RESEARCH ARTICLE

Avidity within the N-terminal anchor drives α-synuclein membrane interaction and insertion

Ersoy Cholak¹ | Katrine Bugge² | Adree Khondker³ | Kimmie Gauger⁴ | Elena Pedraz-Cuesta⁴ | Morten Enghave Pedersen⁵ | Saskia Bucciarelli¹ | Bente Vestergaard¹ | Stine F. Pedersen⁴ | Maikel C. Rheinstädter³ | Annette Eva Langkilde¹ | Birthe B. Kragelund²

¹Department of Drug Design and Pharmacology, University of Copenhagen, Copenhagen, Denmark
²Structural Biology and NMR Laboratory, The Linderstrøm-Lang Centre for Protein Science and Department of Biology, University of Copenhagen, Copenhagen, Denmark
³Department of Physics and Astronomy, McMaster University, Hamilton, ON, Canada
⁴Section for Cell Biology and Physiology, Department of Biology, University of Copenhagen, Copenhagen, Denmark
⁵Fida Biosystems ApS, Copenhagen, Denmark

Correspondence
Birthe B. Kragelund, Structural Biology and NMR Laboratory, The Linderstrøm-Lang Centre for Protein Science and Department of Biology, University of Copenhagen, 2200 Copenhagen N, Denmark.
Email: bbk@bio.ku.dk
Annette Eva Langkilde, Department of Drug Design and Pharmacology, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark.
Email: annette.langkilde@sund.ku.dk
Maikel C. Rheinstädter, Department of Physics and Astronomy, McMaster University, ABB-241 1280 Main Street West, Hamilton, ON, Canada.
Email: rheinstadter@mcmaster.ca

Funding information
The Novo Nordisk Foundation, Grant/Award Number: NNF15OC0016670;

Abstract
In the brain, α-synuclein (aSN) partitions between free unbound cytosolic and membrane bound forms modulating both its physiological and pathological role and complicating its study due to structural heterogeneity. Here, we use an interdisciplinary, synergistic approach to characterize the properties of aSN:lipid mixtures, isolated aSN:lipid co-structures, and aSN in mammalian cells. Enabled by the isolation of the membrane-bound state, we show that within the previously described N-terminal membrane anchor, membrane interaction relies both on an N-terminal tail (NTT) head group layer insertion of 14 residues and a folded-upon-binding helix at the membrane surface. Both binding events must be present; if, for example, the NTT insertion is lost, the membrane affinity of aSN is severely compromised and formation of aSN:lipid co-structures hampered. In mammalian cells, compromised cooperativity results in lowered membrane association. Thus, avidity within the N-terminal anchor couples N-terminal insertion and helical surface binding, which is crucial for aSN membrane interaction and cellular localization, and may affect membrane fusion.

Abbreviations: Ac-P₁₋₁₄, N-terminal acetylated 14-residue N-terminal peptide of aSN; Ac-aSN, N-terminal acetylated aSN; aSN, α-synuclein; CD, circular dichroism; CSPs, chemical shift perturbations; DAPI, 4,6-diamidino-2-phenylindole, dihydrochloride; d₀, membrane head-head distance; dₛ, lamellar spacing; EDTA, ethylenediaminetetraacetic acid; FIDA, flow-induced dispersion analysis; HSQC, heteronuclear single quantum coherence; IDP, intrinsically disordered protein; L:P, lipid to protein ratio; LBs, Lewy bodies; MD, molecular dynamics; MQ, Milli-Q; NMR, nuclear magnetic resonance; NR, neutron reflectometry; NTA, N-terminal acetylation; NTT, N-terminal tail; P₁₋₁₄, 14-residue N-terminal peptide of aSN; PD, Parkinson's disease; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol); POPC, 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine; PRE, Paramagnetic relaxation enhancement; R₉, radius of gyration; RT, room temperature; SAXS, small-angle X-ray scattering; SEC, size exclusion chromatography; SUVs, small unilamellar vesicles; TCEP, tris(2-carboxyethyl) phosphate; Ve, elution volume; XRD, X-ray diffraction.

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1 | INTRODUCTION

α-synuclein (aSN) is a small (140 residues in human, Figure 1A) intrinsically disordered protein (IDP) primarily expressed in the brain where it is predominantly located at presynaptic termini of neurons. In vivo, aSN partitions between the cytosol and cellular membranes, and both in vivo and in vitro, aSN may form amyloid fibrillar structures. In vivo, these materialize as intracellular aSN deposits, also known as Lewy Bodies (LBs), which are pathological hallmarks of a number of neurodegenerative disorders, including Parkinson’s Disease (PD) and LB dementia. LBs consist mainly of aSN amyloid fibrils as well as membrane constituents. However, new studies show significant amounts of non-fibrillar forms of aSN which may, together with membrane fragments and disordered organelles, form the structural basis for LBs. Although the biological function of aSN is still debated, several studies have shown that aSN interacts with membrane surfaces. Membrane interaction induces helical structures of aSN involving the ~100 N-terminal residues. In cells, aSN is predominantly N-terminally acetylated (Ac-aSN), and while the role of the modification is unclear, it has been shown that N-terminal acetylation (NTA) affects membrane binding affinity, leading to increased helicity in the bound state of Ac-aSN. Recent studies have proposed that a kink positioned around Y39-K45 midway along the helical structure enables membrane-bound aSN to bridge between membranes, thereby supporting fusion of neurotransmitter-containing vesicles with the presynaptic plasma membrane, resulting in neurotransmitter release.

The primary structure of aSN is tripartite with a positively charged N-terminal region hosting most of the seven imperfect sequence repeats, a hydrophobic non-amyloid-β component (NAC) domain and an acidic C-terminal (Figure 1A). The dominating view is that aSN is a monomeric IDP that partially folds upon binding to lipid membranes consisting of anionic lipids, and that this transition is modulated by the first approximately 25 N-terminal residues of aSN have indicated that aSN has a multitude of membrane binding modes, including induction of either two helices, or a single, elongated α-helix. The mode of binding depends highly on experimental conditions such as the lipid to protein molar ratio (L:P), the presence of salt and the membrane composition. Furthermore, aSN binding may change membrane conductance, synaptic neurotransmission, or membrane rupture, and thus, may be associated with pathological changes in neurodegenerative diseases. Depending on these factors, different interaction modes of aSN on the membrane surface have been reported. Recently, the first approximately 25 N-terminal residues of aSN have been suggested to enable membrane anchoring by initiating the association of aSN to the vesicle surface and being essential for the equilibrium between membrane bound and unbound states. Paramagnetic relaxation enhancement (PRE) NMR experiments have implied that the N-terminal anchor region resides on the surface of the lipid bilayer with no insertion, whereas molecular dynamics (MD) simulations and solid-state NMR data, as well as NR suggested that the first 12-15 N-terminal residues partially reside in the bilayer interface with a tilt angle of 12°. Similarly, using deletion variants of aSN it has been shown that helix formation in aSN upon membrane binding depends on the first 25 residues. However, this was highly dependent on the type of membrane mimetic, and the anchor region is therefore not sharply defined. When studied in yeast, deletion of residues 2-11 of aSN resulted in a dramatic impairment of membrane co-location as concluded from co-immunofluorescence, and resulted in increased cellular toxicity. However, since membrane complexity is very different between yeast and mammalian cells, the role of the N-terminal anchor for membrane localization in mammalian cells remains unaddressed. Furthermore, the use of different membrane mimetics and different length peptides has pointed toward the membrane affinity of the anchor region being strongly dependent on the type of mimetic, a conundrum that has not been fully explained. Finally, the structural properties of the N-terminal anchor, and its binding determinants, remains to be fully elucidated and the role of NTA for anchoring and insertion has not been addressed.

