (D) Solid-state NMR structure of recombinant α S fibrils (PDB: 2noa). (E) Cryo-EM structure of MSA patient α S fibrils (PDB: 6xyo).

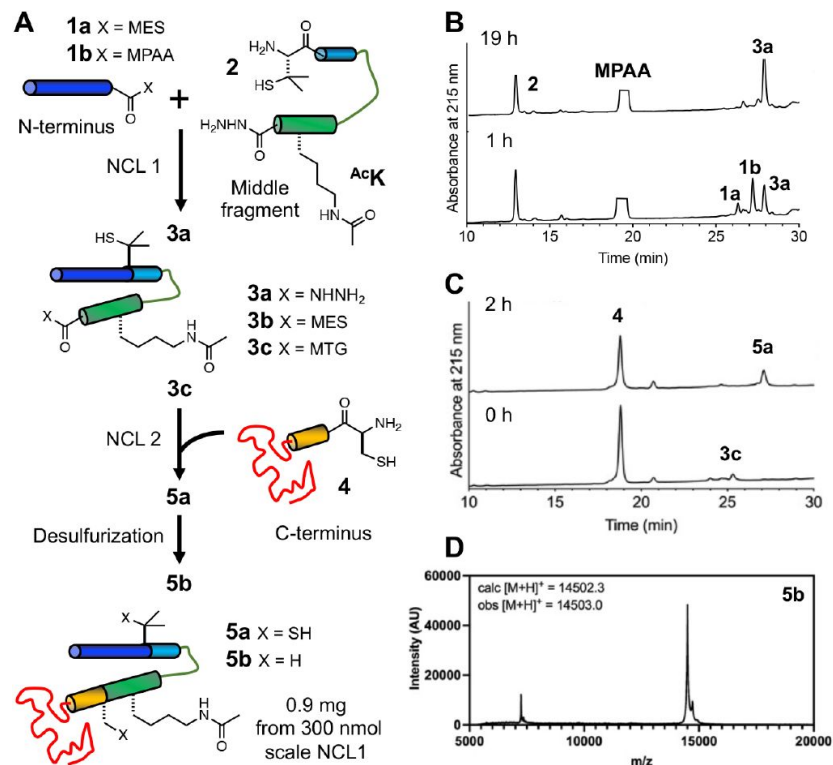


Figure 2.

Semi-synthesis of αS-AcK₈₀.

(A) Acetylation is introduced through peptide synthesis, and the peptide is combined with expressed peptide fragments using NCL. (B) Analytical HPLC trace for the first ligation. **1a**: αS₁₋₇₆-MES, **1b**: αS₁₋₇₆-MPAA, **2**: αS₇₇₋₈₄-Pen₇₇^{AcK₈₀}-NHNH₂, **3a**: αS₁₋₈₄-Pen₇₇^{AcK₈₀}-NHNH₂. (C) Analytical HPLC trace for the second ligation. **3b**: αS₁₋₈₄-Pen₇₇^{AcK₈₀}-MES, **3c**: αS₁₋₈₄-Pen₇₇^{AcK₈₀}-MTG, **4**: αS₈₅₋₁₄₀-C₈₅, **5a**: αS-Pen₇₇C₈₅^{AcK₈₀}. (D) MALDI MS of HPLC-purified αS-AcK₈₀ (**5b**).

The thiazolidine derivatives were deprotected with methoxyamine to give a free *N*-terminal cysteine (4.40 mg/L, SI Figure S1B). The middle acyl hydrazide peptide αS_{77-84} -Pen^{Ac}₇₇-K₈₀-NHNH₂ (**2**, Pen: penicillamine (Haase et al., 2008 [DOI](#))) was synthesized through SPPS (Yield: 12.4 mg, 12 μ mol, 48%, SI Figure S1A).

αS_{1-76} -MES (**1a**) and αS_{77-84} -Pen^{Ac}₇₇-K₈₀-NHNH₂ (**2**) were ligated overnight under routine NCL conditions (NCL1) in the presence of 4-mercaptophenylacetic acid (MPAA). (Yield: 1.46 mg, 172 nmol, 57%, **Figure 2B** [DOI](#)). The purified product (**3a**) was activated by oxidation to form a MES thioester (**3b**) (Yield: 1.29 mg, 126 nmol, 73%, SI Figure S1C). The second ligation (NCL2) between αS_{1-84} -Pen^{Ac}₇₇-K₈₀-MES (**3b**) and αS_{85-140} -C₈₅ (**4**) to form αS -Pen^{Ac}₇₇-C₈₅-K₈₀ (**5a**) as performed in the presence of methyl thioglycolate to allow for desulfurization without intermediate purification (**Figure 2C** [DOI](#)). (Huang et al., 2016 [DOI](#)) The product, αS -^{Ac}K₈₀ (**5b**), was obtained in 43% yield (0.90 mg, 62 nmol, **Figure 2D** [DOI](#)). Although we successfully completed this synthesis, it is notable that we encountered solubility issues of the intermediate fragments (**3b**, **5a**) and the product (**5b**), after lyophilization.

Experiencing difficulties in sample handling and considering the inefficiency of applying NCL to scan 12 lysine acetylation sites distributed throughout the protein, we then sought to access site-specifically acetylated αS through ncAA mutagenesis (**Figure 3A** [DOI](#)). We recombinantly expressed αS with lysine acetylation at site 80 in *E. coli* through amber codon suppression. We used a previously reported pair of aminoacyl tRNA synthetase (chAcK3RS with IPYE mutations) and cognate tRNA to incorporate ϵ -acetyllysine at a position dictated by an amber stop (TAG) codon (Bryson et al., 2017 [DOI](#)). In addition to 10 mM ϵ -acetyllysine, 50 mM nicotinamide, an inhibitor to endogenous deacetylases, was added to the media before inducing αS expression. The protein was expressed as an intein fusion as reported before for easy removal of truncated protein through affinity purification (Batjargal et al., 2015 [DOI](#)). After traceless intein cleavage with 2-mercaptoethanol, the ^{Ac}K-containing protein was purified by reverse phase high performance liquid chromatography (RP-HPLC) and exchanged into appropriate buffers for biophysical assays (**Figure 3B** [DOI](#)).

We obtained 0.65 mg of pure αS -^{Ac}K₈₀ per L of bacterial culture (**Figure 3C,3D** [DOI](#)), a yield comparable to that obtained by NCL, but because no lyophilization or handling of ligation intermediates is required, we did not encounter the solubility problems observed in the NCL process. Therefore, we deemed the ncAA mutagenesis approach at least comparable to NCL for producing a specific construct. Since we wished to study 12 sites distributed throughout the αS sequence, ncAA mutagenesis was also advantageous because we avoided having to generate constructs for several different ligation sites and could simply perform site-directed mutagenesis to insert TAG codons for each new αS -^{Ac}K_n variant.

Bolstered by our success with ^{Ac}K₈₀, we generated TAG mutants at sites 12, 21, 23, 32, 34, 43, 45, 58, 60, 96, or 102. We expressed and purified these proteins, observing successful ncAA mutagenesis at each site (Figs. S2-S12), however, the yield varied significantly between different sites (0.11-1.5 mg). This is an interesting result in light of the large number of sites that were tested in the same protein and the fact that αS is an intrinsically disordered protein, so protein folding should not affect incorporation. Examination of the local RNA sequence context of the amber (TAG) codon did not explain the varied suppression efficiency, based either on previously identified sequence impacts (Pott et al., 2014 [DOI](#)) or by comparing the sites within αS (Figure S13). Given the pseudo-repeat nature of the αS sequence, many sites feature similar sequences, and a comparison of 21 and 58 is particularly striking with a 10-fold difference in expression levels despite near identity in the flanking sequences. While these observations are notable for users of ncAA technology, in the context of this study, our approach allowed us to acquire sufficient amounts of the 12 different authentically modified αS constructs for biophysical experiments.

