

Nonaggregated α -Synuclein Influences SNARE-Dependent Vesicle Docking via Membrane Binding

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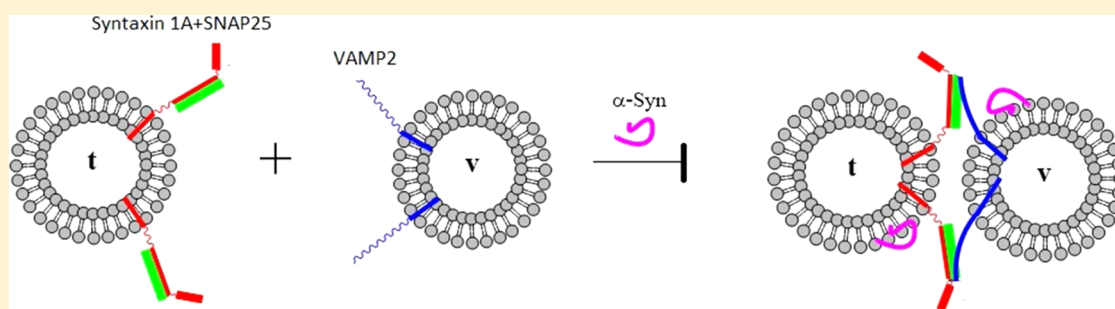
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S Supporting Information



ABSTRACT: α -Synuclein (α -Syn), a major component of Lewy body that is considered as the hallmark of Parkinson's disease (PD), has been implicated in neuroexocytosis. Overexpression of α -Syn decreases the neurotransmitter release. However, the mechanism by which α -Syn buildup inhibits the neurotransmitter release is still unclear. Here, we investigated the effect of nonaggregated α -Syn on SNARE-dependent liposome fusion using fluorescence methods. In ensemble in vitro assays, α -Syn reduces lipid mixing mediated by SNAREs. Furthermore, with the more advanced single-vesicle assay that can distinguish vesicle docking from fusion, we found that α -Syn specifically inhibits vesicle docking, without interfering with the fusion. The inhibition in vesicle docking requires α -Syn binding to acidic lipid containing membranes. Thus, these results imply the existence of at least two mechanisms of inhibition of SNARE-dependent membrane fusion: at high concentrations, nonaggregated α -Syn inhibits docking by binding acidic lipids but not v-SNARE; on the other hand, at much lower concentrations, large α -Syn oligomers inhibit via a mechanism that requires v-SNARE interaction [Choi et al. *Proc. Natl. Acad. Sci. U. S. A.* 2013, 110 (10), 4087–4092].

INTRODUCTION

α -Syn is a major component of the Lewy body that is generally found in the brain of the Parkinson's disease (PD) patients and is implicated in the familial PD.² Point mutants of α -Syn also have been suggested to be related with the autosomal-dominant form of PD.³ α -Syn is a small and peripheral membrane-binding protein⁴ that is widely expressed in central neurons, and is specifically localized and bound to the synaptic membrane,⁵ which might be important for synaptic plasticity.⁶

It was shown that overexpression of α -Syn both in yeast⁷ and in *Drosophila*⁸ inhibits vesicular transport from endoplasmic reticulum to the Golgi complex. Overexpression of α -Syn in chromaffin and rat brain cells largely decreased the neurotransmitter release.⁹ However, knockout of α -Syn showed little effect on the release,^{9c} which might be due to the functional

redundancy among synuclein isoforms α , β and γ . Indeed, a recent triple knockout study revealed the increased release at least for young mice,¹⁰ indicating that synucleins might function as a regulator for neuroexocytosis.

The neurotransmitter release at the synapse requires the fusion of vesicles with the presynaptic plasma membrane. It is believed that synaptic vesicle fusion is mediated by SNARE proteins. Target plasma membrane (t-) SNAREs syntaxin 1A and SNAP-25, and vesicle (v-) SNARE synaptobrevin/vesicle-associated membrane protein (VAMP) 2 associate to form the SNAREpin, which bridges two membranes, facilitating fusion.¹¹

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Although it was recently shown that the C-terminal region of α -Syn interacts with VAMP2 and promotes SNARE assembly,¹² this interaction appears to be a different function of α -Syn, not affecting the neurotransmitter release.¹³ It was however shown that α -Syn reduces exocytosis in yeast and causes the decrease of the copy number of the SNARE complex in mammalian cells,¹⁴ raising the possibility that α -Syn might affect SNARE-dependent fusion directly. Most recently, an in vitro study using ensemble lipid mixing reported the inhibition effect of α -Syn in neuronal SNARE-mediated membrane fusion.¹⁵

