

Figure S1

Figure S1. sEVs promote the growth of the longest neurite in a concentration-dependent manner. Related to Fig. 1. (A) A schematic of the morphological changes in neurons cultured *in vitro*. (B-K) Dissociated E15.5-16.5 mouse cortical neurons were treated with various concentrations (0.05-10 $\mu\text{g}/\text{mL}$) of sEVs purified from the indicated fibroblast cell lines including L cells (B, C), MEFs (D, E), HDFn (F, G), NHLF (H, I) and BJ (J, K), 4 h after plating. Neurons were fixed at 24 and 33 h, and neuronal morphology was examined in Tuj1 stained neurons. Representative images (B, D, F, H, J) and quantifications (C, E, G, I, K) are shown. Arrowheads mark the longest neurite. Scale bar, 40 μm .

Neurite lengths are quantified from a minimum of 90 neurons per condition from 3 independent experiments and plotted as a violin plot with values from each experiment distinctly colored and the median marked by a black line. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using one-way ANOVA with Dunnett's post-test.

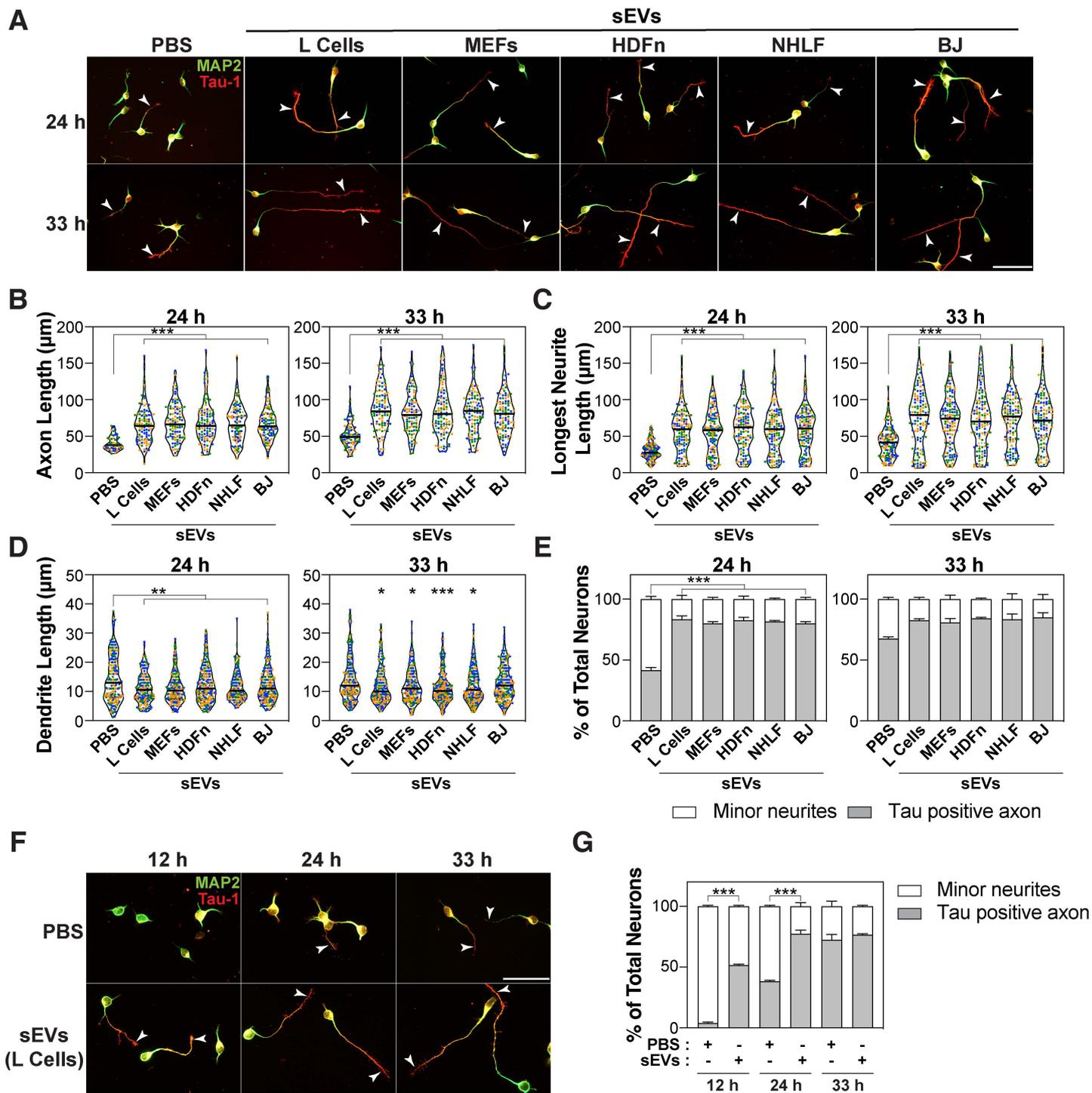


Figure S2

Figure S2. sEVs promote axon outgrowth and polarized neuronal morphology. Related to Fig. 1. (A-G) Dissociated E15.5-16.5 mouse cortical neurons were treated with sEVs purified from the indicated fibroblast cell lines (A-E) or only L cells (F-G), 4 h after plating. Neurons were fixed at 24 and 33 h (A-E) or 12, 24 and 33 h (F-G), and neuronal morphology was examined in neurons immunostained for MAP2 (dendrites, green) and Tau-1 (axons, red). (A, F) Representative images are shown. Arrowheads mark Tau-1 positive axons. Scale bar, 50 μ m. (B-E) The length of the Tau-1 positive axons (B), longest neurite (independent of Tau-1+ staining, C) and individual dendrite length (MAP2 positive, D) is quantified. (E, G) Percent of neurons containing only minor neurites (MAP2 positive) or a single Tau-1-positive axon, is plotted.

Neurite lengths are quantified from a minimum of 45 (for 24 h in panel B) or 90 (for all others) neurons per condition from 3 independent experiments and plotted as a violin plot with values from each experiment distinctly colored and the median marked by a black line. In panel B, for the 24 h control group (PBS treated), less data points were available due to greater percentage of neurons lacking a Tau-1 positive axon. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using one-way ANOVA with Dunnett's post-test (B, C, D), or two-way ANOVA with Tukey's post-test (E, G).

