α-Synuclein assembles into higher-order multimers upon membrane binding to promote SNARE complex formation

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Physiologically, α-synuclein chaperones soluble NSF attachment protein receptor (SNARE) complex assembly and may also perform other functions; pathologically, in contrast, α-synuclein misfolds into neurotoxic aggregates that mediate neurodegeneration and propagate between neurons. In neurons, α-synuclein exists in an equilibrium between cytosolic and membrane-bound states. Cytosolic α-synuclein appears to be natively unfolded, whereas membrane-bound α-synuclein adopts an α-helical conformation. Although the majority of studies showed that cytosolic α-synuclein is monomeric, it is unknown whether membrane-bound α-synuclein is also monomeric, and whether chaperoning of SNARE complex assembly by α-synuclein involves its cytosolic or membrane-bound state. Here, we show using chemical cross-linking and fluorescence resonance energy transfer (FRET) that α-synuclein multimerizes into large homomeric complexes upon membrane binding. The FRET experiments indicated that the multimers of membrane-bound α-synuclein exhibit defined intermolecular contacts, suggesting an ordered array. Moreover, we demonstrate that α-synuclein promotes SNARE complex assembly at the presynaptic plasma membrane in its multimeric membrane-bound state, but not in its monomeric cytosolic state. Our data delineate a folding pathway for α-synuclein that ranges from a monomeric, natively unfolded form in cytosol to a physiologically functional, multimeric form upon membrane binding, and show that only the latter but not the former acts as a SNARE complex chaperone at the presynaptic terminal, and may protect against neurodegeneration.

α-Synuclein is an abundant presynaptic protein that physiologically acts to promote soluble NSF attachment protein receptor (SNARE) complex assembly in vitro and in vivo (1–3). Point mutations in α-synuclein (A30P, E46K, H50Q, G51D, and A53T) as well as α-synuclein gene duplications and triplications produce early-onset Parkinson’s disease (PD) (4–10). Moreover, α-synuclein is a major component of intracellular protein aggregates called Lewy bodies, which are pathological hallmarks of neurodegenerative disorders such as PD, Lewy body dementia, and multiple system atrophy (11–14). Strikingly, neurotoxic α-synuclein aggregates propagate between neurons during neurodegeneration, suggesting that such α-synuclein aggregates are not only intrinsically neurotoxic but also nucleate additional fibrilization (15–18).

α-Synuclein is highly concentrated in presynaptic terminals where α-synuclein exists in an equilibrium between a soluble and a membrane-bound state, and is associated with synaptic vesicles (19–22). The labile association of α-synuclein with membranes (23, 24) suggests that binding of α-synuclein to synaptic vesicles, and its dissociation from these vesicles, may regulate its physiological function. Membrane-bound α-synuclein assumes an α-helical conformation (25–32), whereas cytosolic α-synuclein is natively unfolded and monomeric (refs. 25, 26, 31, and 32; however, see refs. 33 and 34 and Discussion for a divergent view). Membrane binding by α-synuclein is likely physiologically important because in in vitro experiments, α-synuclein remodels membranes (35, 36), influences lipid packing (37, 38), and induces vesicle clustering (39). Moreover, membranes were found to be important for the neuro-pathological effects of α-synuclein (40–44).

However, the relation of membrane binding to the in vivo function of α-synuclein remains unexplored, and it is unknown whether α-synuclein binds to membranes as a monomer or oligomer. Thus, in the present study we have investigated the nature of the membrane-bound state of α-synuclein and its relation to its physiological function in SNARE complex assembly. We found that soluble monomeric α-synuclein assembles into higher-order multimers upon membrane binding and that membrane binding of α-synuclein is required for its physiological activity in promoting SNARE complex assembly at the synapse.

