Expanded View Figures

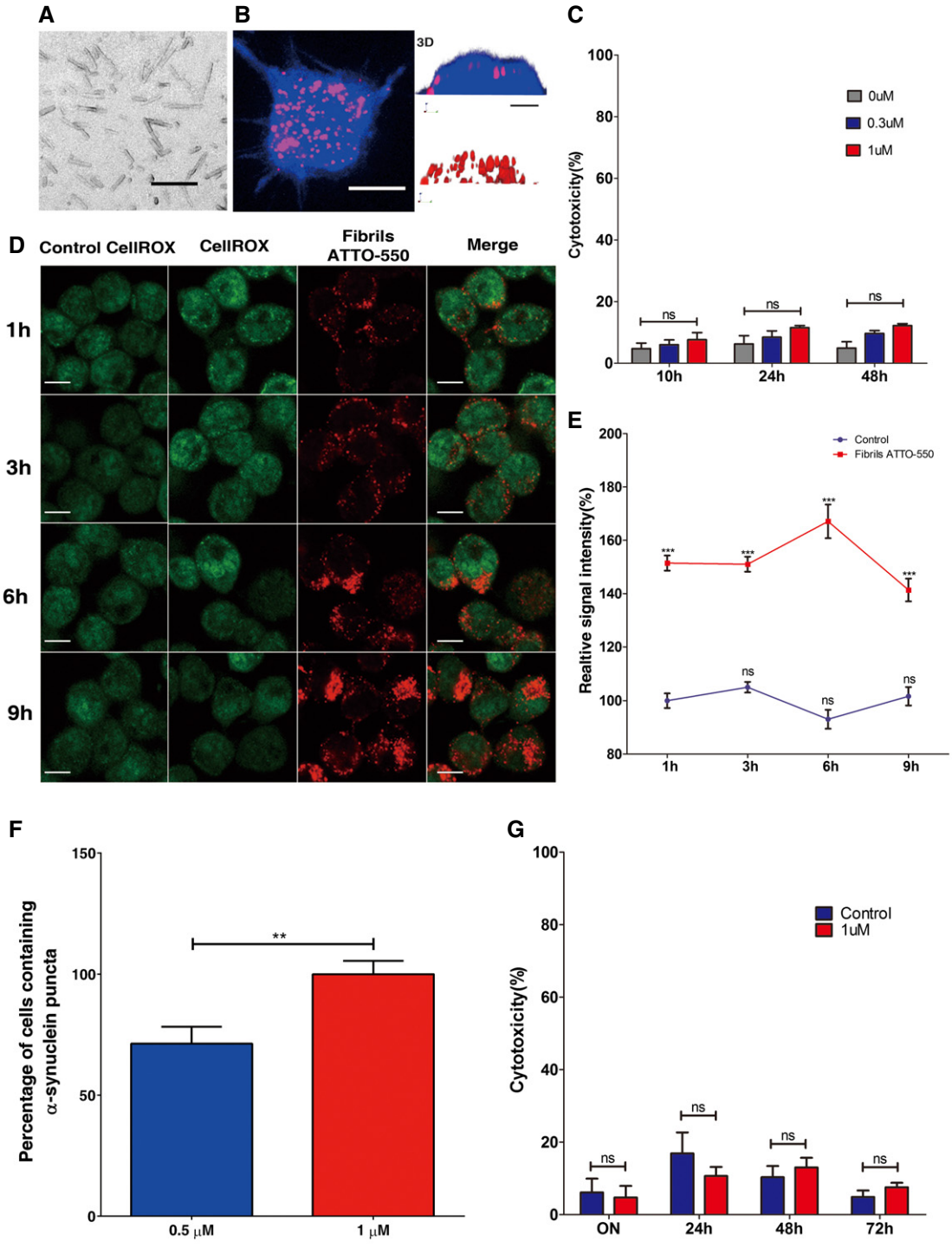

Figure EV1.

