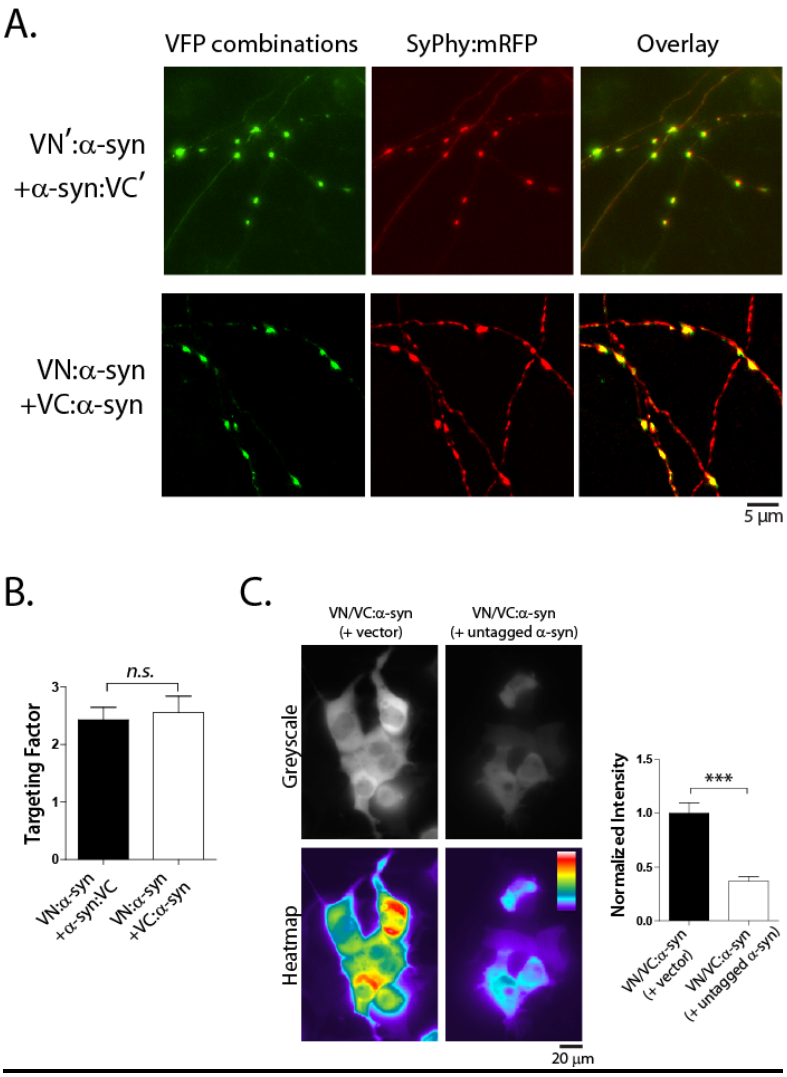
SUPPLEMENTARY FIGURES

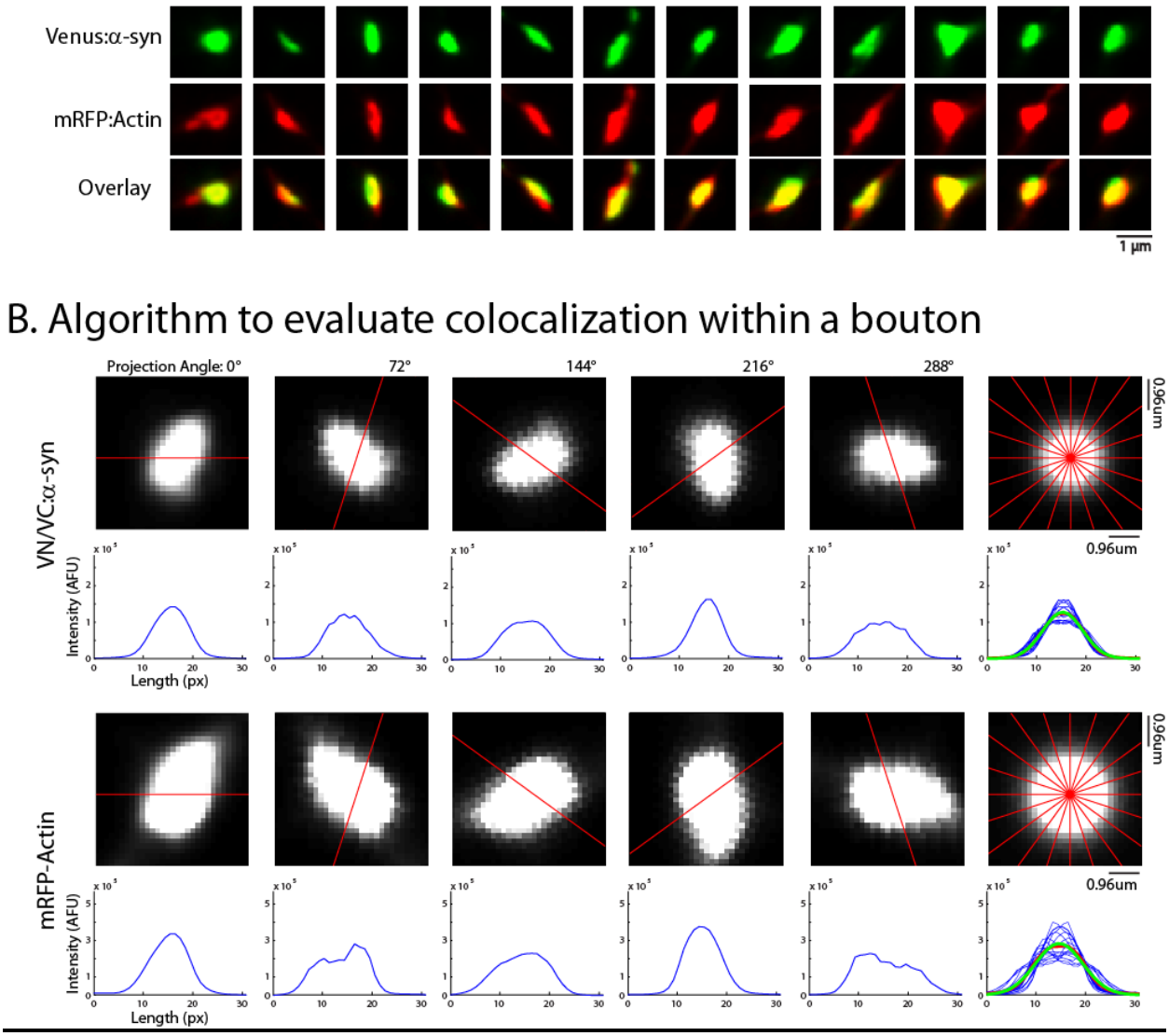


<u>Supplementary figure S1</u>: [Related to figure 1]

(A) Two other VN/VC combinations (marked VN', VC', see "methods" for sequence details) also show widespread and robust synaptic fluorescence. Cultured neurons (~ DIV-14) were co-transfected with VN/VC α-syn's and SyPhy:mRFP and imaged live; representative images are shown.

(B) Targeting factors (see "methods") of different VN/VC-tagged α-syn's were similar (p=0.77; unpaired t test).