Here, we have taken an interdisciplinary approach and identify, isolate, and characterize aSN in a complex ensemble with small unilamellar vesicles (SUVs), which we term aSN:lipid co-structures. We find that N-terminal insertion of aSN into the anionic lipid membrane and on-membrane formation of helical structure, cooperatively enable membrane
binding via avidity. Deletion of the 14 N-terminal residues (aSNΔ1-14) abolishes the affinity for anionic lipid membranes and inhibits formation of aSN:lipid co-structures. Introducing the aSNΔ2-14 variant in mammalian cells caused a shift in the equilibrium away from the membrane, substantiating the functional relevance of the N-terminal tail (NTT). Modulation of any of these binding events will impact not only the lifetime of the membrane-bound state, but also important aspects such as membrane specificity and curvature sensing, and thus, detailed subcellular localization.

2 | MATERIALS AND METHODS

2.1 | Expression and purification of wild type, N-terminal acetylated, Y133C, and Δ1-14 α-synuclein

The gene coding for human aSN and the Y133C variant were cloned into the ampicillin resistant expression vector p-ET11a vector (a kind gift from Bioneer, Hørsholm, Denmark) and the proteins were recombinantly expressed in E. coli BL21
(DE 3) by the addition of IPTG (100 μM) at OD_{600nm} = 0.5-0.6. N-terminal acetylated aSN (Ac-aSN) was co-expressed with components of the N-α-acetylttransferase B (NatB) complex following the protocol described by Johnson et al.

The pNatB (pACYCduet-Ac-aSN 20-Ac-aSN 25) plasmid was a gift from Dan Mulvihill (Addgene #53613). The cells were harvested by centrifugation at 5000 g for 15 minutes and resuspended in an osmotic shock buffer (30 mM Tris, 40% sucrose, 2 mM Ethylenediaminetetraacetic acid (EDTA), pH 7.2). The lysate was centrifuged at 10 000 g for 20 minutes and the pellet resuspended in cold Milli-Q (MQ) H_{2}O adding 37.5 μL saturated MgCl_{2} solution to disrupt the cells. The lysate was then centrifuged at 10 000 g for 20 minutes and the supernatant subsequently boiled at 95°C for 5 minutes to remove impurities. The supernatant was dialyzed overnight against 20 mM Na_{2}HPO_{4}, 2.5 mM EDTA, pH 6.5, and loaded onto a 2 × 5 mL HiTrap Q FF anion-exchange column equilibrated in the dialysis buffer and eluted with a linear gradient of a final salt concentration of 0.5 M NaCl. Fractions containing aSN were identified by SDS-PAGE, pooled and injected in 4 mL aliquots onto a HiLoad Superdex 16/600 200 pg (GE Healthcare) column equilibrated in 0.5 mM NaCl, 20 mM Na_{2}HPO_{4}, pH 7.4 (phosphate buffer). The collected fractions were stored at ~20°C until use. Protein concentration was determined using a Nanodrop UV-Vis spectrophotometer (Thermo Scientific) and the extinction coefficient of 5960 M^{-1}cm^{-1} for aSN. The variant Y133C was purified following the same protocol, with addition of 1 mM of tris(2-carboxyethyl) phosphine (TCEP) before the gel filtration step.

To purify the aSNΔ1-14 variant, the expression construct was purchased from GenScript (United States) with the gene coding for His_{6}-SUMO aSNΔ1-14 inserted into an ampicillin resistant pET-11a expression vector. Expression of aSN in E. coli BL21 (DE 3) was initiated by adding IPTG (1 mM) at OD_{600nm} = 0.6. The cells were harvested by centrifugation at 8000 g for 20 minutes and the pellet resuspended in buffer A (50 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole, pH 8.0) with one tablet of EDTA-free protease inhibitor cocktail (Roche). The cell suspension was lysed by pressurization at ~1.8 kbar using a cell disruptor (Constant Cell Disruption Systems, UK) precooled to 4°C. The lysate was centrifuged at 12 000 g for 20 minutes at 4°C and the supernatant loaded onto a 5 mL HisTrap FF column preequilibrated with buffer A. Proteins were eluted with a stepwise gradient using the following steps: (1) 50 mM imidazole (6%) (2) 250 mM imidazole (46%) (3) 500 mM imidazole (100%). Fractions were pooled, and His_{6}-ULP1 purified according to Ref [41] was added to a final concentration of 0.1 mg/mL. The solution was dialyzed against 50 mM Tris-HCl, 150 mM NaCl, 17.25 mM of imidazole, 2 mM of 2-β-mercaptoethanol pH 8.0, at 4°C and loaded the following day onto a 5 mL HisTrap FF column, flow-through collected and concentrated to 4 mL using an Amicon Ultra 15 centrifugal filter (Millipore) with a molecular weight cutoff (MWCO) of 10 000 Da. The concentrated protein solution was loaded onto a HiLoad 16/600 Superdex 75 column preequilibrated in phosphate buffer and eluted over 1.5 column volume (CV) with a flowrate of 1 mL/min, collected in 1 mL fractions. aSN was stored at −20°C until use. For solution-state NMR, {^{15}}N and {^{13}}C/^{15}N labeled aSN, Ac-aSN, and aSN_{Δ1-14} were prepared using the same protocols and purified from E. coli grown in minimal M9 media supplemented with the trace element solutions, {^{15}}N-NH_{4}Cl and {^{13}}C/D-glucose, respectively (Sigma-Aldrich). For X-ray diffraction (XRD) experiments, the fractions collected using gel filtration were furthermore pooled and dialyzed three times against MQ-H_{2}O. Purified aSN was lyophilized and stored at −20°C until use.

Peptides of the 14 N-terminal residues of aSN and Ac-aSN were purchased in lyophilized form (amino acid sequence: MDVFKGLSKAKEG; >98% purity) from GenScript (United States).

2.2 | Covalent conjugation of aSN-Y133C

aSN-Y133C was mixed with a 10 × molar excess of TCEP and incubated on ice for 30 min. TCEP was removed using a 10 000 Da MWCO spin concentrator (Vivaspin) before adding 10 × molar excess of Alexa-488 maleimide, prepared according to protocol (Thermo Scientific). The mixture was transferred to a glass vial and left to react overnight in a dark cold room (4°C), with gentle magnetic stirring. aSN-Y133C- Alexa488 was separated from the unreacted dye using a Superdex HiLoad 200 16/600 column. The concentration of the purified product (pooled) was 40 μM and degree of labeling ~100%. The aSN_{Y133C-Alexa488} conjugate was aliquoted and stored at −20°C until use.

2.3 | Preparation of small unilamellar vesicles

1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (POPC) and 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1’rac-glycerol) (POPG) (Avanti Polar Lipids [Alabaster, AL]) were dissolved in chloroform and used without further purification. Aliquots of 1.6 mL of phospholipid from a 25 mg/mL stock were transferred to round flasks and evaporated by a rotary evaporator for 30 minutes to form a lipid film. The lipid film was stripped with 2 mL absolute ethanol (Sigma-Aldrich) for three times followed by overnight evaporation under a nitrogen stream. Subsequently, the lipid film was hydrated with HEPES buffer (20 mM HEPES, 200 mM KCl, 0.06 mM CaCl_{2}, 0.02 mM EDTA, pH 7.4) and sonicated for 5 minutes.
The mixture was vortexed for 2 minutes and 8 min off cycles for six times followed annealing of the lipids for 1 hour. To form SUVs, the solution was extruded at least 10 times through an 80 nm polycarbonate filter-membrane (Whatman) using a Lipex extruder (Northern Lipids Inc) to obtain a narrow and well-defined size distribution. The size of the resulting liposomes was assessed using a Malvern NanoZS (Malvern Instruments). The loss of lipids during extrusion was approximately 10%-15% as assessed by phospholipid content determination as described.43 SUVs were stored at 4°C until use and maximum 1 week.