Results

Membrane-Bound Multimeric α-Synuclein Chaperones SNARE Complex Assembly. Promotion of SNARE complex assembly by α-synuclein requires binding of α-synuclein to the N terminus of the v-SNARE protein synaptobrevin-2 (3, 45), suggesting that α-synuclein promotes SNARE complex assembly in its membrane-bound state. Whether membrane binding is actually required for this activity, however, remained unknown. To address this question, we used a reconstituted system composed of purified recombinant proteins (3) (Fig. L4). In these experiments, we measured SNARE complex assembly in the absence and presence of α-synuclein under

Significance

Physiologically, α-synuclein promotes soluble NSF attachment protein receptor (SNARE) complex assembly during synaptic exocytosis. Pathologically, however, α-synuclein forms neurotoxic aggregates that promote neurodegeneration and represent hallmark features of Parkinson’s disease and other synucleinopathies. α-Synuclein exists in a monomeric unfolded state in solution and in an α-helical folded state upon binding to membranes. Yet the relation between these conformational states and their physiological and pathological roles remain unknown. Here, we demonstrate that α-synuclein multimerizes during membrane binding and that the membrane-bound, multimeric form of α-synuclein mediates SNARE complex assembly in presynaptic terminals. Our data delineate a folding pathway for α-synuclein that ranges from a monomeric unfolded form in cytosol to a physiologically functional multimeric form that is membrane bound and chaperones SNARE complex assembly, and that may protect against neurodegeneration.

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latter experiments, we included as a further negative control N-terminally truncated synaptobrevin-2 that still assembles into SNARE complexes but does not bind α-synuclein (3, 45).

We found that, in the absence of liposomes, α-synuclein did not detectably enhance SNARE complex assembly (Fig. 1 B−F). However, α-synuclein significantly increased SNARE complex assembly when negatively charged liposomes were added to the SNARE fragments; this increase was observed even when soluble fragments of all three SNAREs were used. In contrast, negatively charged liposomes alone had no effect on SNARE complex assembly in the absence of α-synuclein (Fig. 1 B−F). Using reconstituted full-length synaptobrevin-2 as opposed to the synaptobrevin-2 fragment alone increased SNARE complex assembly in the absence of α-synuclein, presumably because the immobilized synaptobrevin-2 has a higher effective concentration than a soluble synaptobrevin-2 fragment (Fig. 1 B−F). Nevertheless, α-synuclein again enhanced SNARE complex formation even in this experimental configuration. Thus, only membrane-bound multimeric α-synuclein increases SNARE complex assembly. Importantly, when we used N-terminally truncated synaptobrevin-2 (which lacks 28 N-terminal residues and does not bind α-synuclein) in these experiments instead of wild-type synaptobrevin-2, α-synuclein had no effect on SNARE complex assembly under any condition (Fig. 1 E and F). Because α-synuclein still binds to negatively charged liposomes even in the absence of synaptobrevin binding (3, 45), these results together show that α-synuclein not simply enhance SNARE complex assembly by binding to the liposomes and crowding out the SNAREs, but performs an active role in accelerating SNARE complex assembly by binding simultaneously to the liposomes and to synaptobrevin-2.

**α-Synuclein Multimerizes upon Membrane Binding.** Extensive studies showed that soluble α-synuclein is monomeric (refs. 25, 26, 31, and 32; however, see refs. 33 and 34 for a divergent view), but it is unknown whether membrane-bound α-synuclein is also monomeric. To address this question, we chose chemical cross-linking experiments because it is difficult to measure multimerization of native, unmodified proteins on a membrane with other techniques (e.g., gel filtration, scanning transmission electron microscopy, or analytical ultracentrifugation). Because chemical cross-linking experiments depend on the chance event that reactive groups in a protein complex are separated from each other by a precise distance corresponding to the cross-linking agent, chemical cross-linking experiments are inherently inefficient and depend on the size of the cross-linking agent (47, 48). Moreover, at high concentrations, chemical cross-linking agents nonspecifically link proteins to each other in a diffusion-controlled reaction that is used for tissue fixation. Thus, analysis of protein complexes by chemical cross-linking requires tests of a range of cross-linker concentrations, and use of multiple cross-linking agents with different linker lengths (Figs. 2 and 3, and Fig. S2).

We found that, in mouse brain homogenates obtained in the absence of detergents, four different cross-linking agents [glutaraldehyde, dimethyl suberimidate (DMS), dimethyl adipimidate (DMA), and dimethyl pimelimidate (DMP)] caused cross-linking of native α-synuclein into higher order multimers at different concentrations (Fig. 24 and Fig. S24). Low-percentage SDS/PAGE identified multimers containing eight or more α-synuclein subunits (Fig. 2B and Fig. S2B). α-Synuclein multimerization was not a biochemical artifact of cross-linking, as no multimers of control proteins were observed, demonstrating specificity (Fig. 2F and Fig. S2F).