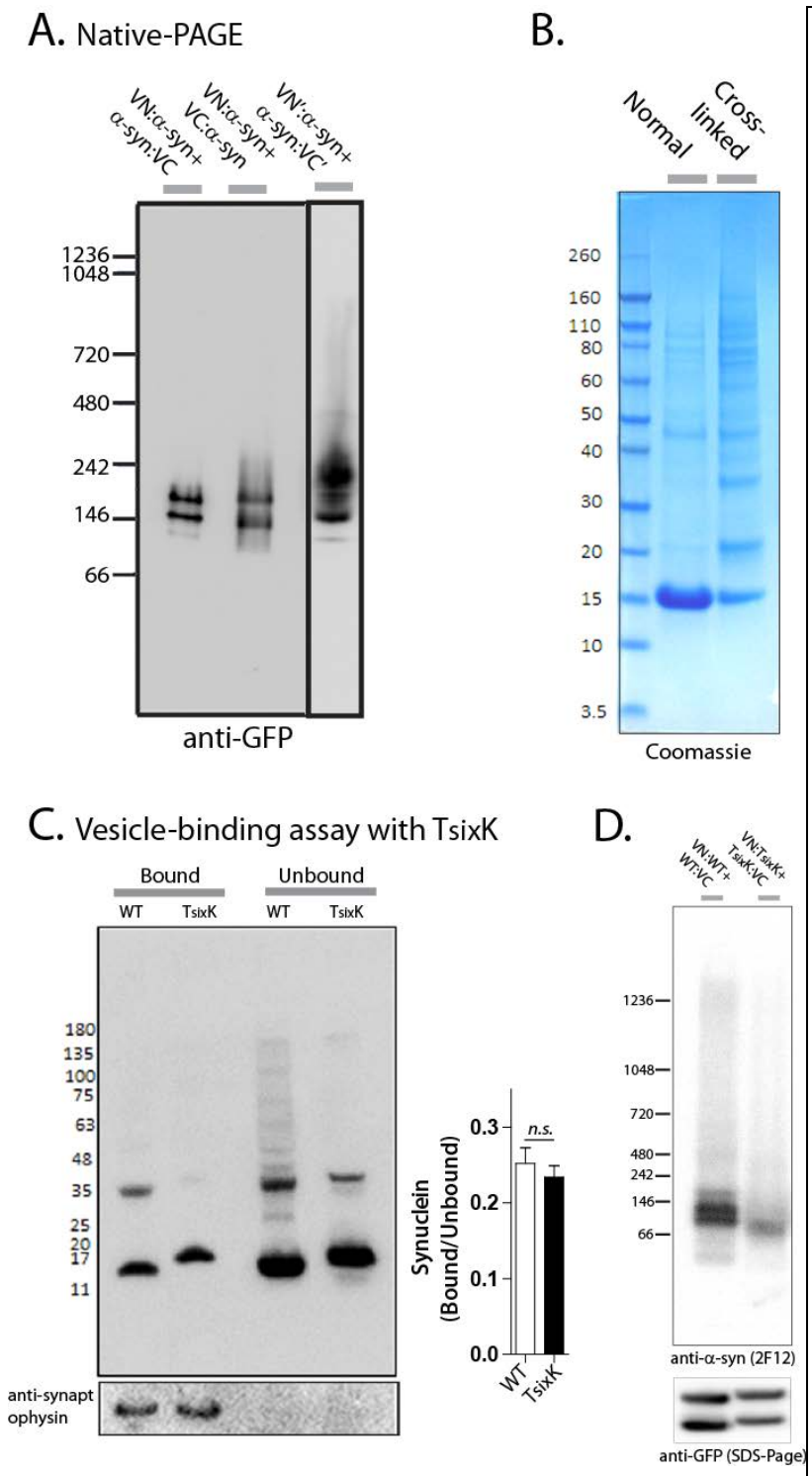
(C) HEK-293 cells were transfected with either VN/VC:α-syn's + empty vector, or VN/VC:α-syn's + untagged α-syn. Fluorescence-intensities in the latter group was visibly lower, quantified on right (N~ 50 cells analyzed for each group).



Supplementary figure S2: [Related to figure 2]

(A) Bouton-crops from neurons transfected with Venus:α-syn and mRFP:Actin (to label entire bouton). Note that the Venus α-syn fluorescence occupies the entire bouton-area, unlike VN/VC:α-syn's (compare with fig. 2A).

(B) Illustrative examples of custom algorithm to evaluate synapse-widths. Data from a single bouton expressing VN/VC:α-syn (top) and mRFP:Actin (bottom) is shown. For each bouton, the algorithm generated linescans at 20 circumferential angles around a weighted centroid (only 5 such angles are shown for clarity). For each linescan, linear fluorescence projections were obtained. Finally, mean of the 20 resultant line scans was approximated with a first order Gaussian, and the synaptic width (FWHM, see "methods") was calculated from these data. Frame on extreme right shows the synthetic data from the algorithms with the overlaid linescans.