2.4 | Size exclusion chromatography (SEC) for isolation of aSN:lipid co-structures

The experiments were devised to isolate the formed aSN:lipid co-structures of different aSN variants, for example, aSN, Ac-aSN, aSNΔ1-14, and pure SUVs. Aliquots of each aSN variant at a final concentration of 100 μM were mixed with SUVs made of POPG or POPC, respectively, at a L:P of 20:1 in a 1:1 mixture of two buffers: phosphate and HEPES buffer (20 mM HEPES, 200 mM KCl, 0.06 mM CaCl2, 0.02 mM EDTA, pH 7.4). The mixture was incubated at room temperature (RT) for 20 or 24 hours. The sample was then loaded onto the preequilibrated HiLoad Superdex 16/600 200 pg SEC column (GE Healthcare) in phosphate buffer by means of a 1 mL loop to ensure equal loading amounts each time. The reaction was followed by absorbance at 215 nm (backbone and SUVs) and 280 nm (aromatics). As controls, SUVs and all aSN variants were individually incubated at RT for 20 hours and loaded onto the column under the exact same conditions. Fractions were collected and analyzed by circular dichroism (CD) and NMR.

To calculate the fraction of unbound aSN in the aSN:lipid co-structures, the area under the peak corresponding to free aSN in the SEC profiles was considered. The boundaries used for the integration of the data were Ve, peak ± 3 mL, where Ve, peak is the aSN peak at 84 mL.

The concentration of aSN and lipid in the co-structures were determined in a parallel study44 to estimate the stoichiometry of the aSN:lipid co-structures.

2.5 | CD spectropolarimetry

Backbone conformation of aSN, Ac-aSN, and aSNΔ1-14, and the two peptides (P1-14 and Ac-P1-14) in the absence and presence of POPG SUVs was assessed by far-UV CD in a 1:1 mixture of phosphate and HEPES buffer. CD measurements were performed on a J-100 Series spectropolarimeter (JASCO, Japan) in a 1 mm quartz cuvette at RT. Data were collected in the wavelength range of 260-190 nm in 1 nm steps. The integration time was adjusted such that the applied High-Tension Voltage (HT) values remained below 600 V to avoid a poor signal-to-noise ratio. All protein samples were measured at the exact same settings as their corresponding buffers averaged in five and the buffer data subtracted. Only data with HT < 600 V are shown.

2.6 | Small angle X-ray scattering (SAXS) data collection and analysis of aSN:lipid bulk samples

In-house SAXS experiments were performed on a Xenocs BioXolver L (Sassenage, France) equipped with a liquid Gallium MetalJet X-ray source (λ = 1.34 Å). For each sample, two different sample detector-distances (d1 = 525.2 mm and d2 = 1475.2 mm) were used, allowing us to cover a q-range of 0.01-0.5 Å⁻¹ (q = 4πsin(θ/2)/λ where 2θ is the scattering angle). The temperature of the exposure cell and of the sample storage unit was kept constant at 22°C using a water bath. About 10 μL samples were loaded automatically from a 96-well tray by the sample handling robot of the BioXolver. Data were collected in multiple 60 seconds frames (for the samples: 15 frames in the setting with d1 and 40 frames in the setting with d2; for the buffer: 40 frames (d1) and 60 frames (d2), respectively). Each frame was initially corrected for background radiation and for direct beam intensity and 2D-averaging applied to yield the total scattering intensity. After buffer subtraction, data from the two settings were merged to cover the full q-range of interest. All data reduction was performed using the software RAW.45,46 Water was used as a secondary standard for absolute scaling of the data.

First, solutions of pure aSN and pure liposomes composed of POPG or POPC were measured. Liposomes and aSN were then mixed in a molar ratio of 20:1 and the solutions were measured immediately after mixing. Pure liposomes and mixed solutions were also measured after ~5, ~9, and ~14 hours incubation at 22°C and pure aSN after 22 hours. The buffer for all samples was a 1:1 mixture of phosphate and HEPES buffer and concentration of the free aSN and liposomes, was the same as the corresponding concentration in the mixtures. SAXS data of mixed samples were analyzed using the software OLIGOMER.48

2.7 | NMR sample preparation

Lyophilized aSN was weighted out and dissolved in phosphate buffer and filtered through 0.20 μm spin filters (Millipore). Ac-aSN and aSNΔ1-14, were directly collected from SEC. Protein solutions and POPG SUVs were dialyzed in the same beaker overnight against phosphate buffer. To
remove possible aggregates, aSN was centrifuged at 9600 g for 3 minutes. SUVs were added to the proteins to reach a L:P of 20:1 at a final protein concentration of 150 μM for 15N-aSN, 150 μM for 15N-Ac-aSN, and 120 μM for 15N-aSNΔ1-14. All NMR samples contained 0.7 mM of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), 10% (v/v) of D2O, and 0.02% (w/v) of NaN3 in total volumes of 350 μL and proteins were dissolved in 1xPBS.

2.8 | Solution state NMR

To minimize amide exchange, all NMR spectra were acquired at 283 K on Bruker AVANCE III 600- or 750-MHz (1H) spectrometers equipped with cryogenic probes. Free induction decays were transformed and visualized in NMRPipe or Topspin (Bruker Biospin) and analyzed using the CcpNmr Analysis software. Assignments of backbone nuclei of 13C, 15N-aSN in the free state were performed manually from the analysis of 15N-1H-HSQC, HNCACB, HNCOCA CB, HN(CA)CO, HNCO, and HN(CA)NNH spectra acquired with nonuniform sampling using standard pulse sequences. Proton chemical shifts were referenced internally to DSS at 0.00 ppm, with heteronuclei referenced by relative gyromagnetic ratios.

Time-course 15N-1H-HSQC spectra of 15N-aSN, 15N-Ac-aSN, and 15N-aSNΔ1-14, all with and without POPG SUVs at L:P = 20:1, were acquired over a period of 45 hours, with each 15N-1H-HSQC spectrum consecutively acquired over 23.5 minutes. Peak intensity ratios for the free aSN and variants over time were calculated relative to the 15N-1H-HSQC spectrum acquired from 0.4 hours of the free variants. Peak intensity ratios for the POPG added samples were calculated relative to the same timepoint for the free variant. Weighted CSPs were calculated between 15N-1H-HSQC spectra with and without POPG or between variants as the weighted Euclidean distance between the peaks using |γN|/|γH| = 0.154.

2.9 | X-ray diffraction (XRD) experiments

Highly oriented multi lamellar membranes were prepared on a single-side polished silicon wafer as previously described. POPOP (Avanti) and aSN were mixed 1:1 in 2,2,2-trifluoroethanol:chloroform (v/v) mixtures at a concentration of 20 mg/mL at desired molar ratios. The wafers were sonicated in 1,2-dichloromethane for 30 min, and then rinsed with alternating methanol and MQ water. The wafers were dried, and 80 μL of solution was deposited inside an orbital shaker (VWR) heated at 37°C for uniform spreading of the lipid:protein solution. Membrane complexes were placed in a vacuum for 16 hours at 37°C for the evaporation of the organic solvent. Membranes were annealed in a humidity-controlled chamber at 97% RH for 48 hours. This procedure ensures that 3000-5000 highly oriented bilayers are present on the silicon surface.

XRD data were obtained using the Biological Large Angle Diffraction Experiment (BLADE) at McMaster University. BLADE uses a 9 kW (45 kV, 200 mA) CuKα rotating anode at a wavelength of 1.5418 Å using a Rigaku HyPix-3000 2D semiconductor detector with an area of 3000 mm² and 100 μm pixel size. All samples were prepared and measured in replicates to check for consistency.

