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Supplementary Information for

α-Synuclein oligomers induce early axonal dysfunction in human iPSC-based models of synucleinopathies

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Supplementary Information Text

SI Material and methods

Vectors

All vectors used are listed in Table S1.

Cells and cell culture

Human iPSC from a PD patient carrying α-Syn Dupl were kindly provided by Douglas Galasko, UCSD (clones SDi1-R-C3 [Dupl1] and SDi1-R-C11 [Dupl2]). A female PD patient with a disease onset at the age of 58 years had a progressive disease course characterized by muscle cramping, tremor and has developed dementia. The α-Syn gene duplication was confirmed in PD Dupl iPSC cells using Multiplex Ligation-dependent Probe Amplification analysis (P051/P052 probemixes, MRC-Holland), which revealed α-Syn gene dosage increase of 3 copies corresponding to a heterozygous duplication. iPSC from two healthy Caucasian individuals with no history of neurologic disease served as controls (Ctrl1.1, clone UKERi33Q-R1-016; and Ctrl1.2, clone UKERi33Q-R1-06; and Ctrl2.1, clone UKERi1E4-R1-016, previously described in (1)). iPSCs were reprogrammed from fibroblasts, differentiated into NPC and, finally, into neuronal cells as described previously (1). One or two NPC lines were generated from each iPSC clone. The results of Ctrl NPC lines were pooled in axonal transport measurements.

Primary human cerebellar astrocytes (HA-c, ScienCell Research Laboratories) were cultured as described by the manufacturer. Co-cultures of NPC with human primary astrocytes were performed at the ratio of 2:1 (NPC:astrocytes) in the presence of 0.5% Fetal bovine serum (FBS). Lentiviral infection was performed 2 days post-seeding at the Multiplicity of infection (MOI) 1. The MOI 1 was chosen based on the absence of significant toxicity in human neuronal cells at the resulting level of α-Syn expression (2). NPT100-18A was added to neuronal cultures, where indicated, at 10 µM during the whole differentiation time. DMSO served as a diluent control in all NPT100-18A experiments. NPT100-18A was kindly provided by Wolfgang Wrasidlo (UCSD) and Eliezer Masliah (NIH).

All experiments with human iPSC-derived cells were reviewed and approved by the Institutional Ethics Review Board (Nr. 4120: *Generierung von humanen neuronalen Modellen bei neurodegenerativen Erkrankungen*) and were carried out in accordance with the declaration of Helsinki. Written informed consents were received from the participants prior to inclusion in the study at the movement disorder clinics at the Department of Molecular Neurology, Universitätsklinikum Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg (Erlangen, Germany) and at the Department of Neurosciences, UCSD (San Diego, CA, USA).

Immunocytochemistry (ICC)

Cells were fixed with 4% paraformaldehyde (PFA) 20 min at 37°C, washed three times with phosphate-buffered saline (PBS; Invitrogen) and permeabilized/blocked by incubating in ICC blocking solution (0.3% Triton X-100, 3% donkey serum [both from Sigma-Aldrich] in PBS) 2 hours (hr) at room temperature (RT). Primary antibodies (Abs) (see Table S2) were incubated in the ICC blocking solution overnight at 4°C. Fluorescently labelled secondary Abs (all from Thermo Fisher Scientific) were incubated in the ICC blocking solution 1 hr at RT. Cell nuclei were stained with 1 µg/ml 4',6-

diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Coverslips with stained cells were mounted on glass microscope slides (Thermo Fisher Scientific) in Aqua Polymount (Polysciences). Images were acquired using a fluorescence microscope Axio Observer.Z1 and a confocal microscope LSM 780 (both from Carl Zeiss). Images were evaluated using ImageJ 1.47f (NIH) and ZEN (Carl Zeiss) software.

