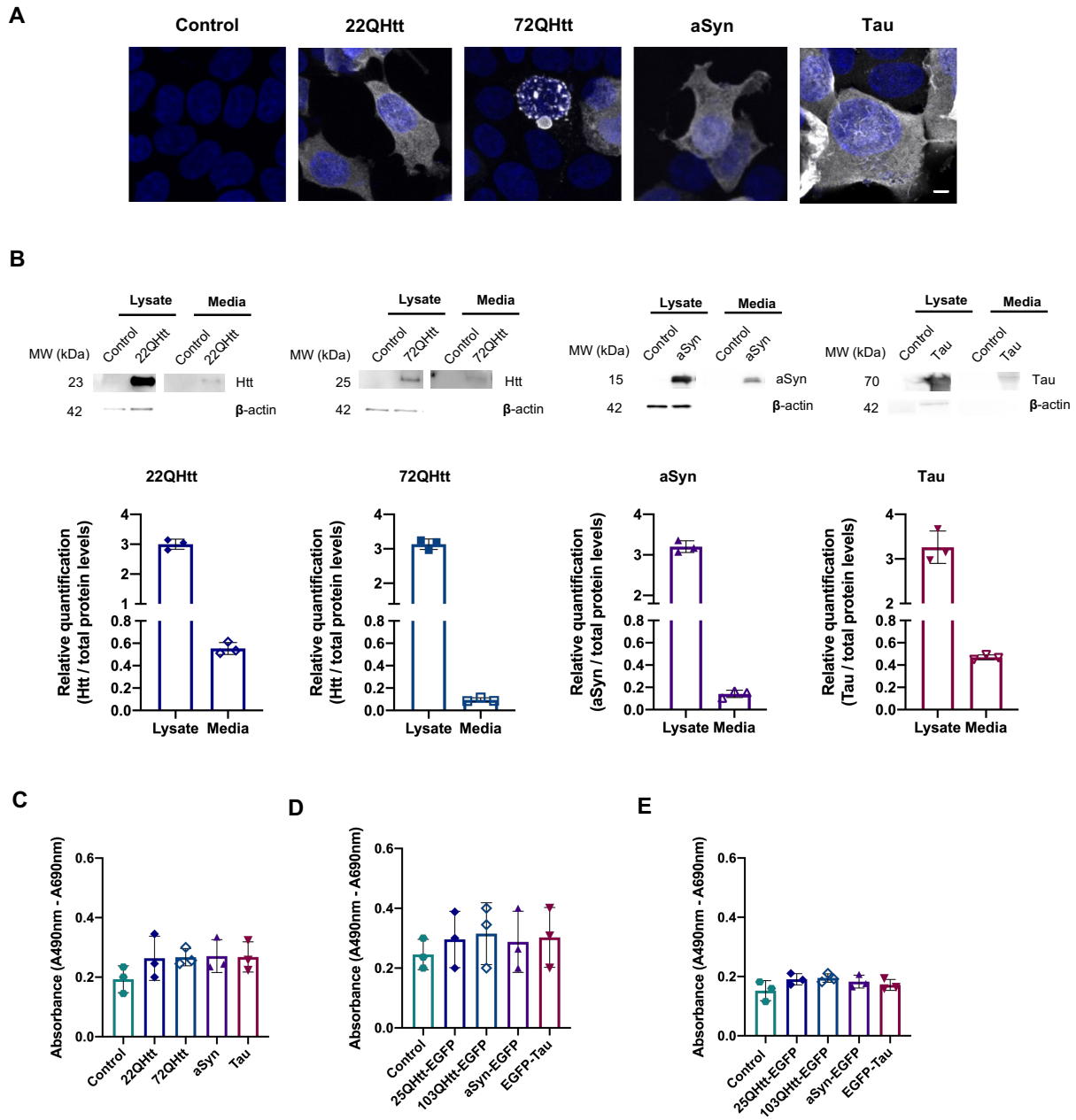
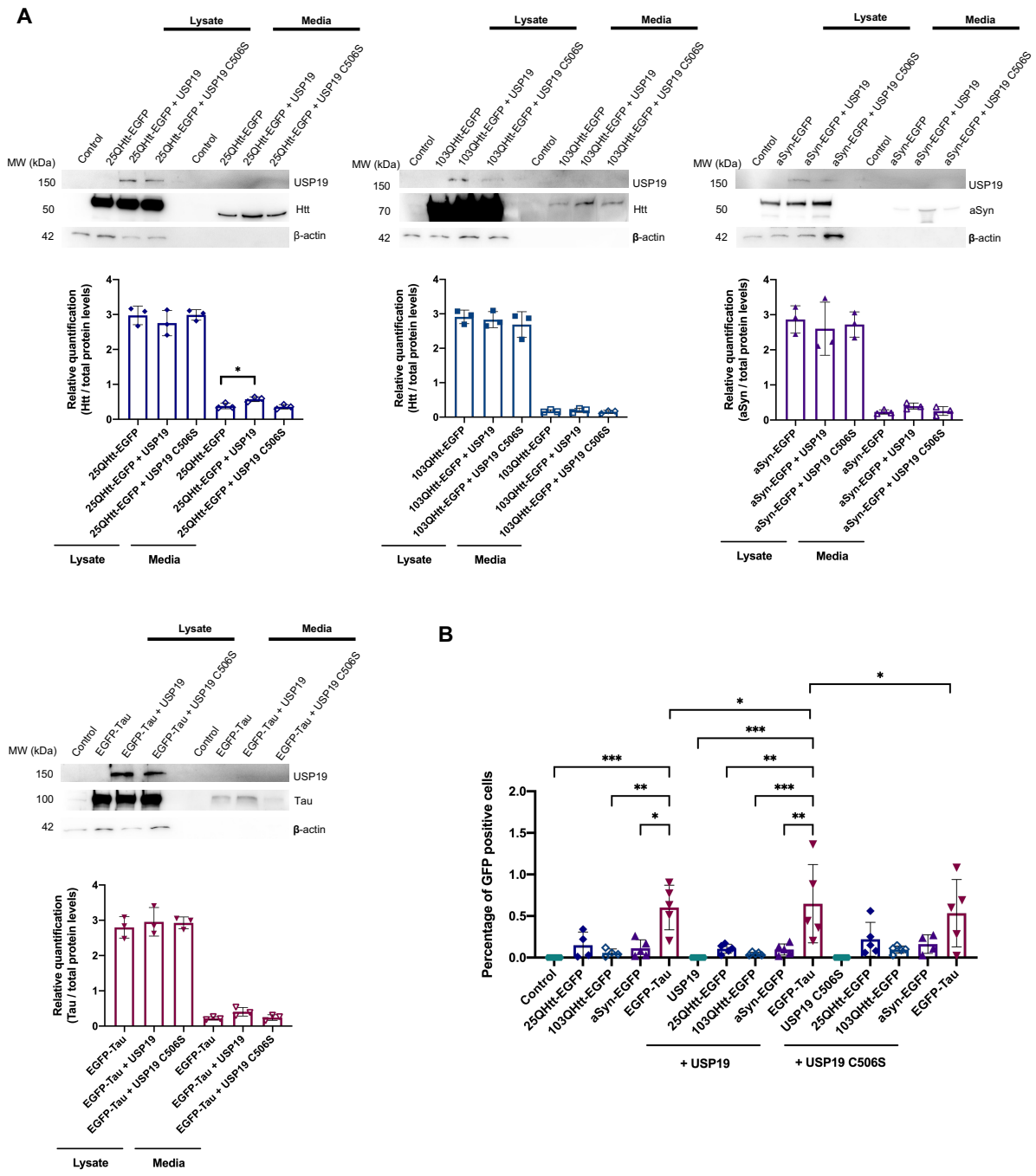
Supplementary Material

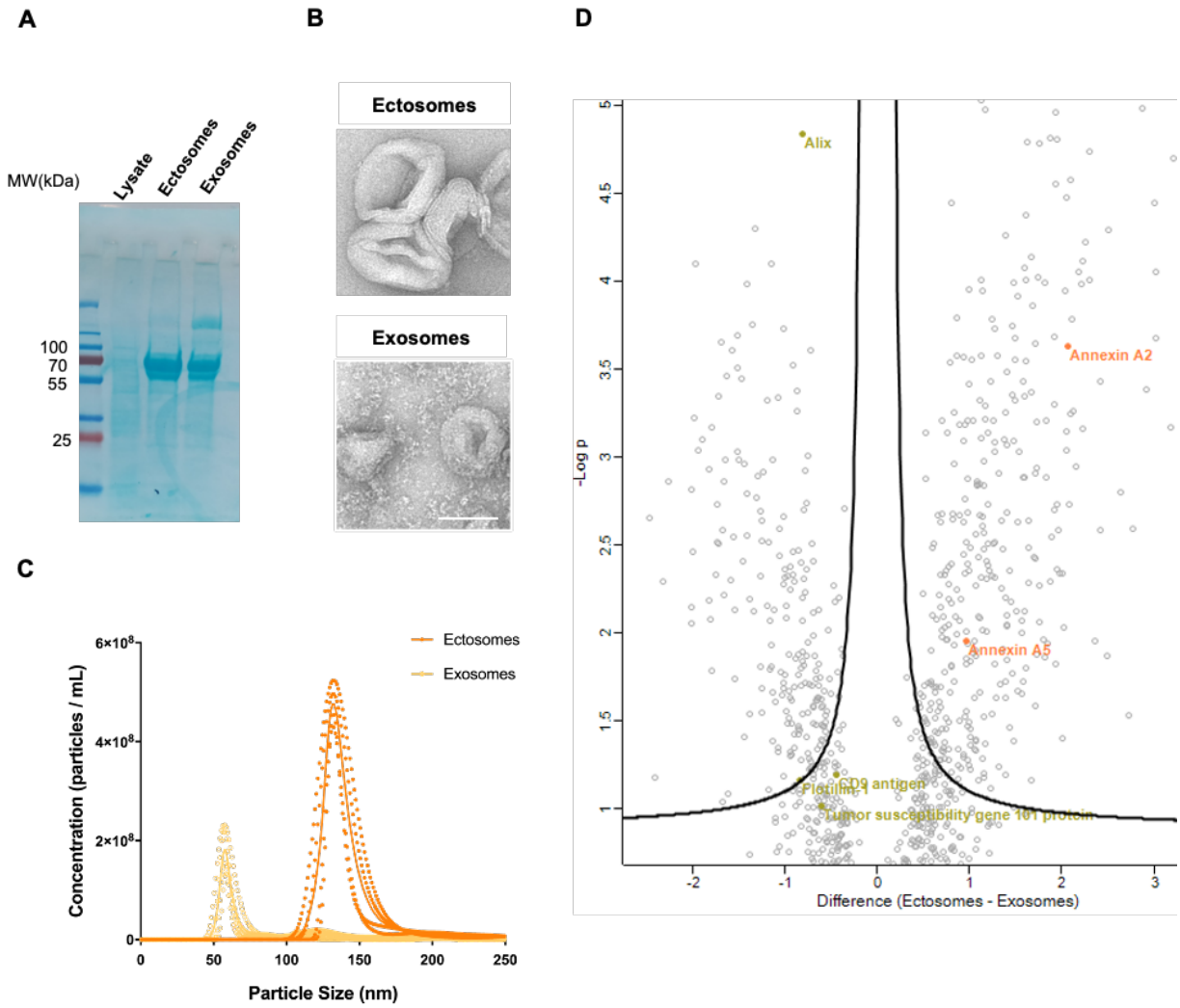
Molecular Mechanisms Mediating the Transfer of Disease-Associated Proteins and Effects on Neuronal Activity



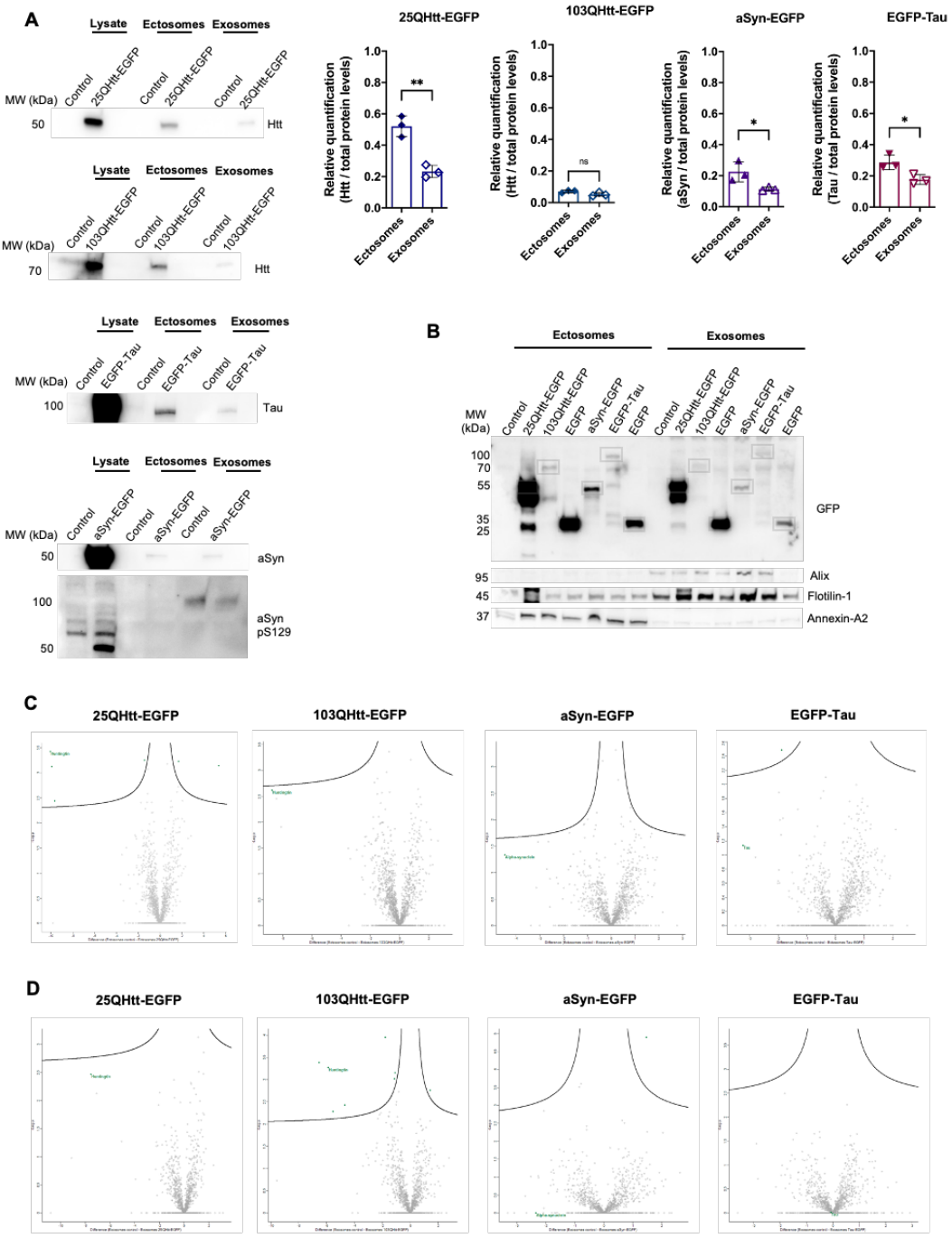
Supplementary Figure 1. Different disease-related proteins are secreted to the extracellular space. A) Representative images of HEK cells transfected with plasmids encoding untagged 22QHtt, 72QHtt, aSyn or Tau. Control cells were transfected with an empty plasmid. Scale bar 5 µm. B) Western blots showing the protein levels in the lysates and released to the cell media of the different cells. Quantifications were normalized to total protein levels using MemCode (*n=3*). Immunoblots were cropped for space purposes. C-E) LDH measurements confirm the absence of cell toxicity and cell death in (C) cells transfected with disease-relate proteins without tag (*n=3*), (D) in HEK cells stably expressing 25QHtt-EGFP, 103QHtt-EGFP, aSyn-EGFP or