The relative electron density, ρ(z), can be approximated by a one-dimensional Fourier analysis,

\[ \rho(z) = \frac{2}{d_z} \sum_{n=1}^{\infty} N \sqrt{I_n q_n} \nu_n \cos \left( \frac{2\pi n z}{d_z} \right) \]

where N is the highest order of the Bragg peaks observed in experiment. The integrated peak intensities, I_n, are multiplied by q_n to receive the form factors, F(q), which is in general a complex quantity, is real-valued in the case of centro-symmetry. The phase problem of crystallography, therefore, simplifies to the sign problem, F(q) = ±|F(q)| and the phases, ν_n, can only take the values ±1. The phases ν_n are needed to reconstruct the electron density profile from the scattering data following Equation (1), and are well-defined by previous literature. When the membrane form factor, F(q), is measured at several q values in a continuous fashion, T(q), which is proportional to F(q), can be fit to the data.

\[ T(q_z) = \sum_N \sqrt{I_n q_n} \nu_n \nu_z \cos \left( \frac{1}{2} d_z q_z - \pi \nu_n \right) \]

In order to determine the phases quantitatively, the form factor has to be measured at different q values using the so-called swelling technique or by measuring the bilayer at different contrast conditions when using neutron diffraction. In this work, the phases, ν_n, were assessed by fitting experimental peak intensities and comparing them to the analytical expression for T(q) in the above equation.

To determine the partitioning coefficient, P, of each peptide, the distinct populations of inserted and external aSN are discriminated by their respective position in the bilayer. The width of the POPG bilayer was experimentally determined to be 37.6 Å. The fraction of aSN within one leaflet (within 18.8 Å as measured from the bilayer center) was defined as membrane-bound inserted; exterior contributions as external protein population. Thus, the partitioning coefficient can be determined as

\[ P = \frac{F_{\text{Inserted}}}{F_{\text{External}}} = \frac{\int_{0}^{18.8} \rho(z) dz}{\int_{0}^{37.6} \rho(z) dz} \]

(3)
For shorter peptide sequences, distinct populations of peptides are typically observed and the partitioning can be directly calculated from diffraction experiments.\(^{56}\)

### 2.10 Flow-induced dispersion analysis (FIDA) assays

FIDA was used for characterizing the interaction between aSN and POPG SUVs, both directly and in competition in the presence of an array of aSN variants. The FIDA experiments were performed using a FIDAlzyer instrument (Fida Biosystems ApS, Copenhagen, Denmark) utilizing laser-induced fluorescence (LIF) detection (ZETALIF Evolution, Picometrics, Labège, France) with excitation wavelength 488 nm (Melles Griot Diode laser, Picometrics) in connection with an optical high-pass filter. A standard fused silica capillary (inner diameter: 75 µm, outer diameter: 375 µm, length total: 100 cm, length to detection window: 84 cm, Fida Biosystems ApS) was used. The capillary was temperature-controlled to 21°C inside the FIDAlzyer instrument, excluding the minor part connected to the LIF detector. Furthermore, capillary inlet and samples were also temperature-controlled to 21°C. A 1:1 mixture of phosphate and HEPES buffer was used for the assay.

A stock solution of 2 µM aSN\(\text{Y}_{133}\text{C-Alexa488}\) was prepared in phosphate buffer and subsequently diluted to a fixed indicator concentration of 200 nM in the analyte concentration range of 0-200 µM POPG liposomes. All samples were preincubated for >10 min, to attain equilibrium prior to analysis. For competitive binding assays; a stock solution of 500 µM POPG liposomes was diluted to a fixed concentration of 100 µM, in the following competitor concentration ranges; 0-68.6 µM for aSN, and 0-63 µM for Ac-aSN, aSN\(\Delta_{1-14}\) and P\(\text{1-14}\), respectively. Finally, aSN\(\text{Y}_{133}\text{C-Alexa488}\) was added to reach a fixed indicator concentration of 200 nM. Each competition assay was performed separately for one competitor, and all samples were preincubated for >10 min to attain equilibrium prior to analysis. The capillary was rinsed and equilibrated prior to each sample analysis with 1 M NaOH and assay buffer, at 1500 mbar for 2 and 3 minutes, respectively. The analyte sample was injected at 1500 mbar for 45 s, subsequently the indicator sample (aSN\(\text{Y}_{133}\text{C-Alexa488}\), mixed with varying analyte concentrations) was injected at 50 µbar for 10 seconds (39 nL, corresponding to 1% of the capillary volume). The injected indicator sample was then mobilized toward the detection point with the analyte sample at 100 mbar for 600 seconds. All samples were analyzed in duplicate.

The Taylorgrams were processed using FIDA data analysis software (Fida Biosystems ApS, Copenhagen, Denmark) in order to calculate the hydrodynamic radius, compensated for viscosity fluctuations. Subsequently, the binding curves were then plotted in the software, to calculate the affinity constant (\(K_d\)) assuming 1:1 binding stoichiometry. The equations utilized by the software have been described previously.\(^{57}\) Note that in this work, the binding curves are generated from the binding isotherm based on diffusion coefficients instead of hydrodynamic radii.\(^{57}\)

### 2.11 Cell culture and transfection

AP1-pH-PLC-GFP cells were maintained in α-minimum Essential medium (Sigma, M8042), supplemented with 10% of fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin, and 600 µg/mL of G418. Cells were kept at 37°C, 5% CO\(_2\) and split every 3-4 days by gentle trypsinization. Full-length aSN and aSN\(\Delta_{2-14}\) were cloned into the pIREShneo (Clontech) vector and expressed in AP1-PH-PLC-GFP cells using 0.5-1 µg DNA/well of a 6-well dish, and Lipofectamine 3000 (Invitrogen, #L3000-015), according to the manufacturer’s instructions.

### 2.12 Western blot analysis

48 hours after transfection, aSN expression was assessed by Western blotting, essentially as in Ref. [58]. Briefly, cells were grown to ~70%-80% confluence in 6-well plates. After one wash in ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na\(\text{2HPO}_4\)-2H\(_2\)O, and 1.5 mM K\(\text{HPO}_4\)), cells were lysed (lysis buffer: 1% SDS, 10 mM Tris-HCl, pH 7.5), sonicated, centrifuged for 5 minutes at 20,000 g, protein concentration determined, loading buffer added (NuPage LDS buffer (Life technologies) plus 25 µM DTT), and samples heated at 95°C for 5 minutes. Equal amounts of protein per lane were separated on NuPAGE 10% bis-tris gels, and transferred to nitrocellulose membranes using the Novex gel System (Novex). Membranes were stained with Ponceau S, blocked at 37°C for 1 hour (5% nonfat dry milk in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20)), and incubated with primary antibody in blocking buffer overnight at 4°C. Antibodies used for Western blotting were: monoclonal mouse antibody against aSN (1:100, Santa Cruz Biotechnology, #sc-69977), and, for detection of loading controls, monoclonal antibody against β-actin (1:20 000, Sigma, #A5441). Membranes were washed in TBST, incubated with Horse Radish Peroxidase (HRP)-conjugated secondary antibodies (1:2000 Goat anti-Mouse (Agilent DAKO, P0447)) for 2 hours, at RT, washed in TBST, and visualized using the Clarity Western ECL Blotting Solutions (Bio-Rad) and a FUSION FX Spectra VIBLER.
2.13 | Immunofluorescence analysis of aSN membrane localization

Immunofluorescence analysis was performed essentially as in Pedraz-Cuesta et al.59 48-64 hours after transfection as above, cells seeded on glass coverslips were washed with ice-cold PBS and fixed with 4% of paraformaldehyde (PFA, Sigma #47608) in TBST for 15 minutes at RT. The cells were permeabilized with 0.1% Triton-X-100 (Sigma, #T8787) in TBST for 5 minutes followed by blocking for 30 minutes with 5% Bovine Serum Albumin (BSA, Sigma, #A7906) in TBST. Samples were incubated with primary antibodies against aSN (1:100 BD Bioscience, #61787, 1:50 Santa Cruz Biotechnology, #sc-69977) and against GFP (1:1000 Abcam Ab1218) in 1% BSA in TBST overnight at 4°C, washed 3 × 5 minutes with 1% BSA in TBST, and incubated with fluorescently labeled secondary antibody (1:600) in 1% BSA in TBST for 1.5 hours. Coverslips were washed for 5 minutes with 1% BSA in TBST, incubated with 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen, Waltham, USA, #C10595) in TBST for 5 minutes, washed 3 × 5 minutes with 1% BSA in TBST, mounted using N-propyl-gallate mounting medium (2% (w/v) in PBS/glycerol, and sealed. Cells were visualized using the 60X objective of an Olympus Bx63 microscope utilizing Olympus CellSens dimensions software and processed in ImageJ. Quantitative analysis was performed using ImageJ software and the “ColorProfiler” plugin: Using ImageJ, a line starting extracellularly was drawn across an area with visible aSN fluorescence. From this line, the membrane region was defined as the region between the point with the highest GFP intensity and the end of the downward slope of the GFP signal. The cytosolic region was defined from a line drawn in continuation of the first line, and spanning twice the length of the membrane line. Only areas in which a clear intensity increase in membrane fluorescence was detectable to distinguish between membrane and cytosol areas qualified for analysis. From the line scans, the average aSN intensity in the two regions was determined, and a membrane localization ratio calculated was as average aSN intensity in the membrane region/average aSN intensity in the cytoplasm region. This value was determined for 10 cells per condition from each of four independent experiments, for both WT aSN and aSNΔ2-14.