In this work, we attempt to explore the direct impact of α -Syn on SNARE-dependent membrane fusion in a more defined setting. The classical ensemble fluorescence fusion assay employing SNARE-reconstituted proteoliposomes with on lipid-mixing indicators confirmed the inhibition role of α -Syn. The known pathotype mutants α -Syn E46K, A53T, and A30P are shown to have a varying degree of membrane affinities.¹⁶ Interestingly, it was found that the rank order of the inhibition potency is consistent with the rank order of their membrane affinities. Furthermore, by using a more powerful single-vesicle fusion assay unambiguously detecting different stages of fusion including docking, hemi and full fusion, we found that α -Syn does not interfere with the fusion step. Instead, α -Syn significantly reduces SNARE-mediated vesicle docking, which appears as inhibition of fusion in ensemble assays. Moreover, we found that C-terminal truncated α -Syn, which blocks the interaction between α -Syn and VAMP2, has similar inhibitory effect as wild-type α -Syn, suggesting that α -Syn interacting with VAMP2 is not required for its inhibition in lipid mixing. Finally, similar inhibition in vesicle docking was also observed in yeast SNARE mediated ensemble lipid mixing further supporting the conclusion that this impairment in vesicle docking by α -Syn is not from specific protein interaction. However, when the membrane binding affinity of α -Syn was eliminated by removing the phosphatidylserine (PS) from the membrane, α -Syn was no longer effective in inhibiting lipid mixing. Thus, our results suggest that α -Syn's membrane binding is the main cause of the inhibition of SNARE-dependent lipid mixing.

MATERIALS AND METHODS

Plasmid Constructs and Site-Directed Mutagenesis.

DNA sequences encoding syntaxin 1A (amino acids 1-288 with three cysteines replaced by alanines), VAMP2 (amino acids 1-116 with C103 replaced by alanines), SNAP-25 (amino acids 1-206 with four native cysteines replaced by alanines), SNAP-25 BoNT/E (amino acids 1-180), soluble VAMP2 (amino acids 1-96), α -synuclein (amino acids 1-140), α -synuclein (1-95) (amino acids 1-95), Sso1p (amino acids 185-290), for which the N-terminal α -helix Habc domain was deleted, and Snc2p (amino acid 1-115) were inserted into the pGEX-KG vector as N-terminal glutathione S-transferase (GST) fusion proteins. Sec9c (amino acid 401-651 of Sec9) was inserted into the PET-28b vector as C-terminal His6-tagged fusion protein. We used the Quick Change site-directed mutagenesis kit (Stratagene) to generate mutants, including α -Syn A30P, α -Syn E46K, and α -Syn A53T; DNA sequences were confirmed by the Iowa State University DNA Sequencing Facility.

Protein Expression and Purification. All N-terminal GST recombinant neuronal and yeast SNARE proteins and α -Syn were expressed in *Escherichia coli* Rosetta (DE3) pLysS (Novagene). The His-tagged proteins were expressed in an *E. coli* BL21 (DE3) (Novagen). Details of purification can be found in our previous work.^{23,27}

Membrane Reconstitution. The mixture of POPC (1-palmitoyl-2-dioleoyl-*sn*-glycero-3-phosphatidylcholine), DOPS (1,2-dioleoyl-*sn*-glycero-3-phosphatidylserine), cholesterol, and DiI (t-vesicles) or DiD (v-vesicles) (molar ratio of 43:15:40:2) in chloroform was dried in a vacuum and was resuspended in a buffer (25 mM HEPES/KOH, 100 mM KCl, pH 7.4) to make the total lipid concentration of about 10 mM. Protein-free large unilamellar vesicles (~100 nm in diameter) were prepared by extrusion through polycarbonate filters (Avanti Polar Lipids). For net neutral charge lipid mixing, 15 mol % DOPS was replaced by equimolar quantity of POPC.

Syntaxin 1A and SNAP-25 (or Sso1p for yeast SNARE mediated lipid mixing), in a molar ratio of 1:1.5, were premixed, and the mixture was left at room temperature for 1 h to form the complex before the reconstitution. For membrane reconstitution, proteins were mixed with vesicles at the protein to lipid molar ratio of 1:200 with ~0.8 g per 100 mL OG in the buffer at 4 °C for 15 min. The mixture was diluted two times with dialysis buffer (25 mM HEPES, 100 mM KCl, pH 7.4), and this diluted mixture was then dialyzed in 2 L dialysis buffer at 4 °C overnight.

Ensemble Lipid Mixing Assay. For neuronal SNARE dependent lipid mixing, reconstituted t-vesicle and v-vesicle were mixed at a ratio of 1:1. The total lipid concentration in the reaction is 0.1 mM. The fluorescence intensity was monitored in two channels with the excitation wavelength of 530 nm and emission wavelengths of 570 and 670 nm for DiI and DiD dye pairs, respectively. Fluorescence changes were recorded with the same Varian fluorometer. All measurements were performed at 35 °C. The initial rate was calculated by analyzing the slope value within the beginning 150 s, and the initial rate of control group was normalized to 1.