EGFP-Tau (*n=3*), and (E) in primary cortical neurons expressing 25QHtt-EGFP, 103QHtt-EGFP, aSyn-EGFP or EGFP-Tau (*n=3*). Significant differences were assessed by one-way ANOVA followed by multiple comparisons with significance between groups corrected by Bonferroni procedure.



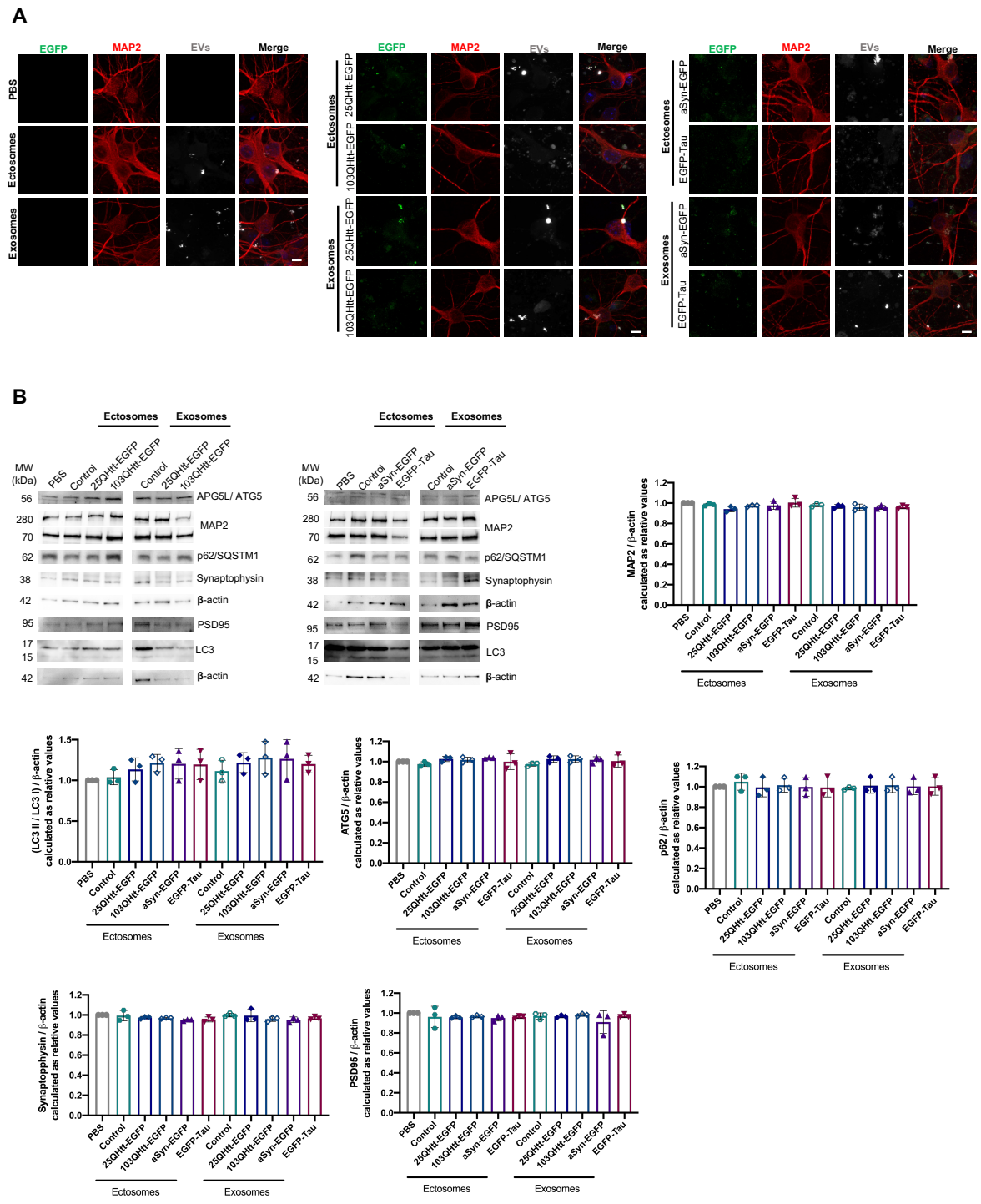
Supplementary Figure 2. USP19 promotes the secretion of 25QHtt-EGFP to the extracellular space. A) Immunoblots showing protein levels in cell lysates and released to the cell media of cells expressing the different proteins. HEK cell stably expressing 25QHtt-EGFP, 103QHtt-EGFP, aSyn-EGFP, and EGFP-Tau were transfected with USP19 or with the catalytic inactive form USP19 C506S. Quantifications were normalized to total protein levels using MemCode (*n=3*). Immunoblots were cropped for space purposes. B) Tau is more strongly internalized by naïve cells. Percentage of EGFP positive cells after incubation with media from cells co-expressing 25QHtt-EGFP, 103QHtt-EGFP, aSyn-EGFP, or EGFP-Tau together with USP19 or