Figure EV1. α-synuclein fibril characterization and internalization in neuron-like cells and cortical primary neurons.

A Electron micrographs of α-synuclein fibrils used throughout this study. The scale bar represents 100 nm. Fibrillar α-synuclein was adsorbed to a carbon-coated copper grid and stained with freshly prepared 1% uranyl acetate. Samples were imaged using a JEOL 1400 electron microscope equipped with an LaB6 filament and operated at 80 kV, and 10,000× magnification, images were recorded with a Gatan Orius CCD camera (Gatan).

B The left panel shows representative Z-stack projection of confocal images showing neuron-like CAD cells after loading with α-synuclein fibrils for 16 h. Cells were then trypsin-washed, fixed and labelled with HCS CellMask Blue; scale bar is 10 μm. The right panel depicts the (z, y) three-dimensional reconstruction (3D) of a cell loaded with fibrils shown in the confocal image (left panel); scale bar is 5 μm. As seen on the three-dimensional images, α-synuclein fibrils are present only in the cytosol and not at the plasma membrane.

C Cell toxicity was measured by LDH release in neuron-like cells after 10, 24 and 48 h of loading increasing concentrations of sonicated α-synuclein fibrils. The bar graph represents the percentage of cytotoxicity normalized to control values. There were no significant differences between control and α-synuclein-loaded cells at any of the time points or concentrations evaluated. Ns, not significant by two-way Student's *t*-test.

D Representative images showing intracellular ROS levels in neuron-like cells upon addition of 1 μM of α-synuclein fibrils for up to 9 h. Intracellular ROS was measured by CellRox Green fluorescence in control and α-synuclein-loaded cells (red). Scale bars are 10 μm.

E The graph shows the percentage of the relative fluorescence intensity of intracellular ROS at different time points in α-synuclein-loaded CAD cells and controls. The values show ~50% increase in ROS production at all time points. ***$P < 0.001$ compared to the control condition. Ns, not significant by two-way Student's *t*-test.

F Quantification of the percentage of primary cortical neurons containing α-synuclein puncta after 16 h of incubation with 0.5 μM (blue bar) and 1 μM (red bar) of α-synuclein fibrils. **$P < 0.001$ by two-way Student's *t*-test.

G Cell toxicity was measured by LDH release in primary neurons on addition of 1 μM of sonicated α-synuclein fibrils for 16, 24, 48 and 72 h. The bar graph represents the percentage of cytotoxicity normalized to control values. There were no significant differences between control (blue bar) and α-synuclein-loaded (red bar) cells at any of the time points evaluated. Ns, not significant by two-way Student's *t*-test.

Data information: Data in (C and E–G) represent the mean ± s.e.m. of three independent experiments. Whereas at least 100 cells were scored for each experiment of internalization and ROS, values for toxicity experiments are derived from triplicates of three different experiments.

Figure EV2. Schematic of the experimental design of co-culture experiments.

A Experimental set-up used for the co-culture experiment (also referred to as a transfer experiment). CAD neuron-like cells are loaded for 16 h with human fluorescent ATTO-550 α-synuclein fibrils. Cells are trypsin-washed and are used as "Donor cells" since their cytosol is loaded with α-synuclein fibrils. Donor cells are mixed with GFP-transfected cells referred to as "Acceptor cells" for 24 h. Then, the co-culture is fixed and imaged and (i) the percentage of cells containing ATTO-550 α-synuclein fibrils and (ii) the average number and size of ATTO-550 α-synuclein fibrils per cells are quantified using ICY software.

B Experimental set-up used for the conditioned medium experiment. This experiment allows investigating the contribution of secretion to cell-to-cell α-synuclein fibril transfer. Here, donor cells are obtained as described in (A) (i.e. loading followed by trypsin wash) and then cultured for 24 h. The medium of donor cells referred to as conditioned medium (CM) is entirely collected and used as is to culture GFP-transfected acceptor cells for 24 h. The same analysis described in (A) is performed (i.e. percentage of cells containing α-synuclein fibrils, number and size of α-synuclein fibrils per cells) but also quantitative analysis of the amount of fibrils within donor cells and the culture medium by filter trapping on cellulose acetate membranes.