Supplementary figure S3:
[Related to figure 3]

(A) Native-PAGE gels from all VN/VC:α-syn combinations are similar. HEK-293T cells were transfected with VN:α-syn + α-syn:VC; VN:α-syn + VC:α-α-syn, or VN':α-syn + α-syn:VC'. After ~ 24h, cells were lysed, run on a Native-PAGE gel and immunoblotted with an anti-GFP antibody.

(B) WT-α-syn expressed and purified from bacteria (Coomassie). A major ~ 15kD band is seen as expected, as well as a minor band corresponding to dimers (also see [S1]. Note increase in high molecular-weight bands upon cross-linking.)

(C) Purified WT or TsixK α-syn protein was incubated with synaptic vesicles/cytosol as described in the "results" and "methods". Note that relative amounts of protein bound to synaptic vesicles were similar in both groups.

(D) HEK293T cells were transfected with VN/VC:WT-α-syn or VN/VC:TsixK-α-syn and lysates were analyzed by Native-PAGE after ~ 24h. Note that the TsixK mutations attenuate higher-order multimers (GFP-staining of SDS-treated samples confirm equivalent loading). Experiments were repeated five times with similar results.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

DNA Constructs and antibodies

VN/VC constructs were as follows. 1) VN:α-syn + α-syn:VC: VN (1-158)-IDGGGGSGGGGSLK-α-syn and α-syn-LE-VC (159-239) [S2]; (2) VN':α-syn + α-syn:VC': VN (1-172)-QAS-α-syn and α-syn-PRARDPPVAT-VC (155-238) [3] and 3) VN:α-syn + VC:α-syn: VN (1-158)-IDGGGGSGGGGSLK-α-syn + VC (159-239)-IDGGGGSGGGGSLK-α-syn. The VN:TsixK-α-syn and TsixK-α-syn:VC were generated by replacing the WT-α-syn in the VN: α-syn and α-syn:VC with TsixK-α-syn. Constructs obtained from other laboratories are noted in acknowledgements. All constructs were verified by sequencing. A guinea-pig polyclonal antibody was used for immunostaining total (mouse + human) α-syn [S4, S5]. Antibodies used for immunoblotting were: BD-α-syn (Clone 42/a-syn, BD-Transduction Lab., 610786), 2F12 [S1], anti-GFP (polyclonal, Abcam, ab290), anti-tubulin (Clone DM1A, Sigma, 9026). All chemicals were from Sigma unless specified otherwise.

Hippocampal Cultures, transfection and viral transduction

Primary hippocampal cultures were obtained from P0-P1 mouse pups and transiently transfected with Lipofectamine 2000 (Invitrogen) as described previously [S5, S6]. All animal studies were performed in accordance with University of California guidelines. For vGLUT1-pHluorin and synaptic-vesicle dispersion experiments, neurons were electroporated with the respective constructs before plating using an Amaxa 4D-Nucleofector™ System (Lonza Inc., Walkersville, MD) with the P3 Primary Cell 4D-Nucleofector X Kit S (V4XP-3032) and program CL-133. The volume of the cell suspension was 20 µl per reaction and the cell density ranged from 1×10^7 to 1.5×10^7 cells/ml. Cells were plated at a density of 60,000 cells/cm^2 onto poly-D-lysine coated coverslips after electroporation and cultured to maturity (DIV14-DIV21) before imaging. For transduction of adeno-associated virus (AAV) constructs, cultured DIV-7 neurons (60,000 cells/cm^2) were transduced with AAV-VN: α-syn ($1.49\text{x}10^{13}$) and AAV-α-syn:VC ($1.94\text{x}10^{13}$) at multiplicity of infection (MOI) equals to $1.5\text{x}10^5$. The vast majority of neurons were infected after 7 days, when lysates were collected for biochemistry.