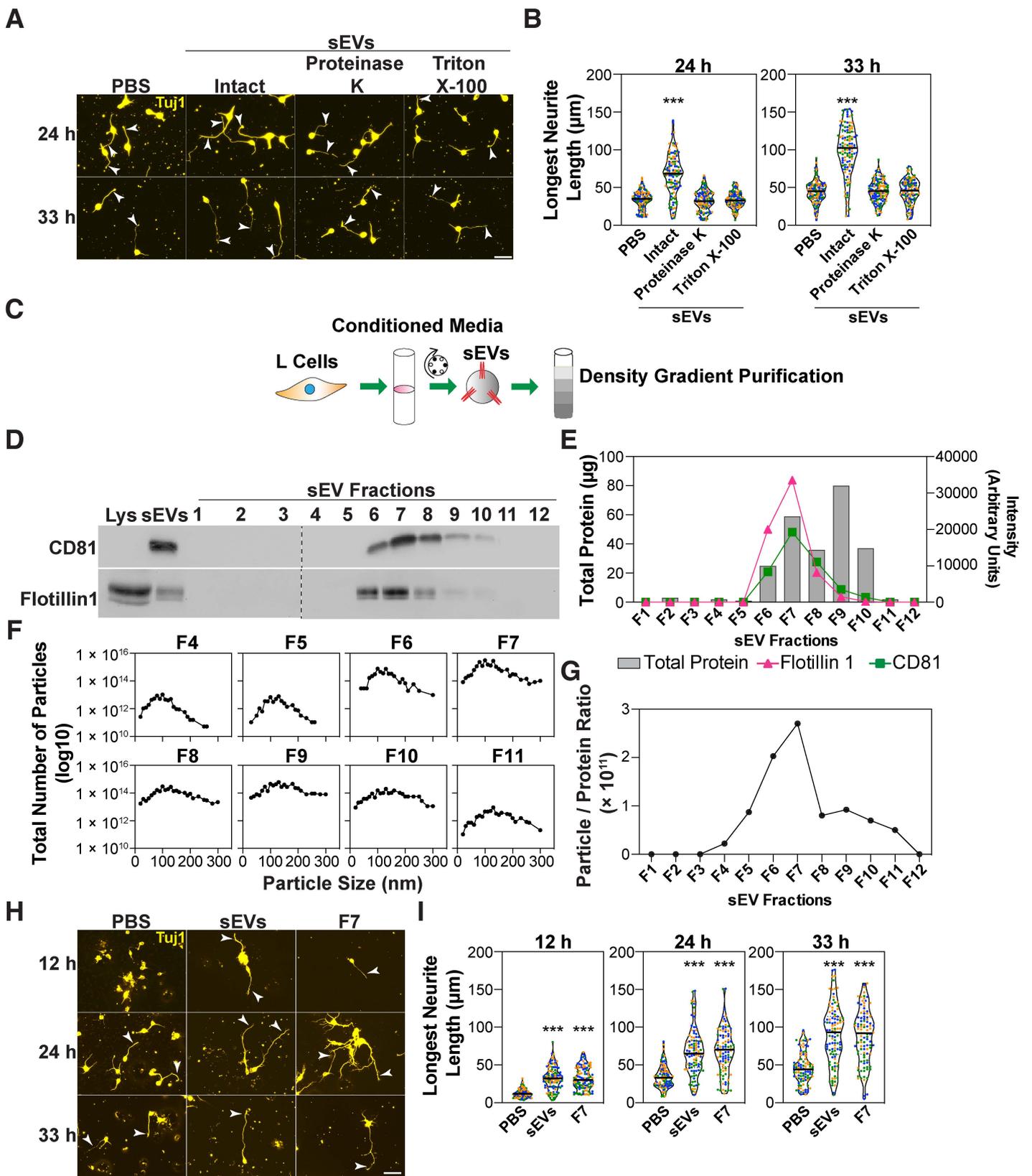


Figure S3

Figure S3. sEVs isolated using a discontinuous density gradient promote the growth of the longest neurite. Related to Fig. 1. (A-B, H-I) Dissociated E15.5-16.5 cortical neurons were treated with the intact sEVs, Proteinase K treated and Triton X-100 treated sEV pellet (**A-B**) or sEV pellet and the F7 fraction (both at 2×10^8 particles/mL) (**H-I**), 4 h after plating. Neuronal morphology was examined in Tuj1 stained neurons at 24 and 33 h (**A-B**) or 12, 24 and 33 h (**H-I**). Representative images (**A, H**) and quantifications (**B, I**) are shown. Arrowheads mark the longest neurite. Scale bar, 40 μ m. (**C**) A schematic of the experimental set up. sEV pellets (100,000 x g) purified from the conditioned media (CM) of L cells using differential centrifugation were subjected to iodixanol discontinuous density gradient purification. (**D**) Twelve fractions were collected starting from the top of the discontinuous gradient and were analyzed by immunoblotting for the indicated EV markers. (**E**) Intensity of the bands of EV markers from (**D**) is plotted against the protein concentration. (**F**) Particle size distribution was determined using NTA. (**G**) Fraction 7 (F7) has the highest particle number/protein ratio. Neurite lengths are quantified from a minimum of 90 neurons per condition from 3 independent experiments and plotted as a violin plot with values from each experiment distinctly colored and the median marked by a black line. Statistical significance: *** $p < 0.001$ using one-way ANOVA with Dunnett's post-test.