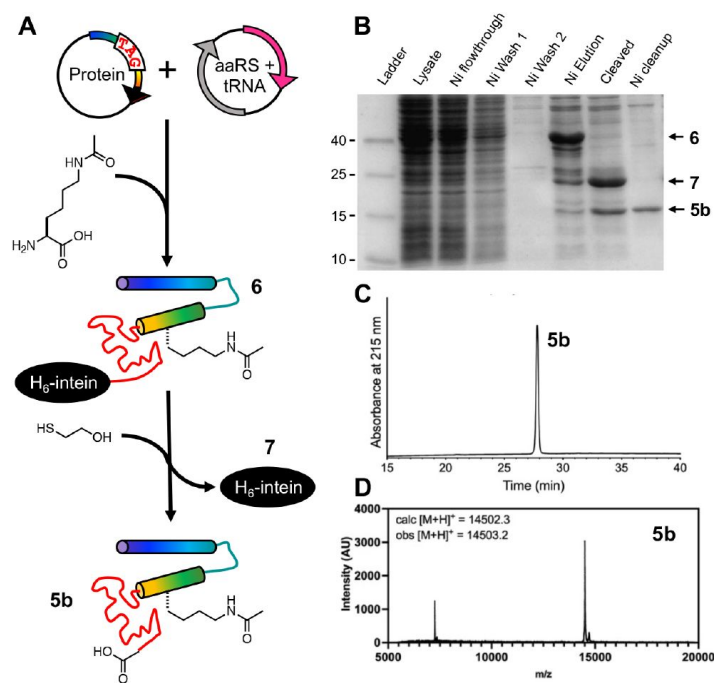


Figure 3.

Expression of α S-AcK₈₀ through ncAA mutagenesis.

(A) An orthogonal aminoacyl tRNA synthetase (aaRS)/tRNA pair site-specifically incorporates acetylysine in recombinant α S. Intein tagging at the C-terminus allows for traceless purification of the full-length product. (B) SDS-PAGE gel (Coomassie stain) showing Ni-affinity purification of recombinant α S-AcK₈₀. Purified α S-AcK₈₀ (5b) characterized with (C) analytical HPLC and (D) MALDI MS.

Thus, in spite of low expression yields for some sites, ncAA mutagenesis was a preferred method for this work, due to the better efficiency in scanning 12 different modification sites and the ease of handling aggregation-prone protein fragments. The expression-based strategy also allows for low-cost access to isotopically labeled, PTM-modified α S constructs, as we have demonstrated previously.(Pan et al.)

Effects on α S Helicity on Micelles and Aggregation

α S is known to bind to lipid surfaces and form helical structures, part of its physiological role in modulating neurotransmitter vesicle trafficking(Ramirez et al., 2023). More specifically, on micelles, an NMR structure showed that micelle-bound α S forms a broken helix, where two helical strands are connected with a loop region (Figure 1B, PDB: 1xq8)(Ulmer et al., 2005). A helical wheel model, created based on this structure, shows that lysine residues are aligned on the membrane surface, and that they are likely involved in enhancing binding by interactions with negatively charged lipid head groups(Meade et al., 2019).

With each acetylated α S variant, we first examined the effects of Lys acetylation on the secondary structure of α S in the presence of micelles by wavelength scan circular dichroism (CD) spectroscopy. Each acetylated α S was compared to unmodified wild type (WT) α S in phosphate-based buffer, pH 7.4, with a large excess of sodium dodecyl sulfate (SDS). We normalized the molar ellipticity at 222 nm of each acetylated construct to that of WT to compare the effect on helicity at each site. We found that significant reduction of helicity was caused only by acetylation at site 43 and that acetylation at other sites had only minor effects on helicity (Figure 4, Figure S14). This study implies that only this site could potentially perturb α S function in neurotransmitter trafficking.

We then investigated whether lysine acetylation at different sites has impacts on α S in pathological contexts. To do this, we first performed *in vitro* aggregation experiments and assessed site-specific effects. A plate-based approach was taken to efficiently perform the assay, and each aggregation reaction was seeded by mixing with α S WT pre-formed fibrils (PFFs) that constituted 10% of the total monomer concentration. The monomer samples were prepared by mixing α S WT with acetylated α S at either 10% or 25% of the total monomer concentration. These concentrations were chosen because our quantitative studies of other PTMs in patient samples indicated that most were present in this range, rather than stoichiometrically(Zhang et al., 2023; Zhao et al., 2022) (see additional discussion in Conclusions). Aggregation was carried out at 37 °C with shaking and kinetics and thermodynamics (final fibril amounts) were monitored.

To examine the effects on aggregation kinetics, we took advantage of the change in fluorescence of the amyloid binding dye, thioflavin T (ThT), during aggregation to monitor the process *in situ*. We found that the effects differ between different modification sites (Figure 5, Figure S15-16). For Lys acetylation at 12, 23, 43, 80 and 102 we observed differential slowing effects – the effects were particularly significant at sites 12, 43 and 80, and the effects at 12, 23 and 43 were dose dependent. While we observed acceleration of aggregation for site 32 both at 10% and 25%, the effects were similar between the different dosages.

To confirm that these effects were not the result of reduced monomer incorporation, we isolated fibrils at the endpoint of 10% or 25% aggregations and SDS-PAGE gels were run and stained with the Coomassie Brilliant Blue dye to quantify total monomer incorporation into the fibrils. We found that there were no consistent reductions in monomer incorporation, and in fact there were some moderate apparent enhancements of incorporation. However, these were generally not consistent between the 10% and 25% aggregation experiments, except in the case of $^{Ac}K_{34}$ (Figure S17-18). Taking all of the aggregation kinetics and monomer incorporation data into account, we chose to investigate the kinetically perturbed sites 12, 43, and 80 further, since the cellular process will be unlikely to reach equilibrium and our previous study had shown that the Gln mimic mutation at position 34 did not alter aggregation in cells.(Zhang et al., 2023)

Figure 4.

Effects of lysine acetylation on micelle-bound α S.

Molar ellipticity at 222 nm was normalized to WT value to quantify helicity on SDS micelles. Mean with SD, R=3

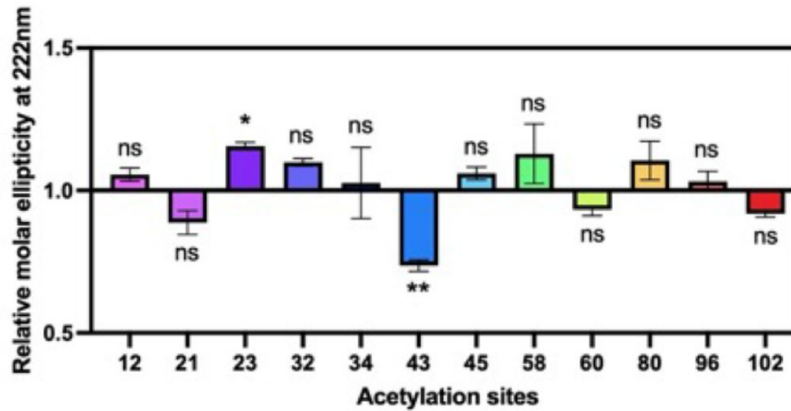
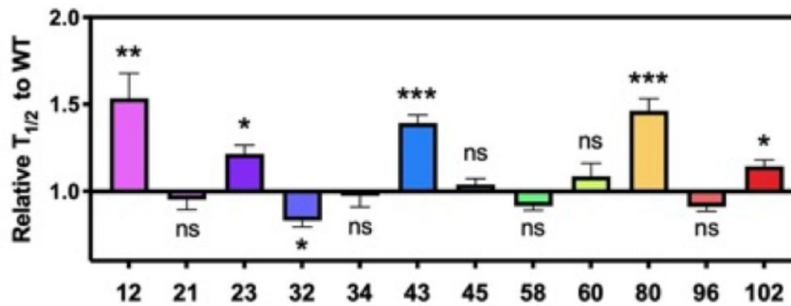


Figure 5.