Single-Vesicle Assays. A quartz slide was cleaned using the 5% Alconox solution followed by 1 M potassium hydroxide and then coated with 99:1 (mol/mol) mPEG:biotin-PEG (Laysan Bio). This PEG-treated quartz slide was placed as the bottom surface of a microfluidic chamber to be used as the imaging surface of our prism-type total internal reflection fluorescence (TIRF) microscopy (based on IX-71, Olympus). To monitor interactions between single v- and t-vesicles, the v-vesicles (30 mM [lipids]) were attached on this quartz imaging surface via neutravidin (Invitrogen) that acts as molecular glue between biotin-PEG and biotinylated lipids. The t-vesicles (10 mM [lipids]) and the Ca^{2+} ion solution were mixed together just prior to the main reaction. This t-vesicles- Ca^{2+} mixture was introduced into the flow chamber for the fusion reaction, and the temperature was maintained at 37 °C during the reaction for half an hour.

In the docking number analysis, we used a computer algorithm that detects local Gaussian maxima in the TIRF images that were recorded by an electron-multiplying charge-coupled device (iXon DU897E, Andor technology) to count the number of the single vesicle complexes in a given area (programs available at <http://bio.physics.illinois.edu/>). Our TIRF microscopy monitored the imaging area of 45*90 mm² at a time, and we calculated the ratio of docked t-vesicles and total anchored v-vesicles as docking probability. Therefore, it could be directly compared for different molecular conditions. Using the same algorithm as used for the docking number analysis, each vesicle-vesicle docking event and the subsequent fusion process were individually identified. We quantified the FRET efficiency using the equation, $I_a/(I_d + I_a)$ where I_d and I_a are the donor and acceptor fluorescence intensities, respectively.

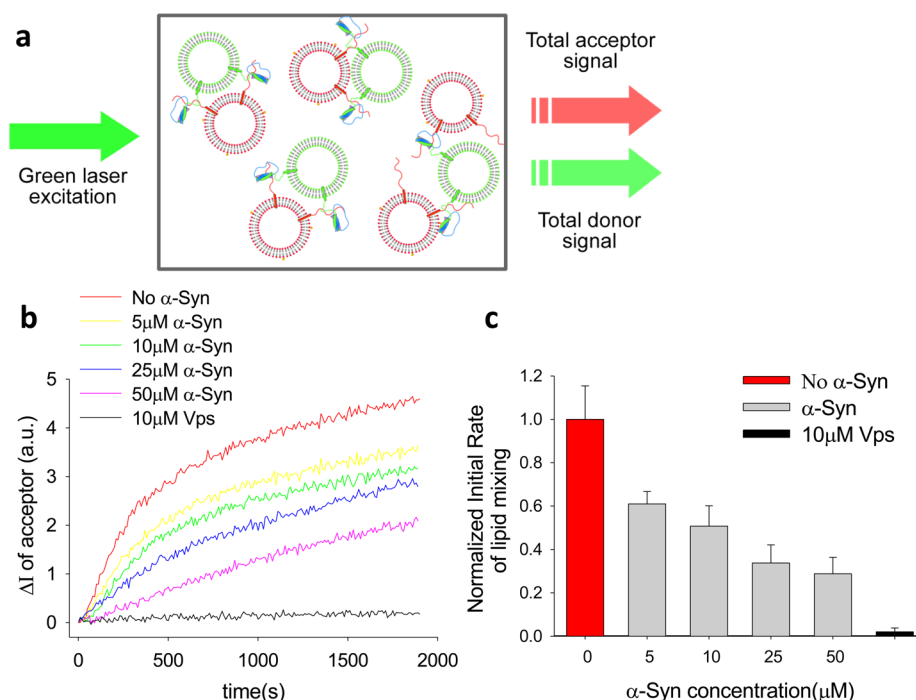


Figure 1. α -Syn inhibits SNARE-induced ensemble lipid mixing. (a) Scheme of ensemble lipid mixing assay involving SNARE-reconstituted vesicles. (b) The change of fluorescence intensity of DiD reflects lipid mixing. The red trace is the control with t-liposome reconstituted with Syn/SNAP-25 and v-liposome reconstituted with VAMP2. The yellow trace represents the lipid mixing for 5 μ M α -Syn, the green trace for 10 μ M α -Syn, the blue trace for 25 μ M α -Syn, and the pink trace for 50 μ M α -Syn. The dark red trace is the lipid mixing control with 10 μ M soluble VAMP2 (Vps). (c) Normalized initial rates of the lipid mixing assays at different α -Syn concentrations. Error bars (the standard deviation) were from 3 independent measurements with 3 different preparations.

Fusion percentage was calculated by the percentage of fusion events with FRET efficiency higher than 0.5.

For the real-time imaging of small sulforhodamine B content release, the same PEG surface was used as above. After the surface was coated with neutravidin, the sulforhodamine B containing v-SNARE vesicles were immobilized on the PEG-coated surface. After two rounds of 1 mL buffer wash, empty t-SNARE vesicles were injected into the channel incubating with v-SNARE vesicles. After 30 min incubation at room temperature ($\sim 25^\circ\text{C}$), the dialysis buffer with or without 500 μ M Ca^{2+} was injected into the flow chamber at a speed of 33 $\mu\text{L/s}$ by a motorized syringe pump.