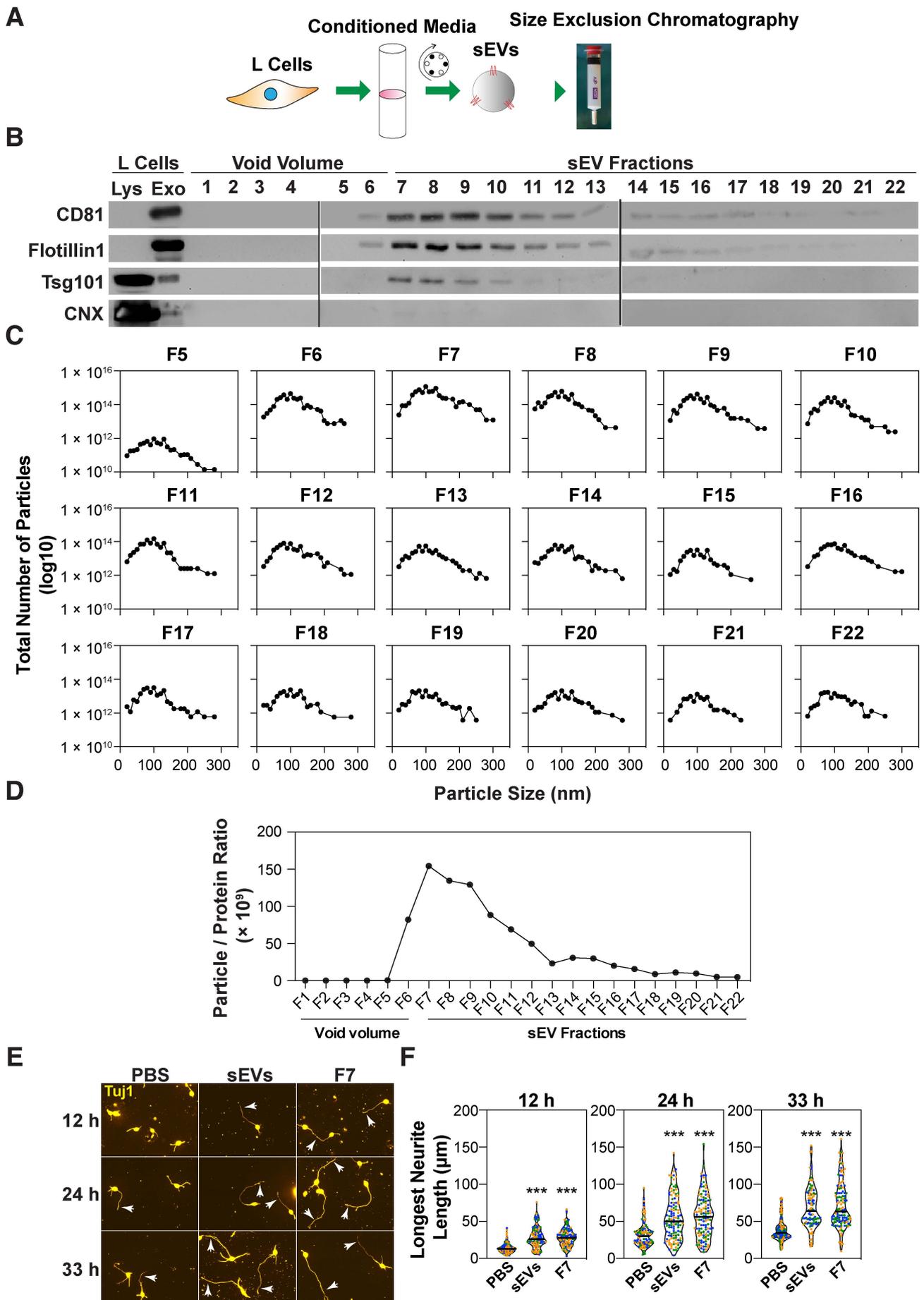
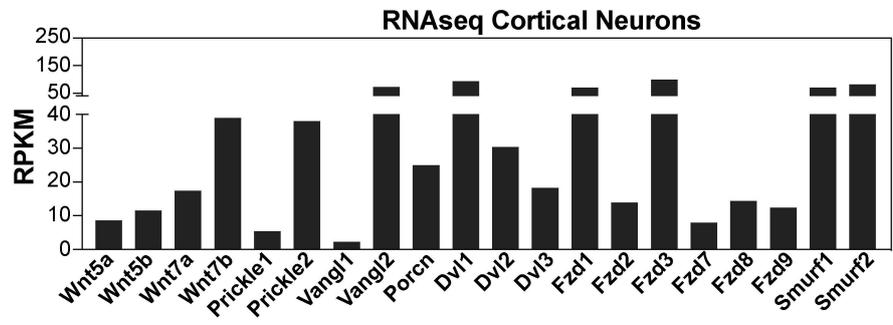


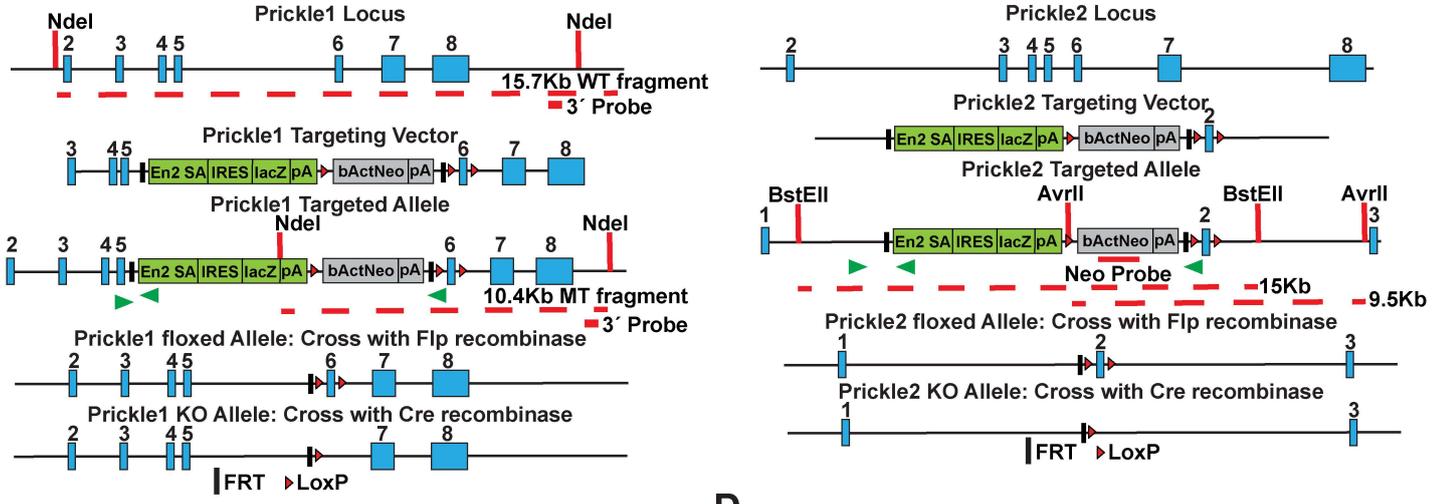
Figure S4

Figure S4. sEVs isolated using size exclusion chromatography promote the growth of the longest neurite. Related to Fig. 1. (A) A schematic illustration of experimental setup. sEVs purified from the CM of L cells were subjected to size exclusion chromatography. (B) A total of 22 fractions were collected and subjected to immunoblotting for EV markers. (C) Particle size was measured using NTA. (D) Fraction 7 (F7) has the highest particle number/protein ratio. (E) F7 fraction promotes the growth of the longest neurite. Dissociated E15.5-16.5 cortical neurons were treated with resuspended sEV pellet (5 $\mu\text{g}/\text{mL}$) and F7 fraction (5 $\mu\text{g}/\text{mL}$), 4 h after plating. Neuronal morphology was examined in Tuj1 stained neurons at various time points: 12, 24 and 33 h. Representative images are shown. Arrowheads mark the longest neurite. Scale bar, 40 μm . (F) The length of the longest neurite was quantified from a minimum of 90 neurons per condition from 3 independent experiments and plotted as a violin plot with values from each experiment distinctly colored and the median marked by a black line. Statistical significance: *** $p < 0.001$ using one-way ANOVA with Dunnett's post-test.