Western blot (WB) of neuronal cells

Neuronal cells (20 days of differentiation) were homogenized in a detergent-free lysis buffer (50mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA und protease inhibitor cocktail [all from Roche]) in a Potter dounce homogenizer on ice. Protein content was determined using a BCA assay (Thermo Fisher Scientific). 30 µg total protein of the homogenate were mixed with Laemmli-SDS buffer and separated in a 12% SDS-Polyacrylamide gel electrophoresis (PAGE) gels. Wet-transfer onto nitrocellulose membranes was performed using a Mini Trans-Blot system (BioRad). To control protein loading, a total protein staining with ponceau was performed. Primary Abs against α-Syn and Tubb3 (see Table S2) were incubated overnight at 4°C in WB blocking solution (5% Blotting-Grade Blocker [BioRad] in TBS/0.1% Tween-20). Horseradish peroxidase-conjugated secondary Abs were incubated in WB blocking solution for 1 hr at RT. Blots were developed digitally by using Luminol based chemiluminescent solutions (ECL blotting solution, GE Healthcare Life Sciences) and Fuji LAS-1000 Luminescent Image Analyzer (Fujifilm). Signal intensities were generated by the Image Lab 5.0 software (Biorad).

Microfluidic chamber cultures and time-lapse video imaging

To analyze axonal transport, neurons were cultured in microfluidic chambers (SND450, Xona Microfluidics) for 20 days. Microfluidic chambers were assembled according to the manufacturer's instructions. 80000 NPC and 10000 astrocytes were seeded on the soma side, and 20000 astrocytes on the axonal side. Cells in the soma side were lentivirally infected 2 days post-seeding. Axonal transport of Mito-DsRed-positive mitochondria was documented by live-cell time-lapse imaging, when the axons had projected through the 450 µm-long microgrooves reaching the axonal side. Live-cell imaging was performed on axons in the microgroove area at stable temperature and balanced CO_2 conditions using a Nikon Eclipse Ti inverted microscope (Nikon) equipped with an EMCCD camera (model DU-885, Andor Scientific Cameras) and a NIS-Elements AR 3.2 software (Nikon). For live-cell imaging, the culture media was replaced by a live-cell imaging buffer (10 mM Hepes, 144 mM NaCl, 2.5 mM KCl, 10 mM Glucose, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$) with an osmolarity equal to the osmolarity of the culture media (310 mmol/kg). Time-lapse recording duration was 10 minutes (min) with one snap per second (s).

Axonal transport evaluation

Time-lapse recordings were analyzed by creating kymographs for single recorded neurite by plotting mitochondria positions at each single time point over a total recording time (600 s) within the pictured neurite length (190 µm) using ImageJ 1.47f and Multiple Kymograph plugin. Total mitochondria numbers per axon length were calculated using kymographs. Mitochondria transport parameters (speed and transport direction) were obtained from single tracks of moving events on the kymographs and the number of

moving versus stationary events per axon were calculated. Mean velocities were calculated considering the speeds of all moving mitochondria in the respective neuronal line. Finally, moving mitochondria were ranked according to their velocities to estimate velocity frequencies. At least 20 axons were analyzed and three to four microfluidic chambers were performed per neuronal line. The thresholds for transport events were considered at a minimum displacement length of ≥ 5 µm and if mitochondria were tractable for a minimum of 3 min (1/3 of a total imaging length) as described earlier (1).
The percentages of delta values of movement incidence and directionality changes after application of NPT100-18A were calculated as (NPT100-18A – DMSO)/NPT100-18A*100%. All experiments with the application of NPT100-18A were performed with 2 independent iPSC clones from Ctrl1.

