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α-Synuclein Can Inhibit SNARE-Mediated Vesicle Fusion through Direct Interactions with Lipid Bilayers

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Supporting Information

ABSTRACT: The native function of α-synuclein is thought to involve regulation of synaptic vesicle trafficking. Recent work has also implicated a role in neurotransmission, possibly through interactions with the proteins involved in synaptic vesicle fusion. Here, we demonstrate that α-synuclein inhibits SNARE-mediated vesicle fusion through binding the membrane, without a direct interaction between α-synuclein and any of the SNARE proteins. This work supports a model in which α-synuclein plays a role in the regulation of vesicle fusion by modulating properties of the lipid bilayer.

α-Synuclein (αS) is a 14.4 kDa neuronal protein implicated in Parkinson's disease (PD), where it is the major component of the Lewy body plaques found in affected brain tissue. αS is localized to synaptic termini, existing in equilibrium between the cytosol and synaptic membrane.[1] In vitro, the ability of αS to bind synthetic lipid bilayers is well-established, and preferences for both specific lipids and highly curved membranes have been observed.[2] Binding of αS significantly alters membrane properties; it can induce curvature, thinning of the lipid bilayer, and tubulation.[3−5] These varying effects of αS when it binds to lipid bilayers allude to potential roles for αS in processes such as membrane fusion that depend on transitions through states of high membrane curvature.

There is also a growing body of work that supports a role for αS in the regulation of synaptic vesicle fusion (reviewed in ref 6). Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins are responsible for synaptic vesicle fusion as well as most other fusion events within cells.[7] In neurons, v-SNAREs (VAMP2 or synaptobrevin) associate with vesicle membranes while t-SNAREs (syntaxin and SNAP-25) are found in target membranes.[8] In a vesicular fusion event, the t- and v-SNAREs assemble into a four-helix bundle, pulling the two membranes together to cause fusion.[9] Multiple animal models show a decrease in the level of neurotransmitter release upon overexpression of αS,[10−12] suggesting that αS may act as a regulator of neurotransmission, altering or disrupting the SNARE-driven fusion of synaptic vesicles. An increase in the rate of induced dopamine release is found in mice lacking all three (α, β, and γ) synuclein proteins.[13]

Here we investigate the role of αS in regulating SNARE-mediated vesicle fusion. To directly assess the effects of αS, we used an in vitro SNARE fusion assay. Vesicles were prepared containing either v- or t-SNARE components (Supporting Information). Fusion was initiated by mixing the two types of SNARE vesicles in the absence or presence of αS and monitored by a fluorescence increase upon lipid mixing.

αS inhibits SNARE-mediated vesicle fusion in a concentration-dependent manner (Figure 1A). Inhibition is observed at αS:accessible lipid ratios of <1:500. The extent of inhibition increases with an increasing αS concentration, and saturation of the effect occurs at an αS:accessible lipid ratio of ∼1:20. Fusion is inhibited a maximum of ∼50% relative to the control. Effects of a similar magnitude have been observed for established regulators of SNARE-mediated fusion.[14,15] αS does not appear to stall the fusion process at hemifusion (Figure S1).

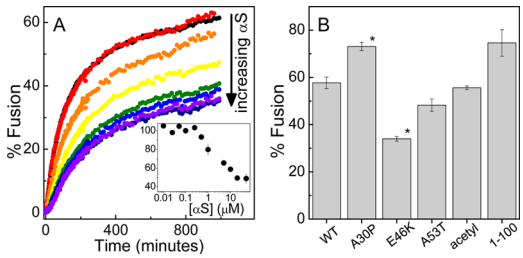

Figure 1. αS inhibits vesicle fusion. (A) SNARE-mediated vesicle fusion as a function of increasing αS concentration (800 μM lipid). The inset shows the quantification of the extent of inhibition normalized to fusion in the absence of αS. (B) Comparison of αS variants (5 μM αS, 800 μM lipid). *$P < 0.01$ compared to the WT.