Native PAGE gels

Native-PAGE was performed using standard protocols. All steps were carried out on ice and detergent free environment to keep the native interactions intact. Briefly, neurons and HEK293 cells expressing VN/VC: α-syn were lysed in buffer containing 50 mM NaCl, 50 mM Tris-Cl, 5 mM EDTA, pH 8.0 and protease inhibitors. After 12000 xg centrifugation, the supernatant was collected and quantified using BCA kit. 20 µg of total protein was resolved on Bis-Tris native-PAGE (Invitrogen). After transferring, PVDF membranes were fixed in PFA [S7] followed by Western blot analysis.

Recombinant expression, chemical crosslinking and purification of human synuclein

Human WT and TsixK mutant synucleins were cloned in pET28b+ vector and expressed in *E. coli* BL21(DE3) using 1 mM IPTG. For crosslinking, 1 mM DSG was added to the cell suspension in PBS with protease inhibitors before lysis for 1 h at 37 °C. Protein purification was performed according to standard protocols using ammonium sulfate precipitation followed by Q-Sepharose anion exchange chromatography. Desalted purified monomers and the mutineers were used for various biochemical analyses.

HEK293T Cultures and Transfections

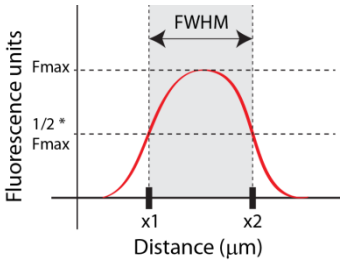
HEK293T cells (ATCC) were maintained in DMEM medium (Invitrogen) containing 10% FBS (Thermo Scientific, San Diego, CA) and passaged every 3-4 days. Cells were transfected with DNA of interest by electroporation using Amaxa 4D system with SF Cell Line 4D-Nucleofector X Kit L (V4XC-2024) and program CM-130. The volume of the cell suspension was 100µl per reaction and the cell density ranged from 1×10^7 to 3×10^7 cells/ml. Cells were cultured for 24 hours post transfection and cell lysates were used for Native-PAGE analysis.

Microscopy, live cell Imaging and immunofluorescence

For synaptic targeting experiments (figs. 1 and 2), neurons were imaged live (maintained at 37°C) on an inverted motorized epifluorescence microscope (Olympus, IX81) fitted with a CoolSNAP HQ^2 camera [S6]. The filter sets used for YFP and RFP imaging were set 49003 and 42005 (Chroma). The excitation wavelength maximums (Emax) were 535 nm for YFP (520-550 nm) and 600 nm for RFP (570-626 nm) respectively; and there was no spectral overlap in our imaging conditions (data not shown). Z-stack images were obtained as previously described [S5] and all images were acquired and processed with MetaMorph software and MATLAB as noted in the text. The synaptic-vesicle dispersion assay and the pHluorin assays were done at room temperature on an inverted motorized epifluorescence microscope (Nikon, Ti Eclipse, Garden City, NY) fitted with an exquisitely-sensitive QuantEM 512SC EMCCD camera (Photometrics, Tucson, AZ) and LED excitation (Lumencor LED, Spectra X). For the vGLUT1-pHluorin experiments, coverslips with cultured neurons were mounted into a Chamlide EC magnetic chamber (Live cell Instrument, ON, Canada), and the entire system was gravity-perfused with Tyrode solution (pH 7.4) containing (in mM): 119 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 25 HEPES, 30 glucose, 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, TOCRIS bioscience, Bristol, UK), and 50 µM D,L-2-amino-5-phosphonovaleric acid (AP5, TOCRIS bioscience). For field-stimulation, 10 V pulses were applied at 10 H_Z for 60 seconds using an SD9-square-pulse stimulator (Grass Instruments, Middleton, WI). Incident excitation (Lumencor LED, Spectra X) was attenuated 10-fold and images were acquired with 500 ms exposures at either three second intervals for three minutes (vGLUT1-pHluorin) or six second intervals for 84 seconds (vesicle-dispersion assay).