α-Syn solubility assay

α-Syn aggregation was evaluated by assessing α-Syn solubility in a sequential extraction as previously described with minor modifications (3). Briefly, differentiated neurons were homogenized in a detergent-free lysis buffer (see WB section) in a Potter dounce homogenizer on ice. The lysates were centrifuged at 100000 g for 1 hr at 4°C and the supernatants were collected as soluble fractions (S1). Pellets were incubated with Tris-buffered saline (TBS: 50mM Tris pH 7.4, 150 mM NaCl) containing 1% Triton X-100 and, after centrifugation (100000 g, 1 hr, 4°C), the Triton X100-soluble fractions (S2) were collected. The Triton X-100-insoluble pellets were re-suspended with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 175 mM NaCl, 5 mM EDTA, 1% NP-40, and 0.5% sodium deoxycholate) containing 1% SDS. After selection of RIPA-soluble S3 fractions as supernatants after centrifugation (100000 g, 1 hr, 4°C), the RIPA-insoluble pellets were solubilized in 8 M urea/ 5% SDS (P3). α-Syn content within different fractions was analyzed by WB using an anti-α-Syn Ab (see Table S2). Intensity of bands corresponding to α-Syn monomer and oligomers were generated by the Image Lab 5.0 software (Biorad). α-Syn in each fraction was calculated by summing up the intensities of monomeric (by ~17 kDa) and oligomeric bands (higher molecular species). Solubility of α-Syn in different cells was evaluated as a proportion of α-Syn in each fraction within total α-Syn pool. Three independent experiments were performed. For the evaluation of α-Syn aggregation in α-Syn mutant neurons after NPT100-18A treatment, S1 (soluble) and S2 (insoluble) fractions were analysed.

Sucrose density gradient centrifugation (SDGC) and size exclusion chromatography (SEC)

SDGC and α-Syn detection were performed as described previously (4). 150 µg protein of cell lysates were applied.
SEC was performed as described in (5) with modifications. Briefly, neuronal cells were homogenized in the detergent-free buffer as described above for WB and the solubility testing. 30 µg pre-formed α-Syn oligomers (derived by nitration of recombinant α-Syn and used to calibrate the SEC column (5)) and cell lysates with 50-250 µg total protein were centrifuged at 100000 g for 1 hr at 4°C prior to loading onto a Yarra SEC 3000 column (Phenomenex). The SEC was performed by using 30 mM Tris/HCl pH 7.4, with 0.2 M NaCl as an eluent at a flow rate of 0.5 ml/min and by monitoring the Ultraviolet (UV) absorbance at 280 nm. A total of 25 fractions were collected with 1 ml per fraction

(fractions 1-5) and 0.5 ml per faction (fractions 6-25). For detection of α-Syn, collected fractions were applied to a nitrocellulose membrane using a Minifold Dot-Blot System (Schleicher & Schuell) and probed with an anti-α-Syn Ab (Table S2). The signals were visualized with the SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific). The size of α-Syn oligomers in neuronal cells was estimated according to retention times of pre-formed oligomers containing monomers, dimers, trimers, tetramers, and higher molecular weight oligomers. For quantification, intensity of signals corresponding to different α-Syn species was normalized to the intensity of monomeric α-Syn in each cell lysate. Three independent experiments were performed.

Transmission electron microscopy (EM)

For ultrastructural analyses, neuronal cells were differentiated on plastic coverslips for five weeks coated with Matrigel (BD Biosciences). Transmission electron microscopy was performed as previously described (6). Ultrathin horizontal sections were stained with uranyl acetate and lead citrate and were examined with a transmission electron microscope EM 906E (Carl Zeiss NTS).

ATP measurements

Neurons were differentiated for 20 days in white flat-bottom 96-well plates and ATP was measured using CellTiter-Glo Luminescent Cell Viability Assay (Promega). In parallel, neurons were differentiated under the same conditions in transparent flat-bottom 96-well plates for viability testing using Image-iT DEAD Green Viability Stain (Thermo Fisher Scientific). ATP and viability measurements were performed according to manufactures' instructions. Luminescence was measured at the Centro LB 960 (Berthold Technologies). Cells stained with iT DEAD Green marker were counterstained with DAPI to quantify viable cells. Relative ATP amount per cell was estimated by normalization of ATP signal to the frequency of viable cells. Three independent experiments in triplicates were performed.

