α-Synuclein Can Inhibit SNARE-mediated Vesicle Fusion Through Direct Interactions with Lipid Bilayers

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Supporting Information

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Methods:

Protein Expression and Purification. αS was recombinantly expressed in E. coli (BL21 DE3**).** Purification was performed as previously described (*1*). Briefly, cells were lysed by incubation in buffer (20 mM Tris-HCl pH 8.0, 40 mM NaOH, 1 mM EDTA, 0.1% Triton X-100) with protease inhibitors (Complete) and DNase treated. Following lysis, cellular debris was removed by centrifugation. The supernatant was further fractionated by ammonium sulfate precipitation. Pelleted protein was resolubilized in buffer (25 mM Tris-HCl pH 8.0, 20 mM NaCl, 1 mM EDTA) and ammonium sulfate was removed by dialysis. The protein was then further purified by anion exchange chromatography followed by size exclusion chromatography. Point variants of αS (A30P, E46K, A53T, T33C) were created using a QuikChange mutagenesis kit (Stratagene). N-terminally acetylated αS was made as described previously (*2*) and purified by the same protocol outlined here.

SNARE proteins were purified using a modified version of the protocol described in (*3*). His-6 tagged SNARE proteins were recombinantly expressed in E. coli (Rosetta). Cells were lysed by incubation in buffer (25 mM HEPES-KOH, 400 mM KCl, 10% glycerol, 0.2 mM TCEP, 1 mg/ml lysozyme, 4% Triton X-100) and lysate was treated with protease inhibitors and DNAse. Cellular debris was removed by centrifugation and the supernatant was incubated with Ni-NTA resin. After overnight incubation, the resin was washed with 5 volumes of buffer (25 mM HEPES-KOH, 400 mM KCl, 10% glycerol, 0.2 mM TCEP) containing 1% octyl β-D-glucopyranoside (OG) detergent and 40 mM imidazole to remove non-specific contaminants. The SNARE protein was then eluted with the same buffer containing 500 mM imidazole. Further purification occurred upon incorporating the SNAREs into vesicles. Soluble SNARE constructs,

CDV (VAMP2 residues 1-95) and sol-t (syntaxin residues 1-265 and full-length SNAP-25), were purified following the same protocol but reducing Triton X-100 to 1% in the lysis buffer and omitting the OG detergent in the wash and elution steps.

Vesicle Preparation. Vesicles were prepared as described (*4*). Chloroform stocks of lipids were mixed to the desired composition and dried to a film. The film was resuspended by gentle shaking in buffer (25 mM HEPES-KOH pH 7.4, 100 mM KCl, 0.2 mM TCEP, 10% glycerol) containing 1% OG and the appropriate concentration of SNARE protein to achieve a 1:100 protein to lipid ratio for v-SNAREs and a 1:200 protein to lipid ratio for t-SNAREs. The lipid solution was diluted 3 fold to reduce the OG below its critical micelle concentration and then dialyzed exhaustively to remove OG. The vesicle solution was mixed with an equal volume of buffer containing 80% Nycodenz. The vesicles were overlaid with a buffer solution with 30% Nycodenz, followed by buffer alone. The vesicles were centrifuged at 55000 rpm for 4 hrs in a SW41ti rotor (Beckman) and the SNARE containing vesicles were removed from the interface of the 30% and buffer layers by pipetting. Vesicles were then extruded 21 times through two 50 nm diameter pores membranes (Whatman Nucleopore Track-Etch, Maidstone, UK) in a Liposofast extruder (Avestin, Ottawa, Canada). Vesicle stocks were used within two days or flash frozen and stored at -80°C for several weeks. Vesicle samples without SNARE proteins were made using the same protocol, omitting the SNARE proteins.