When we examined α-synuclein in brain cytosol (which lacks membranes), we detected no cross-linking with any chemical agent (Fig. 2C and Fig. S2C). This result suggests that the chemical cross-linking of α-synuclein in brain homogenates required the presence of membranes for α-synuclein binding. Indeed, simply adding to the brain homogenate Triton X-100, a detergent that dissolves lipid membranes, blocked α-synuclein cross-linking at all concentrations. Therefore, we believe that cross-linking under these conditions is a chemical artifact of cross-linking, as no multimers of control proteins were observed, demonstrating specificity (Fig. 2F and Fig. S2F).
We next asked whether chemical cross-linking of α-synuclein could also be obtained in native brain tissue. We cut brain slices from mice immediately after they had been killed, and performed chemical cross-linking experiments on these acute slices (Fig. 3). We observed a similar α-synuclein cross-linking pattern in the slices as in brain homogenates (Fig. 3A). Again, the specificity of the cross-linking reactions was validated with control proteins with which we observed only cross-linking when expected, as for example for the SNARE proteins SNAP-25 and syntaxin-1 (Fig. 3B).

Together, these experiments suggest that α-synuclein forms multimers when bound to phospholipid membranes. To test whether these multimers are homomultimers, we examined recombinant α-synuclein and pure liposomes. We found that purified recombinant α-synuclein could be efficiently cross-linked into higher-order multimers upon incubation with phospholipid membranes (Fig. 4). Cross-linking was observed only with negatively charged phospholipids, but not in buffer alone, or with neutral liposomes composed of 100% PC (Fig. 4). This result is consistent with the binding of α-synuclein only to negatively charged but not to neutral phospholipids (19, 20). Moreover, we found that α-synuclein truncations and mutations associated with PD reduce, but do not block α-synuclein cross-linking, in agreement with the generally modest effect of these mutations on phospholipid binding (Fig. S3 and refs. 22, 45, 50, and 51).

Again, to exclude nonspecific aggregation, which is occasionally observed with recombinant α-synuclein, we performed parallel cross-linking experiments without liposomes or with uncharged liposomes, but detected only background levels of cross-linking (Fig. S3 D and E).

**α-Synuclein Multimerizes on Phospholipid Surfaces in an Antiparallel Orientation.** Although chemical cross-linkers can probe for formation of multimers, they do not reveal a particular multimer configuration. To confirm multimerization of α-synuclein upon membrane binding by an independent method, and to investigate the configuration of α-synuclein molecules in the multimers, we used fluorescence resonance energy transfer (FRET) experiments.

We introduced single cysteine residues into recombinant α-synuclein at defined positions, labeled the cysteines with fluorescent Alexa 488- or Alexa 546-maleimide, and purified the labeled proteins (Fig. S2 A and B). Liposome-binding experiments revealed that the cysteine modifications did not alter phospholipid binding except for the fluorescent modification of G41C, which produced a modest decrease in phospholipid binding (Fig. S3 and refs. 22, 45, 50, and 51).

We incubated pairs of Alexa 488- and Alexa 546-modified α-synucleins with liposomes composed either of 30% PS and 70% PC (negatively charged liposomes), or of 100% PC (neutral liposomes, used as a negative control), and measured the fluorescence emission spectrum with an excitation at 490 nm. FRET manifests as a decrease in donor (Alexa 488) and an increase in acceptor (Alexa 546) emission, and can be quantified as donor fluorescence quenching by calculating the ratio of total to donor fluorescence. This experimental design allowed us to test the effect of liposomes on FRET between any given pair of Alexa 488- and Alexa 546-labeled α-synucleins. In these experiments, we argued that multimerization of α-synuclein should only occur in the presence of negatively charged phospholipids because α-synuclein only binds to charged phospholipids (19, 20, 27–30). Thus, the effect of the neutral liposomes—if any—provides a tool to distinguish specific from nonspecific effects. If FRET can be specifically observed with a particular combination of labeled α-synucleins (i.e., is only detectable in the presence of negatively charged liposomes), such FRET must be due to multimerization of α-synuclein. Moreover, if some pairs of labeled α-synucleins do and some do not produce FRET, the mapping of the fluorescent probe position onto the α-synuclein sequence gives insight into the orientation of the α-synuclein molecules in the oligomers.