Effects of lysine acetylation on *in vitro* aggregation.

Aggregation kinetics were monitored by fluorescence intensity change of ThT. Time it takes to reach 50% fibrilization ($T_{1/2}$) for each condition was normalized to that of WT. Seeded aggregation was performed with α S monomers where acetylated α S was mixed with α S WT at 25%:75% ratio. SEM, R=6



Fibril Seeding in Neurons

To investigate the impact of Lys acetylation on aggregation in more physiologically relevant contexts – in cultured neurons – we followed the approach that we have done previously with arginylated α S (Pan et al., 2022; Zhao et al., 2022). We prepared PFFs with the following compositions: α S WT or α S WT mixed with 25% acetylated α S, $^{Ac}K_{12}$, $^{Ac}K_{43}$ or $^{Ac}K_{80}$. Mouse primary hippocampal neurons were grown for 8 days on a coated plate, to which 50 ng/ μ L PFFs were added, following established protocols (Haney et al., 2016; Luk et al., 2009; Marotta et al., 2021). After 2 weeks, intracellular α S aggregates were quantified by staining with an antibody that recognizes phosphoserine 129 (pS₁₂₉), a commonly used pathological marker (Figure 6, Figure S19). Compared to the WT PFFs, all the acetylated PFFs tested resulted in significantly reduced aggregation seeding: the pS₁₂₉ signal (AU $\pm$ SEM (arbitrary units, standard error of the mean)) of PFF-seeded α S aggregates was 2347 ± 107.1 (WT), 1730 ± 83.67 ($^{Ac}K_{12}$), 1854 ± 70.79 ($^{Ac}K_{43}$), 1698 ± 54.41 ($^{Ac}K_{80}$) with respect to DAPI. Notably, acetylation at these sites also slowed seeded aggregation in the *in vitro* fibrilization experiment, but did not reduce aggregates quantified at the endpoint. This supports the idea that aggregation in a cellular context is unlikely to reach saturation.

Structural Characterization of α S Monomers

Having demonstrated that acetylation at K_{12} , K_{43} or K_{80} significantly reduced α S aggregation *in vitro* and in cells, we wished to gain information on the structural impact of acetylation at these sites. First, to give insights into the effects on monomer conformation, we acquired proton-nitrogen correlation spectra (1H , ^{15}N – HSQC) for $^{Ac}K_{12}$, $^{Ac}K_{43}$, or $^{Ac}K_{80}$, an experiment that is facile with nAA mutagenesis, but challenging to perform via NCL due to the high cost of isotopically-labeled amino acids for SPPS. To access ^{15}N -labeled α S, we expressed the acetylated α S and α S WT in M9 minimal media containing ^{15}N -labeled ammonium chloride (SI Figures S20–22). This afforded comparable protein yields to expressions in LB media. It is notable, however, that sub-stoichiometric isotopic labeling was observed at some Lys sites, depending on batches of expression, which could be due to deacetylation in *E. coli* cells followed by incorporation at Lys codons. Overlaying the HSQC spectra for α S-WT and α S- $^{Ac}K_{12}$, $^{Ac}K_{43}$ or $^{Ac}K_{80}$, peak shifts were observed only in signals from surrounding residues, suggesting that the structural change was local and there is no major impact of lysine acetylation on monomer structure (SI Figures S23–S24).

Biophysical Characterization of Lipid Binding

We next wished to learn the effects of Lys acetylation at K_{12} , K_{43} or K_{80} on the native function of α S by investigating its lipid binding mode. To quantify conformational changes of α S upon vesicle binding, we acquired 1H , ^{15}N – HSQC spectra for WT, $^{Ac}K_{12}$, $^{Ac}K_{43}$ or $^{Ac}K_{80}$ in the presence of small, unilamellar vesicles (SUVs) that are composed of 60:25:15 1,2-dioleoyl-sn-glycero-3-phosphocholine/1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine/1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPC/DOPE/DOPS). The NMR peak chemical shifts were similar for all constructs and consistent with spectra previously reported for WT α S (Eliezer et al., 2001). There was no notable chemical shift perturbation at the surrounding residues of each acetylation site (Figure S25).

In the presence of vesicles, a reduction of intensity for residues 1–100 was observed for all the constructs, which is caused by binding of this portion of α S to the slowly tumbling lipid vesicles and is again consistent with previous observations (Bodner et al., 2009; Bussell and Eliezer, 2004). α S- $^{Ac}K_{80}$ had a similar intensity change to WT (~40%, Figure 7A, Figure S26), whereas α S- $^{Ac}K_{43}$ had a smaller intensity change, suggesting weaker vesicle binding (~20%, Figure 7A, Figure S26). This is consistent with the acetylation effects observed with SDS micelles (Figure 4). α S- $^{Ac}K_{12}$ had an intermediate intensity reduction (~30%, Figure 7A, Figure S26), a more significant effect of K_{12} acetylation on vesicle binding than what was observed with SDS micelles

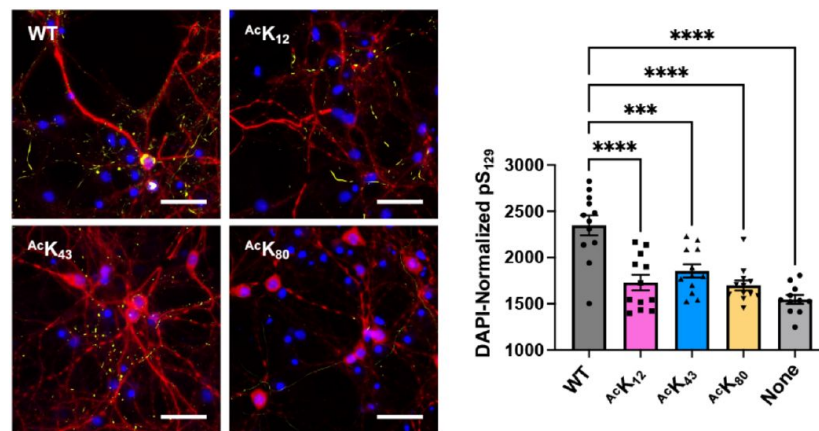


Figure 6.

Effects on aggregation seeding in primary neuron cells.

Left: representative images of neuron cultures treated with unmodified or 25% acetylated αS PFFs, stained with an anti-pS₁₂₉ antibody (yellow), DAPI (blue), and an anti-MAP2 antibody (red). Scale bar = 50 μm. Right: quantification of DAPI-normalized anti-pS₁₂₉ area of intracellular aggregates seeded by different αS PFFs. Mean with SE, R= 11-12. *** = 0.001 < p-value < 0.0001; **** = 0.00001 < p-value < 0.0001

(Figure 4). It is possible that this is due to the differences in curvature and headgroup between the micelles and the vesicles, which is known to result in different α S binding modes (Cholak et al., 2020; Georgieva et al., 2010; Middleton and Rhoades, 2010; Rhoades et al., 2006; Trexler and Rhoades, 2009). It is also possible that this is due to the increased sensitivity of NMR to subtle differences in binding.

