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Supplemental information

Cholesterol determines the cytosolic entry and seeded aggregation of tau

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Figure S1 (related to Figure 1)

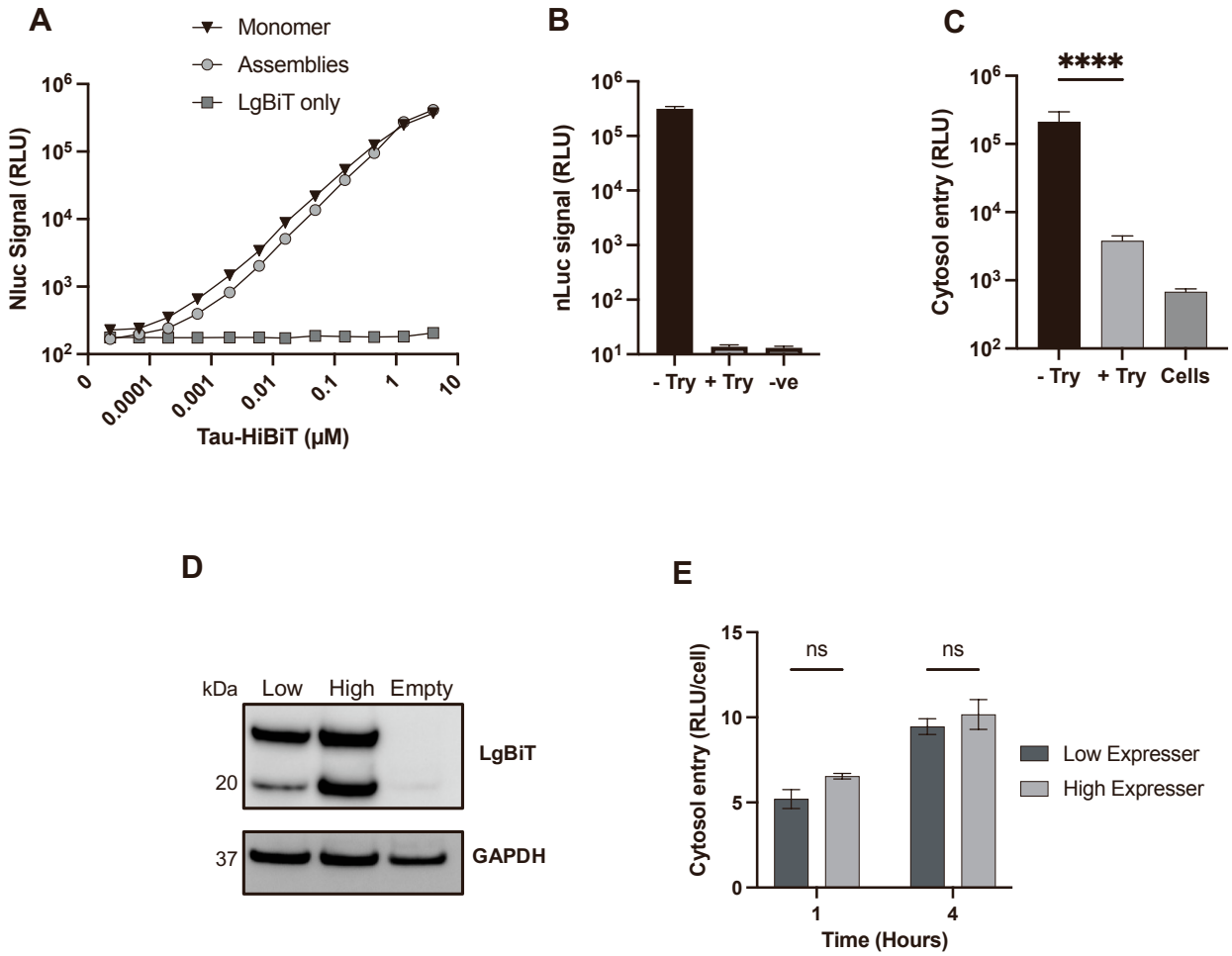


Figure S1 (related to Figure 1): Extracellular signal is sensitive to trypsin and LgBiT concentration is not limiting.
A) Titration of tau-HiBiT monomer or heparin-induced assemblies in the presence of recombinant LgBiT; n=3. Error denotes SEM.
B) In vitro (cell free) reconstitution of NanoLuc with 4 μM tau-HiBiT assemblies complexed with recombinant LgBiT in the presence or absence of trypsin (Try); n=2-9. Error denotes SEM.
C) 10 minutes trypsin protease treatment of HEK-LgBiT after incubation with 250 nM tau-HiBiT assemblies for 1 h, n=6. Error denotes SEM. **** $p < 0.0001$ by one-way ANOVA with Tukey's multiple comparisons.
D) Western blotting of mCherry-LgBiT HEK cells with low and high expression. The upper band corresponds to mCherry-LgBiT (43 kDa) and the lower band LgBiT (19 kDa). Empty refers to 293T cell lysate only.
E) Signal following application of 10 nM tau-HiBiT assemblies to low and high expressing mCherry-LgBiT cells over 4 h, n=4. Error denotes SEM.