(Fig. 2D and Fig. S2D). Moreover, addition of brain membranes to brain cytosol in the ratio present in brain homogenate, restored cross-linking of α-synuclein (Fig. 2E and Fig. S2E). Note that some cross-linking agents appear to increase the size of α-synuclein, probably because they covalently attach phospholipids to α-synuclein, and that cross-linked multimeric α-synuclein seems to exhibit an increased immunoreactivity, likely because cross-linked α-synuclein adheres better to blotting membranes (Fig. 2 and Fig. S2) (49).

Fig. 2. Native α-synuclein multimerizes on the surface of brain membranes. (A–E) Brain homogenates containing intact membranes (A and B; 900 μg of protein), brain cytosol (C; 150 μg of protein), brain homogenates whose membranes were solubilized by addition of Triton X-100 (D; 900 μg of protein), and brain cytosol with readdition of brain membranes (E; 150 μg of cytosolic protein plus 750 μg of membrane protein) were exposed to increasing concentrations of chemical cross-linking agents [Left, glutaraldehyde (0.01%); Right, dimethyl suberimidate (DMS) (0–25 mM)], and equal volumes were analyzed by immunoblotting (arrowheads, α-synuclein oligomers as determined by SDS/PAGE). For additional cross-linking experiments, quantitations, cross-linker concentrations used, size calibrations, and controls, see Fig. S2. (F) Control cross-linking experiments. Brain homogenates were exposed to the same increasing concentrations of glutaraldehyde and analyzed as described above (GDI, guanine-nucleotide dissociation inhibitor; SGT, small glutamine-rich tetratricopeptide repeat-containing protein; Synt-1, syntaxin-1; arrowheads, protein monomers and homooligomers or heterooligomers as determined by SDS/PAGE migration analysis; asterisks indicate nonspecific immunosignals).
We found that, of 15 potential FRET pairs tested, 6 pairs produced significant FRET (Fig. 5 D and E, and Fig. S4B). These data confirm that α-synuclein multimerizes on the phospholipid surface, and suggest that it does so in a defined orientation. Because the cross-linking experiments suggested that α-synuclein multimerizes on the lipid surface into at least octamers (Fig. 2), multimers are not simply dimers.

A potential concern about our FRET experiments is that the α-synuclein added to liposomes may so densely populate the liposome surface with α-synuclein molecules that even α-synuclein molecules that are not multimerized may produce a FRET signal. However, this concern is inconsistent with the selective FRET observed: only some combinations of labeled α-synuclein molecules produced FRET, whereas others did not (Fig. 5 D and E, and Fig. S4B). To further address this concern, we performed titrations of a constant amount of donor-labeled α-synucleins (L8C or A140C) with increasing concentrations of acceptor-labeled α-synuclein (Fig. 6 A–D). These experiments showed that combinations that exhibited no FRET in the experiments of Fig. 5 were also unable to produce FRET at any concentration tested, whereas the positive control pair (L8C–L8C) exhibited robust FRET in a concentration-dependent manner if the acceptor was present in a ratio of at least 0.5 (Fig. 6 A–D). Thus, the FRET we observe is not due to nonspecific crowding of α-synuclein on the phospholipid surface.

α-Synuclein is thought to adopt a bent, hairpin-like conformation on the lipid surface that is composed of two α-helices connected with a flexible linker (27–29, 52). The pattern of positive vs. negative FRET pairs of labeled α-synuclein molecules allows mapping the interactions of α-synuclein molecules in the membrane-bound multimer (Fig. 6 E). This map reveals a striking organization of interactions, suggesting a model for the arrangement of α-synuclein molecules in the multimer (Fig. 6 F).