While NMR is a very valuable technique for characterizing vesicle binding with a non-perturbing label, to measure affinity, we turned to FCS, a well-established method for rigorously determining vesicle apparent dissociation constants ($K_{d,app}$) (Rhoades et al., 2006). To enable this experiment, we expressed the acetylated α S constructs at site 12, 43, or 80 or a non-acetylated construct ("WT"), bearing a Cys mutation at site 114 (α S- $^{Ac}K_{12}C_{114}$, α S- $^{Ac}K_{43}C_{114}$, α S- $^{Ac}K_{80}C_{114}$, and α S- C_{114}) to allow for fluorescent labeling (Figure 7B, SI Figures S27-30). The fluorophore Atto488-maleimide was reacted with purified Cys mutants overnight at 4 °C or for a few hours at room temperature to yield labeled constructs (9, α S- $^{Ac}K_{12}C_{114}^{Atto488}$, α S- $^{Ac}K_{43}C_{114}^{Atto488}$, α S- $^{Ac}K_{80}C_{114}^{Atto488}$, and α S- $C_{114}^{Atto488}$), and the conversion was almost quantitative (SI Figures S27-30). We prepared synthetic lipid vesicles containing 50:50 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine/1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPS/POPC). The diffusion times of free α S and of the vesicles were obtained first, and in assessing the α S-vesicle binding, we added the same quantity of α S to varied concentrations of vesicles and then determined the protein fractions bound by fitting a two-component autocorrelation function. The fraction bound values at each vesicle concentration were used to fit a binding curve for each α S construct. We found that acetylation at site 43 leads to two-fold weaker binding and acetylation at site 12 or 80 did not significantly affect binding (Figure 7C, SI Figures S31-32; $K_{d,app}^{WT} = 3.2 \pm 0.5 \mu M$, $K_{d,app}^{AcK12} = 3.9 \pm 0.3 \mu M$, $K_{d,app}^{AcK43} = 6.3 \pm 1.3 \mu M$, $K_{d,app}^{AcK80} = 4.0 \pm 1.0 \mu M$).

Slightly reduced binding due to acetylation at site 43 correlates with the reduced helicity we observed in the CD wavelength scan and the differences in NMR peak intensities in the presence of vesicles. The NMR experiments showed that $^{Ac}K_{43}$ reduced vesicle binding more significantly than $^{Ac}K_{12}$ (moderate) or $^{Ac}K_{80}$ (little to none). The FCS experiments supported this, showing that $^{Ac}K_{43}$ led to weaker binding than $^{Ac}K_{12}$ or $^{Ac}K_{80}$, which were similar to WT. Previous NMR experiments suggested that the N-terminal helix of α S (residues 6-25) drives association with lipid membranes and the 26-97 region modulates the affinity, depending on lipid composition (Fusco et al., 2014). The different effect between $^{Ac}K_{43}$ and $^{Ac}K_{12}$ or $^{Ac}K_{80}$ suggests that K_{43} is more important in modulating the binding affinity.

Taken together, our results show that among all the disease-relevant acetylation sites, $^{Ac}K_{12}$, $^{Ac}K_{43}$, and $^{Ac}K_{80}$ each inhibit aggregation, but that $^{Ac}K_{43}$ also inhibits membrane binding (as does $^{Ac}K_{12}$, to a lesser degree). Thus, in the case of $^{Ac}K_{43}$, the potential benefits of reduced amyloidogenicity may be offset by compromising function in neurotransmitter release.

Structural Characterization of α S Fibrils

To get preliminary insights into fibril structure effects, we performed TEM imaging on fibrils formed from acetylated α S (α S- $^{Ac}K_{12}$, $^{Ac}K_{43}$ or $^{Ac}K_{80}$), mixed with α S WT, at 25% of the total monomer concentration (SI Figure S33). Interestingly, we observed mixed morphology for PFFs prepared with α S- $^{Ac}K_{12}$, with some very narrow fibrils. Both 25% $^{Ac}K_{12}$ and 25% $^{Ac}K_{43}$ PFFs have minimal helical twist, making them difficult to characterize by cryo-EM. On the other hand, for PFFs prepared with 25% α S- $^{Ac}K_{80}$, we observed a slightly more twisted fibril morphology, so we attempted to solve a structure by single particle cryo-EM methods. We were able to solve a structure to 2.88 Å resolution, and comparison to previously published α S WT fibril structures (see SI for experimental details, density images, and atomic models, SI Figure S34) shows that the backbone fold is similar to the two-stranded polymorph typified by PDB ID 6a6b (Figure 8, inset, 6a6b) (Li et al., 2018b) similar to PDB IDs 6cu7 (Li et al., 2018a) and 6h6b (Guerrero-Ferreira et al., 2018), which all have the "Greek key" protein fold first reported in solid state NMR studies of single stranded fibrils under PDB ID 2n0a (Tuttle et al., 2016). However, the density clearly shows

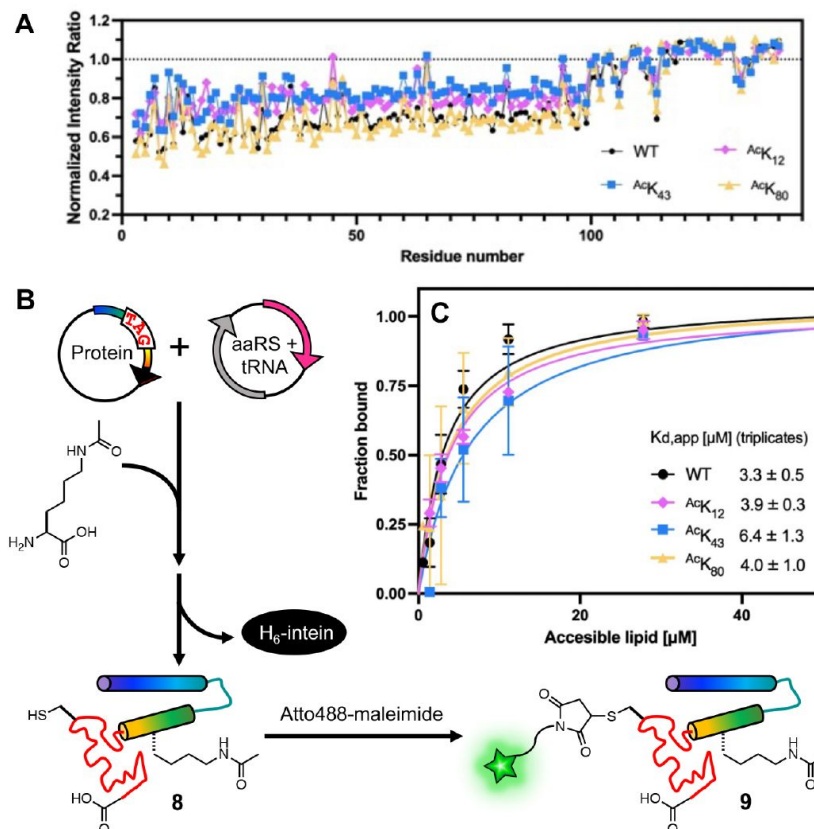


Figure 7.

Effects of lysine acetylation on vesicle binding affinity.