3 | RESULTS

3.1 | Bulk aSN interactions with a POPG membrane system using a L:P of 20:1

The many studies of aSN with membrane mimetics have revealed system dependent affinities and structural properties, so we first established a single experimental condition allowing us to decompose any anchor properties. We analyzed bulk properties by adding aSN to synaptic vesicle mimics, namely POPG and POPC SUVs. This was done at a fixed L:P of 20:1. We combined CD, NMR, SAXS, and flow-induced dispersion analyses (FIDA) (Figure 1), assuring homogeneity of aSN using SAXS, giving a radius of gyration ($R_g$) of 34 Å (Figure S1, Table S1). The disordered state was confirmed by far-UV CD analysis of aSN alone and in the presence of zwitterionic POPC SUVs (Figure 1B) and in agreement, the $^{15}$N-$^1$H heteronuclear single quantum coherence (HSQC) NMR spectrum of aSN showed low dispersion of the $^1$H chemical shifts (Figure 1C). Changes in the aSN HSQC peak intensities were monitored over time to assess sample stability and no changes were seen over ~45 hours (Figure 1D).

After incubation with POPG SUVs for 18 hours, the far-UV CD spectrum of aSN was consistent with $\alpha$-helical structure populated to ~30% (estimated as described in Ref. [60] Figure 1B). Incubation of aSN with POPG SUVs at L:P = 20:1 at 10°C for 45 hours reduced the intensity of the vast majority of NMR peaks from residues 1-131, as expected13,61 (Figure 1E). The reduction was exacerbated toward the N-terminal to ~95% intensity reduction, while chemical shift perturbations (CSPs) were only modestly detectable in the region from residues 4-22, (Figure 1E). The peak intensity reduction is consistent with previous results,13,20 and highlight a membrane-bound state inaccessible to solution-state NMR. No further intensity reduction in the NMR peak intensities was detected after ~30 hours (Figure 1D). The affinity of aSN for POPG SUVs was determined using FIDA. For this, Y133 in the C-terminal region of aSN, not involved in POPG interaction as judged from NMR, was mutated to cysteine and labeled with Alexa-488 to generate aSNY133C-Alexa488. Changes in the diffusion coefficient upon addition of increased amounts of POPG SUVs allowed the determination of a $K_D = 2.5$ μM (Figure 1F). Finally, the overall structural changes in the bulk sample were followed by in-house SAXS on aSN in the presence of POPC or POPG SUVs. The data of the bulk sample of aSN:POPG could not be fitted with the form factors of the individual species (Figure 1G) suggesting formation of new species. The same analysis was repeated with aSN and POPC SUVs, and here, the experimental curve could be fitted by a linear combination of the individual form factors, showing no interaction between aSN and POPG SUVs (Figure S2).

In line with previous studies, aSN forms $\alpha$-helical structure upon membrane interaction, perturbing NMR peaks of the ~120 most N-terminal residues. Decomposition analysis of the bulk sample of aSN:POPG using SAXS suggests formation of one or more new species different to monomeric aSN and POPG SUVs and using diffusion, we determine an average $K_D$ of 2.5 μM.
3.2 |Bulk decomposition using SEC can isolate the membrane-bound aSN population

To isolate the membrane-bound species and dissect their properties, we performed SEC on aSN incubated with POPG SUVs. Alone, aSN eluted as a monomeric protein at \( V_e = \sim 84 \) mL, while vesicles eluted in the void (Figure 2A). aSN also eluted at \( V_e = \sim 84 \) mL after 20 hours of incubation with POPC SUVs. In contrast, incubation for 20 hours with POPG SUVs led to the formation of new species consisting of aSN and SUVs, which we term aSN:lipid co-structures. These eluted over a range of volumes from \( \sim 44 \) to \( \sim 62 \) mL (Figure 2B), and their kinetics of formation, L:P stoichiometry, and influence on fibrillation was determined in a parallel study.\(^{44}\) The fractions containing co-structures were further analyzed by far-UV CD. Helical structure started to appear at \( V_e = \sim 45 \) mL and the ellipticity kept decreasing at 222 nm until \( V_e = \sim 49 \) mL (Figure 2C). Based on normalized ellipticity values, the \( \alpha \)-helical structure was more populated in the selected fraction compared to that of the bulk (\( \sim 60\% \) compared to \( \sim 30\% \)) (see methods for details). The development in the ellipticity at 222 nm did not follow the change in \( A_{280} \) (Figure 2D) suggesting either formation of species with different aSN/lipid ratios or that aSN exhibits different degrees of helicity in different membrane-bound states. In our parallel study, we determined the stoichiometry...
Finally, 15N-1H-HSQC spectra were recorded on Ac-aSN (Figure 3B), but their kinetics of formation was faster.44 Structures formed similarly to the aSN:lipid co-structures of the SEC chromatograms at 280 nm, Ac-aSN:lipid co-structures with POPG SUVs for 18 hours resulted only in a slightly lower helicity as judged by CD (Vc = ~49 mL) was analyzed by NMR (Figure 2E). A complete loss of NMR signals from the 120 most N-terminal residues was seen, suggesting a higher population of vesicle bound aSN than in bulk (Figure 2F), in line with the CD data. The NMR spectrum remained constant over time suggesting that once formed, the bound species—the co-structure—remained stable. The SEC profile also suggested that the POPG bound state of aSN only constituted ~25% of the total aSN, as judged from the ratio of the area under monomeric peak of aSN alone and in the presence of POPG SUVs.

The total area under the curve of the peak for the aSN monomer was estimated ~4300 abs*mL while that of the aSN:POPG mixture (L:P = 20:1 after 20 hours incubation) was depleted to ~3200 abs*mL. Thus, after aSN:lipid co-structures are formed, the unbound protein fraction was estimated to ~75%. These results show that isolation of the vesicle bound state of aSN is possible by SEC and that the bulk state, without purification, consists of a significant fraction of unbound aSN (~75% by SEC) in solution. The membrane-bound state remained stably bound as judged from the NMR peak intensities and has a helical content of approximately 60%, likely distributed over several membrane-bound conformations, the aSN-lipid co-structures.44 We observe membrane association of the first 1-120 residues of aSN while the C-terminal remains disordered. Based on elution volumes, the new species formed have different properties than the SUVs.

### 3.3 aSN:lipid co-structures also form when aSN is N-terminally acetylated

In vivo, the majority of aSN in the brain is N-terminally acetylated.17,18 To understand if this modification affects the ability of aSN to form aSN:lipid co-structures, we repeated the SEC using N-terminally acetylated aSN (Ac-aSN) (Figure S3). aSN and Ac-aSN were analyzed in parallel and individually incubated with the same POPG SUVs batch for 18 hours, and then, loaded onto the SEC column consecutively. Incubation of Ac-aSN with POPG SUVs for 18 hours resulted only in a slightly higher α-helical content than for aSN (33% vs 29%, respectively) (Figure 3A). Judged by the peak absorbance of the SEC chromatograms at 280 nm, Ac-aSN:lipid co-structures formed similarly to the aSN:lipid co-structures (Figure 3B), but their kinetics of formation was faster.44 Finally, 15N-1H-HSQC spectra were recorded on Ac-aSN alone and on Ac-aSN:POPG at L:P = 20:1 at 10°C for 45 hours (Figure 3C). Similar to what was observed for non-acetylated aSN, peak intensities decreased gradually from residue 131 toward the N-terminal (Figure 3D,E). Again, CSPs were modest and restricted to residues 4-22 (Figure 3D) and saturation of NMR peaks was achieved at ~30 hours. Thus, NTA of aSN does not change the overall properties of the aSN:lipid co-structures as judged from the NMR and SEC analyses.