To gain further insight into this phenomenon, we exploited previous work in our lab that quantified differences in membrane binding affinity between αS variants associated with familial forms of PD.[2] Each of the variants inhibits SNARE-mediated fusion, although to differing extents relative to that of the wild type (WT): E46K > WT, A30P < WT, and A53T ≈ WT (Figure 1B). Importantly, the extent to which each of these variants inhibits fusion correlates with its affinity for the lipid bilayer and thus with the amount of αS associated with the vesicles. To illustrate, E46K binds more tightly to membranes and also inhibits more strongly than the WT, while A30P binds membranes more weakly and inhibits fusion less effectively than WT.[2] αS from mammalian sources is acetylated at its N-terminus;[16] this modification does not interfere with its ability to inhibit fusion (Figure 1B). αS truncated at residue 100 is also capable of inhibiting fusion (Figure 1B), although to a lesser extent than predicted on the basis of its binding affinity.[3]

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While the C-terminus does not interact directly with the lipid bilayer, this supports a role for it in inhibiting fusion, perhaps through electrostatic repulsion. It may also reflect a more general ability of amphipathic α-helical membrane-binding proteins to alter lipid bilayer properties and affect processes such as fusion and tubulation. Lastly, of note is a very recent study in which oligomeric αS was reported to inhibit SNARE-mediated vesicle fusion.[17] Monomer αS was not found to have the same effect in this study, likely due to the low content of anionic lipids used in the vesicles (Supporting Information).

The results of the fusion assays indicate a correlation between the extent of inhibition and the amount of αS bound to the vesicles, strongly supported by our previous work with pure lipid vesicles.[2] However, the presence of the t- and v-SNARE proteins could be expected to alter the partitioning of αS to SNARE vesicles, as would binding of αS to either of the SNARE proteins.[18] To directly assess the impact of t- and v-SNARE proteins, we measured binding of αS to vesicles containing t- and v-SNAREs. αS was labeled at residue 33 with NBD, an environment-sensitive fluorophore (αS-NBD). The intensity of the αS-NBD fluorescence changes upon insertion into the lipid bilayer, reporting on binding (Figure S2).

αS binds with lower affinity to vesicles containing either v- or t-SNARE proteins as compared with pure lipid vesicles (Figure 2A). The results are quantified in Table S1. Using a floatation assay as an orthogonal approach, we screened a broader range of lipid compositions and found similar results for all compositions tested (Figure S3). There are several mechanisms by which the presence of SNARE proteins could impact binding of αS to vesicles. The simplest explanation is that the SNARE proteins occupy lipid binding sites that would otherwise be accessible to αS, thereby decreasing the level of protein binding. Electrostatics are important for binding of αS to pure lipid vesicles,[2,19] and the presence of the SNARE proteins, particularly the highly acidic t-SNARE, will alter the effective charge of the vesicles. However, given that αS binds with a higher affinity to more negatively charged lipid vesicles and has also been observed to interact with a variety of negatively charged macromolecules,[20−22] we might expect an increase in binding t-SNARE vesicles, rather than the observed decrease. Lastly, direct binding of αS to VAMP2, mediated by the C-terminus of αS and the N-terminus of VAMP2, both of which should be accessible in the membrane-associated forms of the proteins,[23,24] has been proposed to enhance assembly of the SNARE fusion complex.[18] Our expectation is that binding of αS to VAMP2 would result in a net decrease in the measured K_d for v-SNARE vesicles relative to lipid-only vesicles, whereas we observe a nearly 3-fold increase in K_d (Table S1).

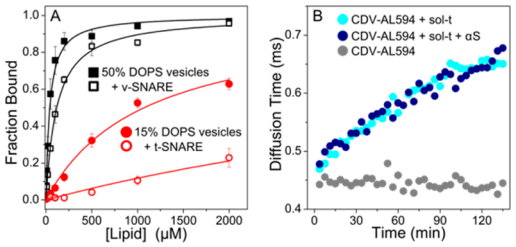

Figure 2. αS does not bind to SNARE proteins. (A) αS (500 nM) binds more weakly to SNARE vesicles (empty symbols) than to comparable pure lipid vesicles (filled symbols). (B) An increase in diffusion time is observed by FCS upon mixing of CDV-AL594 (gray) with sol-t (cyan) reflecting SNARE complex formation. An excess of αS (blue) does not affect the extent or rate of complex formation.