(A) NMR intensity ratio for each residue calculated from ^1H - ^{15}N HSQC spectra collected with ^{15}N -labeled αS variants in the presence or absence of SUVs, normalized by the average ratio for residues 101-140. (B) αS with a TAG codon at the acetylation site of interest and a Cys mutation at a labeling site (**8**) was co-expressed with an aaRS/tRNA plasmid for acetyllysine incorporation. After intein cleavage, labeling with an Atto488 dye was performed through Cys-maleimide chemistry to give an acetylated, labeled protein (**9**) for FCS. (C) Vesicle binding affinity determined by fluorescent correlation spectroscopy measurements. For each construct, measurements were performed on three separate days. Mean with SD, R=3

that K₈₀ is not acetylated in this structure, implying that the WT fibrils are forming this 6a6b-type fibril, while the ^{Ac}K₈₀ fibrils are forming a separate, minority population that we are not able to resolve. In order to more clearly observe the structural impact of K₈₀ acetylation, we prepared fibrils with 100% ^{Ac}K₈₀ αS in Tris-buffered saline (TBS) for cryo-EM studies. For these fibrils, we were able to solve structures of two different polymorphs, both composed of two strands (**Figure 8**, ^{Ac}K₈₀-A and ^{Ac}K₈₀-B, see SI for experimental details and density images, and atomic models, SI Figures S35, S37, S38). The protein fold is essentially the same in both polymorphs, but they differ in strand-strand packing. Since it is well-documented that differences in buffer composition and aggregation methods can lead to differences in fibril morphology, we also prepared ^{Ac}K₈₀ αS fibrils in phosphate-buffered saline (PBS), the same conditions used in our aggregation kinetics studies. Gratifyingly, the ^{Ac}K₈₀ αS fibrils prepared in PBS exhibited the same two polymorphs seen for TBS ^{Ac}K₈₀ αS fibrils, with identical protein folds and strand-strand packings (**Figure 8**, and SI Figure S39). We were also able to solve cryo-EM structures of WT αS fibrils generated under the same conditions in TBS. We observed two WT polymorphs (**Figure 8**, WT-A and WT-B, see SI for experimental details and density images, and atomic models, SI Figures S35, S36, S38) which exhibited similar folds and strand-strand packings to the ^{Ac}K₈₀ polymorphs, but with a notable change in morphology around K₈₀. Acetylation of K₈₀ disrupts a salt-bridge interaction with E₈₃ that can be clearly seen in the WT-B polymorph (**Figure 8**, WT Fold) and neutralizes the sidechain charge, allowing it to pack in a hydrophobic pocket formed by Ala₆₉ and Val₇₁ (**Figure 8**, ^{Ac}K₈₀ Fold). This leads to a twist of the backbone in the T₇₅-A₉₀ segment, generating a modest change in the protein fold. Given that the ^{Ac}K₈₀ protein fold is fairly similar to the WT protein fold, it is not surprising that they exhibit similar strand-strand packings and that K₈₀ acetylation has a moderate impact on aggregation rates. We can use these structures to consider ^{Ac}K₈₀ effects in the context of other structural studies of αS fibrils.

Our WT-A and WT-B structures resemble those first reported under PDB IDs 6rtb and 6rto (**Figure 8**, inset, 6rtb; see SI for additional overlays and structural comparisons, Figure S40). (Guerrero-Ferreira et al., 2019) These polymorphs have been observed by several investigators for WT αS fibrils formed at near-neutral pH, along with the commonly observed 6a6b/2n0a form (**Figure 8**, inset, 6a6b). (Li et al., 2018b) Our ^{Ac}K₈₀ αS fibril structures, ^{Ac}K₈₀-A and ^{Ac}K₈₀-B, resemble those recently reported for WT αS fibrils formed at pH ≤ 6.5 under PDB IDs 8pix and 8pic (**Figure 8**, inset, 8pix). (Frey et al., 2024) In the two fibril polymorphs commonly populated at pH 7, K₈₀ makes key stabilizing salt bridge interactions. For the 6a6b polymorph, K₈₀ makes a salt bridge with E₄₆; for the 6rtb polymorph, it makes a salt bridge with E₈₃. Disruption of these salt bridges by acetylation would destabilize either fold, favoring the ^{Ac}K₈₀ fold that we observe. The fact that a similar fold (8pix) has been seen at lower pHs can be rationalized by assuming that acidification leads to protonation of the E₄₆ or E₈₃ sidechains, weakening their interactions with K₈₀ just as acetylation does to drive a change fibril polymorph. While pH 6.5 is significantly above the pK_a of a typical glutamate sidechain, it is possible that the pK_as are perturbed in the local environment of the fibril, and full deprotonation would not be required to destabilize interactions with K₈₀. Thus, one can rationalize our observation of a polymorph like 8pix for our ^{Ac}K₈₀ at pH 7, when it had only been previously observed at lower pHs. It is further notable that the ^{Ac}K₈₀ fold is robust to changes in buffer (PBS vs. TBS).

Finally, it is worth comparing the effects that we observe for ^{Ac}K₈₀ acetylation to the structural and biophysical effects observed with other αS PTMs which have been structurally characterized, such as Y₃₉ phosphorylation (pY₃₉) or S₈₇ phosphorylation (pS₈₇) and N-Acetyl glucosamine glycosylation (gS₈₇). For pY₃₉ αS, a 4-fold increase in aggregation rate has been observed for αS 100% phosphorylation, with nuanced effects at lower phosphorylation percentages. We have shown that pY₃₉ leads to only modest changes in monomer conformation, based on NMR and single molecule FRET studies. (Pan et al.; Pan et al., 2020b) In contrast, Zhao et al.'s cryo-EM structure shows a dramatic rearrangement of the fibril polymorph. (Zhao et al., 2020) This indicates that the effect of Y₃₉ phosphorylation is primarily at the fibril level, similar to our findings here for ^{Ac}K₈₀ acetylation. Both pS₈₇ and gS₈₇ modifications inhibit aggregation much