Discussion

α-Synuclein is an abundant presynaptic protein that physiologically acts as a nonclassical chaperone for SNARE complex assembly, and pathologically contributes to the pathogenesis of PD and

Fig. 3. α-Synuclein forms multimers in brain slices. (A) Acute brain slices from wild-type mice were exposed to increasing concentrations of cross-linking agents [Left, glutaraldehyde (0–0.03%); Right, dimethyl suberimidate (DMS) (0–3 mM)]. Brain slices were homogenized in sample buffer after cross-linking, and equal volumes of cross-linked proteins were analyzed by immunoblotting (Upper, 15% gels; Lower, 10% gels). The arrowheads indicate α-synuclein multimers as determined by SDS/PAGE migration analysis; the asterisks indicate nonspecific immunosignals (means ± SEMs; n = 3). (B) Control cross-linking experiments. Acute brain slices were exposed to same increasing concentrations of the cross-linker DMS and analyzed as described above (GDI, guanine nucleotide dissociation inhibitor; SGT, small glutamine-rich tetratricopeptide repeat-containing protein; Syb2, synaptobrevin-2; Synt-1, syntaxin-1).
other neurodegenerative disorders (1–18). α-Synuclein is largely cytosolic, but readily binds to membranes, and associates with synaptic vesicles in the presynaptic terminal (22, 23). In the present study, we have asked three questions that are central to synaptic vesicles in the presynaptic terminal (22, 23).

In the cytosolic, but readily binds to membranes, and associates with other neurodegenerative disorders (1). α-Synuclein is largely cytosolic, but readily binds to membranes, and associates with synaptic vesicles in the presynaptic terminal (22, 23). In the present study, we have asked three questions that are central to an understanding of the role of α-synuclein in health and disease: (i) Does α-synuclein function as a SNARE complex assembly chaperone in its soluble or membrane-bound form? (ii) Is α-synuclein bound to membranes a monomer, or does it form a multimer upon membrane binding? (iii) Is α-synuclein normally associated with all synaptic vesicles, or only with those vesicles that are docked and engaged in SNARE complex formation?

Our data provide initial answers to these questions. We demonstrate that α-synuclein acts as a SNARE chaperone only when bound to membranes, that membrane binding causes α-synuclein to form higher-order multimers larger than octamers that have a defined orientation, and that α-synuclein is only stably associated with vesicles that are docked to the presynaptic plasma membrane and are thought to be engaged in SNARE complex formation. Thus, our results suggest a physiological folding pathway for α-synuclein whereby unstructured soluble α-synuclein not only adopts an α-helical conformation upon membrane binding, but simultaneously associates into multimers on the membrane surface (Fig. 8). These multimers are the functionally active form of α-synuclein in promoting SNARE complex assembly. Consistent with this function, α-synuclein is stably bound to synaptic vesicles only when these vesicles are docked and are thought to be in the process of forming SNARE complexes. We believe that these observations not only define an unanticipated multimeric state of α-synuclein that is selectively formed upon membrane binding but also localize the point of action of α-synuclein to the site of synaptic vesicle fusion (Fig. 8).

Our results raise new questions. First, how does α-synuclein work? The multimers cluster synaptobrevin-2/VAMP2 and thus increase the local concentration of synaptobrevin molecules, which would be expected to enhance SNARE complex formation. We believe that these observations not only define an unanticipated multimeric state of α-synuclein that is selectively formed upon membrane binding but also localize the point of action of α-synuclein to the site of synaptic vesicle fusion (Fig. 8).

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Second, how do our results relate to recent prominent studies on the multimerization of α-synuclein? Initially, soluble α-synuclein was described as a natively unfolded monomer (25, 26). This view was challenged when α-synuclein purified from human erythrocytes was found to be a stably folded tetramer, as judged by analytical ultracentrifugation (33). Confusingly, however, in the same study electron microscopy failed to detect a monodisperse population of α-synuclein tetramers, and high-resolution NMR studies also did not observe a stable tetramer of α-synuclein, but revealed a dynamic multimerization of α-synuclein molecules at very high concentrations (54). Subsequent work, moreover, demonstrated that both recombinant α-synuclein and native cytosolic α-synuclein purified from brain were monomeric and natively unfolded, suggesting that, at least in brain (where α-synuclein is most highly expressed and its pathological role in neurodegeneration is most important), soluble α-synuclein is a natively unfolded monomer (31, 32).