SNARE Vesicle Composition. t-SNAREs were incorporated into vesicles containing 15% 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) and 85% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) molar ratio and v-SNAREs were incorporated into vesicles containing 50% DOPS and 50% POPC unless otherwise noted. Higher concentrations of DOPS than found in many published fusion assays were used in v-SNARE vesicles to enhance αS interactions with

the vesicles. αS binding to vesicles comprised of a lower fraction of anionic lipids was sufficiently weak that effects on fusion were not pronounced. We found that αS had minimal effects on fusion when both v-SNARE and t-SNARE vesicles contained only 15% DOPS. This is consistent with a very recent study where inhibition of fusion is not observed by monomer αS when vesicles contain only 7% anionic lipid (*5*). Interestingly, in this study an oligomer of αS is observed to inhibit fusion, which we do not address in this work. Increasing the DOPS concentration beyond 15% in t-SNARE vesicles resulted in excessive aggregation of the proteoliposomes. For vesicle fusion assays 1.5% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (PE-NBD) and 1.5% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (PE-Rhodamine) were substituted for 3% of the POPC in v-SNARE vesicles. In all floatation assays, a small fraction (0.1% PE-Rhodamine) was substituted for POPC lipid. (All lipids from Avanti Polar Lipids)

Protein Labeling. αS and CDV were site specifically labeled for use in floatation assays, FCS, and binding studies. Labeling was performed by introduction of cysteine point mutations (αS T33C and CDV 96C) by Quikchange mutagenesis (Stratagene). Purified protein was treated with 1 mM DTT for 30 minutes at 4°C. The DTT was removed using two coupled HiTrap desalting columns (GE Healthcare). The protein was incubated with excess fluorescent dye (Alexa 488-maleimide, Alexa 594-maleimide, or NBD-iodoacetamide, Invitrogen) for 4 hours at 4°C. Following incubation unreacted dye was removed using two coupled desalting columns. Labeling efficiency was quantified using fluorophore absorbance to calculate dye concentration. Protein absorbance at 280 nm corrected for dye absorbance as well as a Bradford assay were used to calculate protein concentration. Labeling efficiency was greater than 90% in all cases.

SNARE-Induced Vesicle Fusion Assays. Vesicle fusion assays were based on published protocols (*6*). For measurements, 40 µl t-SNARE vesicles (1 mM lipid) were mixed with αS and buffer to a total volume of 50 µl in wells of a 96 well plate. The plate was pre-warmed to 37°C in a Synergy 4 fluorescence platereader (Biotek). 5 µl of fluorescent v-SNARE vesicles (1 mM lipid) were then added to each well and mixed. The plate was sealed and the fluorescence emission of NBD was collected (Excitation=460 nm, Emission=525 nm) as a function of time. Upon fusion, the density of labeled lipids in the v-SNARE vesicles is diluted with unlabeled lipids in the t-SNARE vesicles, resulting in a decreased Förster resonance energy transfer (FRET) between the donor (NBD) and acceptor (Rhodamine) fluorophores, visualized through an increase in NBD fluorescence. Following the assay, the lipids were solubilized using triton X-100, and the NBD fluorescence was recorded. Each curve is normalized to the fully solubilized intensity. In Figure 1, N=3 and the error is the standard error of the mean; *$P < 0.01$ compared to WT by a Student's *t* test.

Saturation of binding sites was determined very roughly by estimating the amount of αS bound to v-SNARE vesicles under the conditions of the fusion essay; because the K_d for t-SNARE vesicles is ~20 fold lower, we did not consider that a significant amount of αS was bound to them. Based on the measured K_d of ~120 µM (Figure 2A in manuscript), we estimate that ~40% of the αS is bound to the v-SNARE vesicles. For 10 µM total protein, we calculate 4 µM bound protein. For the ~90 µM total lipid in the v-SNARE vesicles used in these measurements, this corresponds to a protein to lipid ratio of ~1:22. This value is within a factor of two of the ~1:40 ratio observed previously under saturating binding conditions (*7*). We note that it is highly likely that there is some non-specific adsorption of αS to the 96 well plate chambers and thus the amount of active αS is somewhat lower than the added amount, and that

the saturating measurements done previously were in different buffer conditions. We consider these two numbers to be relatively close and evidence that we can expect that most of the lipid binding sites on the vesicles are occupied upon saturation of the inhibitory effect on fusion.

The maximal fusion differs between Figure 1 and Figure S1 because of improvements in technique over time. Specifically, minimizing the amount of density medium extracted with the vesicles when removing vesicles from the gradient improved fusion. Data in Figure 1 was collected earlier than that in Figure S1 and likely has more density medium present during fusion. Care was taken to only compare data using the same prep and data was normalized to fusion in buffer alone to further reduce the effect of these differences.