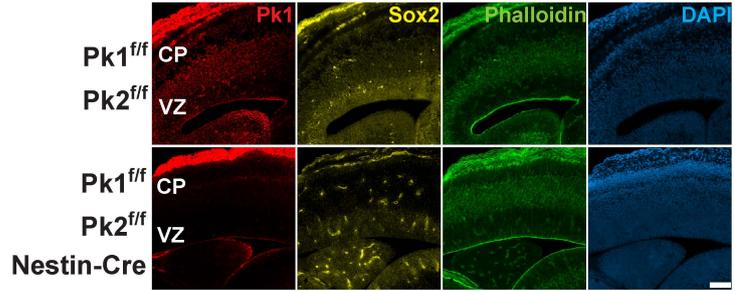
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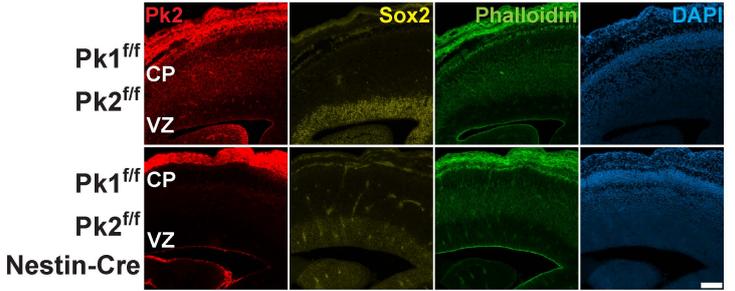
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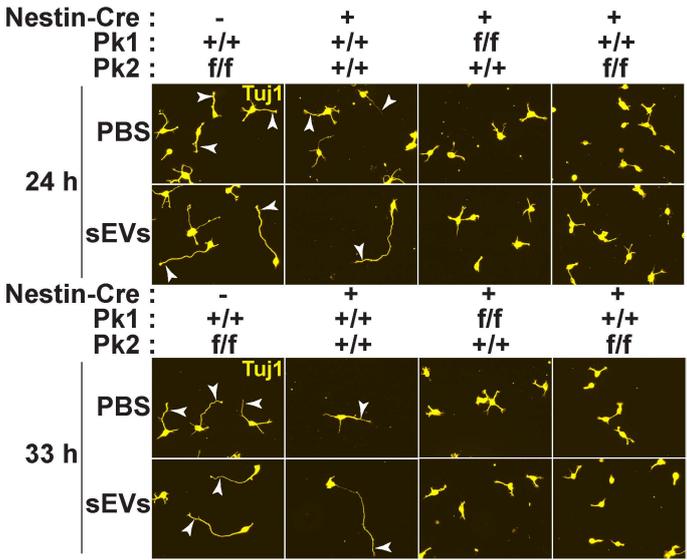
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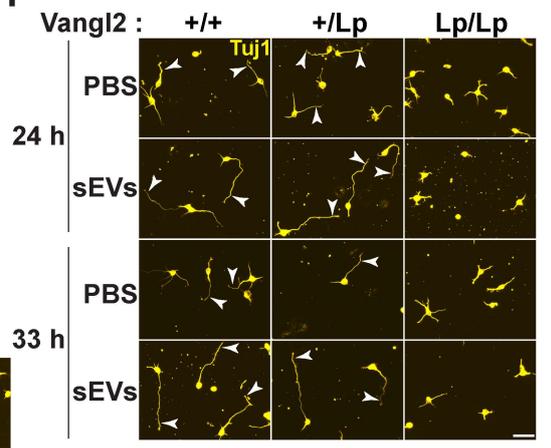
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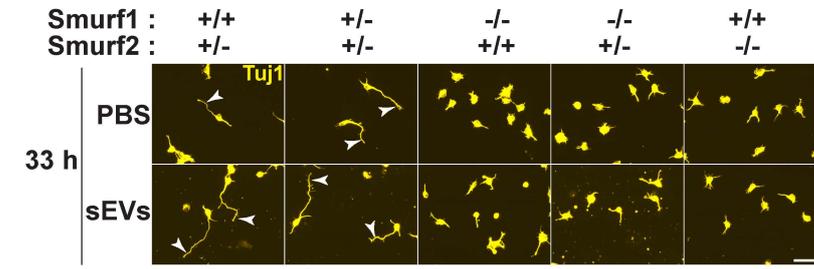
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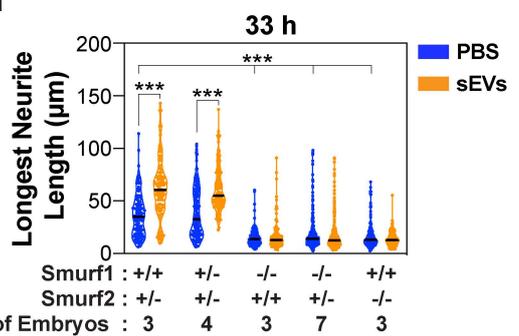


Figure S5

Figure S5. The PCP components, Pk1/2 and Vangl2, are required for sEV-induced growth of the longest neurite. Related to Fig. 2. (A) Expression of PCP genes and Wnts in cortical neurons. Total RNA was isolated from E15.5-16.5 cortical neurons after 33 h of *in vitro* culturing. An RNA sequence library was constructed, and high-quality reads were aligned to the *M. musculus* genome and expression of the indicated genes plotted. RPKM, Reads per kilobase of transcript per million mapped reads. (B) Schematic maps of the Pk1 and Pk2 wild type allele, targeting vector, targeted allele, floxed allele after crossing with Flp recombinase and knockout allele after crossing with Cre recombinase are shown. Green arrowheads indicate the position of PCR primers for genotyping. (C, D) Brain cryosections of E15.5 mouse embryo were stained with antibodies for Pk1 and Sox2 (C) or Pk2 and Sox2 (D) along with phalloidin. CP: cortical plate, VZ: ventricular zone. Scale bar, 100 μ m. (E-H) Cortical neurons (E15.5-16.5) were isolated from Pk1 and Pk2 conditional knockout mice (E) or Vangl2 mutant littermates (F) or Smurf1 and Smurf2 knockout mice (G, H), and treated with sEVs from L cells, 4 h after plating. Neurons were fixed at 24 and 33 h and stained with Tuj1. Representative images are shown. Arrowheads mark the longest neurite. Scale bar, 40 μ m. (H) The length of the longest neurite was quantified from 30 neurons per embryo, and the total number of embryos analyzed was indicated below the genotypes. Statistical significance: *** $p < 0.001$ using two-way ANOVA with Tukey's post-test.