USP19 C506S for 24 h. Cell counting was performed using flow cytometry (*n=5*). Significant differences were assessed by one-way ANOVA followed by multiple comparisons with significance between groups corrected by Bonferroni procedure. Differences were considered to be significant for values of $p<0.05$ and are expressed as mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.



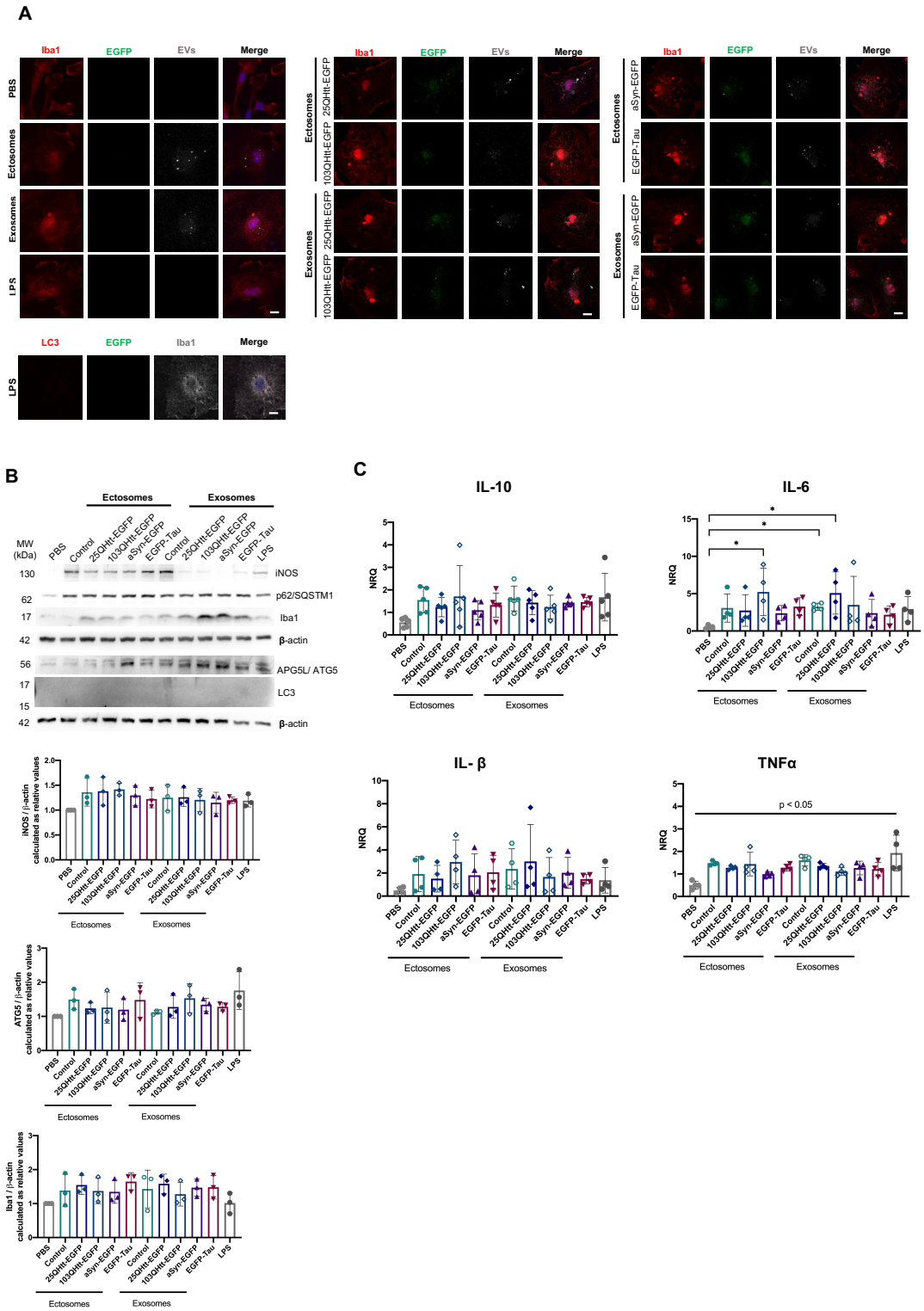
Supplementary Figure 3. Purification and characterization of secreted EVs using differential centrifugation. A) MemCode staining demonstrates the total protein levels present in each fraction. Exosome-depleted cell media was collected from HEK cells after 24 h and subsequently centrifuged at different speed. B) Whole-mount electron microscopy analysis of each pellet showing representative images of ectosomes and exosomes (scale bar 100 nm) (*n=3*). C) Nanoparticle tracking analysis (NTA) measurements of particle concentrations and average size distributions of ectosomes and exosomes. Average is represented with