### 3.4 Structure of the membrane-bound aSN from X-ray diffraction

To address the properties of the NMR-inaccessible membrane-bound state of aSN, we used wide-angle XRD on highly oriented lipid bilayer systems containing POPG as schematically shown in Figure 4A and using aSN variants. The collected 2D X-ray intensity images (Figure 4B) were analyzed to determine the overall molecular structure of the aSN:lipid co-structures along the out-of-plane (qz) axis. A single series of Bragg peaks along qz is indicative of a lamellar membrane phase (Figure 4C). The experimentally obtained results in reciprocal space can be converted to real space by Bragg’s Law, dz = 2π/Δqz, with dz being the lamellar spacing (dL). The addition of aSN to POPG membranes resulted in additional peaks along qz, which indicated a secondary membrane phase. By fitting the diffraction pattern to two distinct series of Lorentzian peaks, the pattern was decoupled into two phases: A membrane phase and a membrane-embedded protein phase with spacings of 62.2 ± 0.1 Å and 94.4 ± 0.1 Å, respectively (Figure 4D, E). By integrating the intensity of each peak, the electron density profile, ρ(z), of each phase was determined (Figure 4F). By comparing the membrane-embedded protein phase with the pure POPG membranes, the electron density was fitted by a theoretical model of a membrane and different locations and orientations of aSN. The best fit of this model to the data determined the penetration of the molecule into the bilayer and in this way the position of aSN in the aSN:lipid co-structures was determined. aSN and Ac-aSN showed similar properties, with an increased electron density at |z| < 20 Å, suggesting that a fragment of aSN is inserted into the membrane. The data and the results fit were consistent with an insertion of the 14 most N-terminal residues into the bilayer. We refer to this as the NTT. As seen in Figure 4F, aSN electron density penetrated ~5.8 Å into the membrane. Assuming these residues form an α-helical structure, the model fitting is consistent with an insertion angle approximated to 17° in the head-tail interface of the membrane. By comparing the electron density with respect to the lipid head-to-head distance of ~19 Å, Ac-aSN was found to partition ~2 Å deeper into the membrane than aSN. Furthermore, as shown in Figure 4F, two distinct populations of aSN were found in...
The unit cells centered around $|z| = 20$ and $40$ Å, corresponding, respectively, to a membrane-bound and a free state. The membrane-bound state was populated to $>80\%$, differing from the SEC analyses. However, the flow in SEC likely underestimates the bound population, whereas XRD likely overestimates it due to lower hydration, which can make the bound states more favorable. The membrane-bound electron density of aSN spans from 12-28 Å around the bilayer, which may suggest that a large region of aSN is localized on the surface of the bilayer (ie, residues 15-100).

Together, these results can be compiled into a two-phase model for aSN in POPG membranes, where there is an equilibrium between a free and a membrane-bound state, Figure 4G. The bound state anchors the NTT into the lipid head group layer and a large region of the protein is bound on the membrane surface.

3.5 | Avidity in membrane interaction

To understand the importance of the N-terminal insertion for membrane affinity, we investigated a 14-residue N-terminal peptide of aSN (P1-14) corresponding to the NTT. We analyzed both this peptide and the acetylated counterpart (Ac-P1-14). The CD spectra of P1-14 and Ac-P1-14 were characteristic of disordered chains, with a minimum at 200 nm and in the presence of POPG SUVs, (20:1) both peptides remained disordered (Figure 5A). Thus, alone, the NTT either lacks affinity for POPG SUVs (as supported by earlier studies on similar aSN peptides$^{62}$), or the peptides interact with the membrane without structure induction. From electron density profiles of P1-14 and Ac-P1-14 with POPG membranes obtained by XRD (Figure S3A), we observed an increased electron density in the lipid head groups, indicative of N-terminal contact with
FIGURE 4  The N-terminal of aSN and Ac-aSN inserts into the anionic lipid membranes. A, Schematic of X-ray diffraction set-up. B, 2-dimensional intensity image from POPG + 1 mol% aSN (97% relative humidity). The dashed lines show the slice along the out-of-plane axis \( q_z \) used for further analysis. C, Out-of-plane profile of each sample studied; POPG alone (black), POPG + aSN (red), POPG + Ac-aSN (green) D, Phase decomposition of POPG + aSN sample and component Lorentzian peaks for both POPG and POPG + aSN phases, respectively. E, Position of Bragg peaks and membrane spacing for POPG and POPG + aSN phases. F, Resulting electron density profile for each sample studied, illustrated membrane in background corresponds to position in bilayer. G, Summary of diffraction results which shows aSN between the stacked membranes with lamellar spacing \( d_z \) and membrane head-head distance \( d_{HH} \).
FIGURE 5 Truncation of the first N-terminal 14 residues prevents aSN:lipid co-structure formation. A, Far-UV CD analysis of the peptides, for example, P1-14 and Ac-P1-14, in the presence of POPG (100%) SUVs. P1-14 and Ac-P1-14 were incubated 20 hours at RT with POPG SUVs at L:P of 20:1. P1-14 alone (red line), Ac-P1-14 (gray line), P1-14:POPG (red dashed line), Ac-P1-14:POPG (gray dashed line). B, Membrane partitioning. C, SEC analysis of aSN (black) and aSNΔ1-14 (red) incubated with POPG SUVs at a L:P = 20:1 for 20 hours at RT monitoring A280. Monomeric aSN and SUVs elute at V_e of ~84 and ~42 mL, respectively. D, Far-UV CD analysis of aSN and aSNΔ1-14 in the presence of POPG (100%) SUVs. aSN and Ac-aSN were incubated 20h at RT with POPG SUVs at L:P of 20:1. aSN alone (black line), aSNΔ1-14 (red line), aSN:POPG (black dashed line), aSNΔ1-14:POPG (red dashed line). E, Perturbations of NMR resonances. Top: Weighted CSPs between aSN and aSNΔ1-14, plotted against residue number. Middle top: CSPs of aSNΔ1-14, resonances after 44.4 hours at 10°C in the absence and presence of POPG at L:P = 20:1 plotted against residue number. Middle bottom: Ratios of NMR peak-intensities of aSNΔ1-14, after incubation at 10°C for 44.4 hours relative to resonance intensities measured at timepoint 0-0.4 hours (I(f, 0.4 hours)) plotted against residue number. Bottom: NMR resonance intensity ratios of aSNΔ1-14, after incubation at 10°C for 44.4 hours in the presence of POPG at L:P = 20:1. I(P) relative to resonance intensities measured at the same timepoint for free aSNΔ1-14, plotted against residue number. "**" highlight unassigned residues or prolines, and the gray horizontal bar the absent residues 1-14. F, Out-of-plane profiles for POPG and POPG + aSNΔ1-14 with assigned Bragg peaks. G, Position of peaks as a function of a number of Bragg peaks to calculate membrane width (shown in insert). Errors are from linear fit. H, Changes in the diffusion coefficient of prebound aSNY133C-Alexa488:POPG samples after competition with different aSN variants to monitor the binding affinity of aSNY133C-Alexa488 toward lipid membranes in the presence of different aSN variants. The IC50 was determined as described in materials and methods.
the lipid bilayers. However, significant increases in electron density were not seen in the hydrophobic core of the bilayer at |z| < 12 Å suggesting that full insertion did not occur. From quantification of the partitioning, only a small population (<10%, P1-14) and <16%, Ac-P1-14) inserted into the membrane contrasting the >80% insertion seen for the full-length aSN (Figure 5B). In line with the studies of the full-length proteins, a slightly deeper insertion of Ac-P1-14 compared to P1-14 was observed (Figure S3A). Together, these data suggest that insertion of the NTT of aSN into the membrane is dependent on binding of the rest of the N-terminal anchor to the membrane surface.