Western Blot. Purified α -syn was resolved by 12% SDS-PAGE and then transferred to PVDF membrane (Millipore). The membrane was blocked with 5% (w/v) nonfat dry milk in TBST (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 1 h and incubated with α -Syn primary antibody (1:1,500, 701085, Life Technologies) at 4°C overnight. The membrane was then washed with TBST and incubated with Peroxidase anti-Rabbit-IgG secondary antibody (1:3,000, 1213156A, Invitrogen) at room temperature for 1 h. Then the membrane was washed with TBST again and reacted with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The bands were visualized by ChemiDOC system (Bio-Rad).

Cosedimentation Assay. The binding properties of α -Syn to proteoliposomes (VAMP2- and Snc2p-vesicles) and protein-free vesicles were measured through the high-speed cosedimentation assay using Airfuge Air-Driven Ultracentrifuge (Beckman). Membrane reconstitution was performed the same as the sample used in our fluorescence assay except 20 mol % DOPS was used in the negative charged vesicles. 200 μL

each vesicle (200 μL) was incubated with 8 μM α -Syn in dialysis buffer (25 mM HEPES pH 7.4, 100 mM KCl) for 1 h at room temperature, and then the mixture was subjected to Airfuge at 90,000 rpm and 30 psi for 30 min at 4°C . Pelleted vesicles and/or vesicle-bound α -Syn were resuspended using the same buffer and resubjected to centrifuge for three times. Finally, the pellets were resuspended in 60 μL of the same buffer, and 5 μL of each sample was analyzed by Western blot using α -Syn antibody.

RESULTS

α -Syn Inhibits Ensemble SNARE-Induced Lipid Mixing. Previous *in vivo* studies suggested that overexpression of α -Syn inhibits neurotransmitter release.^{9,17} Therefore, we asked if α -Syn interferes with the lipid mixing step during membrane remodeling. To answer this question, we carried out the fluorescence lipid mixing assay, in which wild-type t-SNARE (a 1:1 mixture of Syntaxin 1A and SNAP-25) was reconstituted to t-vesicles that contained 2 mol % fluorescence donor lipid DiI, while wild-type VAMP2 was reconstituted to v-vesicles containing 2 mol % fluorescence acceptor lipid DiD for the ensemble fluorescence detection of lipid mixing (Figure 1a). With this setup the fusion between t- and v-vesicles caused the increase of the acceptor DiD signal due to Förster (fluorescence) resonance energy transfer (FRET) (the red trace in Figure 1b). To show that lipid mixing probed by FRET between DiI and DiD was SNARE-dependent we carried out lipid mixing without SNAP-25 or in the presence of the SNAP-25E mutant that is the product of the proteolytic cleavage of SNAP-25 by botulinum neurotoxin E (BoNT/E). In both cases we did not observe noticeable lipid mixing, indicating that lipid mixing here was SNARE-dependent (the black and cyan traces