C Experimental set-up used for the filter experiment. This set-up was designed to separate donor and acceptor cells to investigate the contribution of (i) secretion or/and (ii) cell contact to transfer. The co-culture is performed similarly as described in (A) with the exception that donor cells are plated in the well, and then a transwell filter is placed on top of which acceptor cells are plated. After 24-h co-culture, the same analysis is performed (see A).

D Experimental set-up used for the seeding experiment. Here, donor cells loaded with α-synuclein fibrils Alexa-488 (and trypsin-washed as described in A) were co-cultured with acceptor cells overexpressing ChFP-α-synuclein for 72 h. The number of ChFP-α-synuclein fibril puncta as well as the co-localization rate between α-synuclein fibrils Alexa-488 and ChFP α-synuclein fibril puncta was quantified.

E Schematic of the experimental design of exogenous α-synuclein fibril internalization in co-cultured cells. Donor cells previously loaded with ATTO-550 α-synuclein fibrils were co-cultured with untransfected acceptor cells for 24 h. After 12 h of co-culture, cells were challenged with α-synuclein fibrils Alexa-488 (i.e. exogenously added α-synuclein fibrils) for an additional 12 h.

F Schematic of the experimental design of α-synuclein fibril internalization and transfer between primary neurons. Donor neurons pre-loaded with ATTO-550 α-synuclein fibrils were co-cultured with CTG-labelled acceptor neurons for 72 h. Acceptor neurons were prepared from a different dissection and labelled in suspension before adding them on top of the donor neurons. After 72 h, the cells are fixed and imaged and (i) the percentage of cells containing α-synuclein puncta and (ii) the number and average size of α-synuclein puncta per cell are quantified using ICY software.