Indeed, the multimers that we identified here form only upon membrane binding and are very different from the stable tetramer proposed for α-synuclein purified from erythrocyte cytosol (33). We find no evidence for a stable tetramer (Figs. 2–4) but observe that the membrane-induced multimerization produces large α-synuclein assemblies containing more than eight subunits (Figs. 2–4). Moreover, we find that α-synuclein multimers are absent from cytosol—simple disruption of membranes by detergents disrupts all multimers (Fig. 2D)—whereas the previous postulate was that α-synuclein tetramer is a soluble cytosolic species. Thus, our data are fully consistent with the studies that concluded that α-synuclein is a natively unfolded monomer in solution and are inconsistent with the notion of a stable tetramer.

Third, the arguably most important question raised by our data is how the α-synuclein multimers that are physiologically assembled on phospholipid membrane surfaces and promote SNARE complex assembly relate to pathological oligomers and fibrils. Does binding and multimerization of α-synuclein on synaptic vesicles induce aggregation or protect against aggregation? Although the relatively high concentration of α-synuclein on the synaptic vesicle membrane may increase its propensity to aggregate, the formation...
of α-helices upon lipid binding may impede misfolding of α-synuclein into β-sheet–containing conformations, and thereby hamper pathological oligomerization and aggregation into fibrils. Regardless of which of these possibilities will turn out to be true, our data support the notion that α-synuclein is subject to constant conformational switches associated with binding to and dissociation from synaptic membranes in vivo.

Our study also has several limitations. Given that we were able to demonstrate the multimerization of α-synuclein upon membrane binding by two independent methods that have little in common (chemical cross-linking with multiple different agents and FRET with fluorescently labeled α-synuclein), and that both of these methods showed the absolute requirement for membrane binding in order for multimerization to occur, we think that the possibility for artifacts is low. Moreover, we found similar cross-linking patterns in brain slices as with purified α-synuclein binding to liposomes (Figs. 3 and 4), demonstrating that the α-synuclein multimers are present in vivo and that they constitute homomultimers. However, we do not know the tertiary structure of α-synuclein on the membrane surface. Although the FRET data are intriguing in suggesting an ordered array, they obviously do not conclusively characterize such an array at the atomic level. Moreover, we do not yet understand the dynamics of the α-synuclein multimers on the lipid surface and have no insight into how their assembly and dissociation are regulated. Independent of the answers to these open questions, the multimerization of α-synuclein on the lipid surface that we reveal here suggests a mode of action connecting protein and lipid interactions to each other, and broadly resembles that of other lipid-binding proteins such as BAR-domain proteins (55, 56).

In summary, viewed together our data describe a folding pathway whereby monomeric cytosolic α-synuclein physiologically folds into α-helical multimers upon phospholipid-membrane binding in presynaptic terminals (Fig. 8). The membrane-bound multimeric α-synuclein is composed of ordered array and promotes SNARE complex assembly, whereas the monomeric α-synuclein lacks such activity, suggesting a dynamic, activity-dependent fluctuation in the state and conformation of α-synuclein that contributes to maintaining nerve terminal function.

Materials and Methods

Fractionation of Brain Tissue and Synaptic Vesicle Isolation. One mouse brain was homogenized in 2 mL of homogenization buffer (PBS, pH 7.4, for glutaraldehyde cross-linking experiments, 150 mM NaCl, 50 mM Hepes-NaOH, pH 8.5, for imidoester cross-linking experiments) including EDTA-free protease inhibitors (Roche). For experiments with brain homogenate, samples were directly used. For further fractionation, nuclei were removed by centrifugation for 15 min at 4 °C and 1,000 × gav. Supernatants were then separated into cytosolic and membrane fractions using ultracentrifugation for 1 h at 280,000 × gav and 4 °C. Supernatants were removed, and pellets were homogenized in an equal volume of homogenization buffer. Separation was repeated three times to remove any trace residues of cytosol in membrane fractions, and vice versa. To solubilize membranes, brain homogenate was incubated with 0.5% Triton X-100 for 2 h at 4 °C. Insoluble material was pelleted by centrifugation for 20 min at 10,000 × gav, and 4 °C. Isolation of synaptic vesicles and separation of free and docked vesicle populations was performed as previously described (53).