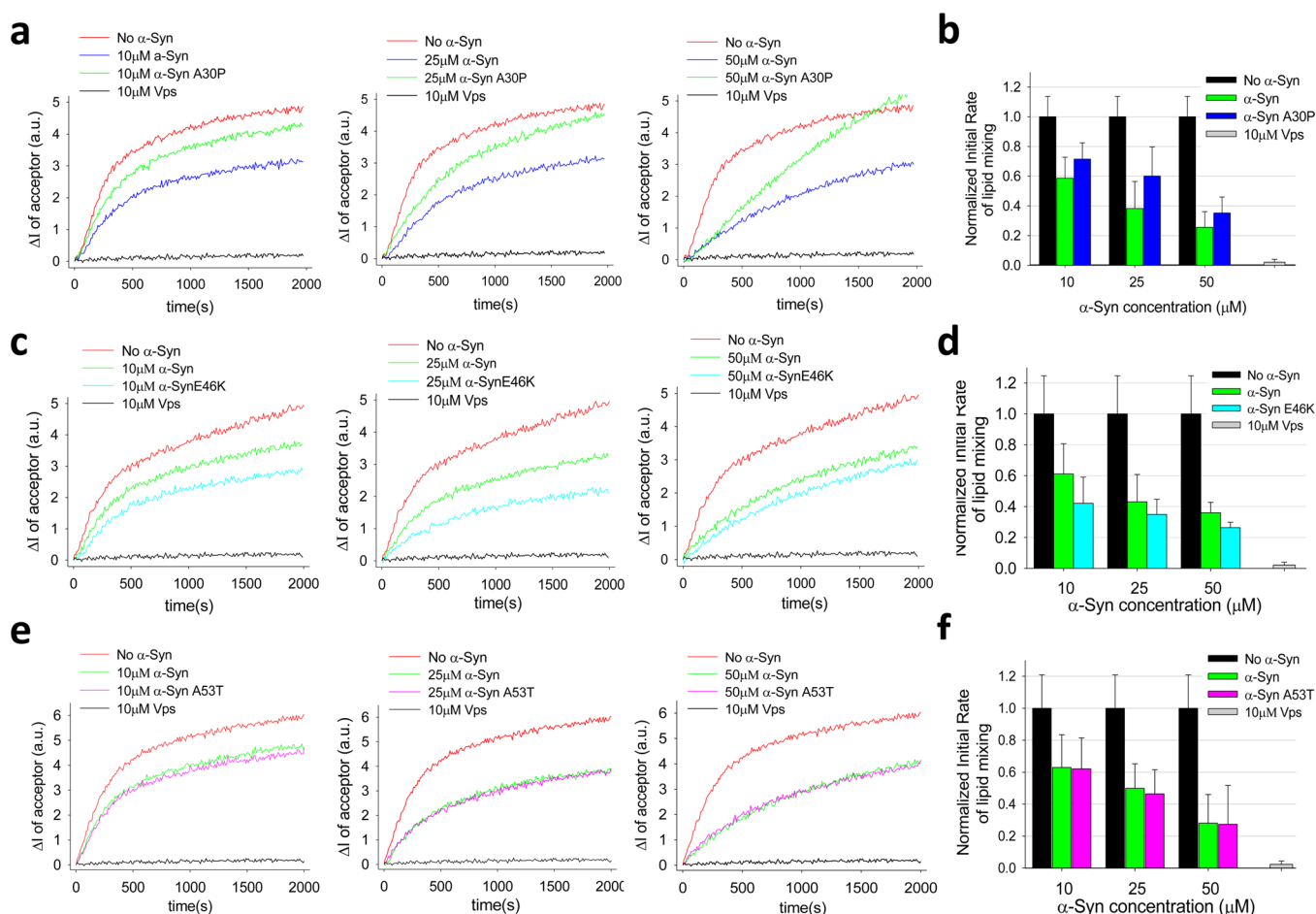


Figure 2. Lipid binding affinity of α -Syn correlates to the inhibition effect. The change of fluorescence intensity of DiD reflects lipid mixing. The red traces are the lipid mixing control group. The green traces represent lipid mixing with α -Syn, while the blue, cyan, and pink traces represent lipid mixing with α -Syn A30P (A), α -Syn E46K (C), and α -Syn A53T (E) at the concentration of 10 μ M, 25 μ M, and 50 μ M, respectively. The black trace is the lipid mixing control with 10 μ M Vps. Normalized initial rates of the lipid mixing assays at different α -Syn and α -Syn A30P (B), α -Syn E46K (D), and α -Syn A53T (F) concentrations. Error bars representing the standard deviation were obtained from 3 independent measurements with 3 different preparations.

in Figure 1b, respectively). Also, soluble VAMP2 (Vps, amino acids 1-96), which is often used as a competitive inhibitor for SNARE-dependent membrane fusion, showed complete inhibition of lipid mixing at 10 μ M (the dark red trace in Figure 1b), further supporting the SNARE-dependency of lipid mixing.

Upon addition of α -Syn lipid mixing was dramatically inhibited, even at the concentration of 5 μ M (the yellow trace in Figure 1b). With the increased concentration of α -Syn, the lipid mixing was attenuated progressively more (Figure 1b). The analysis of the initial rates of lipid mixing indicates that 50 μ M α -Syn can block SNARE-induced lipid mixing as much as 70% (Figure 1c). Because lipid mixing is strictly SNARE-dependent we can rule out the possibility that α -Syn blocks SNARE-independent spontaneous fusion of membranes.

α -Syn Pathotype Mutants Show Parallel Gain- and Loss-of-Functions in Membrane Binding vs Fusion Inhibition. *In vivo* studies indicated that membrane binding ability of α -Syn may be correlated with its inhibition of the neurotransmitter release.^{9c} To investigate whether α -Syn's membrane affinity is related to its inhibition of SNARE-dependent lipid mixing, we carried out the ensemble lipid mixing assays with three mutants of α -Syn, A30P, E46K, and A53T, which are linked to rare inherited PD.³ NMR studies

indicated that α -Syn A30P has a decreased lipid affinity, while α -Syn E46K has an increased affinity when compared with that of the wild-type. Meanwhile, α -Syn A53T is similar to the wild-type in its membrane binding activity.¹⁶ Interestingly, the lipid mixing assay revealed that α -Syn A30P was somewhat less effective in inhibiting SNARE-dependent lipid mixing than wild-type (Figure 2a and 2b). In contrast, α -Syn E46K was a little more potent than the wild-type (Figure 2c and 2d). Meanwhile, α -Syn A53T showed little difference from the wild-type (Figure 2e and 2f). The results show a correlation between the α -Syn's membrane binding activity and its inhibitory activity for SNARE-dependent lipid mixing. Thus, membrane binding may be necessary for the inhibition of SNARE-dependent membrane fusion by α -Syn.