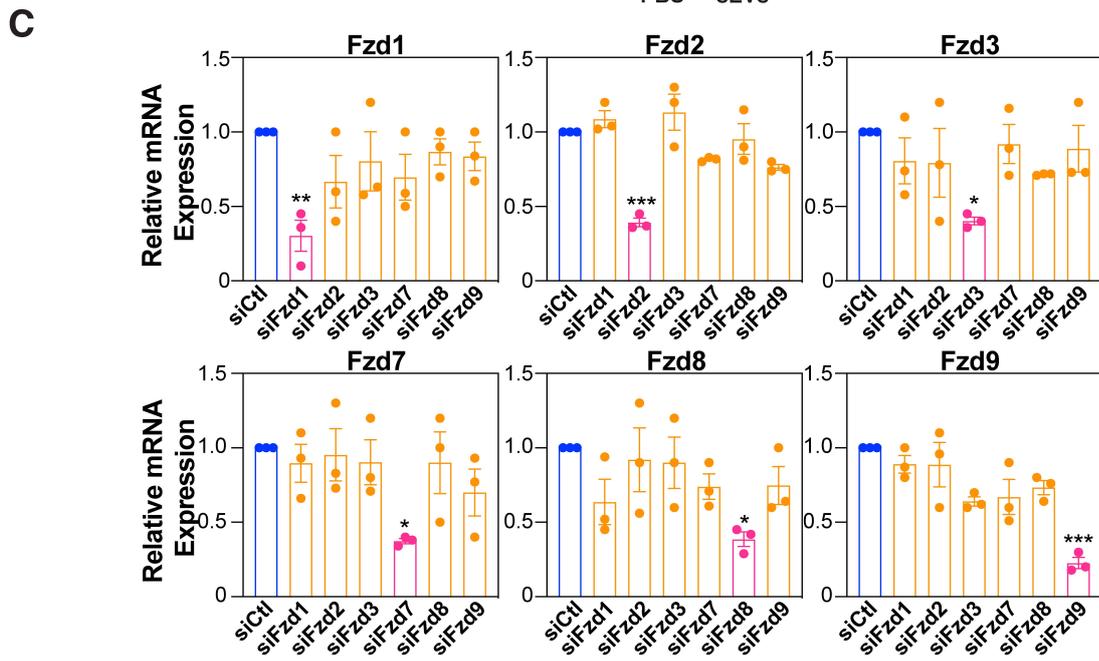
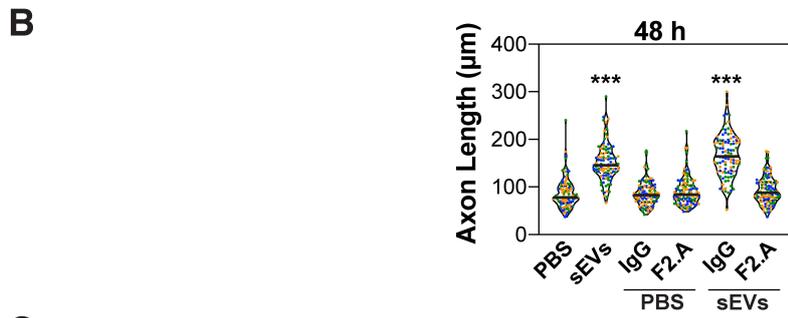
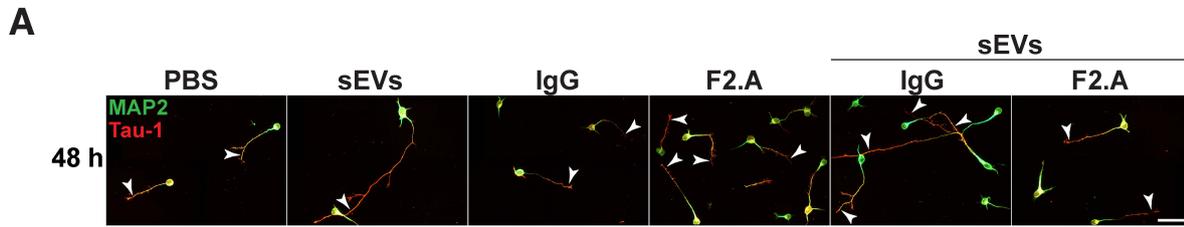


Figure S6

Figure S6. The PCP components, Fzds, are required for sEV-induced growth of the longest neurite. Related to Fig. 3. (A-B) Dissociated E15.5-16.5 mouse cortical neurons were treated with sEVs (5 $\mu\text{g}/\text{mL}$) from L cells and IgG or a Fzd blocking antibody, F2.A (100 nM), 24 h after plating. Neurons were fixed at 48 h, and neuronal morphology was examined in neurons immunostained for MAP2 (dendrites, green) and Tau-1 (axons, red). Representative images are shown **(A)**. Arrowheads mark Tau-1 positive axons. Scale bar, 50 μm . **(B)** The length of the Tau-1 positive axons was quantified from a minimum of 90 neurons per condition from 3 independent experiments and plotted as a violin plot with values from each experiment distinctly colored and the median marked by a black line. **(C)** Knockdown efficiency for Fzds was determined in GFP-positive neurons isolated by FACS. Relative mRNA expression was determined by qPCR and is plotted as the mean \pm SEM from 3 independent experiments. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using one-way ANOVA with Dunnett's post-test.