Patch clamp measurements

Whole-cell patch clamp recordings were performed in neuronal cultures (differentiated for five weeks) at RT using an EPC 10 amplifier (HEKA electronics) in artificial cerebrospinal fluid bubbled with 95% O_2 and 5% CO_2 (125 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 10 mM d-Glucose; pH 7.4). The pipette solution contained 4 mM NaCl, 135 mM K-Gluconate, 3 mM $MgCl_2$, 5 mM EGTA, 5 mM HEPES, 2 mM Na_2-ATP and 0.3 mM Na_3-GTP (pH 7.25).

Synaptosomal preparation and characterization

Neurons were differentiated in co-culture with astrocytes for five weeks. Synaptosomes were isolated as described elsewhere (7). Briefly, cells were washed twice with ice-cold PBS, detached in 400 µl/well buffered sucrose (0.32 M sucrose, 4 mM Hepes, pH 7.5 containing protease inhibitor cocktail) by gentle scraping, and homogenized in a 1 ml glass homogenizer on ice. Total cell homogenates were centrifuged at 1200 g 10 min at 4°C to remove cell debris. The supernatants were centrifuged at 12600 g 20 min at 4°C to result in pelleted synaptosomal fractions and cytosolic fractions (supernatants). Synaptosomal pellets were resuspended in 40 µl buffered sucrose. One part of

synaptosomes from control neuronal cultures were fixed with 4% PFA on coverslips and in suspension and tested by immunostaining and fluorescence-activated cell sorting (FACS), respectively, for the presence of synapsin I and PSD95. Immunostaining was performed as described above for ICC using anti-synapsin I and anti-PSD95 Abs (see Table S2). DAPI was used to control for the presence of nuclei in synaptosomal fractions. For FACS analysis, fixed synaptosomes were permeabilized with FACS-Saponin (0.1% Saponin [Sigma-Aldrich] in FACS-PBS [2% FBS, 0.01% NaN3 in PBS]) and blocked with 8% donkey serum in FACS-Saponin on ice. 1 µg of each anti-synapsin I and anti-PSD95 Abs (see Table S2) were incubated in FACS-Saponin for 30 min at 4°C followed by incubation with fluorescently labelled secondary Abs for 30 min at 4°C. Finally, synaptosomes were washed with FACS-Saponin and resuspended in FACS-PBS for analysis. At least 30000 events were analyzed on a BD FACSCalibur (BD Biosciences). FACS data were evaluated using FlowJo 8.5.3 software. Triton X-100 was added to the synaptosomal and cytosolic fractions to a final concentration 1%, and samples were stored at -80°C until usage. Synaptosomal fractions were further analysed by WB (primary Abs are listed in Table S2). Since individual synaptosomal preparations (six independent experiments) resulted in different total protein yield per sample, equal amount of protein from each sample (mock, WTS, E46, and E57K synaptosomal fractions) within an individual synaptosomal preparation was loaded. From one preparation, 3 µg protein and from five preparations, 15 µg protein of each sample could be loaded. Blots were developed using ECL blotting solution (GE Healthcare Life Sciences) and Fuji LAS-1000 Luminescent Image Analyzer (Fujifilm). Signal intensities were evaluated using ImageJ 1.47f software. Internal normalization of densitometry signals of each sample was performed against total loaded protein amount. To enable a comparison between individual experiments, internally normalized values of α-Syn over-expressing neuronal cultures were normalized to control neurons (Mock).

Statistical analysis

Data were analyzed either by two-tailed Fisher's exact test (all data from Ctrl vs Dupl neurons), and Chi-square test (mitochondria motility data with application of NPT100-18A, subcellular distribution of Miro1), or by two-way (α-Syn solubility and oligomerization analyses, and mitochondrial velocity evaluations, SNPH and KLC1 within neurites), or one-way Analysis of variance (ANOVA; all other data) followed by Dunnett's or Bonferroni's multiple comparison test. All statistical analyses were performed using the GraphPad Prism 5 software (GraphPad Software). Differences were considered statistically significant at p-value (p) ≤ 0.05.