Calcium-Induced Vesicle Fusion. Vesicle fusion assays were based on published protocols (*6*). Measurements were made using 100% POPS vesicles. Unlabeled vesicles were mixed with labeled vesicles (doped with 1.5% PE-Rho and 1.5% PE-NBD) in a 2:1 ratio to a final concentration of 75 μM in the absence or presence of αS.. The sample was pre-warmed to 37°C. After reaching equilibrium, the sample fluorescence (excitation =460 nm, Emission=525 nm, 5 nM slitwidths) was monitored for 100 seconds, then 2 mM $CaCl_2$ was added to initiate fusion. Fluorescence was monitored for an additional 400 seconds. The FRET based increase in NBD fluorescence is identical to the SNARE fusion assay and curves are again normalized to triton-X100 solubilized lipid. αS concentrations necessary to inhibit calcium fusion are much lower because of the lower total lipid content, as well as the high anionic lipid content in the calcium fusion assay which increases αS binding.

Inner leaflet mixing. Inner leaflet mixing assays were performed as described above for the vesicle fusion assays with some modifications (*4*). Immediately before mixing the components, the fluorescently labeled vesicles were treated with a 10 mM sodium dithionite solution for 5

minutes, which reduces the NBD in the outer leaflet such that it is non-fluorescent. The dithionite is removed using HiTrap desalting columns (GE Healthcare) and the vesicles are mixed as in a standard fusion assay. The observed signal is a result of dequenching of only unexposed, inner leaflet NBD. Before normalization, fluorescence intensity is about half the value of untreated samples as expected.

SNARE Vesicle Binding. Direct binding of αS to vesicles with and without SNARE proteins was measured by monitoring the fluorescence change of αS labeled with NBD, αS-NBD. αS was labeled at residue 33, on the hydrophobic side of the amphipathic α-helix (*8*), which should result in insertion of the dye into the lipid bilayer upon binding. The change from an aqueous to a hydrophobic environment results in a large change in fluorescence yield. Samples of 500 nM αS-NBD and increasing lipid concentrations were mixed and incubated for 30 minutes. Emission spectra were collected on a PTI fluorimeter (Excitation=460 nm). Spectra of vesicles alone at all concentrations were collected and subtracted from the αS samples to remove the minor scatter component resulting from high lipid concentrations. In Figure 2A, N=3 and the error is the standard error of the mean.

The 15/85 DOPS/POPC vesicles with and without t-SNAREs did not reach saturation at the maximum lipid concentrations used. Fits of the protein free titration gave a similar saturation maximum as the tighter binding 50/50 DOPS/POPC vesicles and this maximum was assumed to be the actual maximum. For the t-SNARE containing vesicles, binding was so weak that we were unable to determine an approximate saturation point. The given fit assumes saturation at a level equal to the 50/50 DOPS/POPC vesicles with and without v-SNAREs. In this case, a K_d is not calculated in Table S1, but only a lower bound is estimated.

Floatation Assays. Floatation assays were performed using Alexa 488 labeled αS (αS-AL488) to aid in visualization. Pure lipid vesicles, as well as those containing t-SNAREs or v-SNAREs, at a concentration of 1 mM lipid were incubated with 2 μM αS for 1 hour at 4°C. To make a density gradient the vesicles were mixed with an equal volume of 80% Histodenz (Sigma) solution and overlaid with a 30% Histodenz solution followed by buffer. Vesicles were centrifuged at 55,000 rpm for 3 hours in a TLS 55 rotor (Beckman). Vesicles and bound protein are pipetted from the interface of the 30% Histodenz and buffer layers. Free protein remains in the bottom 40% Histodenz layer. Vesicles and free protein samples were separated by SDS PAGE and visualized by fluorescence imaging on a Fujifilm FLA-5100 scanner. Images were quantified using Image Gauge software (Fujifilm).

When comparing data from the NBD-binding and floatation assay, the floatation data (Fig S3) corresponds roughly to the 1000 μM point in Figure 2A. However, the v-SNARE vesicle compositions are different between the two assays, with reduced anionic lipid in the floatation assay which shows greater differences in binding at this lipid concentration. This prevents us from making direct comparisons of the v-SNARE data in these two assays. However, the trend (i.e. reduced binding of αS to vesicles containing VAMP2) is preserved. Comparison of the 15% DOPS lipid only data from the two assays gives similar binding. The floatation assay does give somewhat higher binding than the NBD assay for t-SNARE containing vesicles, but this could be explained by the unknown saturation point of the t-SNARE NBD binding curve discussed above as well as the few data points collected and resulting uncertainty of the t-SNARE floatation assays. Nevertheless, as with the v-SNARE vesicles, the same trend of reduced binding of αS to vesicles containing t-SNARES is observed in both assays.