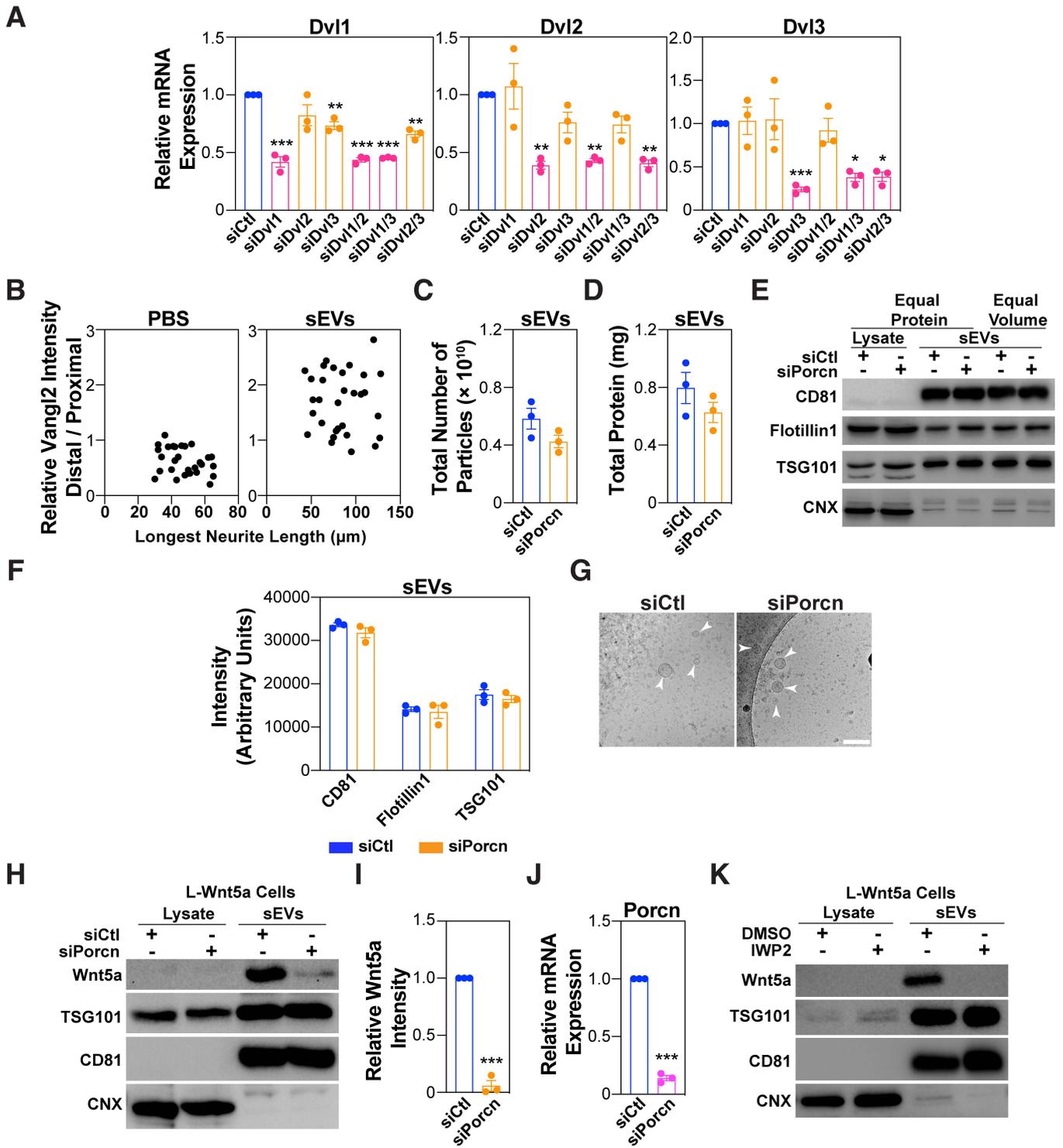


Figure S7

Figure S7. Related to Fig. 4 and 5. (A) Knockdown efficiency for Dvls was determined in GFP-positive neurons isolated by FACS. Relative mRNA expression was determined by qPCR and is plotted as the mean \pm SEM from 3 independent experiments. (B) The ratio of distal/proximal Vangl2 intensity as a function of the longest neurite length in neurons treated with PBS or sEVs is quantified from 30 neurons from 3 independent experiments. (C) The total number of particles in the sEV pellet (100,000 x g) were measured using NTA. (D) The total amount of protein in the sEV pellet was measured by Bradford assay. (E) Characterization of sEVs derived from porcupine-deficient L cells. A representative immunoblot of the cell lysate and 100,000 x g pellet (sEVs) analyzed for EV markers and calnexin (CNX). (F) Quantification of (E). sEVs were collected from an equal number of cells, and equal volumes of sEV pellets (right two lanes) were used for comparison. Protein bands were quantified using ImageJ. (G) Representative TEM images of sEV pellet. Arrowheads indicate round vesicles. Scale bar, 200 nm. (H-K) Porcupine inhibition blocks Wnt5a secretion in sEVs. L-Wnt5a cells were treated with siCtl or siPorcupine (siPorcn) (H-J) and DMSO or IWP2 (10 μ M) (K) followed by isolation of sEVs. A representative immunoblot of sEVs and lysates indicating the level of Wnt5a, EV markers and CNX is shown (H, K). (I) Quantification of (H). Protein bands were quantified using ImageJ. (J) Knockdown efficiency of Porcupine in L-Wnt5a cells. Values are plotted as the mean \pm SEM from 3 independent experiments (A, C, D, F, I, J). Statistical significance: * p <0.05, ** p <0.01, *** p <0.001 using unpaired t-test (C, D, I, J), one-way ANOVA with Dunnett's post-test (A), or two-way ANOVA with Tukey's post-test (F).

