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α-synuclein multimers cluster synaptic-vesicles and attenuate recycling

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SUMMARY

The normal functions and pathologic facets of the small presynaptic protein α-synuclein (α-syn) are of exceptional interest. In previous studies, we found that α-syn attenuates synaptic exo/endocytosis [1, 2]; however underlying mechanisms remain unknown. More recent evidence suggests that α-syn exists as metastable multimers and not solely as a natively-unfolded monomer [11-16]. However conformations of α-syn at synapses – its physiologic locale – are unclear; and potential implications of such higher-order conformations to synaptic function is unknown. Exploring α-syn conformations and synaptic function in neurons, we found that α-syn promptly organizes into physiological multimers at synapses. Furthermore, our experiments indicate that α-syn multimers cluster synaptic-vesicles and restrict their motility – suggesting a novel role for these higher-order structures. Supporting this, α-syn mutations that disrupt multimerization also fail to restrict synaptic-vesicle motility or attenuate exo/endocytosis. We propose a model where α-syn multimers cluster synaptic-vesicles, restricting their trafficking and recycling – consequently attenuating neurotransmitter release.

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SUPPLEMENTARY INFORMATION

Supplemental Information includes three figures and Supplemental Experimental Procedures can be found with this article online at xxxxxxx

Keywords

α-synuclein; synapse; vesicle-trafficking; pHluorin; tetramers

RESULTS AND DISCUSSION

Contemporary insights into α-synuclein biology

The presynaptic bouton is a central communicating hub, where a sequence of well-orchestrated events leads to exocytosis of neurotransmitter-loaded vesicles into the presynaptic cleft. A variety of presynaptic proteins participate, mainly assisting in the organization and trafficking of synaptic-vesicles. One such protein is α-syn; of singular interest due to its involvement in Parkinson's disease and related movement-disorders/ dementias. In previous studies we found that small increments in α-syn-levels lead to suppression of exo/endocytosis [1, 2]; and that α-syn restricts the lateral mobility of synaptic-vesicles between *en-passant* boutons [2], called “superpool” trafficking [3]. Along with other studies [4-9], available data advocate the concept that α-syn physiologically attenuates neurotransmitter release; however underlying mechanisms are unclear. α-syn also binds to VAMP2 and promotes SNARE-assembly [10], but the consequence of these interactions on synaptic physiology is uncertain [7, 10]. Regardless, a clear picture of the physiologic role of α-syn has not emerged yet.

As function often follows form in biology, understanding physiologic α-syn conformations is important. Recent studies offer surprising insights, suggesting that α-syn exists as metastable helical multimers, with predominant tetramers [11]. Though this view has been challenged [12, 13], available data from purified brain α-syn show higher-order multimers and mixed helical conformations [13, 14], consistent with the idea that α-syn exists as metastable conformers, exchanging between a monomeric and multimeric state. Even so, key questions remain unresolved. What is the conformation of α-syn *at synapses*, its normal locale? Do α-syn assemblies influence its function? If so how? Here we couple fluorescence-complementation assays – that selectively stabilize putative α-syn assemblies – with various cell-biological paradigms to evaluate vesicle-trafficking and synaptic function.

Multimeric α-syn conformations at presynaptic boutons

Though recent studies have demonstrated α-syn multimers [11, 15, 16], most experiments used biochemical or biophysical methods that do not provide spatial information; thus α-syn conformations at the presynapse are not entirely clear. We first evaluated the organization of α-syn at synapses of cultured neurons using bimolecular fluorescence complementation (BiFC) – an established method to visualize protein-protein interactions [17]. In this assay, one partner of an interacting-pair is tagged to the N-terminus fragment of the Venus fluorescent protein (VN), while the other partner to the complimentary C-terminus (VC). If and when the two interacting partners associate, the Venus fragments are reconstituted and become fluorescent [see schematic in **fig. 1A (i)**]. Reconstitution is irreversible, thus even transient interactions can be “captured” by these methods [18].