Recombinant Protein Expression and Purification. Full-length human α-synuclein cDNA was inserted into modified pGEX-KG vectors (GE Healthcare), containing an N-terminal TEV protease recognition site. α-Synuclein contained an extra N-terminal glycine after cleavage with TEV protease in addition to the normal N-terminal glycine. α-Synuclein contained an extra N-terminal glycine after cleavage with TEV protease in addition to the normal N-terminal glycine. α-Synuclein contains an extra N-terminal glycine after cleavage with TEV protease in addition to the normal N-terminal glycine. α-Synuclein contains an extra N-terminal glycine after cleavage with TEV protease in addition to the normal N-terminal glycine.
α-Synuclein associates with synaptic vesicles docked at the presynaptic plasma membrane. (A) Separation of synaptic vesicles by sucrose gradient centrifugation into free (fractions 6–13) and docked vesicle pools (fractions 23–32). Equal volumes of each fraction were analyzed by immunoblotting (Syb2, synaptobrevin-2; α-Syn, α-synuclein; Syp1, syntaxin-1; Syb1, synaptophysin 1). (B and C) Quantitation of the distribution of the indicated proteins across the sucrose gradient (B) and of relative protein levels in the free vs. docked synaptic vesicle pool (C). Means ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001 by Student t test; n = 3.

600 nM, and protein expression was induced with 0.05 mM isopropyl β-D-thiogalactoside for 6 h at room temperature. Bacteria were harvested by centrifugation for 20 min at 2,100 × g, and pellets were resuspended in solubilization buffer (PBS, 0.5 mg/mL lysozyme, 1 mM PMSF, DNase, and an EDTA-free protease inhibitor mixture (Roche)). Cells were broken by sonication, and insoluble material was removed by centrifugation for 30 min at 7,000 × g, and 4 °C. Proteins were affinity-purified using glutathione Sepharose beads (GE Healthcare) incubation overnight at 4 °C, followed by TEV protease (Invitrogen) cleavage overnight at room temperature. His-tagged TEV protease was removed by incubation with Ni-NTA (Qiagen) overnight at 4 °C. SNARE complexes were expressed as described (3). Synaptobrevin-2 ΔN lacks 28 N-terminal residues and does not bind to α-synuclein (3). Protein concentrations were assessed using the bicinchoninic acid method according to the manufacturer’s protocol (Thermo Scientific).

Liposome Preparation and Liposome-Binding Assay. Liposomes were prepared as previously described (3). For lipid-binding assays, 1 mg of brain PC (Avanti Polar Lipids) or 0.7 mg of PC and 0.3 mg of brain PS (PCPS) (Avanti Polar Lipids) in chloroform were dried in a glass vial under a nitrogen stream. Residual chloroform was removed by lyophilization for 2 h. Small unilamellar vesicles were formed by sonication 1 mg/mL of lipids in PBS on ice. For lipid-binding studies, 5 μg of protein of α-synuclein were incubated with 100 μg of liposomes for 2 h at room temperature. Samples were then either subjected to a liposome flotation assay (3) or cross-linking experiments.

Chemical Cross-linking. Cross-linking experiments were performed using glutaraldehyde (TCl America), or the imidoesters DMA, DMP, and DMS (all from Thermo Scientific). Glutaraldehyde cross-linking experiments were carried out in PBS, pH 7.4, imidoester cross-linking experiments in 50 mM Hepes buffer, pH 8.5, with 150 mM NaCl. Thirty microliters of brain homogenate (900 μg), 30 μL of brain lysate (900 μg), 15 μL of brain cytosol (150 μg) plus 15 μL of buffer, 15 μL of brain cytosol (150 μg) plus 15 μL of brain membranes (750 μg), or 30 μL of recombinant myc-tagged α-synuclein (2 μg in buffer or on liposomes) were incubated for 5 min with 10 μL of glutaraldehyde. For imidoester cross-linking experiments, samples were incubated with 5 μL of α-synuclein and 2.5 μL of glutaraldehyde. Reaction was stopped by addition of 10 μL of 1 M Tris, pH 8.8. For cross-linking experiments in brain slices, equal tissue amount of 250-μm brain slices from wild-type mice were incubated in 500 μL of PBS with glutaraldehyde for 2 h at room temperature, or in 500 μL of lipo-alpha-β-γ-δ (Avanti) overnight at 4 °C. Reaction was stopped by addition of 20 μL of 1 M Tris, pH 8.8. Supernatants were removed, and brain slices were sonicated in 100 μL of 2x Laemmli sample buffer containing 100 mM DTT. For cross-linking experiments in vitro, 5 μg of recombinant purified α-synuclein were incubated with 100 μg of liposomes in 100 μL of buffer, or in 100 μL of buffer for 5 min with 10 μL of glutaraldehyde for 2 h with 10 μL of DMS. Reaction was stopped by addition of 10 μL of 1 M Tris, pH 8.8. Samples were boiled before separating via SDS/PAGE.