α -Syn Reduces Vesicle Docking without Interfering Lipid Mixing. SNARE-mediated membrane fusion is a process involving multiple steps: docking, hemifusion, and full (complete) fusion. However, the ensemble assay cannot dissect individual steps.¹⁸ Recent studies showed that the rate of ensemble lipid mixing is mainly limited by the docking step,¹⁹ which causes a misinterpretation of fusion since a particular factor that is added to the experiment may increase docking rather than fusion. In order to overcome these limitations of ensemble assays, several new techniques have been developed

for observing lipid mixing at the single-vesicle level.²⁰ By monitoring FRET of individual donor/acceptor vesicle pairs (Figure 3a), the single-vesicle assay can detect different stages

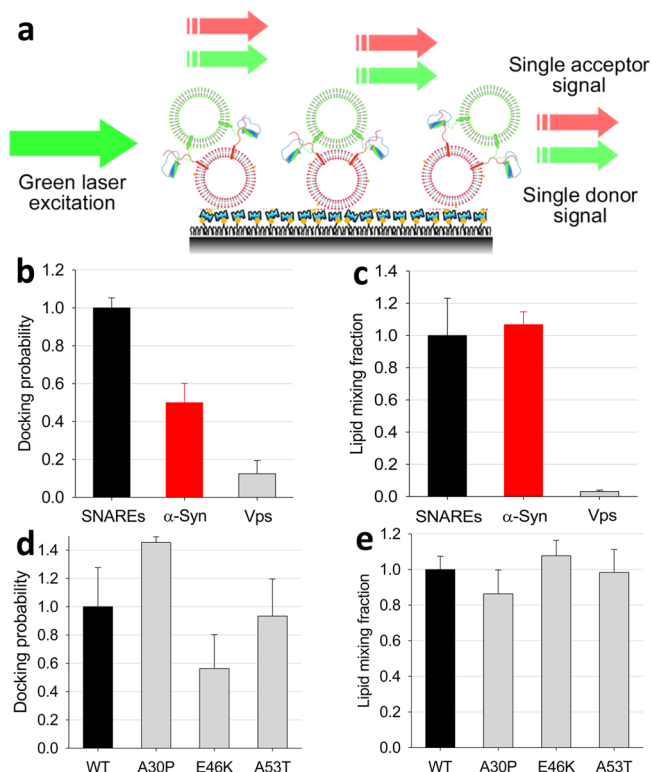


Figure 3. α -Syn specifically inhibits vesicle docking. (a) Scheme of the single-vesicle lipid mixing assay involving SNARE-reconstituted vesicles. (b) Bar graph of the vesicle docking probability (b) or the lipid mixing fraction (c) with or without α -Syn. Different effects on the vesicle docking probability (d) and lipid mixing (e) by α -Syn mutants. Error bars, which represent the standard deviations, were obtained from 3 independent measurements with 3 different preparations.

of fusion including docking and hemi- and full fusion, which was not possible for previous ensemble assays.²¹ Through single-vesicle lipid mixing, we found that α -Syn significantly reduces the vesicle docking induced by SNAREs (Figure 3b). For the full lipid mixing indicated by a high FRET value, the

effect of α -Syn is negligible (Figure 3c). We also tested three mutants of α -Syn, A30P, E46K, and A53T, in the single-vesicle lipid mixing assay. We observe a good correlation between membrane affinities of α -Syn mutants and vesicle docking (Figure 3d). However, similar to wild-type, they have no observable effect on lipid mixing between two lipid bilayers (Figure 3e). Thus, the results show the variation of lipid mixing among α -Syn mutants in the ensemble assay was largely due to the negative influence on vesicle docking.

α -Syn Has No Effect on Ca^{2+} -Triggered Content Release at Single Vesicle Level.

Lipid mixing does not necessarily imply complete fusion since hemifusion alone can also produce mixing of lipid molecules.²² Any assay relying on lipid-mixing readouts can lead to the misinterpretation of membrane fusion. With newly developed single-vesicle content-mixing assay monitoring dequenching of small content dyes (sulforhodamine B), we found that α -Syn does not influence fast content release triggered by Ca^{2+} (Figure 4). For this experiment, we included full-length synaptotagmin 1,²³ the calcium sensor, in our single vesicle assay.

α -Syn Inhibits Lipid-Mixing via Interaction with Negative Charged Lipids but Not VAMP2.