To mitigate concerns related to over-expression, we used a 'molecular replacement strategy', where the exogenous protein is expressed in a knockout background, achieving near-physiologic expression-levels [19]. Specifically, we expressed VN/VC tagged human wild-type α-syn's (VN/VC:α-syn's) in cultured hippocampal neurons (days in vitro-DIV 14) from α-syn null mice and visualized fluorescence after ~14h expression [strategy in **fig. 1A(ii)**]. Expression of human α-syn in cultured α-syn null neurons was similar to the expression of native mouse α-syn in parallel-processed cultures (see below). Three different VN/VC-tagged α-syn combinations were used (see methods). In all cases, robust fluorescence was seen at boutons (**fig. 1B**-top and **Supp. fig. 1A, B**). Co-transfection of VN and VC alone did not show any synaptic fluorescence (**fig. 1B**-bottom and data not shown). Complementation was also seen in nonneuronal cells as reported previously [20], and excess un-tagged α-syn diminished VN/VC:α-syn complementation in HEK cells – presumably by competition – suggesting that complementation was specific for α-syn (**Supp. fig. 1C**). Synaptic fluorescence due to VN/VC:α-syn complementation was widespread, seen in virtually all transfected boutons; overall similar to neurons transfected with Venus:α-syn (**fig. 1B**, graph on right). It is unlikely that the complementation in our experiments is an artifact of over-expression, as fluorescence intensities of transfected VN/VC:α-syn boutons in α-syn -/- neurons is similar to endogenous mouse α-syn fluorescence in WT neurons (**fig. 1C, D** and also see next).

Next we visualized the time-course of accumulation of newly-synthesized VN/VC:α-syn at boutons. We transfected α-syn null neurons with VN/VC:α-syn (or Venus:α-syn) and visualized the entry of newly-synthesized (somatically-derived) fluorescent molecules into boutons (4-5 hours after transfection), adopting an imaging strategy that we recently developed ([21], see schematic in **fig. 1E**). **Figure 1F** shows representative images from one such experiment. Note that the kinetics of VN/VC:α-syn entry into boutons is only slightly slower than Venus:α-syn, quantified in **figure 1G**. These data indicate that multimerization of α-syn is an early event, and likely not a consequence of abnormal long-term intra-molecular associations. Also note that the experimental paradigm (visualizing entry of newly-synthesized proteins into α-syn −/− boutons) further argues that the complementation is unlikely to be a result of over-expression.

α-synuclein multimers cluster synaptic-vesicles

Do α-syn multimers have a physiologic role? While qualitatively comparing the synaptic VN/VC:α-syn fluorescence to the fluorescence of endogenous or Venus:α-syn, we noticed that the reconstituted VN/VC:α-syn fluorescence seemingly occupied only a subset of the total bouton-area. To verify this, we used a previously-described protocol to label the entire bouton-profile (using fluorescent actin, see [22]) and visualized VN/VC:α-syn fluorescence in these boutons. Indeed reconstituted VN/VC:α-syn occupied only a fraction of the bouton-area, as shown in representative bouton-crops (**fig. 2A**). This distribution is unusual, distinct from Venus:α-syn that typically occupies the entire bouton (see **Supp. fig. 2A**). We also developed custom algorithms to quantify these data. Briefly, as bouton shapes vary, we measured the cross-sectional area of each bouton along 20 circumferential angles and then calculated its mean "synapse-width" (see **fig. 2B** for general concept; **Supp. fig. 2B** and methods for details). Compiled data from these analyses are shown in **fig. 2C, D**. Note that

the VN/VC:α-syn fluorescence only occupies a subset of the bouton cross-sectional area and is indistinguishable from the area occupied by the synaptic-vesicle cluster (labeled with synaptophysin:GFP, **fig. 2C**). Furthermore, in neurons co-transfected with VN/VC:α-syn and synaptophysin:mRFP (SyPhy:mRFP), there is a significant overlap of fluorescence (also reflected in correlations between their "synapse-widths", see **fig. 2D**).

Previous studies expressing WT α-syn in yeast revealed a dramatic clustering of vesicles by α-syn [23, 24]. Previously, we found that α-syn restricts synaptic-vesicle motility between *enpassant* boutons [2], suggesting that α-syn might cluster vesicles within synaptic boutons and restrict vesicle-motility. Since α-syn multimers associate with synaptic-vesicles (above), we asked whether stabilized VN/VC:α-syn multimers facilitate clustering of synaptic-vesicles and inhibit vesicle-motility even further. To test this we designed an assay to directly visualize synaptic-vesicle dispersion; based on previous observations that neuronal activity disperses synaptic-vesicles from boutons into flanking axons [25, 26]. Specifically, we asked if stabilized α-syn multimers (reconstituted VN/VC:α-syn's) would attenuate the activity-dependent dispersion of synaptic-vesicles (labeled with SyPhy:mRFP, see **fig. 2E**). Indeed while Venus:α-syn alone inhibited this dispersion (as expected), VN/VC:α-syn's attenuated this dispersion even further (**fig. 2F** and also see **fig. 4C** later). Collectively, these experiments suggest that α-syn multimers associate with synaptic-vesicle clusters and restrict their trafficking.