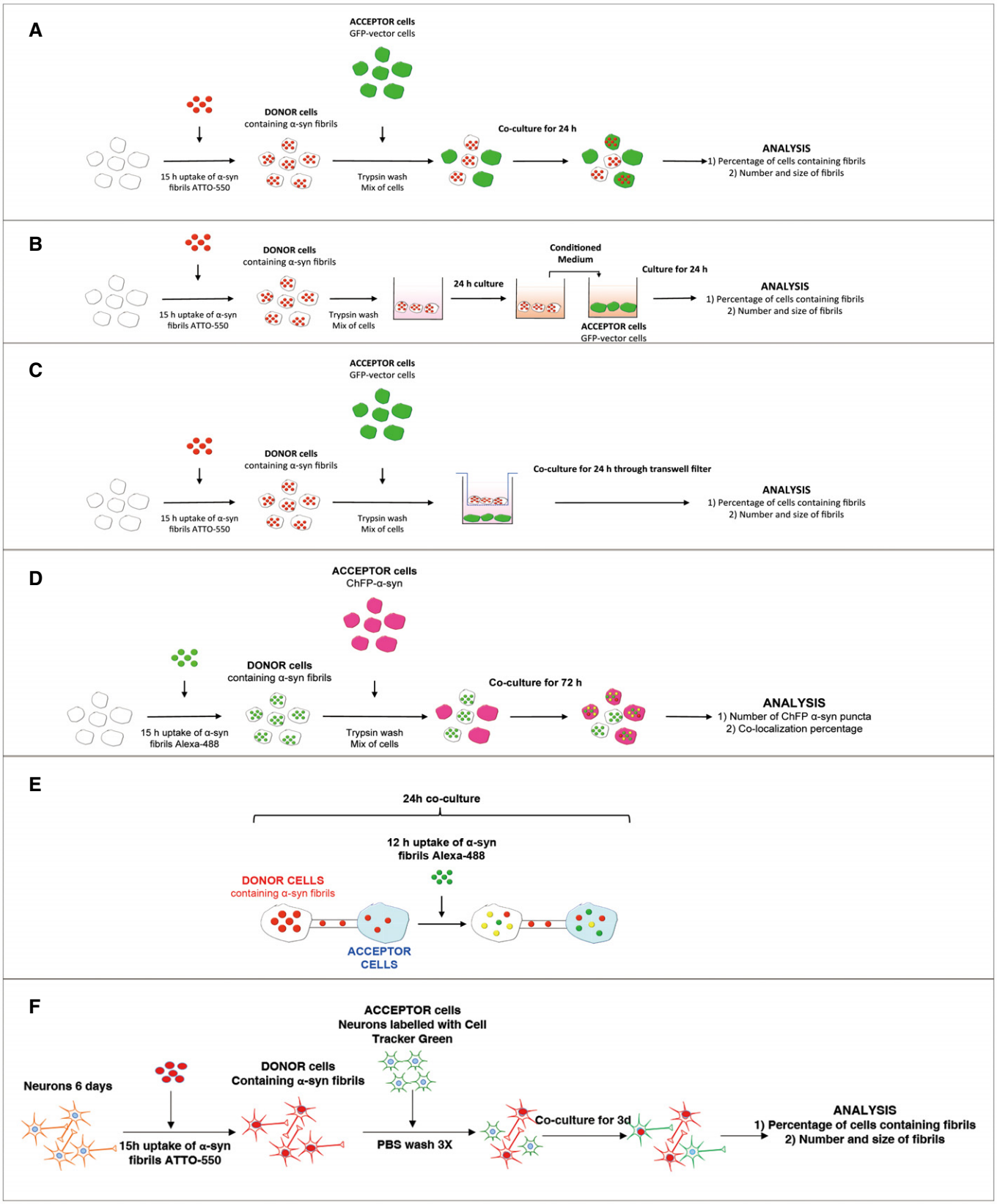

Figure EV2.