SNARE Complex Formation by FCS. Complex formation was assessed by monitoring an increase in diffusion time of Alexa 594 labeled CDV (CDV-AL594) upon binding to sol-t. 50 nM CDV-AL594 was mixed with 1 μM sol-t in buffer (25 mM HEPES-KOH pH 7.4, 100 mM KCl, 5% glycerol, 0.2mM TCEP) with or without 20 μM unlabeled αS. The experiment was also repeated with labeled αS (αS-AL488) at a concentration of 50 nM, with 1 μM each of CDV and sol-t (Fig S4). Photon traces were collected continuously for 2 hours and autocorrelation curves were calculated for every 200 seconds of measurement. The curves were fit using lab-written software in Matlab to an equation for a single component to reflect the average size of diffusing components (*9*) where N is number of molecules, τ_D is the diffusion time, and s accounts for the radial to axial dimensions of the focal volume:

$$G(\tau) = \frac{1}{N}(1+\frac{\tau}{\tau_D})^{-1}(1+\frac{\tau}{s^2\tau_D})^{-1/2}$$

Complex formation by size exclusion. Size exclusion chromatography was performed on mixtures of CDV-AL594 and unlabeled sol-t in the presence and absence of αS to determine the extent of SNARE complex formation. CDV-AL594 and sol-t were mixed and incubated at 4°C for 4 hours. The mixtures were then separated on a Superdex 200 column. Fractions were collected and analyzed by SDS-PAGE and imaged using fluorescence and Coomassie stain.

Supporting Information Table:

Vesicle Type	K_d (μM)
50/50 DOPS/POPC	41 ± 2
50/50 DOPS/POPC + v-SNARE	117 ± 7
15/85 DOPS/POPC	1072 ± 63
15/85 DOPS/POPC + t-SNARE	> 2000

Table S1: K_d values of αS-vesicle binding calculated from fits of binding curves obtained by NBD fluorescence shown in Figure 2A.

Supporting Information Figures:

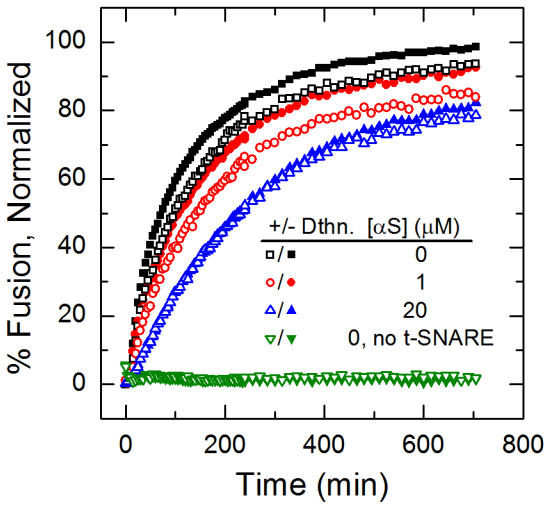

Figure S1. Fusion of inner leaflet. Fluorescence emission timecourses of NBD during SNARE-mediated vesicle fusion with increasing αS concentration. Untreated (filled symbols) and dithionite (Dthn) treated (open symbols) vesicles report on total lipid mixing and inner leaflet mixing respectively. Total and inner leaflet mixing are approximately equivalent.