Biochemical analysis of α-synuclein multimers

To biochemically evaluate α-syn multimers, we transiently introduced VN/VC:α-syn's into HEK-293 cells or cultured neurons (by adenoviral infections) and analyzed cell-lysates by Native/SDS-PAGE gels (**fig. 3A**). As shown in the native gels (**fig. 3B**), only a few higher-order α-syn bands were typically seen. Though precise molecular weights cannot be determined by these methods, these bands run at ~ 146 kD (α-syn tetramers would be expected to run at ~ 114kD in our system – 4 α-syn's + 2 VFP's). These experiments were repeated several times with similar results, and notably, all three VN/VC combinations showed similar biochemical profiles (**Supp. fig. 3A**).

Though our above data (fig. 2) suggest that α-syn multimers associate with synaptic-vesicles, they do not directly show vesicle-binding. To address this, we evaluated the association of monomeric and multimeric α-syn with purified synaptic-vesicles. Based on previously published protocols [27, 28], we incubated α-syn-free synaptic-vesicles and cytosol with purified monomeric or multimeric α-syn (chemically cross-linked, see [15]; **fig. 3C** and **Supp. fig. 3B**). The main advantage of this assay is that brain cytosolic factors known to affect α-syn binding to membranes are available, recapitulating the in-vivo situation [27]. As shown in **figure 3D**, α-syn multimers are indeed capable of binding to synaptic-vesicles, though multimers are also present in cytosolic fractions. Note that these data are in general agreement with Dettmer et al., 2013.

A mechanistic link between α-syn multimerization and synaptic function

The above data suggest that α-syn multimers associate with and cluster synaptic-vesicles. As α-syn suppresses exo/endocytosis [1, 2], one can imagine a scenario where α-syn multimers

cluster synaptic-vesicles, thus restricting vesicle-recycling and consequently, neurotransmitter release. If correct, this model predicts that disrupting α-syn multimers would also diminish α-syn-induced vesicle clustering and abrogate α-syn-induced synaptic attenuation. Though molecular determinants of α-syn multimerization are unknown, we reasoned that the most striking feature of the α-syn molecule – N-terminal repeats – might play a role. The N-terminus of α-syn has seven 11-residue repeats that are predicted to fold into amphipathic alpha-helices, highly conserved among species [29-31]. Recent simulation-models also implicate these repeats in α-syn tetramerization [32]. We used a rationally-designed synthetic mutant, where six threonines (T) – centrally lying along the helical face of the N-terminus – are mutated to lysines (K; known as TsixK, see **fig. 4A** and [33]. These mutations are expected to disrupt significant portions of the extended hydrophobic face of the helix, and greatly diminish the helical conformation of α-syn [33]. Notably, this reduction in helicity occurs despite robust association with vesicles [33], and indeed both WT and TsixK protein bind synaptic-vesicles with equal affinity in our in-vitro assay (**Supp. fig. 3C**).

Accordingly, we tested the predictions of our model by comparing the ability of WT and TsixK mutants to: 1) organize into multimers, 2) cluster synaptic-vesicles, and 3) influence synaptic-vesicle recycling. First we transfected VN/VC pairs of WT α-syn's (VN/VC:WT) or TsixK α-syn's (VN/VC:TsixK) in cultured neurons from α-syn −/− mice as described previously. As shown in **fig. 4B**, fluorescence complementation in the VN/VC:TsixK α-syn's was markedly attenuated. Diminution of higher-order conformers was also seen biochemically (**Supp. fig. 3D**). Next we tested the ability of the TsixK mutant to suppress activity-induced synaptic-vesicle dispersion. As shown in **fig. 4C**, while WT α-syn attenuated synaptic-vesicle dispersion as expected, the TsixK mutant failed to do so. Finally, we asked if TsixK mutations also abrogated the ability of α-syn to attenuate synaptic-vesicle recycling. Towards this we used a pHluorin-based assay that directly reports synaptic-vesicle recycling ([34, 35]; see **fig. 4D**). While WT-α-syn attenuated recycling as reported previously [5], TsixK-α-syn only had a mild (non-significant) effect (**fig. 4E, F**).