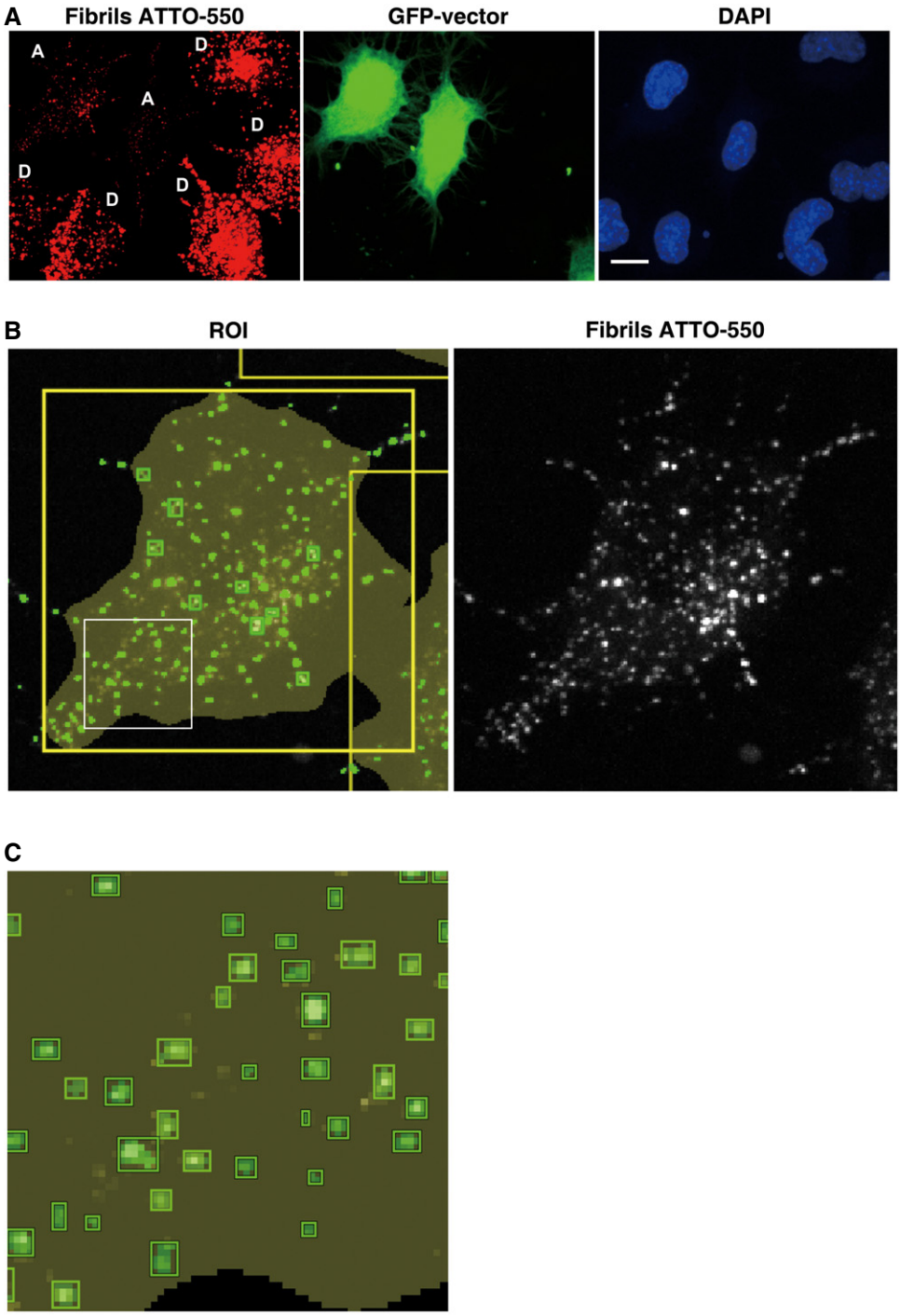

Figure EV3. α-synuclein fibril detection in acceptor cells.

A Representative confocal picture of donor cells loaded with α-synuclein fibrils (D, in red), co-cultured with GFP vector-transfected acceptor cells (A, in green) and stained with DAPI (in blue). Scale bar represents 10 μm.

B, C Representative confocal picture of an acceptor cell from (A) (top left cell) showing cell segmentation (large yellow ROI) and detection of α-synuclein fibrils in small green square ROIs (left panel). The green square ROIs change in size according to the size of the fibrils. Thereby the large fibrils are seen in green squares and the smaller fibrils appear in small green spots which when the magnification is increased resolve into squares as seen in the inset in (C). Note in (B) the correlation between α-synuclein fibrils (in white, right panel) and the spots detected by the software (in green, left panel).