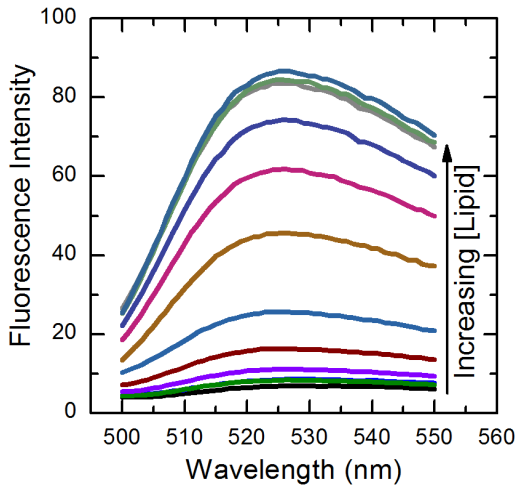

Figure S2. Fluorescence emission spectra of αS-NBD with increasing concentrations of lipids. Data shows representative spectra for αS binding 50/50 DOPS/POPC lipids. All data is quantified in Figure 2A. 500 nM αS-NBD and vesicles were mixed and incubated for 30 minutes prior to data collection. The lipid concentration measured spans from 0 to 2000 μM total lipid.

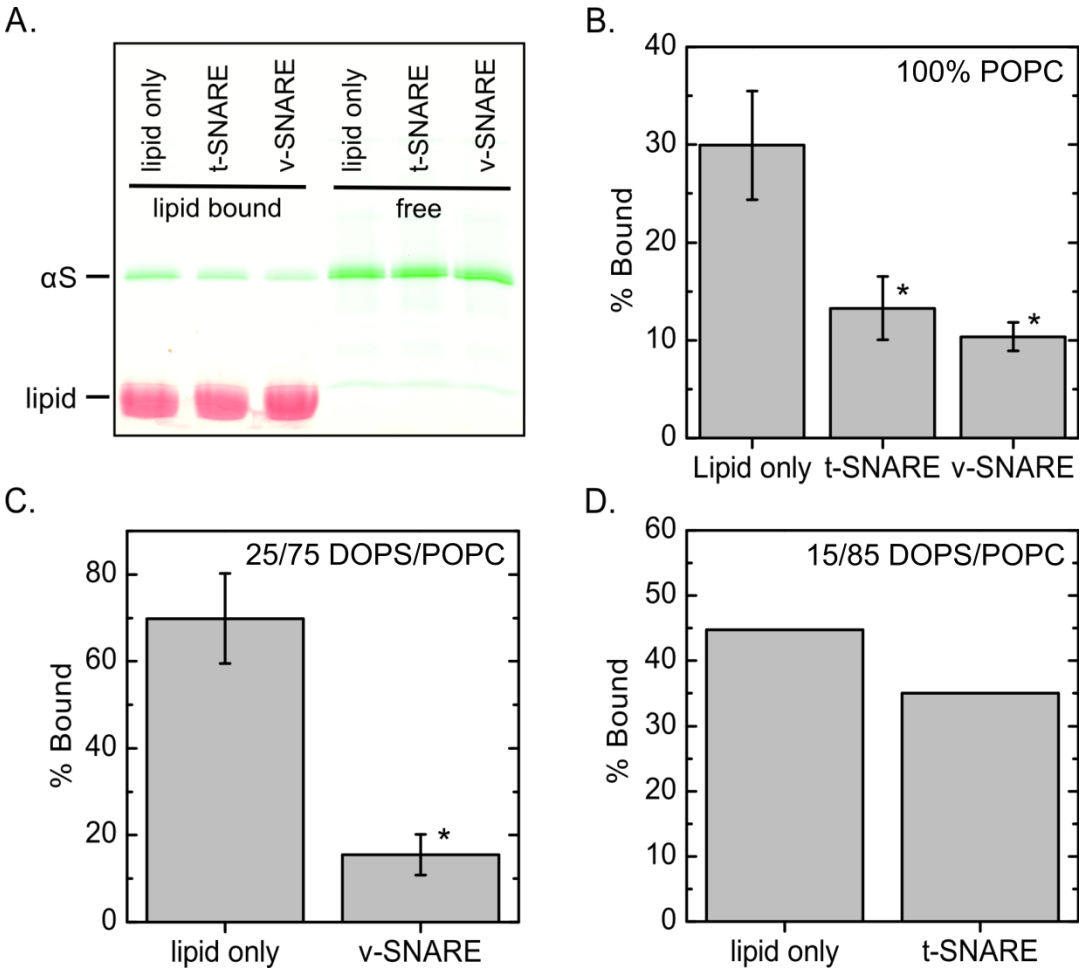

Figure S3. αS binds more weakly to SNARE vesicles. A. Fluorescence gel image of a floatation assay of αS binding to 100% POPC vesicles. αS is colored in green, lipids are colored red. B-D. Quantification of binding by floatation assays for different lipid compositions: B. 100% POPC (as in panel A; n=3), C. 25/75 DOPS/POPC (n=4), D. 15/85 DOPS/POPC (n=2). Ratio of bound to total αS was taken for each lane. error is s.e.m. * indicates $p<0.05$ compared to lipid only by Student's T-test