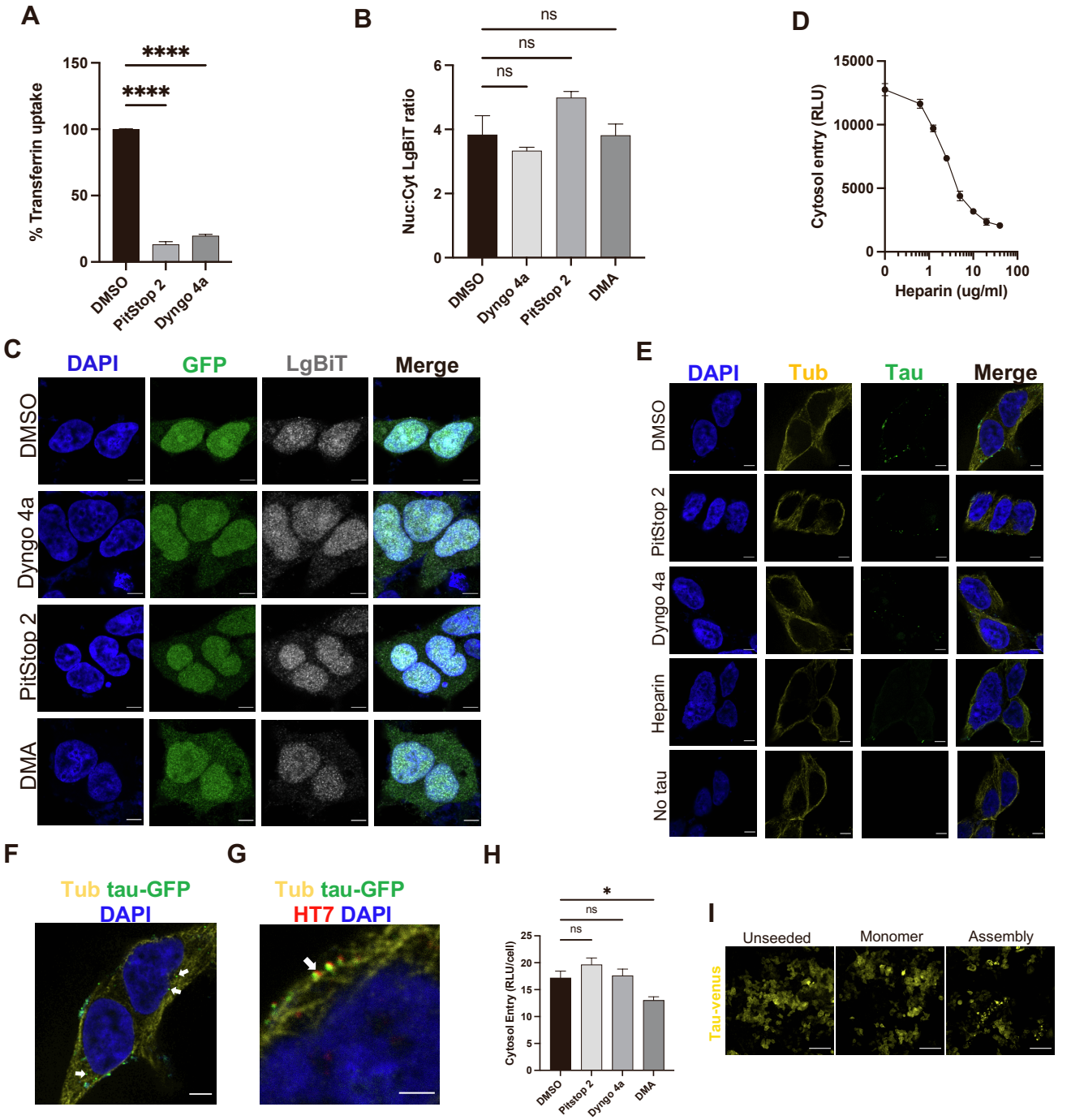


Figure S2 (related to Figure 2): Pathway inhibitors impair tau-GFP uptake and do not affect LgBiT localisation.
A) Uptake of 10 µg/ml alexa-fluor-647-conjugated human transferrin to HEK293T cells, analysed by flow cytometry. Cells were pre-treated for 30 minutes with PitStop 2 or Dyngo 4a (both 20 µM) prior to addition of transferrin for 15 minutes. $1x10^4$ events were recorded from 3 independent experiments. Error denotes SEM. **** $p < 0.0001$ by one-way ANOVA with Tukey's multiple comparisons.
B) Quantification of nuclear:cytosol ratio of LgBiT from confocal microscope images shown in (**C**) after treatment with Dyngo 4a (20 µM), PitStop 2 (20 µM), DMA (500 µM) or solvent (DMSO) for 1 h. Error denotes SEM.
C) Confocal microscopy of HEK-NGL cells treated with drug or solvent as described in (**B**) and immunostained for GFP and LgBiT. Scale bars, 5 µm.