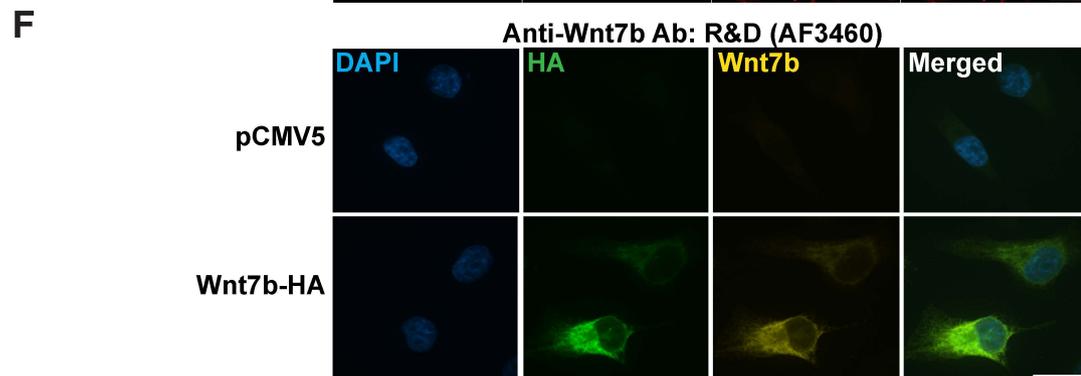
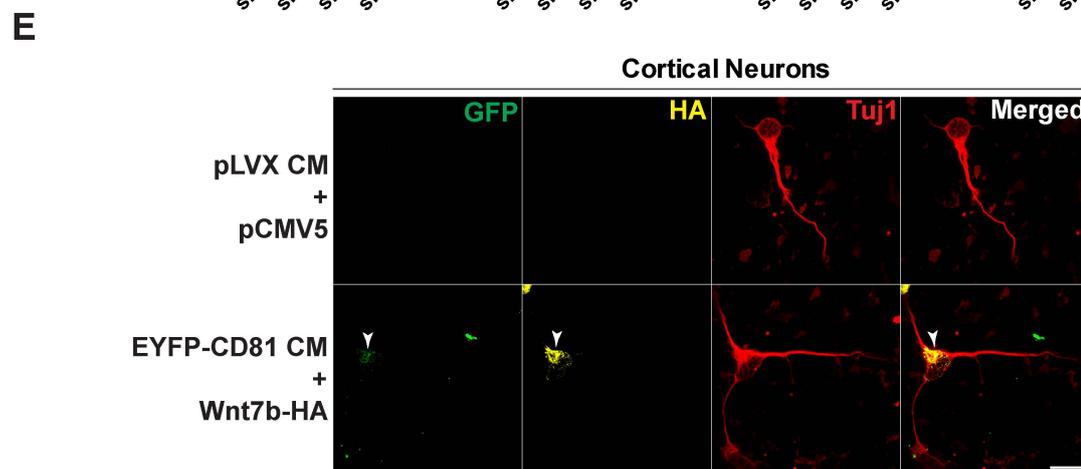
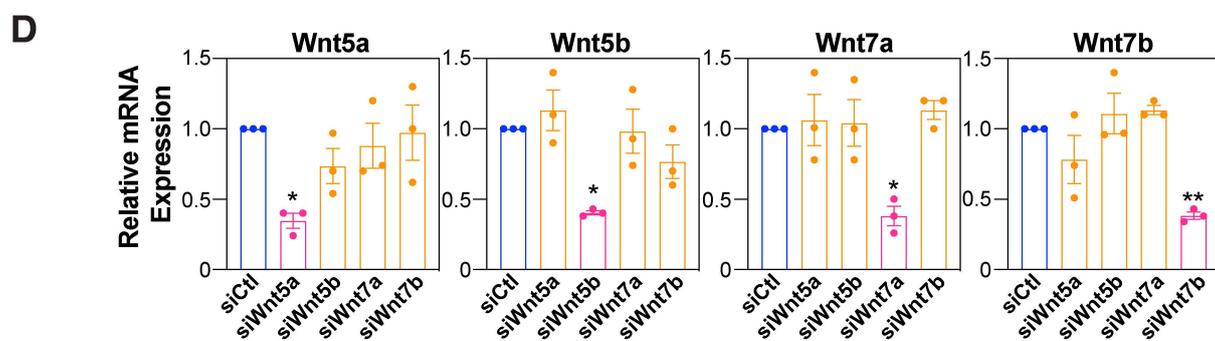
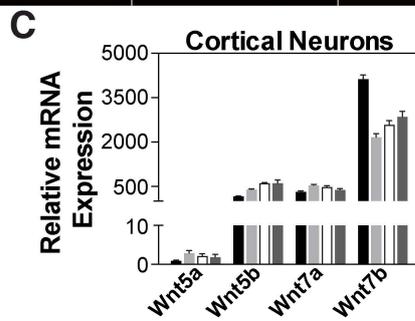
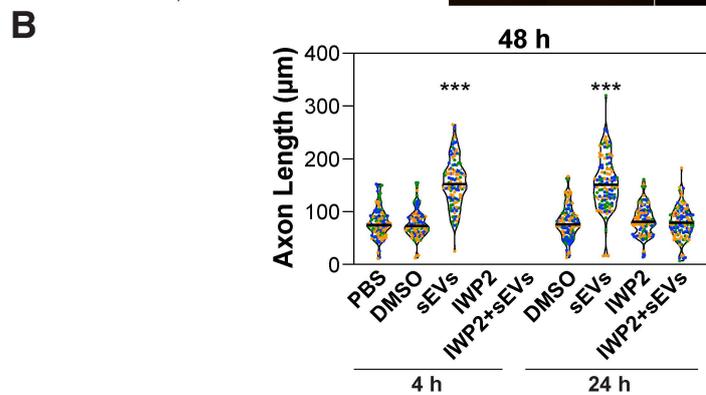
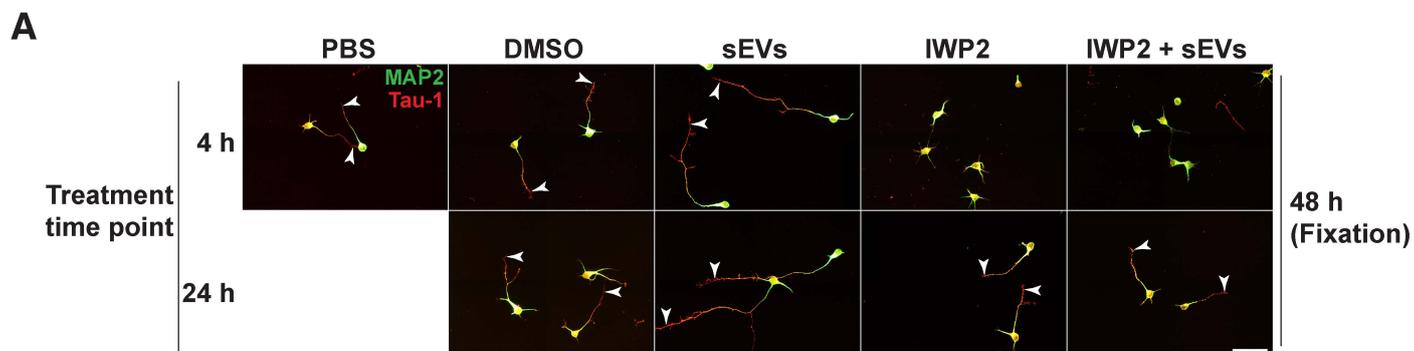


Figure S8

Figure S8. Related to Fig. 5 and 6. (A-B) Dissociated E15.5-16.5 mouse cortical neurons were treated with sEVs (5 $\mu\text{g}/\text{mL}$) from L cells and DMSO or IWP2 (10 μM), 4 h or 24 h after plating. Neurons were fixed at 48 h, and neuronal morphology was examined in neurons immunostained for MAP2 (dendrites, green) and Tau-1 (axons, red). Representative images are shown **(A)**. Arrowheads mark Tau-1 positive axons. Scale bar, 50 μm . **(B)** The length of the Tau-1 positive axons was quantified from a minimum of 90 neurons per condition from 3 independent experiments and plotted as a violin plot with values from each experiment distinctly colored and the median marked by a black line. For IWP2-treated neurons at 4 h, a comparable number of data points were not available due to lack of Tau-1 positive axons. **(C)** Expression of Wnts in cortical neurons. RNA was isolated from cortical neurons at 0, 12, 24 and 33 h of culturing. A representative plot indicating mRNA expression relative to Wnt5a, determined by qPCR from 2 independent experiments is shown. **(D)** Efficiency of siRNA-mediated knockdown of Wnts. RNA was isolated from GFP-positive neurons after FACS. Relative mRNA expression was determined by qPCR. **(E)** Overlap of the localization of sEVs and Wnt7b-HA. Cortical neurons electroporated with 2 μg of either pCMV5 or C-terminal HA-tagged Wnt7b (Wnt7b-HA) were treated with 10X CM containing CD81-EYFP, 24 h after plating for 30 min. Representative images of neurons stained with GFP (green), HA (yellow) and Tuj1 (red) antibodies from 20 neurons from 2 independent experiments are shown. Arrowheads indicate the localization of Wnt7b and sEVs. Scale bar, 20 μm . **(F)** Wnt7b antibody characterization. MDA-MB-231 cells were transfected with 2 μg of either pCMV5 control or C-terminal HA-tagged Wnt7b (Wnt7b-HA). Cells were fixed after 48 h and immunostained with HA (green) and Wnt7b (yellow) antibodies. Scale bar, 20 μm .