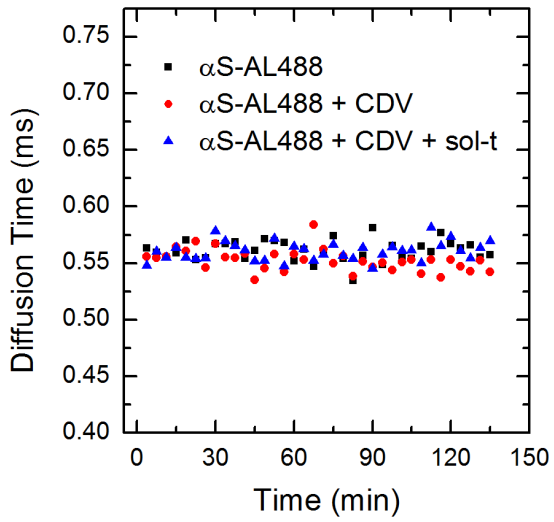

Figure S4. αS is not incorporated into SNARE complex. No increase in the diffusion time of αS-AL488 (black) is observed indicating that it is not incorporated into the SNARE complex (blue) upon mixing with unlabeled CDV and sol-t. SNARE complex formation occurs over this timescale, as visualized in Figure 2B in the main manuscript. There is also no evidence of binding to isolated CDV (red).

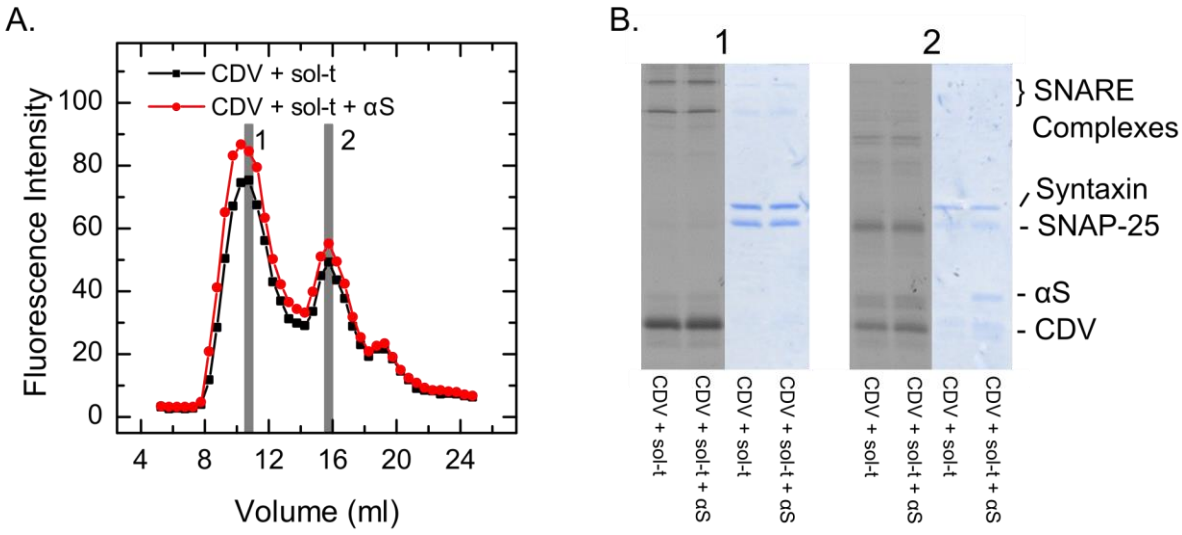

Figure S5. αS does not affect SNARE complex formation. A. Fluorescence traces of a mixture of CDV-AL594 and sol-t incubated with and without αS separated by size exclusion chromatography. B. SDS-PAGE of peak fractions marked by gray bars 1 & 2 in "A" visualized by fluorescence scan (left) and Coomassie staining (right).