D) 1 h tau entry assay with 50 nM tau-HiBiT assemblies in HEK-NGL cells following pre-treatment with increasing concentrations of heparin for 1 h; n=3. Error denotes SEM.
E) Confocal microscopy of HEK 293T cells pre-treated with PitStop 2 (20 µM), Dyngo 4a (20µM), Heparin (40 µg/ml) or solvent (DMSO) for 1 h followed by incubation with 50 nM tau-GFP assemblies for 1 h. Scale bars, 5 µm.
F) Zoomed in view of cells from panel (**E**) demonstrating intracellular tau-GFP assemblies (white arrows).Scale bar, 5 µm.
G) Tau-GFP assemblies co-localised with human tau HT7 antibody. Scale bar, 2 µm.
H) Entry of 50 nM tau-HiBiT monomer to HEK-NGL cells after pre-treatment with PitStop 2 (20 µM), Dyngo 4a (20 µM), DMA (200 µM) or solvent control (DMSO) for 1 h; n=6. Error denotes SEM. * $p < 0.05$ by unpaired t test.
I) Fluorescence microscope images of P301S-tau-venus biosensor cell line 72 h after challenge with 2 nM of tau monomer or assemblies in the presence of 1% lipofectamine. Scale bars, 50 µm.

Figure S3 (related to Figure 3)