the filled line while each dotted line represents one biological replicate. Yellow circles represent exosomes measurements, while orange dots represent ectosomes measurements (*n=4*). D) Proteomic analyses of ectosomes and exosomes using label-free quantitative mass spectrometry demonstrates the enrichment of specific protein markers in each fraction. Yellow dots represent the proteins enrichment in exosomes, while orange dots represent enrichment in ectosomes. Dots above the volcano plot line represent proteins for which differences were significant (false discovery rate [FDR] <0.1). Data represented in "t-test Difference (Ectosomes - Exosomes)" vs. "-Log t-test p-value" (*n=5*).



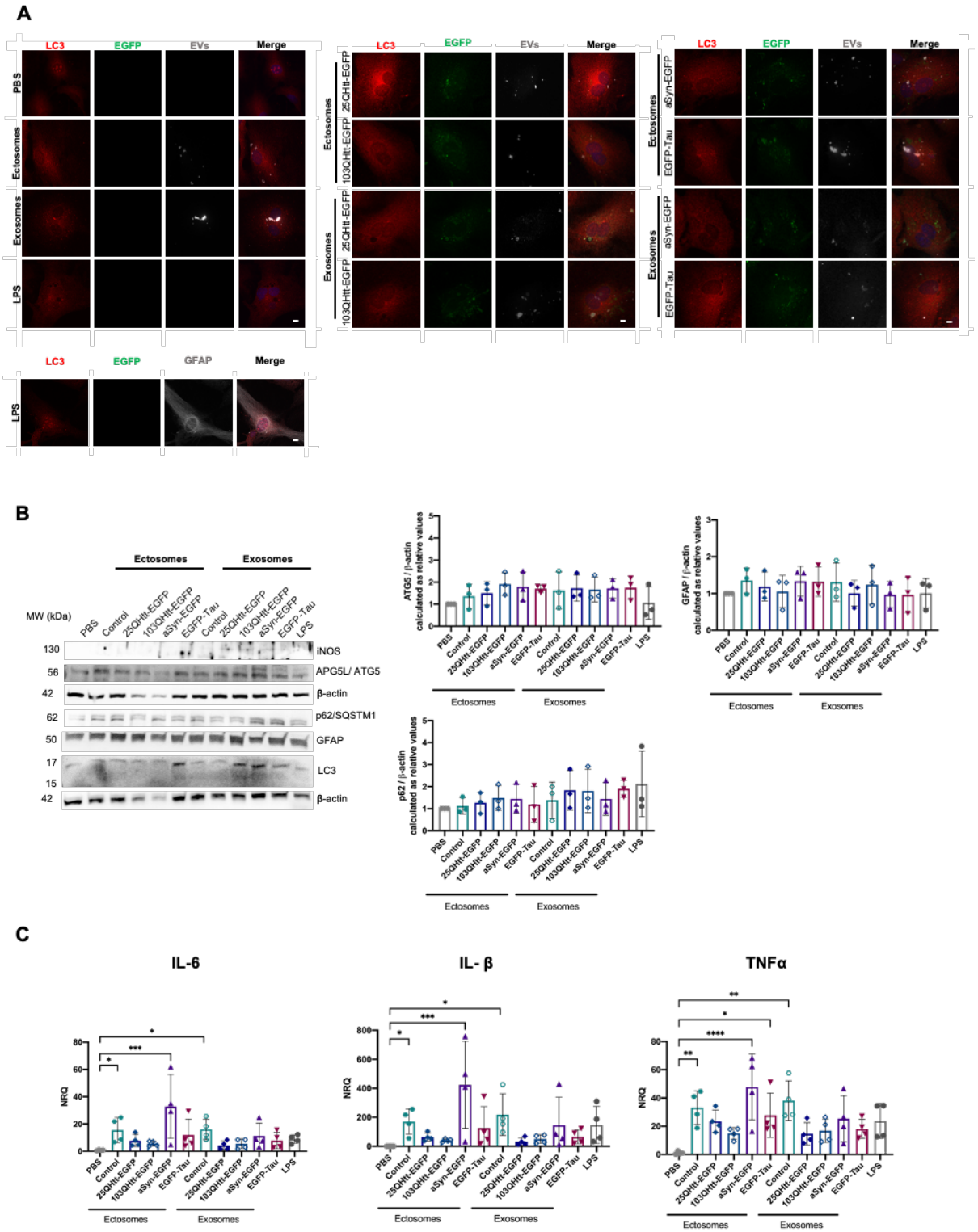
Supplementary Figure 4. Disease-related proteins are enriched in ectosomes. A) Immunoblots of ectosomes and exosomes purified from the media of HEK cells stably expressing 25QHtt-EGFP, 103QHtt-EGFP, aSyn-EGFP, or EGFP-Tau for 24 h. Equal quantities of protein were separated on SDS-PAGE gels, and membranes