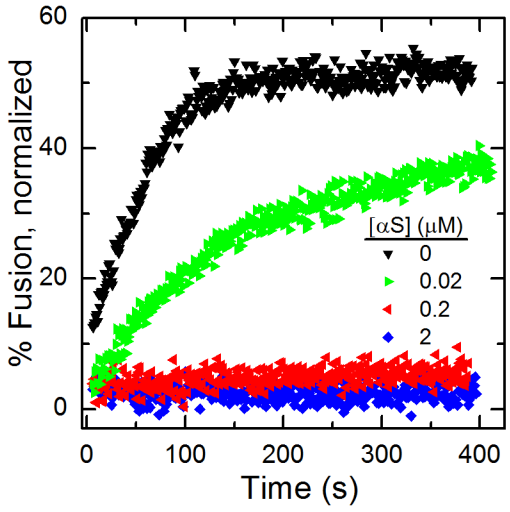

Figure S6. Calcium-mediated vesicle fusion. Fluorescence emission timecourses of NBD dequenching during calcium-mediated vesicle fusion with increasing αS concentration. NBD fluorescence increases as a result of lipid mixing and reduced FRET between NBD and rhodamine upon dilution.

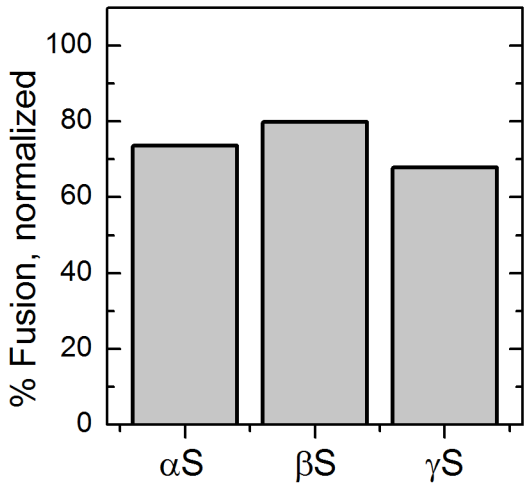

Figure S7. SNARE-mediated fusion is inhibited by β- and γ-synuclein (protein kindly provided by Vanessa Ducas). N=2.

References:

1. Trexler, A. J., and Rhoades, E. (2009) Alpha-synuclein binds large unilamellar vesicles as an extended helix, *Biochemistry 48*, 2304-2306.
2. Trexler, A. J., and Rhoades, E. (2012) N-Terminal acetylation is critical for forming alpha-helical oligomer of alpha-synuclein, *Protein science : a publication of the Protein Society 21*, 601-605.
3. Parlati, F., Weber, T., McNew, J. A., Westermann, B., Sollner, T. H., and Rothman, J. E. (1999) Rapid and efficient fusion of phospholipid vesicles by the alpha-helical core of a SNARE complex in the absence of an N-terminal regulatory domain, *Proceedings of the National Academy of Sciences of the United States of America 96*, 12565-12570.
4. Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H., and Rothman, J. E. (1998) SNAREpins: minimal machinery for membrane fusion, *Cell 92*, 759-772.
5. Choi, B. K., Choi, M. G., Kim, J. Y., Yang, Y., Lai, Y., Kweon, D. H., Lee, N. K., and Shin, Y. K. (2013) Large alpha-synuclein oligomers inhibit neuronal SNARE-mediated vesicle docking, *Proceedings of the National Academy of Sciences of the United States of America 110*, 4087-4092.
6. Struck, D. K., Hoekstra, D., and Pagano, R. E. (1981) Use of resonance energy transfer to monitor membrane fusion, *Biochemistry 20*, 4093-4099.
7. Middleton, E. R., and Rhoades, E. (2010) Effects of curvature and composition on alpha-synuclein binding to lipid vesicles, *Biophysical journal 99*, 2279-2288.
8. Jao, C. C., Hegde, B. G., Chen, J., Haworth, I. S., and Langen, R. (2008) Structure of membrane-bound alpha-synuclein from site-directed spin labeling and computational refinement, *Proceedings of the National Academy of Sciences of the United States of America 105*, 19666-19671.
9. Nath, A., Trexler, A. J., Koo, P., Miranker, A. D., Atkins, W. M., and Rhoades, E. (2010) Single-molecule fluorescence spectroscopy using phospholipid bilayer nanodiscs, *Methods in enzymology 472*, 89-117.