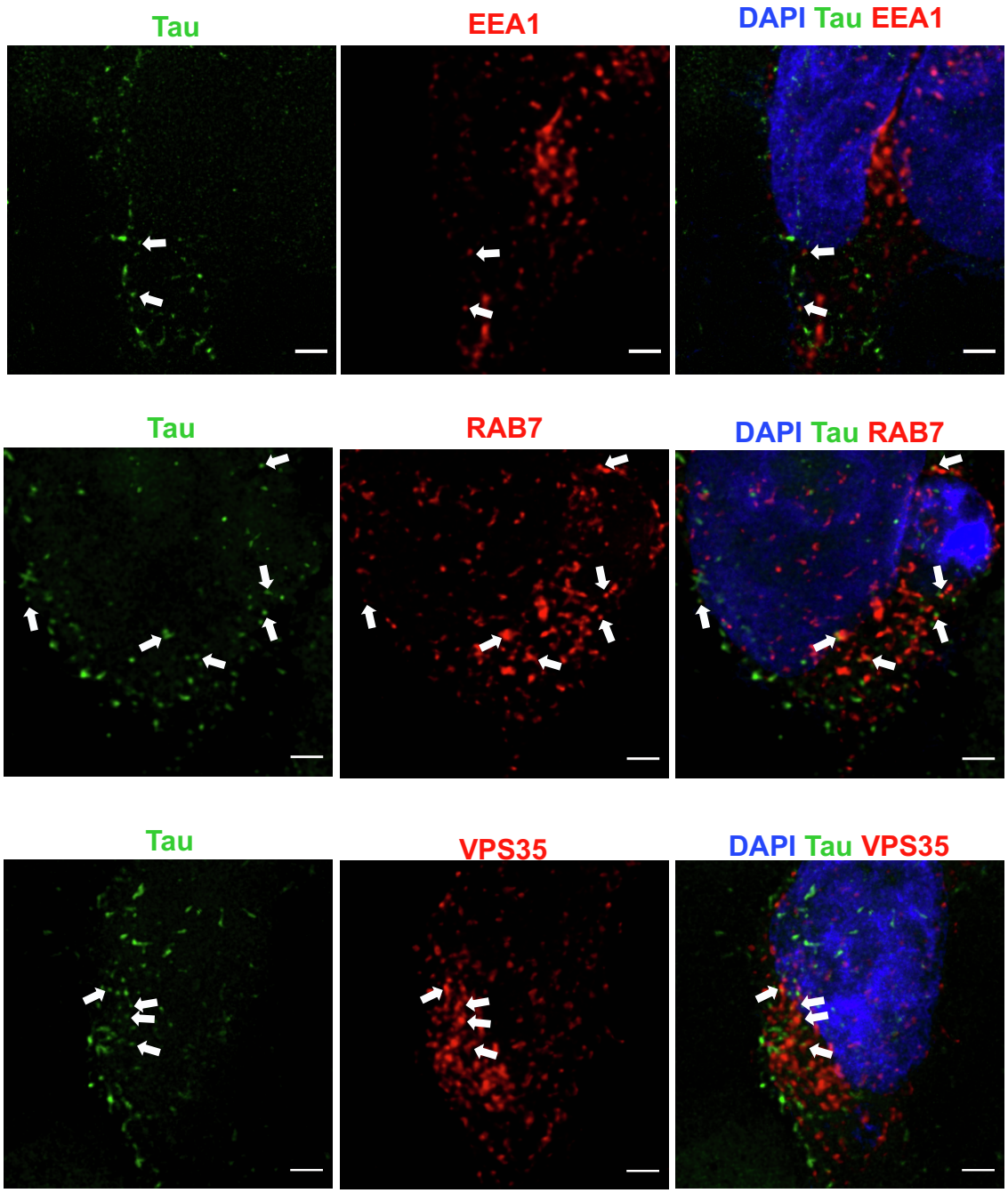


Figure S3 (related to Figure 3): Tau-GFP assemblies co-localise with endolysosomal markers. Confocal microscopy (z-stack) of HEK293T cells after a 1 h uptake assay with 200 nM tau-GFP assemblies. Cells were fixed and stained for early endosomal marker EEA1, late endosome marker RAB7 or retromer associated VPS35. White arrows indicate areas with colocalization. Scale bars, 5 µm.

Figure S4 (related to Figure 4)