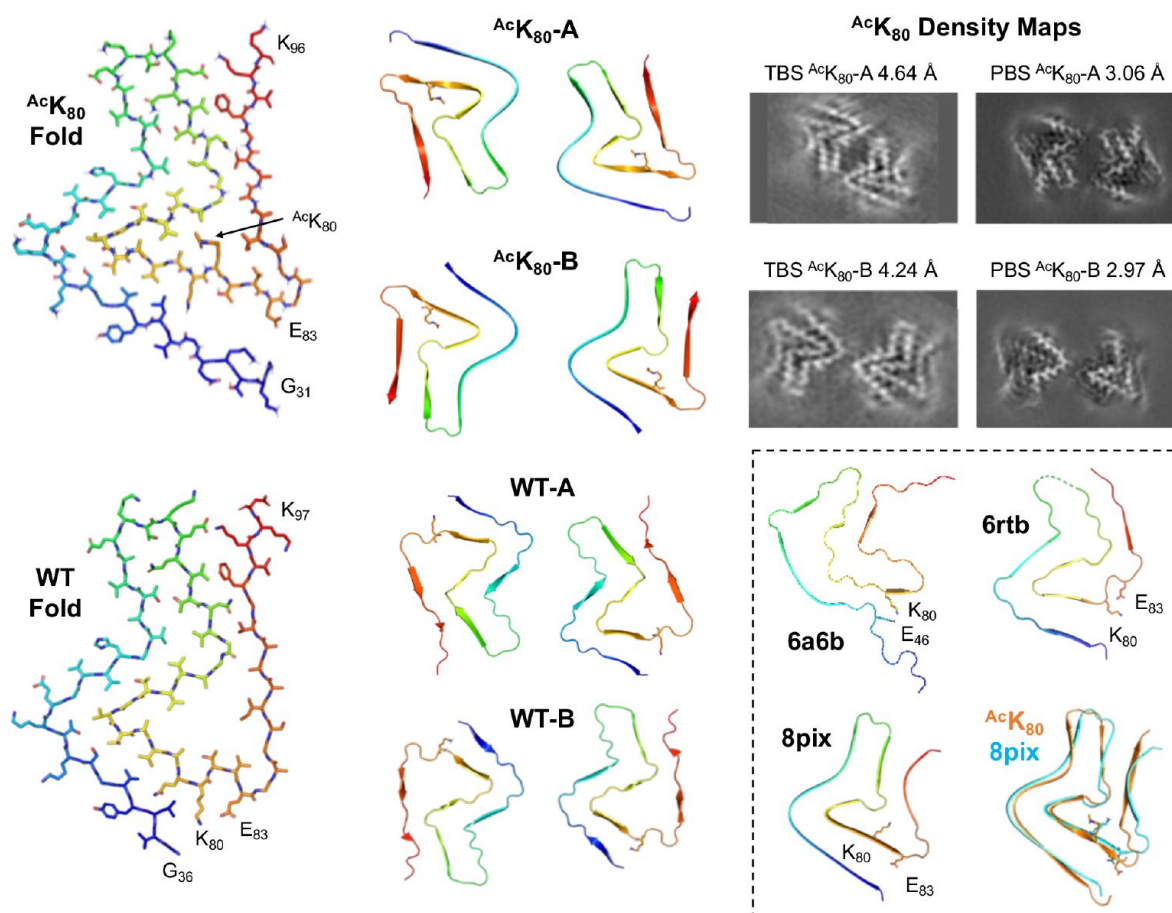


Figure 8.

Structural impact of K₈₀ acetylation on fibril morphology.

AcK₈₀ Fold and WT Fold show the fold of a single αS molecule in the fibrils, viewed down the fibril axis (from AcK₈₀-A PBS and WT-A TBS structures). AcK₈₀-A and AcK₈₀-B show the two fibril polymorphs, with similar protein folds, but different strand-strand packing (from PBS structures). WT-A and WT-B show the two fibril polymorphs, with similar protein folds, but different strand-strand packing (from TBS structures). AcK₈₀-A Density Maps show that the same fibril polymorphs were obtained for fibrils made in TBS and PBS. Inset: The interactions of K₈₀ are shown in three previously αS fibril polymorphs designated by their PDB IDs. (Frey *et al.*, 2024 [DOI](#); Guerrero-Ferreira *et al.*, 2018 [DOI](#); Li *et al.*, 2018b [DOI](#)) The overlay shows the similarity of the AcK₈₀ fold to the 8pix fold.

more drastically than pY₃₉. Two different structures of gS₈₇ αS fibrils have been reported, both showing a significant deviation from reported WT αS polymorphs. (Balana et al., 2024 [↗](#); Hu et al., 2024 [↗](#)) Despite their differences, the structures both provide a clear rationale for the effects of S₈₇ glycosylation. Intriguingly, Hu *et al.* highlight the way in which gS₈₇ changes the structure of the 80-89 region of αS to disrupt the E₄₆-K₈₀, destabilizing the 6a6b fold. (Hu et al., 2024 [↗](#)) The pS₈₇ structure is different from either gS₈₇ structure, and although the residue cannot be observed in the structure, it again demonstrates that a PTM can dramatically alter fibril morphology. Thus, like ^{Ac}K₈₀ studied here, these PTMs seem to primarily exert their influence on αS aggregation through changes in fibril structure, which is sensible given the disordered nature of the αS monomer.

Deacetylase Site Specificity

Since acetylation of K₁₂, K₄₃ and K₈₀ can reduce αS aggregation, we considered the potential of increasing acetylation at these sites by inhibiting a KDAC. Doing so would require that the KDAC had some specificity for these residues. Using previously published methods, (Decroos et al., 2014 [↗](#); Dowling et al., 2008 [↗](#); Osko et al., 2021 [↗](#)) we expressed and purified recombinant human HDAC8, a Zn-dependent HDAC known to act on non-histone proteins, including cytosolic targets like tubulin in HeLa cells (Vanaja et al., 2018 [↗](#)). We treated samples of each of the acetylated αS variants with HDAC8 and monitored deacetylation through a matrix assisted laser desorption ionization (MALDI) MS assay using ¹⁵N-labeled αS as a standard. After 24 h, all of the constructs showed significant levels of deacetylation, but ^{Ac}K₃₄, ^{Ac}K₄₃, ^{Ac}K₄₅, and ^{Ac}K₈₀ retained an average of 44% acetylation, 3-fold greater levels than all other sites (Figure 9 [↗](#)). These preliminary results indicate that inhibition of HDAC8 could increase acetylation levels of αS at specific lysine residues shown to retard aggregation *in vitro* and in PFF-seeded neurons.

Conclusion

In this study, we incorporated ^{Ac}K site-specifically at all 12 disease-relevant sites through nCAA mutagenesis and characterized the effects of this PTM on the physiological and pathological roles of αS using a variety of techniques. The aggregation assays showed that many of the Lys acetylations observed in patient samples have no effect, demonstrating that there is no non-specific effect on protein solubility or electrostatic interactions, at least for single Lys modifications. At sites 12, 43, and 80, Lys acetylation significantly slowed the formation of fibrils, both *in vitro* and in cells. Therefore, increasing acetylation at these sites through the use of KAT stimulators or KDAC inhibitors has potential therapeutic benefits. However, acetylation at Lys 12 or 43 perturbs membrane binding moderately, so increasing acetylation at these sites in αS could disrupt its native function in neurotransmitter vesicle trafficking. Thus, Lys 80 seems like the most promising site for targeted acetylation. Indeed, our previous cell-based studies using Gln mimics have shown that Lys 80 modification reduces aggregation. (Zhang et al., 2023 [↗](#)) By determining cryo-EM structures of ^{Ac}K₈₀ fibrils, we have provided a structural explanation for its inhibitory effects. Collectively, our results imply that strategies that can specifically enhance acetylation at Lys 80, without affecting Lys 12 or Lys 43, would be the most favorable approach to reduce αS aggregation pathology.

It should be noted that it is not clear at this point exactly what the acetylation levels at K₁₂, K₄₃ and K₈₀ are in synucleinopathy patients. Taking advantage of our capability to produce authentically acetylated αS, we determined the extent of acetylation within human protein samples by quantitative liquid chromatography MS (SI, Figures S41-S49). The ^{Ac}K αS standards allowed us to correct for changes in trypsinization and ionization efficiency of acetylated peptides, the latter of which turned out to be very low for the ^{Ac}K₈₀ peptide due to its large size (a result of the missed K₈₀ cut site due to acetylation, SI Table S5). The level of acetylation was variable – no clear trend was observed between healthy control and patients – nor between patients of different diseases (SI, Table S4, Supplementary Data 1). Nevertheless, the MS data suggest that the 10 and