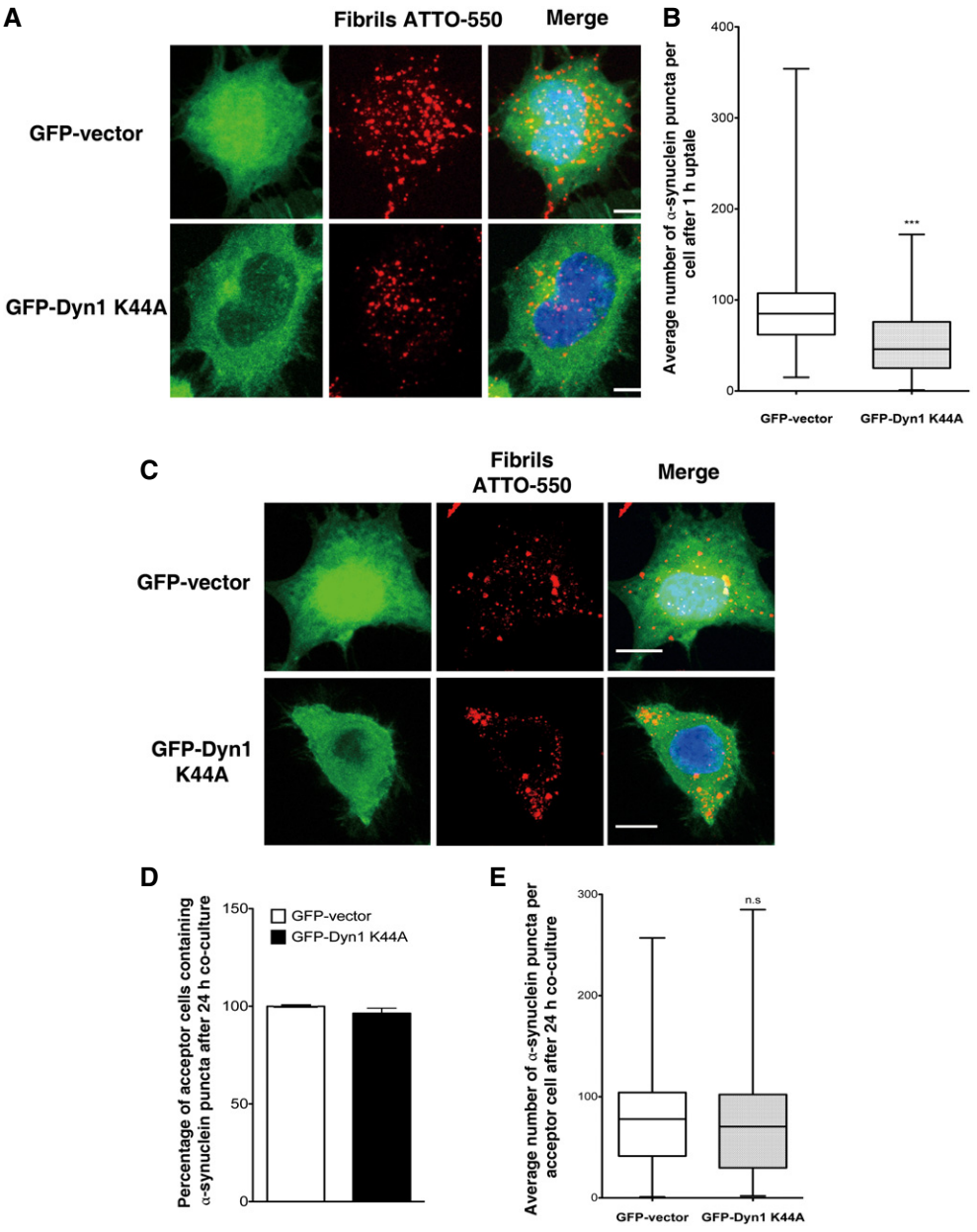

Figure EV4. α-synuclein fibrils are partially internalized in a dynamin-dependent manner.

A Representative images of GFP vector (upper panel) or GFP-dynamin-1 K44A-transfected cells (bottom panel) challenged for 1 h with ATTO-550 α-synuclein fibrils, trypsin-washed and then fixed: in red, α-synuclein fibrils; in green, GFP vector or GFP-dynamin-1 K44A-transfected cells; and in blue, nuclei. Scale bars represent 5 μm. $n = 3$ independent experiments.

B Quantification of the average number of α-synuclein fibrils per cell obtained from experiments in (A) shows a significant decrease in the number of α-synuclein fibrils in dynamin dominant-negative cells (GFP-Dyn1 K44A) compared to control cells (GFP vector) indicating a partial dynamin-dependent mechanism of α-synuclein fibril uptake in neuron-like cells (***$P < 0.001$ by two-tailed Mann–Whitney U-test). $n = 3$ independent experiments.

C Representative images of GFP vector (upper panel) or GFP-dynamin-1 K44A-transfected acceptor cells (bottom panel) after 24-h co-culture with donor cells loaded with α-synuclein fibrils: in red, α-synuclein fibrils; in green, GFP vector or GFP-dynamin-1 K44A-transfected acceptor cell; and in blue, nuclei. Scale bars represent 10 μm. $n = 3$ independent experiments.