Dynamic α-synuclein multimers at synapses

Collectively, the data support a model where synaptic α-syn is organized into metastable conformers that bind to and cluster synaptic-vesicles, restricting their trafficking. We posit that by influencing synaptic-vesicle trafficking, multimeric α-syn conformers restrict recycling, consequently attenuating neurotransmitter release. Using complementation-assays that stabilize putative protein-protein interactions, we found that near-physiologic levels of α-syn result in robust and widespread complementation at synapses (**fig. 1**). Given the transient transfection of proteins into an α-syn null background; the resultant low expression-levels (comparable to endogenous-levels, see **fig. 1D**); and the paradigms used to visualize initial entry of newly-synthesized α-syn into boutons (**fig. 1E-G**), it is unlikely that the complementation seen in our experiments is a result of over-expression. Moreover, data from three different combinations of VN/VC-fragments (tagged to α-syn's) are similar, untagged α-syn appears to compete with fluorescence complementation in HEK cells, and the TsixK α-syn mutant also fails to complement; collectively arguing that complementation is not due to vagaries of the Venus-fragments, but reflect *bona-fide* α-syn interactions.

Clustering synaptic-vesicles and regulating recycling/neurotransmitter release – a potential function of α-syn multimers

Do α-syn multimers have a physiologic role? Stabilized VN/VC:α-syn multimers were associated with synaptic-vesicles (**fig. 2A-D**), and also inhibited the trafficking of synaptic-vesicles (**fig. 2E, F**; and also **fig. 4C**). Our biochemical data also show that α-syn multimers can associate with synaptic vesicles (**fig. 3D**). Notably, the data do not rule out a role for cytosolic α-syn multimers. A recent study showed that purified α-syn protein clusters synthetic vesicles in an in-vitro lipid-binding assay [36]. Though in isolation, the relevance of these in-vitro findings to neurons and synapses is uncertain; in light of data shown here, the collective evidence advocate the concept that α-syn plays a physiologic role in clustering synaptic-vesicles. Supporting the idea that helical folding of α-syn is important for multimerization, Varkey et al. recently showed that incubation of α-syn with lipid-nanoparticles – known to induce helicity – increases intra-molecular FRET of α-syn [37]. However, another recent paper suggests that α-syn is exclusively involved in attenuating endocytosis [38]. Nevertheless, many studies indicate that α-syn influences the exocytic-cycle and SNARE-assemblies, and a more complete dissection of exo- v/s endo-cytosis is warranted. The exact mechanisms by which α-syn multimers restrict vesicle mobility are still unclear. One possibility is that α-syn multimers between adjacent vesicles associate with each other (an “interlocking model”), with perhaps α-syn/VAMP2 interactions also playing a role as suggested by Diao et al. [36] – an open question for future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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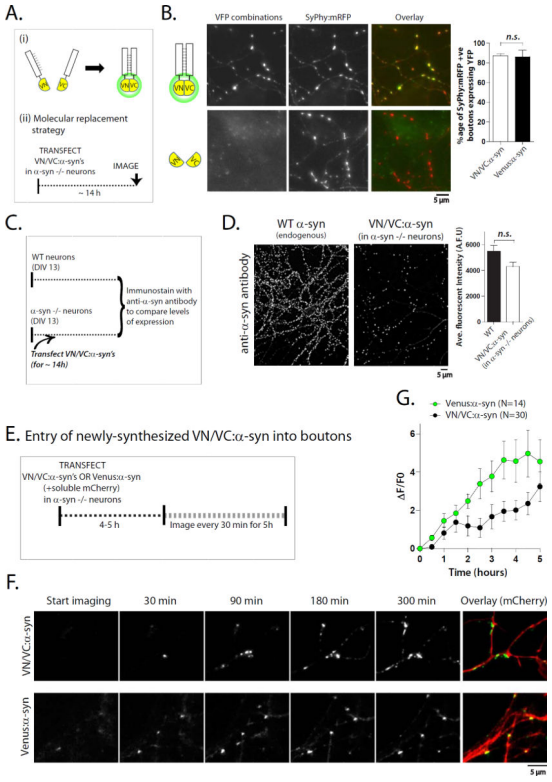

Figure 1. Multimeric α-syn conformations at presynaptic boutons

(A) Schematic of complementation assay **(i)** and molecular replacement strategy (**ii**). Cultured hippocampal neurons from α-syn −/− mice were transiently transfected with various VN/VC-tagged α-syn's (see “results”) and visualized after ~ 14 hours.