To investigate if the remaining part of the N-terminal anchor would be enough to facilitate membrane interaction of aSN, we produced a variant lacking the first 14 residues, aSNΔ1-14, and repeated the SEC, CD, XRD, and NMR analyses in the absence and presence of POPG vesicles. Indeed, as expected, aSN:lipid co-structures formed for aSN, whereas aSNΔ1-14 eluted exclusively as a monomer at Vc = ~85 mL without detectable aSN:lipid co-structure formation (Figure 5C). The symmetric appearance of the void peak of aSNΔ1-14:POPG suggested that aSNΔ1-14 has lost the ability to form aSN:lipid co-structures. Unlike full-length aSN, aSNΔ1-14 was found by CD to be disordered both in the absence and the presence of POPG SUVs (Figure 5D). The NMR spectra of aSNΔ1-14 revealed that deletion of residues 1-14 did not affect the overall aSN ensemble prior to SUV addition (Figures 5E and S3B) and no changes in peak positions or intensities were detected upon addition of POPG SUVs (Figure 5E). Finally, by XRD, only a single series of Bragg peaks was observed for both POPG alone and aSNΔ1-14:POPG membranes (Figure 5F). In contrast to aSN, the d spacing did not significantly increase with the addition of aSNΔ1-14 to the membranes (Figure 5G). The affinity of the aSN variants for POPG SUVs was determined using FIDA where POPG SUVs were co-incubated with 0.2 μM aSNY133C-Alexa488 at L:P = 20:1 as well as with variants. Both aSN and Ac-aSN could outcompete co-added aSNY133C-Alexa488 giving IC50 values of 0.5 μM and 0.3 μM, respectively. However, within the concentration range of 0-78 μM, neither aSNΔ1-14 nor P1-14 were able to outcompete aSNY133C-Alexa488 when co-added to the SUVs (Figure 5H).

In conclusion, neither by SEC, CD, XRD, nor NMR did we observe interactions of aSNΔ1-14 with POPG lipids. Furthermore, aSNΔ1-14 had no ability to form aSN:lipid co-structures. Together with data on the NTT peptides, this suggests that membrane binding is achieved by a cooperation within the N-terminal anchor that couples the NTT and the remainder of the anchor. Thus, avidity within the anchor is important for forming and securing a long-lived membrane-bound state of aSN. This is, to our knowledge, the first demonstration of avidity in IDP:membrane binding and the first demonstration of a specific role for the aSN NTT for mammalian aSN:membrane interaction.

3.6 | Lack of anchoring by the NTT reduces aSN membrane localization in mammalian cells

Previously, in yeast cells, the N-terminal residues 2-11 have been shown to be important for membrane localization, as judged by immunofluorescence imaging.39 To address the biological relevance of avidity in membrane binding in the more complex mammalian cell, and provide a quantitative measure for the effect on membrane localization, we generated AP1-PH-PLC-GFP cells, a CHO-derived cell line stably expressing a GFP-tagged phosphatidyl-inositol-(4,5)-bisphosphate reporter for visualization of the plasma membrane. Full-length wild-type aSN and aSNΔ2-14 were expressed to approximately equal levels in these cells (Figure 6A). aSN localization was assessed by immunofluorescence analysis, which showed that both wild-type aSN and aSNΔ2-14 (magenta) localized to the cytosol as well as to the plasma membrane, assessed as co-localization with GFP (green) (Figure 6B). Validating the specificity of aSN staining, similar results were obtained with two different aSN antibodies (Figure 6B). aSN:plasma membrane interactions were quantified based on line-scan analyses using the two different aSN antibodies (Figure 6C). This approach ensured that low-affinity interactions were maintained and that only plasma membrane interactions (as opposed to interactions with various organelles) were included. Results were similar using the two aSN antibodies and showed that the relative membrane localization of aSNΔ2-14 compared to WT aSN was significantly reduced (Figure 6D-E). Taken together, these results show that the NTT contributes to aSN membrane localization in mammalian cells.

4 | DISCUSSION

It is widely acknowledged that at least part of the physiological role of aSN is associated with its ability to bind to membranes.25 However, studying the interaction between aSN and lipid membranes at the molecular level is challenging due to the intrinsically disordered nature and population heterogeneity of aSN as well as the inherent structural complexity of membranes.63 A number of studies support an emerging view that the N-terminal of aSN plays an anchoring role in membrane interaction, modulating not only the physiological, but also the pathological role of aSN.36,64,65 However, the details of how this anchoring role is executed, its length and its properties have remained elusive. Enabled by an integrative biophysical approach, we isolated and characterized the membrane-bound state of aSN. In the bulk, under...
FIGURE 6  The NTT contributes to the cellular co-localization of aSN. AP1-PH-PLC-GFP cells, which express a GFP-tagged PI(4,5)P₂ binding protein used for visualization of the plasma membrane, were transiently transfected with WT aSN or aSNΔ2-14 and, 48-64 hours later, subjected to either. A, Western blotting for aSN and β-actin as loading control or B, immunofluorescence analysis for aSN (magenta) and GFP (green). DAPI was used to stain nuclei. Scale bars: 20 µm. BD: BD biosciences aSN antibody; SC: Santa Cruz Biotechnology aSN antibody. White lines illustrate the positions of the corresponding line scans in C. C, Line scan profiles were generated in ImageJ using the Color Profiler plugin. Relative aSN membrane expression was determined as the ratio between aSN intensity values in the membrane (first black line from left) and cytosol (second black line from left). D-E, Relative membrane expression based on the BD (D) and SC (E) antibody staining, respectively. Data are shown as means with individual points and SD error bars, for a total of 40 cells, 10 in each of four experiments, per antibody and condition. ****: P < .0001, Students t test
the conditions of this study this state constituted less than 50% of all aSN, but remained stable after isolation as judged by NMR data and showed distinct structural characteristics despite the broad elution profile as shown by SAXS. Using data from isolated species enabled us to further decompose the N-terminal anchor region of aSN into a bipartite cooperative structure. The ~14 most N-terminal residues insert into anionic membranes at a skewed insertion angle, which is coupled to the folding of the helical region onto the surface of the membrane, thereby synergistically establishing the aSN:lipid co-structures. Thus, a key finding of this work is the presence of avidity within the N-terminal anchor.

The energy landscape of aSN is highly dynamic. Hence, it is likely that the membrane-bound state of aSN re-arranges from one conformational state to another, and with different distributions between the on- and off-membrane states, depending also on the type and shape of the membrane with which it interacts. Indeed, also under the conditions investigated here using a more anionic POPG membrane mimetic, we reveal that the aSN:lipid co-structures elute from the SEC column in fractions with distinct structural properties. Experimental parameters, such as aSN concentration, lipid to protein ratio, as well as aSN point mutations are known to affect membrane-binding modes and in a recent study we show how some these properties affect the kinetics of the co-structure formation. Previously, using fluorescence and NR measurements, and later complemented by solid-state NMR and MD simulations, it was suggested that aSN penetrates ~9-14 Å into the outer leaflet of the membrane bilayer, with the 15 N-terminal residues inserted at an angle of 12°. Using XRD, we calculated from modeling of the electron density the number of residues partitioning into the bilayer to be 14 and the angle of insertion to be 17°. This is in line with previous reports, but we extend these studies to further establish that NTA did not change the angle, but led to a slightly deeper insertion. Deletion of the first 14 N-terminal residues of aSN impaired the formation of aSN:lipid co-structures and led to a dramatic reduction of the membrane binding affinity of both the aSNΔ1-14 variant. Likewise, the NTT alone (P1-14) had dramatically reduced affinity for the SUVs, revealing avidity within the N-terminal anchor of aSN. The decomposition of the anchor into two coupled units is consistent with previous NMR data that showed a lower helix population for residues 10-13. Indeed, it has been shown that N-terminal deletion (Δ2-11) causes dramatic impairment of vesicle binding of aSN in vitro, as well as of membrane binding and cellular toxicity in yeast. Here, importantly, we extended these studies to the more complex mammalian cells, showing for the first time quantitatively that deletion of residues 2-14 reduces aSN membrane localization in mammalian cells. While significant, the difference is quantitatively smaller in a cellular context than in vitro. This likely reflects that the cell, and in particular the sub-membranous region where the aSN:plasma membrane interaction occurs, differs completely from the in vitro scenario in terms of crowding, charges, ionic strength and pH, and lipid composition. That significantly reduced membrane association of the N-terminally deleted aSN is observed in the cellular context demonstrates that the N-terminal anchor has an impact in vivo and is therefore physiologically relevant.