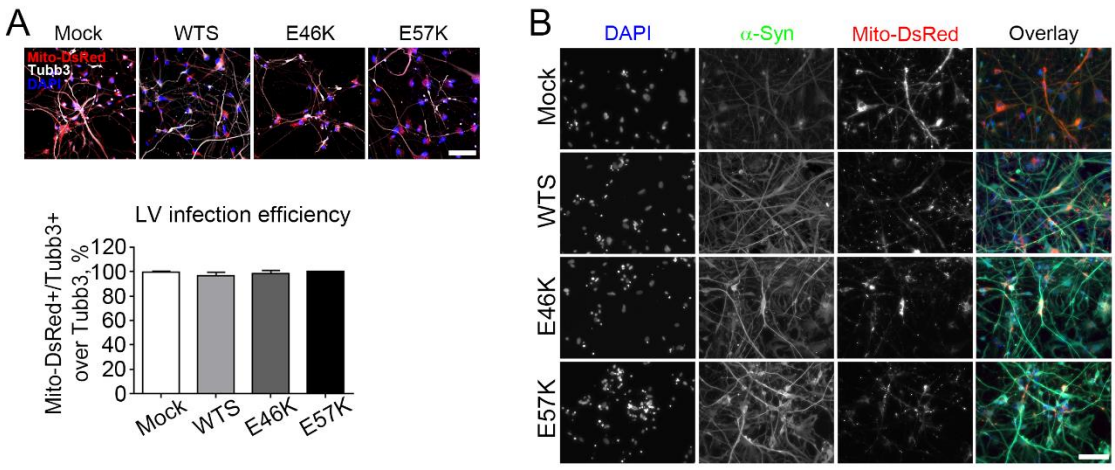

Fig. S1. Lentiviral-induced over-expression of α-Syn in human iPSC-derived neurons. (A) Lentiviral (LV) efficiency was evaluated in 20 day-differentiated iPSC-derived neurons (differentiation time used for axonal transport measurements) by the percentage of Mito-DsRed/β3-tubulin (Tubb3) double-positive neurons within total Tubb3-positive neurons (upper panel). Similarly high LV infection efficiency (97 - 100%) was found in all conditions: Mock, WTS, E46K, and E57K (lower panel). Data are presented as mean ± SD. **(B)** Similar levels of α-Syn over-expression in control iPSC-derived neurons LV infected with WTS, E46K, and E57K using an anti-α-Syn Ab LB509 (green). Mitochondria were visualized by Mito-DsRed (red). Mock neurons were infected with LV construct encoding only Mito-DsRed. Scale bars 100 μm.