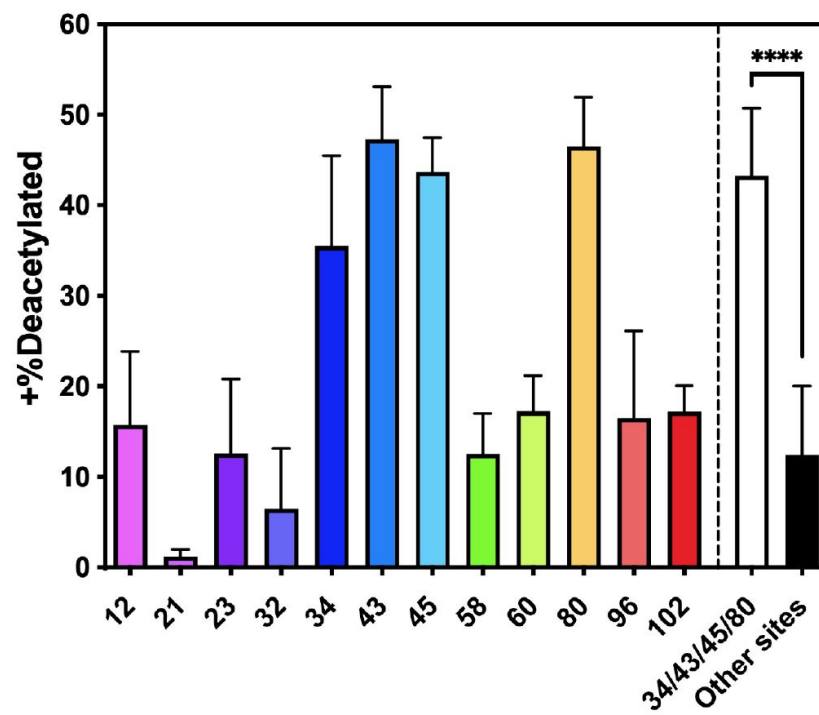


Figure 9.

Site-specificity of HDAC activity.

Samples of each of the acetylated α S variants were mixed with HDAC8 and after 24 h, acetylation levels were checked with a MALDI MS assay using ^{15}N -labeled α S as a standard. Mean with SD, R=3

25% acetylation that we used for aggregation experiments are in the (patho)physiological range. Given the results reported here, it will be valuable to generate antibodies to acetylated peptides for the $^{Ac}K_{12}$, $^{Ac}K_{43}$ and $^{Ac}K_{80}$ epitopes to more easily quantify the levels of acetylation in both soluble and fibrillar αS for immunofluorescence microscopy and Western blotting studies.

More broadly, our experiments show the value of a ncAA mutagenesis approach in systematically investigating a PTM that occurs at many locations in a protein. Since the yields were similar between NCL and ncAA mutagenesis, the ability to scan many sites by simple site-directed mutation to a TAG codon clearly makes ncAA mutagenesis the method of choice for our application. We efficiently scanned 12 different modification sites and fluorescently labeled proteins for binding studies. The ncAA approach was also crucial to generating isotopically labeled, acetylated αS for solution-phase NMR experiments and MS analysis. The isotopic labeling approach could be used in future solid-state NMR experiments to give detailed structural insight into slowed aggregation and distinct fibril morphology.

Our future experiments will include assessing the site-specificity of KATs and other KDACs for sites in αS in a similar fashion to the HDAC8 experiments here, studies enabled by our ability to easily produce ^{Ac}K αS constructs. We can study modulation of HDAC8 and these other enzymes for their ability to specifically increase acetylation at Lys 80 without altering acetylation at Lys 12 or 43. We will also investigate effects on αS aggregation in cellular models in the presence of small molecule modulators of HDAC8 and these other enzymes. Furthermore, combining ncAA mutagenesis and NCL would allow us to study more complex PTM effects in αS , such as the combinatorial effects between multiple lysine acetylations or crosstalk between acetylation and other PTMs.

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Additional files

Supporting Information. Detailed protocols for plasmid mutagenesis, protein semi-synthesis and ncAA mutagenesis, fluorescent labeling, CD, aggregation measurements, neuron seeding, TEM, lipid vesicle preparation, FCS measurements, NMR data acquisition, cryo-EM structure determination, and MS analysis as well as additional figures (PDF and spreadsheet) are provided in Supporting Information. The authors have cited additional references within the Supporting Information. (Anderson, 1946; Delaglio et al., 1995; Dikiy and Eliezer, 2014; Lee et al., 2015; Maciejewski et al., 2017; Volpicelli-Daley et al., 2011; Waxman and Giasson, 2008) [link](#)

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Reviewer #1 (Public review):

Summary:

This paper describes experiments with alpha-synuclein (aS) with acetylated lysines (acK) at various positions. Their findings on how to use non-canonical amino acid (ncAA) mutagenesis to generate aS with acetylated lysines are valuable. The paper then continues with a range of experiments to characterise the acetylated alpha-synuclein constructs at different positions, with the aim of providing insights into which sites are relevant to disease or their function inside cells. The paper concludes these experiments with the suggestion that inhibiting the Zn²⁺-dependent histone deacetylase HDAC8 to potentially increase acetylation at lysine 80 may have therapeutic benefit. However, the relevance of most of these experiments is unclear, mainly as the filaments that form from these constructs are different from those observed in human disease (but see below for more details). Moreover, using the recombinantly produced acetylated versions of alpha-synuclein to normalise mass-spectrometry data, the authors themselves report that acetylation of alpha-synuclein does not differ between individuals with Parkinson's disease or healthy controls.

Strengths:

The authors report difficulties with chemical synthesis, and then decide to make these constructs using non-canonical amino acid (ncAA) mutagenesis, which seems to work reasonably well (yields vary somewhat). In the Conclusion section, the authors report that they used these recombinant proteins to obtain quantitative insights into the levels of

acetylation of lysines in individuals with PD versus healthy controls, for which they find no significant differences. This part of the work is valuable.

Weaknesses:

The authors then use circular dichroism to show that aSyn with acK at position 43 has less alpha-helical content. From this result, they deduce that "only this site could potentially perturb aS function in neurotransmitter trafficking", but no experiments on neurotransmitter trafficking were performed.

Subsequently, they measure the aggregation speed of the variants in seeded aggregation experiments with preformed fibrils (PFFs) from WT aSyn, and conclude that acK at positions 12, 43, and 80 yields slower aggregation. They reach similar conclusions when measuring seeded aggregation in primary cultures. As far as I understand it, the seeding experiments in cells use seeds that are assembled from partially acetylated alpha-synuclein, but that are made of non-acetylated wildtype alpha-synuclein, and the alpha-synuclein that is endogenous in the cells is also non-acetylated (or at least not beyond what happens in these cells at endogenous levels). It is therefore unclear how the cellular seeding experiments relate to the in vitro aggregation assays with (partially) acetylated substrates. Anyway, both aggregation experiments ignore that the structures of aSyn filaments in Parkinson's disease (PD) or multiple system atrophy (MSA) are different from those formed in these experiments, and that, therefore, the observed aggregation kinetics are likely irrelevant for the speed with which disease-relevant filaments form in the brain.

NMR and FCS experiments show that acK at positions 12 and 43 may reduce binding to vesicles, which then leaves only acK80.

Finally, the authors describe the cryo-EM structure of mixtures of acK80:WT aSyn filaments, which are predominantly made of WT aSyn, with a previously described structure. Filaments made of only acK80 aSyn have a modified arrangement of this structure, where the now neutral side chain of residue 80 packs inside a hydrophobic pocket. The authors discuss differences between the acK80 structures and those of other structures from in vitro assembled aSyn filaments, none of which are the same as those observed from PD or MSA brains, nor are any attempts made to transfer observations from the in vitro experiments to the structures of disease. The relevance of the cryo-EM structures for human disease, therefore, remains unclear.

The Conclusion on p.20 mentions an interesting and valuable result: the authors used the acetylated recombinant proteins to determine the extent of acetylation within human protein samples by quantitative liquid chromatography MS (SI, Figures S41-S49). Their conclusion is that "The level of acetylation was variable - no clear trend was observed between healthy control and patients - nor between patients of different diseases (SI, Table S4, Supplementary Data 1)" This result implies that acetylation of aS is not directly related to its pathogenicity, which again adds doubts on the disease-relevance of the results described in the rest of the paper.