(B) Top: Representative images of reconstituted Venus fluorescence in neurons expressing VN/VC:α-syn's (also see Supp. fig. 1A). Note these neurons are co-transfected with synaptophysin:mRFP (SyPhy:mRFP) to label boutons. Bottom: No fluorescence was seen in boutons expressing un-tagged VN + VC alone. Right: The vast majority (~85%) of SyPhy:mRFP-positive boutons also expressed VN/VC:α-syn; comparable to boutons expressing Venus:α-syn and SyPhy:mRFP (N~700 boutons for each group from two separate batches of cultures, p=0.90).

(C) Overall design to compare expression-levels of transfected VN/VC:α-syn to endogenous mouse α-syn. Un-transfected cultured neurons from WT mice and VN/VC:α-syn-transfected cultured neurons from α-syn −/− mice were fixed and immunostained with an anti-α-syn antibody (guinea-pig α-syn antibody). Cell culture and immunostaining of both groups were processed in parallel. Note that while the antibody would recognize mouse α-syn in WT neurons, it would only label transfected α-syn in the VN/VC:α-syn transfected group.

(D) Representative images from the two groups in (C) (left) and quantification of overall average fluorescence intensities (right; N~10 visual fields containing ~ 3000-10,000 boutons; p=0.06). Note that the number of VN/VC:α-syn transfected boutons is much lower than immunostained WT boutons (as expected with transient transfections), but the fluorescence-intensities are similar.

(E) Overall design. Cultured α-syn −/− neurons were co-transfected with VN/VC:α-syn's (or Venus:α-syn) + soluble mCherry, and kinetics of initial α-syn entry and synaptic accumulation was evaluated by long-term imaging (see "results" and [21] for more details).
(F) Representative frames from two time-lapse movies showing pre-synaptic accumulation of VN/VC:α-syn (top) and Venus:α-syn (bottom) over 5 hrs of imaging.
(G) Quantification of average VFP intensities of boutons over 5 hrs. Note that though the kinetics of VN/VC:α-syn accumulation (black dots) is slower than Venus:α-syn (green dots) as expected, the difference is modest, suggesting that complementation is a relatively early event.

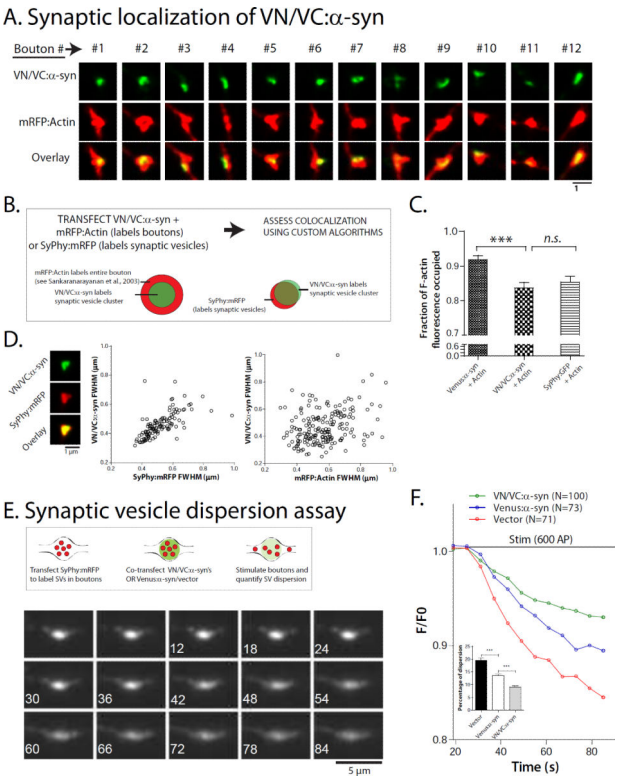

Figure 2. α-syn multimers cluster synaptic-vesicles

(A) Bouton-crops from neurons co-transfected with VN/VC:α-syn and mRFP:Actin (to label entire bouton-profile, see “results”). Note that reconstituted VN/VC:α-syn's only occupy a fraction of the bouton cross-sectional area.