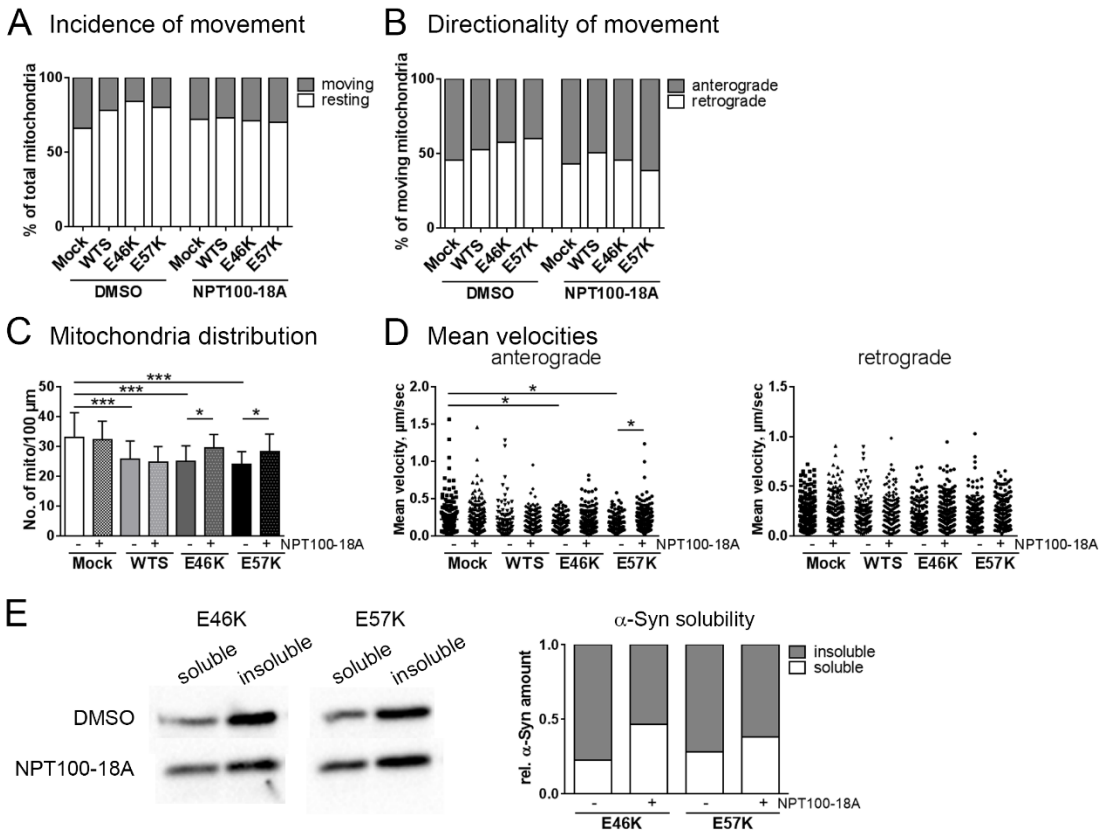

Fig. S2. Reduced mitochondrial distribution and mean anterograde velocities could be rescued by NPT100-18A in α-Syn mutant neurons. (A) Impaired mitochondrial movement incidence in WTS, E46K, and E57K neurons (DMSO) were restored in all α-Syn over-expressing neurons by application of NPT100-18A (NPT100-18A). DMSO was used as a diluent control in NPT100-18A experiments. **(B)** Reduced frequencies of anterograde mitochondrial transport were detected in neurons over-expressing oligomerizing α-Syn mutants compared to Mock (DMSO). Accordingly, NPT100-18A rescued impaired incidence of anterograde transport to a control level in α-Syn mutant neurons, but did not change the transport directionality in WTS neurons (NPT100-18A). **(C)** Less mitochondria per 100 µm axonal length were determined in WTS, E46K and E57K neurons compared to Mock. Application of NPT100-18A restored mitochondrial distribution in α-Syn mutant but not WTS neurons. **(D)** Significantly reduced mean anterograde velocities were measured in E46K and E57K neurons compared to Mock (left panel). NPT100-18A increased mean anterograde velocity only in E57K neurons. No differences in retrograde mean velocities were detected in any α-Syn over-expressing neurons independently on the addition of NPT100-18A (right panel). * - $p \leq 0.05$; ** - $p \leq 0.01$; *** - $p \leq 0.001$. Data are presented as mean ± SD in **(C)**. Data of 2 independent iPSC clones from Ctrl1 are shown together. **(E)** Reduced insoluble α-Syn levels (indicative of reduced α-Syn aggregation) were detected in neurons over-expressing oligomer-prone α-Syn mutants (E46K and E57K) after NPT100-18A treatment. Representative WB signals (left panel) and quantifications of soluble *vs.* insoluble α-Syn levels (right panel). Total α-Syn levels (soluble+insoluble) were set as 1.