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Reviewer #2 (Public review):

Summary:

Shimogawa et al. studied the effect of lysine acetylation at different sites in the alpha-synuclein (aS) sequence on the protein-membrane affinity, seeding capacity in the test tube and in cells, and on the structure of fibrils, using a range of biophysical methods. They use

non-canonical amino acid (ncAA) mutagenesis to prepare aS lysine acetylated variant at different sites.

Strengths:

The major strength of this paper is the approach used for the production of site-specific lysine acetylated variants of aS using ncAA mutagenesis, as well as the combination of a range of biophysical methods together with cellular assays and structure biology to decipher the effect of lysine acetylation on aS-membrane binding, seeding propensity, and fibril structure. This approach allowed the author to find that lysine acetylation at positions 12, 43, and 80 led to lower seeding capacity of aS in the test tube and in cells, but only acetylation at lysine 80 did not affect aS-membrane interaction. These results suggest that lysine acetylation at position 80 may be protective against aggregation without perturbing the proposed functional role of aS in synaptic plasticity.

Weaknesses:

SDS is not a good membrane model to investigate the effect of lysine acetylation on aS membrane-binding because it is a harsh detergent and solubilizes membranes. Negatively charged vesicles or vesicles made of a mixture of lipids mimicking the lipid composition of synaptic vesicles are more accepted in the field to study aS-membrane interactions. The authors used such vesicles for the FCS experiments, and they could be used for the initial screening of the 12 lysine acetylated variants of aS.

It would help the reader to have the experimental details (e.g., buffer, protein/lipid concentrations) for the different assays written in the figure legend.

The authors use an assay consisting of mixing 10% fibrils + 90% monomer to investigate the effect of lysine acetylation on aS. However, the assay only probes fibril elongation and/or secondary processes. The current wording can be misleading, and the term aggregation could be replaced by seeding capacity for clarity. For example, the authors state that lysine acetylation at sites 12, 43, and 80 each inhibits aggregation, but this statement is not supported by the data. Instead, the data show that the acetylation at these sites slows down the fibril elongation and thus decreases the seeding capacity of aS fibrils. In order to state that lysine acetylation has an effect on aS aggregation, fibril formation, the author should use an assay where the de novo formation of fibrils is assessed, such as in the presence of lipid vesicles or under shaking conditions.

It is not clear from the EM data that the structures of the different lysine acetylated variants are different, unlike what is stated in the text.

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Reviewer #3 (Public review):

Shimogawa et al. describe the generation of acetylated aSyn variants by genetic code expansion to elucidate effects on vesicle binding, aggregation, and seeding effects. The authors compared a semi-synthetic approach to obtain acetylated aSyn variants with genetic code expansion and concluded that the latter was more efficient in generating all 12 variants studied here, despite the low yields for some of them. Selected acetylated variants were used in advanced NMR, FCS, and cryo-EM experiments to elucidate structural and functional changes caused by acetylation of aSyn. Finally, site-specific differences in deacetylation by HDAC 8 were identified.

The study is of high scientific quality, and the results are convincingly supported by the experimental data provided. The challenges the authors report regarding semi-synthetic

access to aSyn are somewhat surprising, as this protein has been made by a variety of different semi-synthesis strategies in satisfactory yields and without similar problems being reported.

The role of PTMs such as acetylation in neurodegenerative diseases is of high relevance for the field, and a particular strength of this study is the use of authentic acetylated aSyn instead of acetylation-mimicking mutations. The finding that certain lysine acetylations can slow down aggregation even when present only at 10-25% of total aSyn is exciting and bears some potential for diagnostics and therapeutic intervention.

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Author response:

We thank you for your efforts in reviewing our manuscript. We sincerely appreciate that the reviewers were all enthusiastic about our comparison of native chemical ligation (NCL) and non-canonical amino acid (ncAA) mutagenesis methods for installing acetyl lysine (AcK) in alpha-synuclein, as well as the wide variety of biochemical experiments enabled by our ncAA approach. We respond to the critiques specific to each reviewer here.

Reviewer #1:

Expressed concern that in vitro studies of effects on membrane binding were not followed up with neurotransmitter trafficking experiments. While we certainly think that such studies would be interesting, they would presumably require the use of acetylation mimic mutants (Lys-to-Gln mutations), which we would want to validate by comparison to our semi-synthetic proteins with authentic AcK. Such experiments are planned for a follow-up manuscript, and we will investigate the reviewer's suggested experiment at that time.

Reviewer #1 Noted that the method of in vitro seeding really reports on the impact of acetylation on the elongation phase of aggregation. We will clarify this in our revisions. They also expressed concern that this was different than the role that acetylation would play in seeding cellular aggregation with pre-acetylated fibrils. We will also acknowledge and clarify this in our revisions. Having the monomer population acetylated in cells presents technical challenges that might also be addressed with Gln mutant mimics, and we plan to pursue such experiments in the follow-up manuscript noted above.

Reviewer #1 Criticized the fact that the pre-formed fibrils used in seeding would not have the same polymorph as PD or MSA fibrils derived from patient material. They were also critical of how our cryo-EM structure of AcK80 fibrils related to the PD and MSA polymorphs. Finally, while the reviewer liked the MS experiments used to quantify acetylation levels from patient samples, they felt that our findings then threw the physiological relevance of our structural and biochemical experiments into question. We believe that all of these critiques can be addressed by clarifying our purpose. We are not necessarily trying to claim that our AcK80 fold is populated in health or disease, but that by driving Lys80 acetylation, one could push fibrils to adopt this conformation, which is less aggregation-prone. A similar argument has been made in investigations of alpha-synuclein glycosylation and phosphorylation. Our results in Figure 9 imply that this could be done with HDAC8 inhibition. We will revise the manuscript to make these ideas clearer, while being sure to acknowledge the limitations noted by Reviewer #1.

Reviewer #2:

Expressed concern over our use of SDS micelles for initial investigation of the 12 AcK variants, rather than the phospholipid vesicles used in later FCS and NMR experiments. We

will note this shortcoming in revisions of our manuscript, but we do not believe that using vesicles instead would change the conclusions of these experiments (that only AcK43 produces an effect, and a modest one at that).

We will add additional detail to the figure captions, as requested by Reviewer #2.

Reviewer #2 shared some of the concerns of Reviewer #1 regarding the distinctions of which phase of aggregation we were investigating in our in vitro experiments. As noted above, we will clarify this language.

Finally, Reviewer #2 stated that “It is not clear from the EM data that the structures of the different lysine acetylated variants are different.” We feel that it is quite clear from structures in Figure 8 and the EM density maps in Figure S38 that the AcK80 fold is indeed different. Although the overall polymorphs are somewhat similar to WT, the position of K80 clearly changes upon acetylation, altering the local fold significantly and the global fold more moderately.

Reviewer #3:

Found the results convincing, including the potential therapeutic implications. The only concern noted was that they found the difficulties in semi-synthesis of AcK-modified alpha-synuclein surprising given that it has been made many times before through NCL. Indeed, our own laboratory has made alpha-synuclein through NCL, and the yields reported here are in keeping with our own previous results. However, since NCL did not give higher yields than ncAA methods, and it is significantly easier to scan AcK positions using ncAAs, we felt that ncAAs are the method of choice in this case. We will clarify this position in the revised manuscript.

In conclusion, on behalf of all authors, I again thank the reviewers for both their positive and negative observations in helping us to improve our manuscript. We will revise it to strive for greater clarity as we have noted in this letter.

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