(B) Experimental design: Neurons were co-transfected with VN/VC:α-syn and markers to label the entire bouton-profile (mRFP:Actin) or synaptic-vesicles (SyPhy:mRFP); and extent of overlap was determined by custom algorithms (see “results” and “methods” for details).

(C, D) Both reconstituted VN/VC:α-syn and SyPhy:GFP occupied a smaller fraction of the bouton than Venus:α-syn (~ 200 boutons analyzed for each group from two separate batches of cultures, ***$p < 0.001$). **(D)** Bouton-widths (FWHM, see methods) of VN/VC:α-syn and SyPhy:mRFP were correlated (left; $r=0.36$, $p<0.0001$), unlike VN/VC:α-syn and mRFP:Actin, further indicating associations of complemented VN/VC:α-syn's with synaptic-vesicles (N=120 boutons from two separate batches of cultures).

(E) Top: Schematic of “synaptic-vesicle dispersion assay”. Synaptic-vesicles are labeled by SyPhy:mRFP and neurons are stimulated to disperse synaptic-vesicles (see “results”). Bottom: A time-series showing dispersion of synaptic-vesicles from a bouton (elapsed time in seconds on lower left, asterisk marks the start of stimulation).

(F) Quantification of synaptic-vesicle dispersion using above assay. While Venus:α-syn diminishes dispersion-kinetics (compared to vector), the dispersion is further attenuated by VN/VC:α-syn (note that error bars are too small to be seen). Extent of dispersion quantified in inset (19.5%, 13.6% and 9% of total synaptic-vesicles were dispersed in vector, Venus:α-syn and VN/VC:α-syn groups respectively; ***$p < 0.001$, unpaired t test).

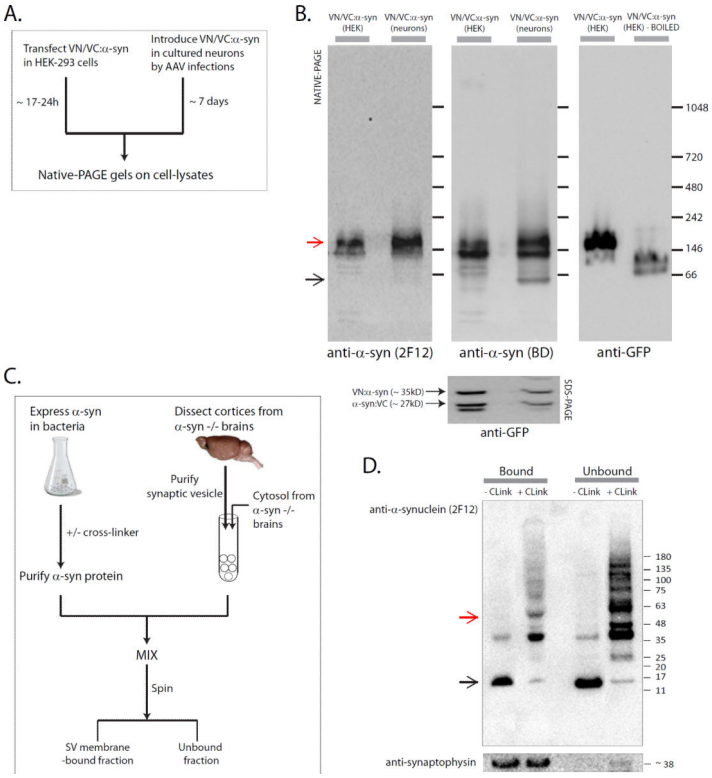

Figure 3. Biochemical analyses of α-syn multimers

(A) VN/VC:α-syn's were introduced into HEK293T cells or neurons (by viruses), expressed for the times indicated, and cell-lysates were analyzed by Native/SDS-PAGE.

(B) Native-PAGE show α-syn higher-order multimers immunoblotted with two α-syn antibodies and an anti-GFP antibody that also recognizes YFP (note disruption upon boiling). The red arrow marks the position where bands are typically seen, black arrow marks putative monomeric α-syn in neurons. An SDS-PAGE immunoblotted with anti-GFP marks the VFP-fragments. Each experiment was repeated 3-5 times with similar results.