FRET Experiments. One hundred micromolar GST-fusion protein of α-synuclein containing a cysteine (M12, L8C, G41C, K96C, or A140C) were captured on GST-ρ-galactosidase beads (GE Healthcare). GST-α-synuclein was reduced with 1 mM DTT in 100 μL of 1 M Tris, pH 8.8. Supernatants were removed, and α-synuclein was eluted from the GST moiety using TEV protease overnight at room temperature. His-tagged TEV protease was removed using Ni-NTA agarose (Qiagen). For FRET experiments, 2.5 μg of Alexa 488-labeled α-synuclein and 2.5 μg of Alexa 564-labeled α-synuclein were incubated with and without 100 μg of α-synuclein. Selective labeling of liposomes was performed using Alexa 546 C5 maleimide (Invitrogen) overnight at 4 °C in the dark. Beads were washed four times with PBS to remove residual unbound dye, and α-synuclein was eluted from the GST moiety using TEV protease overnight at room temperature.
buffer was added, and samples were separated by SDS/PAGE and immunoblotted. For analysis of SNARE complex assembly by communoprecipitation, samples were incubated with polyclonal syntaxin-1 antibody (438B) and 50 μL of a 50% (vol/vol) slurry of protein A-Sepharose beads (GE Healthcare) for 2 h at 4 °C. Following five washes with 0.1% Triton X-100 in PBS, bound proteins were eluted with 2× SDS samples buffer and boiled for 20 min at 100 °C. Coprecipitated proteins were separated by SDS/PAGE, with 5% of the input in the indicated lane.

**Gel Electrophoresis and Protein Quantitation.** *SDS/PAGE.* Protein samples were separated by SDS/PAGE and transferred onto nitrocellulose membranes. Blots were blocked in Tris-buffered saline containing 0.1% Tween 20 (Sigma) and 3% fat-free milk for 2 h at room temperature. The blocked membrane was incubated in blocking buffer containing primary antibody for 1 h, followed by five washes. Membranes were incubated in blocking buffer containing horseradish peroxidase (HRP)-conjugated secondary antibody (MP Biomedicals; 1:5,000) for 2 h at room temperature. HRP immunobLOTS were developed using enhanced chemiluminescence (GE Healthcare).

**Tris-acetate PAGE.** For resolution of high mass range, proteins were separated on 6% Tris-acetate gels and transferred onto PVDF membranes according to the manufacturer’s protocol (Invitrogen). After transfer, membranes were obtained in methanol, and were blocked and incubated with primary antibody as described above. All quantitative immunoblotting experiments were performed with iodinated secondary antibodies (PerkinElmer, 1:1,000) overnight at room temperature as described.121 Blots were exposed to phosphorimager screens (GE Healthcare) overnight and scanned using a Typhoon scanner (GE Healthcare), followed by quantification with ImageQuant software (GE Healthcare).

**Primary Antibodies.** Monoclonal antibodies used were α-synuclein (610786; BD Transduction), GDI (cl. 81.2; SYSY), neurologin-1 (cl. 4C12; SYSY), rab3A (cl. 42.4; SYSY), SNAP-25 (cl. 71.1, SYSY; SM181, Covance), synaptobrevin-2 (cl. 69.1; SYSY), and syntaxin-1 (HPC-1; SYSY). Polyclonal antibodies used were α-synuclein (U1217), syntaxin-1 (438B), and SGT (CHAT33).

**Statistical Analyses.** Curves obtained from cross-linking experiments were analyzed by two-way repeated-measure ANOVA, using GraphPad Prism. Communoprecipitation experiments are shown as recovered protein (relative to the input), which was firstnormalized to the immunoprecipitated protein and then normalized to the control. All other data shown are means ± SEMs and were analyzed by Student’s unpaired two-tailed t test to compare the data groups.

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