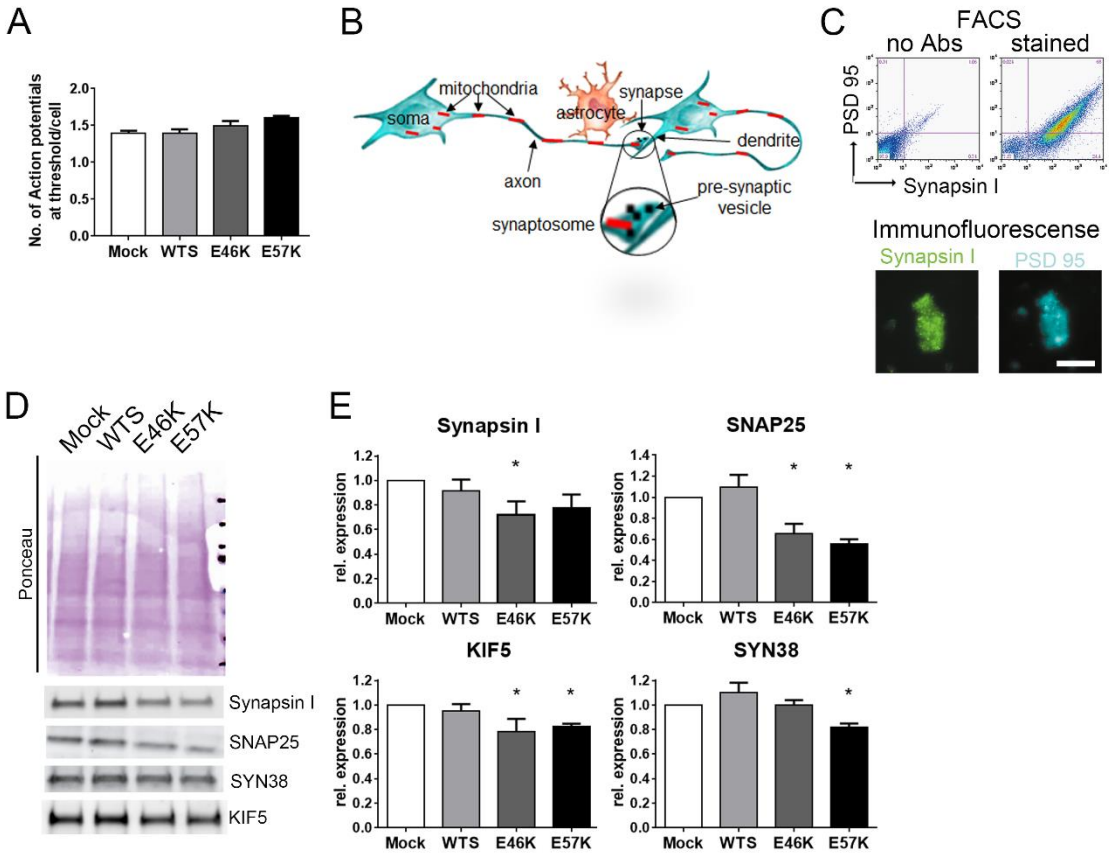

Fig. S3. Alterations of the synaptic compartment in α-Syn over-expressing neurons. (A) Whole-cell patch clamp analysis showed similar firing behaviour of the neurons with regard to evoked action potentials as presented by mean numbers of action potentials at threshold per neuron. **(B)** Synaptosomes consist of pre- and post-synaptic compartments of the neuronal cultures. **(C)** Flow cytometric (FACS) and immunofluorescent characterization of isolated synaptosomes positively stained for synapsin I and PSD95. Lower panel shows a synaptosomal cluster. Scale bar 20 µm. Representative example of synaptosomes from control neuronal culture is shown. **(D)** Representative signals and **(E)** quantifications shown as mean ± SEM of a WB analysis reveal reduced amounts of pre-synaptic proteins (synapsin I, SNAP25, SYN38) and kinesin 1 (KIF5) in synaptic compartment of α-Syn mutant neurons (E46K and E57K). Ponceau staining was used to control for an equal protein loading. * - $p \leq 0.05$.