(C) In-vitro reconstitution assay. Purified synaptic-vesicles and cytosol from α-syn −/− mouse brains were mixed with WT-α-syn purified from bacteria with/without a chemical cross linker (DSG). Vesicle membrane bound and unbound fractions were separated by centrifugation and analyzed by SDS-PAGE.

(D) Both monomeric and cross-linked α-syn multimers bound to synaptic-vesicles (a synaptophysin stain confirms that all synaptic-vesicles are in the bound fraction). Red and black arrows mark positions of putative tetramers and monomers. Experiment was repeated twice with similar results.

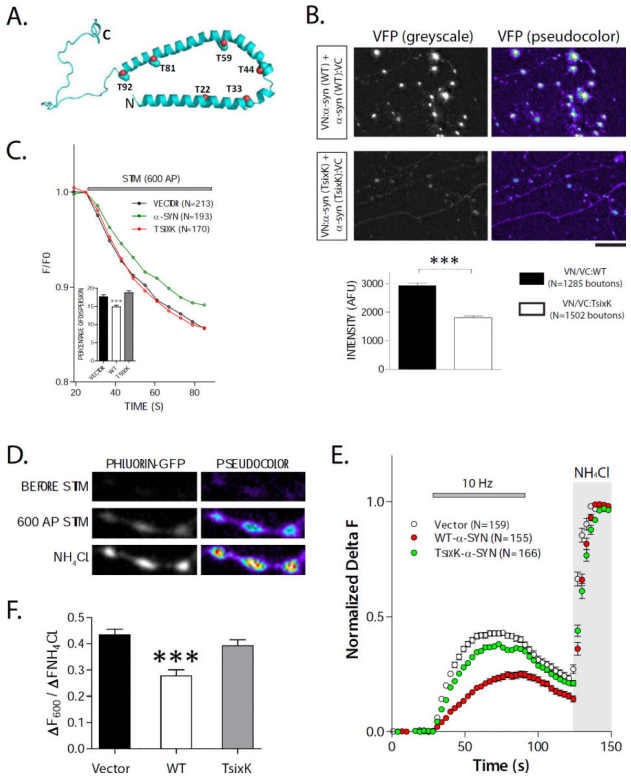

Figure 4. Mechanistic links between α-syn multimerization and synaptic function

(A) Schematic of the α-syn helices (shaded) and position of the six mutations.

(B) Neurons from α-syn −/− mice were transfected with VN/VC:WT or VN/VC:TsixK α-syn's and fluorescence was quantified in boutons. There were clear diminutions in the TsixK datasets as shown in the representative images and quantification below.

(C) "Synaptic-vesicle dispersion" assay: Neurons were co-transfected with SyPhy:mRFP (to label synaptic-vesicles) and untagged WT or TsixK α-syn (or vector alone). Boutons were stimulated and decay of RFP fluorescence from boutons was quantified (see "results"). Note that while WT α-syn attenuates activity-induced synaptic-vesicle dispersion, the TsixK mutant has no effect on vesicle-trafficking (N=number of boutons).

(D) Synaptic recycling evaluated by vGlut-pHluorin assays. Cultured neurons were co transfected with vGlut-pHluorin and either untagged WT α-syn or TsixK α-syn. Fluorescence-change of the pH-sensitive vGlut-pHluorin probe reflects synaptic-vesicle recycling in this assay (see "results" and "methods"). Representative panels show fluorescence intensity change of vGlut-pHluorin upon 600 AP stimulation and NH_4Cl perfusion. Note that NH_4Cl alkalinizes all vesicles, revealing the total (recycling + resting) pool in these neurons.

(E, F) Representative ensemble average of vGlut-pHluorin traces from empty vector, WT α-syn or TsixK α-syn transfected neurons (N=number of boutons). Note that while WT α-syn nattenuates neurotransmitter release and decreases mean recycling-pools compared to vector-controls, TsixK α-syn fails to show this effect; quantified in (**F**) (all data normalized to total pools). Recycling/total pool for vector=43±2.17 %; WT α-syn =28±2.38%; TsixK α-syn =39±2.29% (~ 160 boutons on 7-9 coverslips were analyzed for each group from three separate batches of cultures; ***$p < 0.001$ compared to vector by one-way ANOVA

followed by Dunnet's post hoc test). Total (alkalinized) pools of vector, WT-α-syn and TsixK-α-syn groups were 317.1 ± 16 AFU, 317.5 ± 11 AFU and 376 ± 18 AFU (mean ± SEM) respectively).