D Relative percentage of acceptor cells containing α-synuclein fibrils after co-culture as in (C) revealed that transfer of α-synuclein fibrils does not depend on dynamin-1. Data are shown as mean ± s.e.m. (n.s. with GFP vector raw percentage set to 100%, not significant by Student's t-test).

E Quantification of the average number of α-synuclein fibrils per acceptor cells after co-culture as in (C) showing no significant difference in the number of α-synuclein fibrils in acceptor cells in control (GFP vector) and dynamin dominant-negative (GFP-Dyn1 K44A) conditions. Data are shown as mean ± s.e.m. of three independent experiments (n.s., not significant by two-tailed Mann–Whitney U-test).

Data information: In the box and whisker plots shown in (B and E), boxes extend from the first to the third quartile, the line inside the boxes shows the median and the whiskers represent the min/max value of at least 100 cells scored for each experiment.

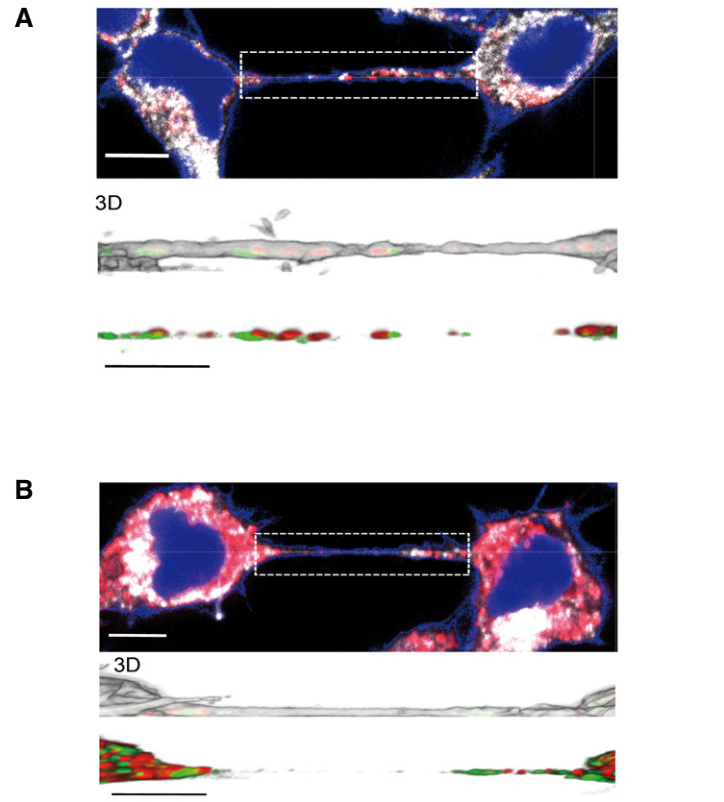

Figure EV5. α-synuclein fibrils are not found in endosomal vesicles inside TNTs and do not co-localize with lysosomal vesicles of acceptor cells after transfer.

A, B Confocal images showing a TNT connecting two cells loaded with red ATTO-550-labelled α-synuclein fibrils immunostained with EEA1 (A) or Vamp3 (B) (in white) and stained with WGA Alexa-488 (in blue). The images represent a Z-projection of the several middle stacks where TNTs are located. Insets show 3D reconstructions of TNTs using ICY software: red, ATTO-550 fibrils; green, EEA1 (A) or Vamp3 (B); white, WGA Alexa-488. Several α-synuclein fibrils are present in the lumen of the TNT but do not co-localize with EEA1 (A)- or Vamp3 (B)-positive vesicles. Scale bars represent 10 μm (confocal images) and 3 μm (insets).

C Representative confocal images of GFP-Rab7-transfected acceptor cells after co-culture (with donor cells containing ATTO-550 α-synuclein fibrils) show no co-localization between transferred α-synuclein fibrils (in red) and GFP-Rab7-positive vesicles (in green). Scale bar represents 10 μm, and the nucleus is in blue (n = 3 independent experiments).