Table S1. Vectors*

Vector name	Application	Source
pEF1a-MCS_IRES_Mito-DsRed	Control vector for NPC infection	cloning
pEF1a-WTS_IRES_Mito-DsRed	WTS encoding vector for NPC infection	cloning
pEF1a-E57K_IRES_Mito-DsRed	E57K encoding vector for NPC infection	cloning
pEF1a-E46K_IRES_Mito-DsRed	E46K encoding vector for NPC infection	cloning
pEF1a-MCS_IRES_GFP	Cloning: exchange of GFP for Mito-DsRed (enzymes XhoI, BspHI, XbaI)	Gift from F.H. Gage, Salk Institute, La Jolla, USA
pEF1a-WTS_IRES_GFP	Cloning: exchange of GFP for Mito-DsRed (enzymes XhoI, BspHI, XbaI)	own laboratory
pEF1a-E57K_IRES_GFP	Cloning: exchange of GFP for Mito-DsRed (enzymes XhoI, BspHI, XbaI)	own laboratory
pCAG-MCS_IRES_Mito-DsRed	Cloning of Mito-DsRed into pEF1a-MCS/WTS/E57K_IRES_GFP (enzymes XhoI, BspHI, XbaI)	Gift from D.C.Lie, Institute of Biochemistry, FAU Erlangen, Germany
pCMV-E46K_IRES_GFP	Cloning of E46K into pEF1a-WTS_IRES_Mito-DsRed instead of WTS (enzymes XhoI, NotI, BamHI)	own laboratory
pCMV-VSVG	Lentiviral packaging	Gift from F.H. Gage
pCMV-MDL	Lentiviral packaging	Gift from F.H. Gage
pCMV-RevRSV	Lentiviral packaging	Gift from F.H. Gage

*MCS – multiple cloning site; GFP – green fluorescence protein; pEF1a – human elongation factor-1 alpha promoter; pCAG - chicken beta actin promoter; pCMV – human cytomegalovirus promoter; NPC – neural precursor cells; WTS – wild type α-Syn.

Table S2. Primary Antibodies*

Specificity	Clone	Host	Dilution	Application	Company
α-Syn	LB509	mouse	1:1000	WB of neuronal lysates	Abcam
α-Syn	LB509	mouse	1:100	ICC	Abcam
α-Syn	Syn1	mouse	1:1000	WB and dot blot in α-Syn solubility, SDGC and SEC analyses	Transduction Laboratories
Tubb3 Tubb3	Tuj1 Tuj1	mouse rabbit	1:500 1:500	WB and ICC ICC	BioLegend Covance
Synapsin I	poly	rabbit	1:1000 1:500 1 µg	WB of synaptosomes; ICC FACS	Synaptic Systems
SNAP25	poly	rabbit	1:10000	WB of synaptosomes	Synaptic Systems
SYN38	SVP-38	mouse	1:500	WB of synaptosomes	Sigma-Aldrich
KIF5	poly	rabbit	1:500	WB of synaptosomes	Sigma-Aldrich
Miro1	CL1083	mouse	1:250	ICC	Abcam
SNPH	poly	rabbit	1:50	ICC	Thermo Scientific
KLC1	poly	rabbit	1:100	ICC	Santa Cruz
Tau	poly	rabbit	1:100	ICC	Dako
pTau	AT8	mouse	1:200	ICC	Thermo Scientific
PSD95	poly	goat	1:500 1 µg	ICC FACS	Abcam

*WB – western blot; ICC – immunocytochemistry; SDGC - sucrose density gradient centrifugation; SEC – size exclusion chromatography; α-synuclein – α-Syn; Tubb3 – β3 tubulin; SNAP25 - synaptosomal-associated protein 25; SYN38 – synaptophysin; KIF5 – kinesin 1; TOMM20 - Translocase Of Outer Mitochondrial Membrane 20; Miro1 - Mitochondrial Rho GTPase 1; SNPH - Syntaphilin; KLC1 - Kinesin Light Chain 1; pTau - phosphorylated Tau; PSD95 - post-synaptic density 95.

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