Image Analysis

Synaptic co-localization algorithms: Registered Z-stack images were deconvolved (Huygens), max-projected and aligned as described previously [S5]. Regions of interest (ROIs) were placed around each bouton puncta, and further analyses were done in MATLAB. The puncta were then digitally-rotated around the weighted centroid at 20 discrete intervals of theta = 2π/20 (rad), and linear fluorescence projections were taken at each interval-angle. The mean of the 20 resulting line scans was approximated with a first order Gaussian, and the full-width half-max (FWHM) was calculated as $|2\sigma|\sqrt{\log(4)}$, where σ is the standard deviation of the Gaussian function (also see Supp. fig. 2B). FWHM is a quantitative measure of synapse-widths in our experiments [S8]. Illustrated by the figure on right, it is the width of the shaded region between points in an intensity-curve where the amplitude has dropped to half (1/2 * Fmax).



Quantification of vGLUT1-pHluorin and synaptic-vesicle dispersion assays. ROIs were placed on each bouton and average intensities were obtained for each frame within the time lapse. For vGLUT1-pHluorin experiments, F_{max} was defined as the average fluorescence of the maximal five frames after NH_4Cl perfusion. Baseline F_0 was defined as the average fluorescence of the initial 10 frames before stimulation [(F_0 = average (F_1:F_{10})]. Fluorescence intensity of a bouton at a given time point (F) was normalized to F_0 and F_{max} and expressed as $(F-F_0)/(F_{max}-F_0)$. For dispersion assays, fluorescence of a bouton at a given time point (F) was normalized to the average fluorescence of the initial five frames [(F_0 = average (F_1:F_5)] and expressed as F/F_0. Percentage of dispersion was expressed as $(F_{max} - F_{min})/F_0$ for each bouton.

Quantification of synaptic targeting factor. Targeting factor was calculated as described previously [S9], with some modifications. Briefly, the GFP-tagged protein of interest and soluble DsRed were co-transfected and fluorescence intensities were measured along lines drawn perpendicularly to the axons and across the boutons. Peak fluorescence along boutons and the adjacent axon were determined by fitting Gaussian functions to the resultant intensity profiles and corrected for background. Targeting factor was expressed as $(Bouton_{Green}/Axon_{Green})/(Bouton_{Red}/Axon_{Red}) - 1$.

In-vitro synaptic vesicle binding assay

Synaptic vesicle and cytosol from alpha-synuclein null mice cortices were isolated according to [S10]. Briefly, brain cortices were homogenized in buffer A (320 mM sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.4) with protease inhibitors. After sequential centrifugation at 1000 xg and 13,300 xg for 10 min, crude synaptosomes were loaded onto a 5%-9%-13% Ficoll gradient prepared in buffer A and centrifuged at 35,000 xg for 35 min. Interface between 9%-13% was washed with buffer B (140 mM NaCl, 5 mM KCl, 20 mM HEPES, 5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM glucose). Resultant pellet was incubated on ice for 10 min in buffer C (10 mM HEPES, 18 mM KOAc, pH 7.2) and centrifuged at 24000 xg to pellet down the

synaptic vesicles. Vesicle pellet was washed with buffer D (25 mM HEPES, 125 mM KOAc, 2.5 mM $MgCl_2$) and stored at -80 till use. To isolate cytosol, cortices were processed in buffer containing 85 mM sucrose, 100 mM KOAc, 1 mM MgOAc and 20 mM HEPES, pH 7.4 and subjected to sequential centrifugation at 15, 000 xg and 100,000 xg. Resultant cytosol was dialyzed in buffer containing 145 mM KOAc and 25 mM HEPES, pH 7.2 and used for the vesicle binding assay. 30 ug purified protein was incubated with purified synaptic vesicles and KO-cytosol for 30 min at 37°C. Content was centrifuged at 24,000 xg for 10 min to separate bound and unbound fractions. After two rinses with buffer D samples were used for gel analysis and western blot.

Statistical Analysis

Statistical analyses were performed using Prism software (GraphPad, La Jolla, CA). Nonparametric Student's t-test was used for comparing two groups, and one-way ANOVA for multiple groups. Results are expressed as mean±SEM. A p value <0.05 was considered significant.

Supplementary References

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