The use of soluble t- and v-SNARE constructs allowed us to examine their interactions with αS in the absence of the lipid bilayer. In particular, we considered the possibility that the excess of lipid relative to protein found in our model system, as compared to actual synaptic vesicles,[25] interferes with or masks evidence of a direct interaction between αS and either of the SNARE proteins. Soluble SNARE constructs were created by removing the transmembrane anchors, resulting in the cytoplasmic domain of VAMP2 (CDV, residues 1−95) and soluble t-SNARE (sol-t, syntaxin residues 1−265 and full-length SNAP-25). SNARE complex formation by these soluble constructs was observed by monitoring an increase in the diffusion time of Alexa 594-labeled CDV (CDV-AL594) upon binding to unlabeled sol-t using fluorescence correlation spectroscopy (FCS). Incorporation of CDV-AL594 into the larger complex results in a more slowly diffusing fluorescent species due to the increased hydrodynamic size (Figure 2B). The addition of an excess of αS does not alter either the kinetics or the extent of complex formation (Figure 2B). Interaction of αS with the SNARE complex was assessed directly by repeating the experiment using Alexa 488-labeled αS (αS-AL488) and unlabeled CDV and sol-t (Figure S4). No change in the diffusion time of αS was observed on the time scale of SNARE complex formation, indicating it is not associated with the complexes. Lastly, complex formation was examined by using size exclusion chromatography to separate SNARE complexes from the free constituent proteins in CDV/sol-t samples in the absence and presence of αS.[26,27] αS was observed to elute as a free monomer, and the extent of SNARE complex formation was independent of αS (Figure S5). This finding is consistent with our FCS measurements and indicates that αS does not appear to have a direct role in chaperoning SNARE complex formation. With these measurements, however, we cannot exclude the possibility that interactions with αS could be mediated by any of the numerous proteins, such as synaptotagmin and complexin, that regulate SNARE complex formation in vivo.

Our results strongly support a model in which αS inhibits SNARE-mediated vesicle fusion through its interactions with lipid membranes. These findings are broadly consistent with the body of in vitro and in vivo work that suggests that the function of αS may derive from its ability to alter lipid bilayer properties, rendering it less fusogenic. The most direct support for this model comes from the observation that αS inhibits calcium-mediated, SNARE-independent vesicle fusion[28](Figure S6), suggesting that decreased membrane fusability may be independent of the fusion mechanism. αS has been observed to anneal defects in bilayers, resulting in a more uniform membrane with a higher energy barrier to fusion.[29] Both experiment and simulations have shown that αS binding thins lipid bilayers and increases their rigidity,[3] an effect that increases the energy required to form the highly curved fusion intermediates.[30] More generally, αS is capable of inducing curvature and tubulation in bilayers composed of a range of lipids.[3−5] Induction of curvature and tubulation of lipid bilayers are also features of many BAR domain proteins that have well-studied roles in remodeling membranes in various endocytic and exoctytic pathways. This last feature is particularly intriguing in light of a recent study that found upregulation of the BAR domain proteins endophilin A1 and endophilin B2 in mice deficient in α-, β-, and γ-synuclein.[5] The endophilins

are implicated specifically in sensing and/or induction of membrane curvature,[31,32] indicating there may be a compensation for the loss of these functions in the absence of all the synucleins (Figure S7).

It is of interest to consider the consequences of our model in interpreting αS functional studies in a variety of model systems. An increased level of dopamine release in αS knockout mice and a reduced level of dopamine release in mice overexpressing αS can both be explained by αS functioning to inhibit fusion.[10−13] In cultured mammalian cells and in yeast, overexpression of αS disrupted ER−Golgi transport,[26,33] a process highly dependent on vesicle fusion. Overexpression of αS in *Caenorhabditis elegans* resulted in an increased level of fragmentation of mitochondria, due to a decreased level of fusion between mitochondria.[28] Moreover, downregulation of αS was associated with mitochondrial elongation, reflecting enhanced fusion. Finally, our observation that αS does not enhance SNARE complex formation is consistent with a study that observed a normal abundance of SNARE complexes in mice lacking all three members of the synuclein family.[13] Our results here help in the mechanistic understanding of altered membrane fusion events observed in vivo and strongly implicate a role for αS in membrane remodeling at the synaptic terminal.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S7 and detailed experimental methods. 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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