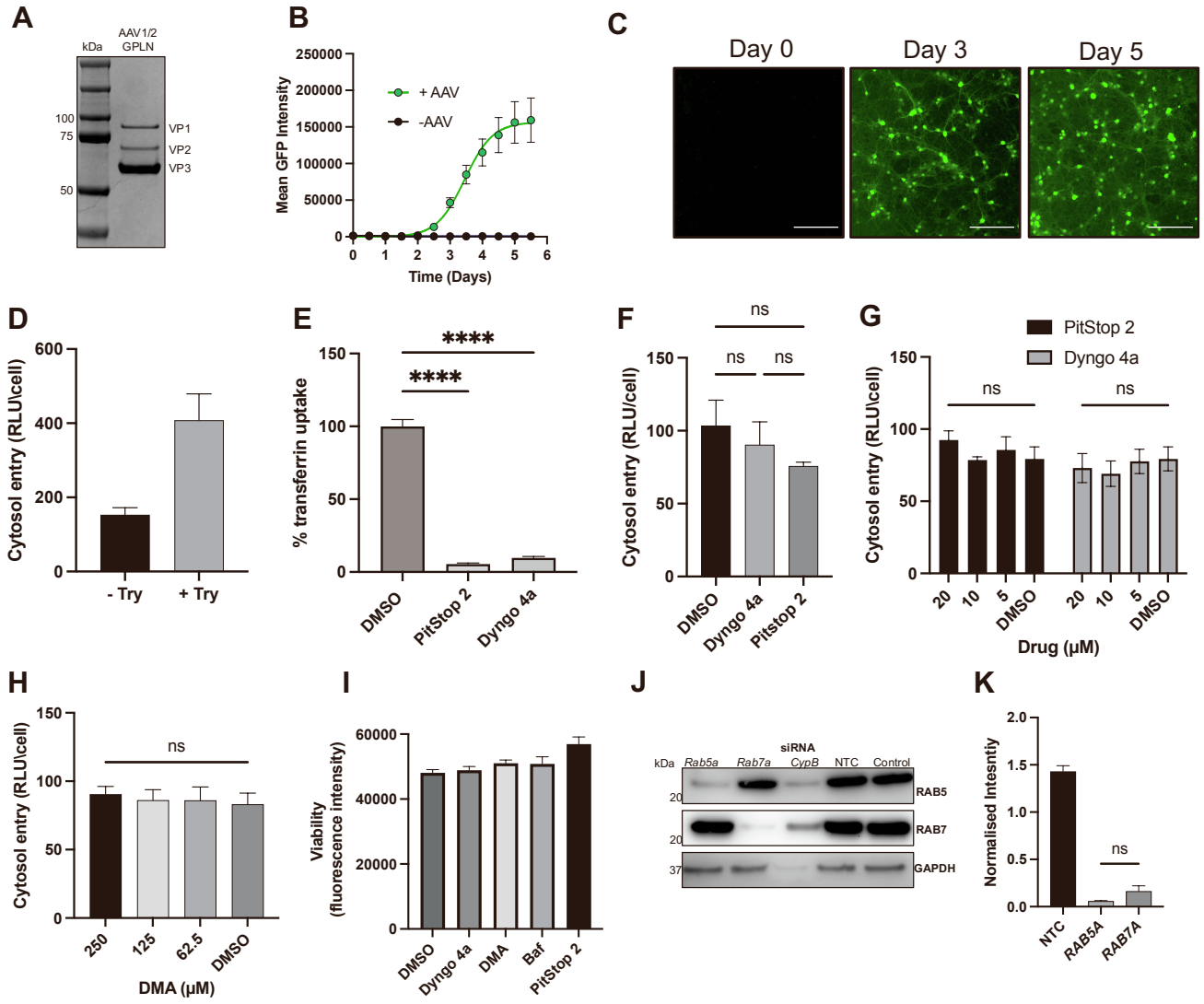


Figure S4 (related to Figure 4): Neuronal entry signal is not reduced by trypsin and monomeric entry is unaffected by pathway inhibitors.

A) Coomassie staining of purified AAV1/2-hSyn-GPLN after SDS-PAGE. Viral proteins VP1, VP2, and VP3 are labelled.

B) Time-course of GFP expression of primary mouse neurons following transduction with AAV1/2-hSyn-GPLN at a multiplicity of 50,000 genome copies/cell over the course of 5 days; n=6. Error denotes SEM.

C) Representative images of AAV transduced neurons from panel **B** at depicted time points. Scale bars, 200 μm.

D) Treatment of GPLN-neurons with or without trypsin (Try) for ten minutes prior to signal acquisition after incubation with 50 nM tau-HiBiT assemblies for 1 h. In the +Try condition we observed rounding of cells, likely amplifying luminescent signal; n=3, N=2 independent experiments. Error denotes SEM.

E) Transferrin uptake assay in DIV 7 primary neurons with 10 μg/ml Alexa-Fluor-647-conjugated human transferrin. Cells were pre-treated for 1 h with PitStop 2 or Dyngo 4a (both 20 μM) prior to addition of transferrin for 15 minutes. $1x10^4$ events were recorded from 3 independent experiments. Error denotes SEM. **** $p < 0.0001$ by one-way ANOVA with Tukey's multiple comparisons.