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using one-way ANOVA with Dunnett's post-test **(B, D)**.

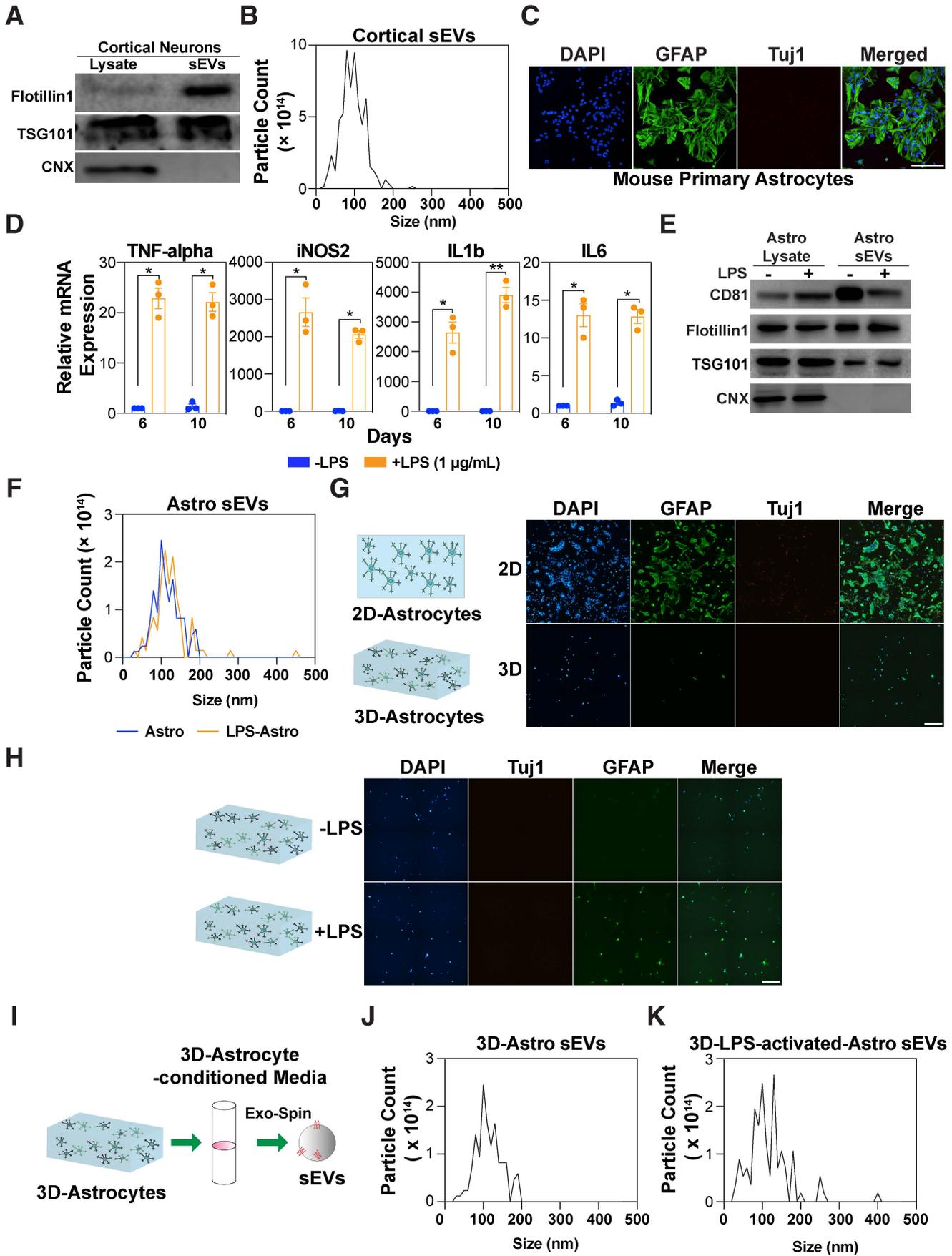


Figure S9

Figure S9. Related to Fig. 7. (A) Immunoblot of sEVs and lysates from cortical neurons. **(B)** NTA of the sEV pellet. **(C)** Purity of primary astrocytes cultures. Enriched cultures of astrocytes from P1-P4 mouse pups were immunostained for the astrocyte marker, GFAP, and the neuronal marker Tuj1. Scale bar, 200 μ m. **(D)** LPS activates mouse primary astrocytes. Astrocytes were treated with LPS (1 μ g/mL), and RNA was isolated after 6 or 10 days. Relative expression of pro-inflammatory genes was measured by qPCR. **(E)** Immunoblot of lysates and the sEV pellet from primary astrocytes. **(F)** NTA of the sEV pellet. **(G)** Astrocytes are activated upon culturing in 2D plates. Representative immunofluorescence images of astrocytes grown either on a glass surface or in a soft collagen gel. Scale bar, 200 μ m. **(H)** LPS activates astrocytes grown in 3D-collagen gels. Astrocytes were treated with LPS (1 μ g/mL), fixed after 48 h and subsequently immunostained with GFAP and Tuj1 antibodies. Representative images are shown. Scale bar, 200 μ m. **(I)** A schematic representation of sEV purification from astrocytes grown in a soft 3D-collagen gel. CM was collected after 6 days and subsequently sEVs were purified using Exo-spinTM columns. **(J and K)** NTA of the sEVs. NTA plots are from 1 representative experiment out of 3 independent purifications **(B, F, J, K)**. Values are plotted as the mean \pm SEM from 3 independent experiments **(D)**. Statistical significance: * $p < 0.05$, ** $p < 0.01$ using two-way ANOVA with Tukey's post-test **(D)**.