Our results showed a correlation between the α -Syn's membrane affinity and its inhibitory activity for SNARE-dependent lipid mixing (Figure 2). Thus, we questioned if α -Syn's fusion-inhibiting activity is mainly due to its membrane interaction or its interaction with VAMP2 which was reported previously.^{1,24} It has been shown that the N-terminal region of VAMP2 interacts with the C-terminal region of α -Syn.¹² To test if this specific interaction plays a role in the α -Syn's inhibition of SNARE-dependent lipid mixing, we used a C-terminal truncated α -Syn mutant (amino acids, 1-95) to block α -Syn's binding to VAMP2. Even in the absence of the interaction with VAMP2, the truncated α -Syn mutant has a similar inhibitory effect on lipid mixing as the α -Syn wild-type (Figure 5a), suggesting that α -Syn interaction with VAMP2 is not required for the inhibition of lipid mixing. Furthermore, we investigated the effect of α -Syn on lipid mixing induced by a yeast SNARE family, which is involved in Golgi-to-plasma membrane trafficking in yeast^{7,8} and is distantly related to the neuronal SNARE family with only less than 30% sequence identities.²⁵ As expected, a significant inhibition of lipid mixing was observed by α -Syn (Supplementary Figure 1a and 1b), and

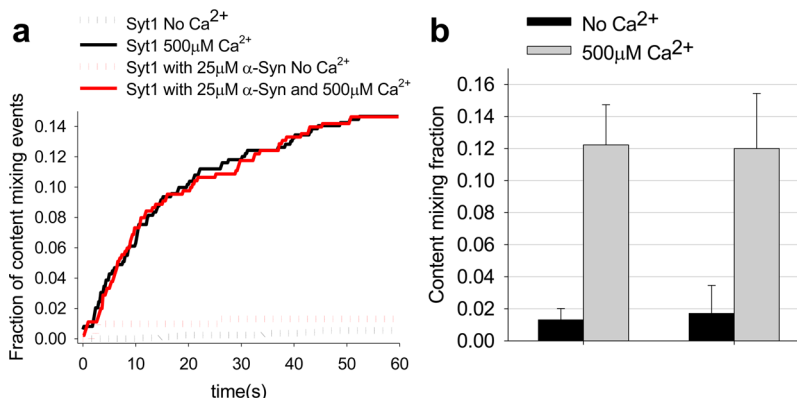


Figure 4. α -Syn has little effect on Ca^{2+} -triggered content mixing. (a) The real-time synaptic vesicle content mixing assay mediated by SNAREs and Syt1. The black lines represent the accumulated content mixing events in the presence of SNAREs and Syt1 only, while the red traces represent content mixing events with 25 μM α -Syn. (b) Fraction of content mixing events happened within 60 s. Error bars, which represent the standard deviation, were obtained from at least $n = 3$ independent measurements with 3 different preparations.

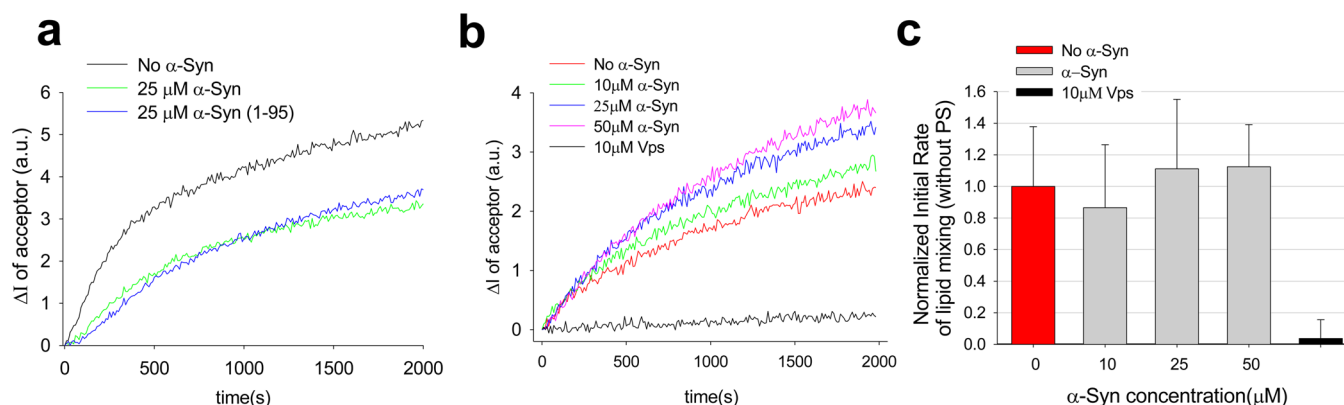


Figure 5. α -Syn inhibits lipid fusion via membrane binding but not interacting with VAMP2. (a) C-terminal truncated α -Syn(1-95) inhibits ensemble lipid mixing as wild-type α -Syn. The black trace is the control with t-liposome reconstituted with Syn/SNAP-25 and v-liposome reconstituted with VAMP2. The green and blue traces represent lipid mixing with 25 μ M α -Syn and α -Syn(1-95), respectively. (b) The inhibition role of α -Syn on ensemble lipid mixing depends on charged lipid molecules, PS. The change of fluorescence intensity of DiD reflects neutral lipid mixing (only POPC and Cholesterol). The red trace is the control with t-liposome reconstituted with Syn/SNAP-25 and v-liposome reconstituted with VAMP2. The green trace represents the lipid mixing for 10 μ M α -Syn, the blue trace for 25 μ M α -Syn, and the pink trace for 50 μ M α -Syn. The black trace is the lipid mixing control with 10 μ M soluble VAMP2 (Vps). (c) Normalized initial rates of the lipid mixing assays at different α -Syn concentrations. Error bars for the standard deviation were obtained from 3 independent measurements with 3 different preparations.