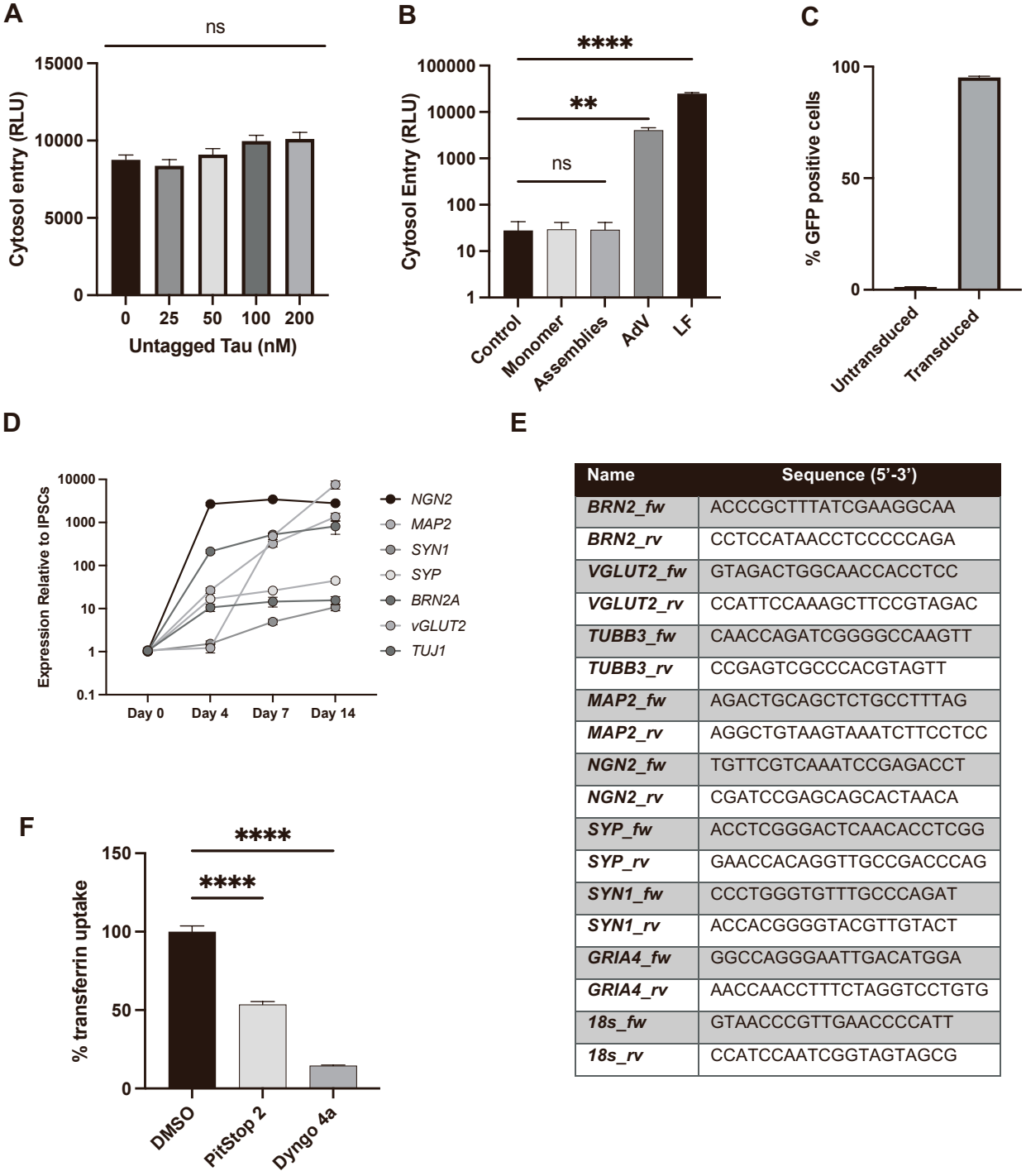
F) Tau entry in DIV 14 GPLN-neurons after pre-treatment with Dyngo 4a (20 μM), PitStop 2 (20 μM) or solvent DMSO for 1 h followed by a 1 h entry assay with 50 nM tau-HiBiT assemblies. Neurons were transduced at DIV 9; n=3. Error denotes SEM.

G and H) Entry of 50 nM tau-HiBiT monomers 1 h after challenge with indicated concentrations of PitStop 2, Dyngo 4a (**G**) or DMA (**H**). GPLN-neurons were pre-treated for 1 h prior to assay; n=3, N=3 independent experiments. Error denotes SEM.

I) Presto blue viability assay of GPLN-neurons after tau entry assay and Dyngo 4a (40 μM), DMA (250 μM), Bafilomycin-A (200 nM), PitStop 2 (40 μM), or solvent control (DMSO) treatment throughout, showing no reduction in viability with any drug compared to control; n=3. Error denotes SEM.

J and K) Full uncropped western blot from **Fig. 4** (main text) (**J**), and quantification of band densitometry (**K**) from 3 independent experiments. Error denotes SEM.