were blotted with the indicated antibodies. Protein levels were normalized to total protein levels using Memcode (*n=3*). Immunoblots were cropped for space purposes. Significant differences were assessed by two-tailed unpaired t test comparison and are expressed as mean ± SD, **p*<0.05, ***p*<0.01. B) Uncut immunoblot of ectosomal and exosomal fractions purified from the media of HEK cells stably expressing 25QHtt-EGFP, 103QHtt-EGFP, aSyn-EGFP or EGFP-Tau for 24 h. Gray boxes highlight the expected MW (kDa) for each protein. C, D) Proteomic analyses of ectosomes and exosomes using label-free quantitative mass spectrometry demonstrates the enrichment of 25QHtt-EGFP, 103QHtt-EGFP, aSyn-EGFP and EGFP-Tau in ectosomes (C) and exosomes (D) compared with the control (proteins are identified in green). C) Data represented in "t-test Difference (Ectosomes – Ectosomes 25QHtt-EGFP/ 103QHtt-EGFP/ aSyn-EGFP/ EGFP-Tau)" vs. "-Log t-test p-value" (*n=3*). D) Data represented in "t-test Difference (Exosomes – Exosomes 25QHtt-EGFP/ 103QHtt-EGFP/ aSyn-EGFP/ EGFP-Tau)" vs. "-Log t-test p-value" (*n=3*). Dots above the volcano plot line represent proteins for which differences were significant (false discovery rate [FDR] <0.1).



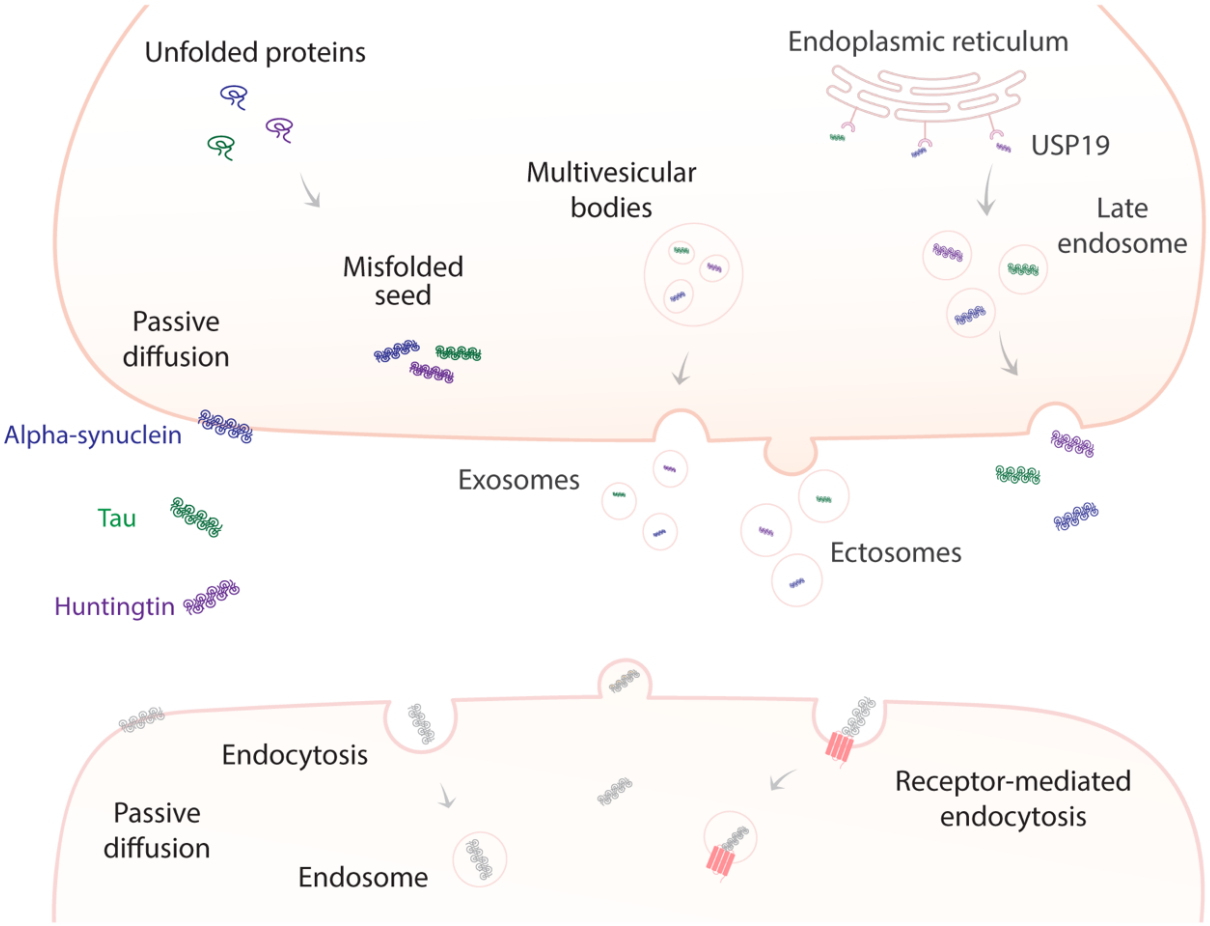
Supplementary Figure 5. Ectosomes and exosomes containing disease-related proteins are internalized by primary cortical neurons. A) Ectosomes and exosomes were labelled with Alexa Fluor 633 C5-maleimide (grey) and applied to neuronal cultures at a concentration of 20 µg/mL for 24 h. Cells were immunostained for MAP2 (red) (*n=4*). Scale bar 5 µm. B) Ectosomes and exosomes containing 25QHtt-EGFP, 103QHtt-EGFP, aSyn-EGFP, or EGFP-Tau were applied to primary cortical cultures at a concentration of 20 µg/mL for 24 h. Immunoblot and protein quantifications of PSD95, synaptophysin, MAP2, APG5L/ ATG5, p62, and LC3 (*n=3*). Immunoblots were cropped for space purposes. Significant differences were assessed by one-way ANOVA followed by multiple comparisons with significance between groups corrected by Bonferroni procedure. Differences were considered to be significant for values of $p<0.05$ and are expressed as mean ± SD.