### 4.1 A coherent model for aSN membrane interaction and cooperativity

Most studies describing aSN interactions with membranes have been performed in the bulk. Here, having isolated and characterized the aSN:lipid co-structures, we were able to extract properties of the bound state that together with previously published data may provide a coherent model of aSN:membrane interactions: First, the repetition of lysine residues embedded in the 11-mer motifs facilitates the initial electrostatic attraction of aSN to the membrane. The preferential binding affinity of aSN for vesicles containing only acidic lipids, but not for those with net neutral charge, highlights the importance of these lysines. This mode of binding is further supported by membrane binding studies on other lysine-containing IDPs such as membrane proteins and membrane curvature sensing proteins. Once encounters with the negatively charged surface occur, the NTT is brought vis-à-vis the headgroups, enhancing the probability of insertion by a local concentration effect. Either the NTT inserts first allowing the remaining anchor region to cooperatively fold into a helical structure on the negatively charged surface, or the helical structure is formed first, allowing the NTT to insert. Both events may likely occur and enhance the overall affinity cooperatively. Once anchoring is established, N-terminal residues outside the anchor folds and unfolds dynamically on the membrane surface, leaving the C-terminal 40 residues mostly disordered and unbound. Using SDS micelles, two antiparallel helices in aSN, H1 (3-37) and H2 (45-92), were assigned and similar helix break observations or different helical binding modes were reported by others. Furthermore, H2 was suggested to be able to detach and engage in interactions with a second membrane, leading to membrane fusion. In the absence of other vesicles/membranes, both helices likely remain dynamically attached on the surface as supported by pulsed EPR and single molecule fluorescence analyses. In our studies, we did not address the helical break, but the variation in properties of the co-structures could be indicative of a dynamic binding. Thus, aSN likely binds to membranes by exploiting a multilayer structure consisting of the NTT (residues 1-14), H1 (residues 15-37) (together likely constituting the anchor), and H2 (residues 45-97). An overview of this model is shown in Figure 7.
aSN has a preference for binding to curved membranes of anionic lipids, a preference that is enhanced by NTA. NNT insertion and the cooperativity between this and helix formation on the membrane surface within the anchor, might explain this preference as membrane curvatures would optimize NTT insertion. The NTT has small side chains located on one side of the helix, with an asymmetric distribution of hydrophobicity, and thus, has more resemblance to a fusion peptide than to a transmembrane helix. Fusion peptides are N-terminal regions of proteins active in viral membrane fusion forming α-helical structure when inserted into membranes and serving as membrane anchors. Furthermore, they appear to destabilize the target membrane locally, providing a starting point for fusion. Thus, insertion of the NTT may affect membrane stability in a similar way, an effect we observed directly as aSN decomposed the SUVs via co-structure formation; an effect not seen for aSNΔ1-14.

The inherent relatively low affinity of aSN for the membrane and the avidity effect described here is likely to be strongly sensitive to mutations, membrane constituents, and posttranslational modification. These factors will affect not
only the equilibrium between membrane bound and free states of the protein, but may also change the curvature sensing ability of aSN, fusion properties, and hence, the subcellular specificities of the protein, all of relevance to pathology. Indeed, changing the threonine in the KTKEGV repeats to more hydrophobic residues, such as phenylalanines or leucines, allowed aSN binding to liposomes regardless of their size or charge, likely via enhanced surface helix binding. Increasing the hydrophobicity by mutating all glycines and alanines to valines in a peptide model of aSN (residues 2-23) led instead to increased curvature sensing. This model lacks the majority of the surface helix, and NTT insertion, thus, becomes more dependent on curvature. In analogy, phosphorylation of Ser87 in H2 decreased membrane affinity, just as familial PD mutations like A30P and A53T located in H1 and H2, respectively, also decreased membrane binding. Thus, any changes to the chemical properties of aSN via changes in the local affinity around the altered residues may hamper either NTT insertion or surface helix formation, and may thus, indirectly weaken—or decouple—the avidity.

A few N-terminal truncations of human aSN have recently been reported in samples from postmortem brain tissue, most of which were missing the entire N-terminal region, and only one with truncation of the first four residues. The role of this form still remains elusive. N-terminal truncation has recently been shown to induce alternative polymorphs of aSN fibrils, suggesting a potential link between altered NTT and phenotype variation in Parkinson’s disease. As shown here, deletion of residues 2-14 quantitatively affected membrane binding in mammalian cells and changes to the NTT may therefore lead to an alteration of the function of the protein in, for example, synaptic vesicle homeostasis and aSN exocytosis. Indeed, since aSN is highly specialized to sense specific lipid types and vesicle sizes, NTT insertion could determine where in the cell aSN operates, but further studies are needed to determine the role of N-terminal truncated forms.

The N-terminal insertion and membrane interaction may influence fibrillation and in a parallel study we have investigated aSN fibrillation in the presence of aSN:POPG co-structures at various incubation times. Initially, with short incubation times, the co-structures appear to recruit additional aSN to the membrane, while the fully formed co-structures increase fibril nucleation, likely through the protruding C-terminal parts mimicking the fibril surface. Given the observed differences in the distribution between the cytosolic and membrane-bound states, we further suggest that avidity within the N-terminal anchor plays a physiological/pathophysiological role, and hence, that avidity should be considered carefully in future studies searching for improved therapeutic avenues. Finally, the role of the NTT as a potential fusion peptide opens for new questions in relation to its potential role in membrane fusion, curvature sensing, and how the biological functions of aSN depends on anchoring. aSN is the first example of an IDP demonstrating avidity in membrane binding, but it is likely that other IDPs harbor similar properties and are active in membrane fusion.

ACKNOWLEDGMENTS
The authors wish to thank Jacob Hertz Martinsen for protein production and Eric Jackson for skilled technical support. This work was supported by the Novo Nordisk Foundation Synergy program (BBK, SFP, BV), the Lundbeck Foundation Initiative BRAINSTRUC (BV, AEL), the Canada Foundation for Innovation (MCR), the Natural Sciences and Engineering Research Council of Canada (MCR), and McMaster University (MCR).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTION
AEL and BBK directed the study with input from SFP and BV. EC carried out SEC and CD studies and data analysis and was involved in SAXS, XRD, and FIDA sample preparation, experimental design, and data collection. KB carried out the NMR studies and data analysis. AK and MR carried out the XRD studies and data analysis. EPC and KG carried out cellular studies and were assisted by SFP in data analysis. SB carried out SAXS studies and data analysis, MEP the FIDA studies and data analysis. All authors discussed the data. EC, AEL, and BBK wrote the manuscript with contributions from all other authors.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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**How to cite this article:** Cholak E, Bugge K, Khondker A, et al. Avidity within the N-terminal anchor drives α-synuclein membrane interaction and insertion. *The FASEB Journal*. 2020;00:1–21. [https://doi.org/10.1096/fj.202000107R](https://doi.org/10.1096/fj.202000107R)