Figure S5 (related to Figure 4)



E

Name	Sequence (5'-3')
BRN2_fw	ACCCGCTTTATCGAAGGCAA
BRN2_rv	CCTCCATAACCTCCCCCAGA
VGLUT2_fw	GTAGACTGGCAACCACCTCC
VGLUT2_rv	CCATTCCAAAGCTTCCGTAGAC
TUBB3_fw	CAACCAGATCGGGGCCAAGTT
TUBB3_rv	CCGAGTCGCCCACGTAGTT
MAP2_fw	AGACTGCAGCTCTGCCTTTAG
MAP2_rv	AGGCTGTAAGTAAATCTTCCTCC
NGN2_fw	TGTTCGTCAAATCCGAGACCT
NGN2_rv	CGATCCGAGCAGCACTAACA
SYP_fw	ACCTCGGGACTCAACACCTCGG
SYP_rv	GAACCACAGGTTGCCGACCCAG
SYN1_fw	CCCTGGGTGTTTGCCCAGAT
SYN1_rv	ACCACGGGGTACGTTGTACT
GRIA4_fw	GGCCAGGGAATTGACATGGA
GRIA4_rv	AACCAACCTTTCTAGGTCCTGTG
18s_fw	GTAACCCGTTGAACCCCATT
18s_rv	CCATCCAATCGGTAGTAGCG

Figure S5 (related to Figure 4): Tau does not mediate its own entry and iPSC-derived neurons express mature markers.

A) Titration of untagged tau assemblies in the presence of 50 nM tau-HiBiT assemblies in HEK-NGL cells and entry quantified after 1 h; n=4. Error denotes SEM.

B) Endosomal lysis assay performed by delivery of NanoLuc-encoding plasmid to HEK293 cells in the presence of excess tau monomer or aggregate (1 µM), adenovirus (MOI 100 IU/cell) or LF (0.2 µl/well); n=3. Error denotes SEM. ** $p < 0.01$ **** $p < 0.0001$ by one-way ANOVA with Tukey's multiple comparisons.

C) Percent positive GFP cells of day 14 GPLN-iNeurons acquired by flow cytometry; n=3, N=3 independent differentiations. 10,000 events minimum recorded. Error denotes SEM.

D) qPCR of neuronal genes at day 0, day 4, day 7 and day 14 of the iPSC differentiation strategy to generate differentiated iNeurons; n=3 from N=3 independent differentiations. Error denotes SEM.

E) Primer sequences for genes targeted in qPCR.

F) Transferrin uptake assay in day 14 iNeurons with 10 µg/ml alexa-fluor-647-conjugated human transferrin. Cells were pre-treated for 1 h with PitStop 2 or Dyngo 4a (both 20 µM) prior to addition of transferrin for 15 minutes. $1x10^4$ events were recorded from 3 independent differentiations. **** $p < 0.0001$ by one-way ANOVA with Tukey's multiple comparisons.

Figure S6 (related to Figure 5)