Supplementary Figure 6. Extracellular vesicles internalization induces autophagy activation and production of inflammatory markers by microglial cells. A) Ectosomes and exosomes containing 25QHtt-EGFP, 103QHtt-EGFP, aSyn-EGFP or EGFP-Tau were labelled with Alexa Fluor 633 C5-maleimide (grey) and applied to microglial cultures at a concentration of 10 µg/mL for 24 h. Cells were immunostained for Iba1 (red) (*n=4*). Scale bar 10 µm. B) Immunoblot and protein quantifications of iNOS, Iba1 and APG5L/ATG5 (*n=3*). Immunoblots were cropped for space purposes. C) EV treatment leads to the activation of the pro-inflammatory markers IL-6 and TNFα in microglia cells after 24 h (*n=4*). Significant differences were assessed by one-way ANOVA followed by multiple comparisons with significance between groups corrected by Bonferroni procedure. Differences were considered to be significant for values of $p<0.05$ and are expressed as mean ± SD, $*p<0.05$.



Supplementary Figure 7. Extracellular vesicles internalization induces production of inflammatory markers by astrocytic cells. A) Ectosomes and exosomes containing 25QHtt-EGFP, 103QHtt-EGFP, aSyn-EGFP, or EGFP-Tau were labelled with Alexa Fluor 633 C5-maleimide (grey) and applied to cell cultures at a concentration of 20 µg/mL for 24 h. Cells were immunostained for LC3 (red) (*n=4*). Scale bar 10 µm. B) Immunoblot and protein quantifications of APG5L/ATG5, GFAP, and p62 (*n=3*). Immunoblots were cropped for space purposes. C) EV treatment leads to the activation of the pro-inflammatory markers IL-6, IL-β, and TNFα in astrocytic cells after 24 h (*n=4*). Significant differences were assessed by one-way ANOVA followed by multiple comparisons with significance between groups corrected by Bonferroni procedure. Differences were considered to be significant for values of $p<0.05$ and are expressed as mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.



Supplementary Figure 8. Graphical abstract.