the vesicle cosedimentation assay shows that α -Syn has little affinity with v-vesicles reconstituted with yeast v-SNARE Snc2p (Supplementary Figure 1c). Thus, it confirms that the α -Syn's interaction with v-SNARE is not necessary for its inhibition in lipid mixing. Meanwhile, in the absence of the negative charged lipid DOPS, we did not observe any inhibition in lipid mixing at various α -Syn concentrations within experimental errors (Figure 5b and 5c), even though there is some binding of the α -Syn to VAMP2 containing v-vesicles (Supplementary Figure 1c). Thus, the results show that membrane binding, but not the VAMP2 interaction, is the main cause of the α -Syn's fusion inhibiting activity.

DISCUSSION

There is compelling evidence that α -Syn buildup in cells at high concentrations interferes with exocytotic pathways: α -Syn block the ER to Golgi trafficking in yeast⁷ and in mammalian cells.^{8,14} Overexpression of α -Syn in Chromaffin cells as well as in mouse neurons inhibits release of neurotransmitters.^{9,17} Although it is most likely that α -Syn interferes with the exocytotic machinery, the molecular mechanism by which high α -Syn blocks the exocytosis has not been known. In this work, using *in vitro* reconstitution, we show that α -Syn at high concentrations (>5 μ M) influences SNARE-dependent membrane fusion by inhibiting the docking step.

The proteoliposome fusion assay at single-vesicle level probes membrane remodeling steps and our results show that α -Syn inhibits specifically the docking step. Meanwhile, several cellular measurements indicate that α -Syn disrupts the upstream trafficking stages including vesicle tethering or vesicle clustering as well, resulting in dispersion of vesicles away from the fusion active zone.^{8,9c} Therefore, it is possible that α -Syn interferes with the neuroexocytotic pathway at the multiple stages, warranting further investigation.

It has been previously argued that inhibition of exocytosis by α -Syn represents its toxicity.^{7,17} If so, we might have expected that the familial PD-causing mutations A30P, E46K, and A53T gave stronger inhibition for SNARE-dependent lipid mixing. On the contrary, our results revealed that there is no clear correlation between its fusion-inhibiting activity and disease-

causing mutations. The findings here are short of revealing the pathogenic mechanism of familial PD. Therefore, we hesitate to conclude yet whether the fusion-inhibiting activity of α -Syn perhaps represents its toxicity or not. It is possible that the familial PD-causing mutants are just more susceptible to the cellular or exogenous factors that lead to transformation of α -Syn to protofibrils, which is believed to be its disease-causing form,²⁶ and their disease characteristics are not fully reflected in our results.

It appears that the inhibition of docking by α -Syn is coupled with the α -Syn's membrane binding but not with the interaction with VAMP2. A line of evidence supports this claim: (1) in the absence of the negatively charged lipid PS, which strongly favors α -Syn's membrane binding, the fusion-inhibiting activity of α -Syn is abrogated; (2) the gain-of-function mutant α -Syn E46K in membrane binding inhibits membrane fusion more than the wild-type, while the loss-of-function mutant A30P inhibits fusion less than the wild-type; (3) the C-terminal truncated α -Syn mutant, without its binding to VAMP2, shows a similar inhibition in lipid mixing as the wild-type; (4) it exhibits a strong inhibitory activity for another SNAREs involved in yeast trafficking, which shows no interaction with α -Syn; and (5) despite some α -Syn's binding v-SNARE in the absence of negatively charged lipid, the fusion inhibitory activity of α -Syn is completely abrogated in the absence of negatively charged PS.

How could the membrane binding of α -Syn inhibit the docking step? A recent report by Sudhof and co-worker shows that α -Syn at high concentration can cluster vesicles, perhaps due to the cross-linking of vesicles by α -Syn's intervesicular interactions using its two membrane binding helices.^{4,24} Although Sudhof and co-workers speculated that the vesicle clustering might be partly due to its binding to VAMP2, our results argue that its interaction with VAMP2 does not play a role in inhibiting vesicle docking. We also emphasize here that the fusion inhibition by α -Syn at high concentration differs from the fusion inhibition α -Syn oligomers at much lower concentrations in that the latter requires α -Syn's interaction with VAMP2.

In conclusion, our *in vitro* results demonstrate that α -Syn has the capacity of influencing SNARE-dependent membrane fusion by reducing vesicle docking, most likely via membrane binding, without interfering with the release of contents.

■ ASSOCIATED CONTENT

■ Supporting Information

α -Syn inhibits yeast SNARE mediated lipid fusion, α -Syn cosedimentation experiment, as well as α -Syn and mutants oligomerization Western blotting analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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