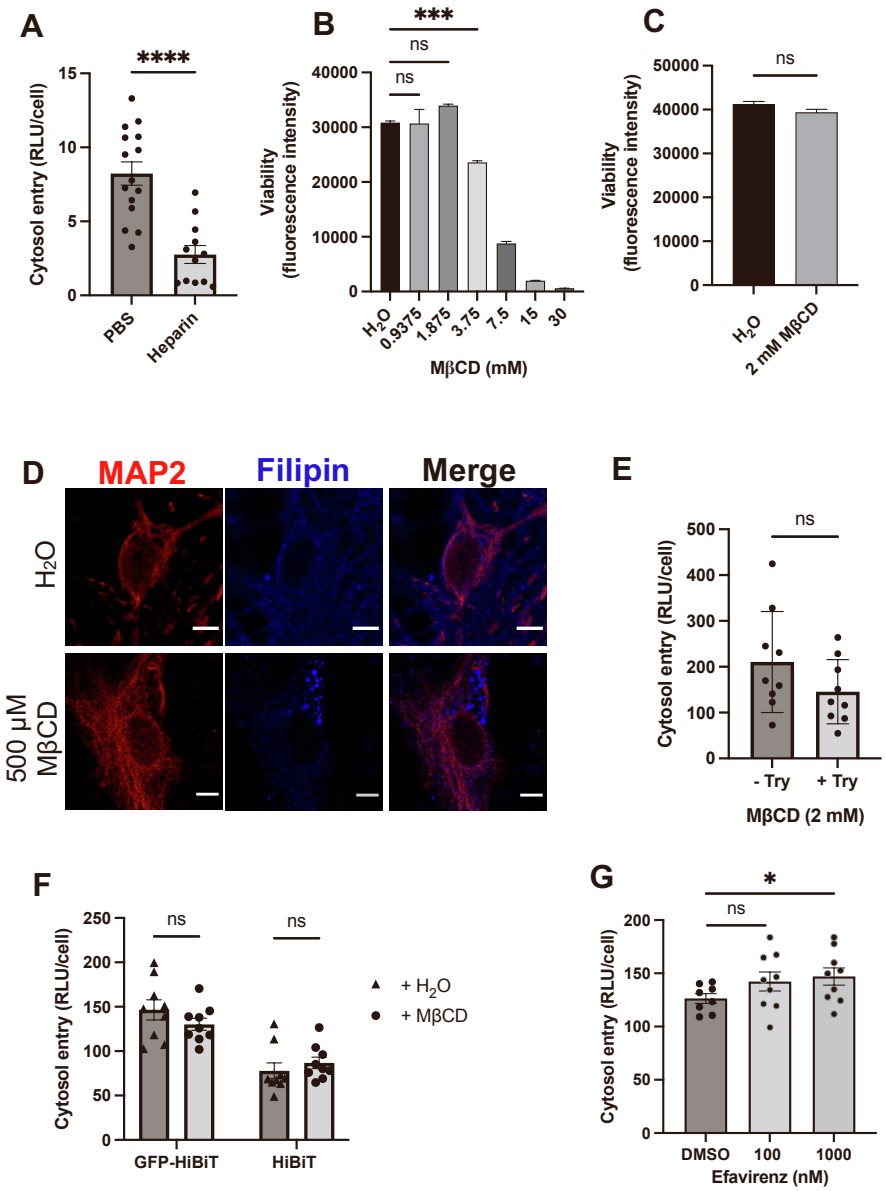


Figure S6 (related to Figure 5): MβCD does not induce cell leakiness and cholesterol-sensitivity is tau specific.
A) Effect of heparin on tau entry in GPLN-iNeurons. Cells were pre-treated with heparin (40 µg/ml) or solvent (PBS) for 1 h prior to assaying the entry of 50 nM tau-HiBiT assemblies in 1 h, n=3-6 from N=3 independent differentiations. Error denotes SEM. **** $p < 0.0001$ by unpaired t test.
B) Presto blue viability assay of GPLN-neurons after 30 minutes pre-treatment with indicated concentration of MβCD followed by a 1 h entry assay; n=3. Error denotes SEM. *** $p < 0.001$ by one-way ANOVA with Tukey's multiple comparisons.
C) Presto blue viability assay of GPLN-neurons after pre-treatment with 2 mM MβCD for 2 h or solvent (water) by a 1 h entry assay; n=6. Error denotes SEM.
D) Total cholesterol staining of DIV 7 primary neurons with Filipin after treatment with solvent (water) or 500 µM MβCD overnight. Scale bars, 5 µm.
E) Effect of trypsin (Try) on entry signal after MβCD treatment. GPLN-neurons were pre-treated for 2 h with MβCD followed by a 1 h entry assay with 50 nM tau-HiBiT assemblies. Cells were then treated with or without Try for 10 minutes at 37ºC and signal acquired; n=3, N=3 independent experiments. Error denotes SEM.
F) Effect of MβCD treatment on the entry of 6xHis-GFP-HiBiT or HiBiT peptide entry alone in GPLN-neurons. Cells were pre-treated for 2 h with 2 mM MβCD or solvent (water) and a 1 h entry assay performed with 50 nM of GFP-HiBiT or HiBiT peptide; n=3 from N=3 independent experiments. Error denotes SEM.
G) Effect of Efavirenz (Efv) on tau entry. GPLN-neurons were pre-treated for 16 h with depicted concentrations of Efv and a 1 h entry assay with 50 nM tau-HiBiT assemblies performed; n=3, N=3 independent experiments. * $p < 0.05$ by unpaired t test.

Figure S7 (related to Figure 6 and 7)

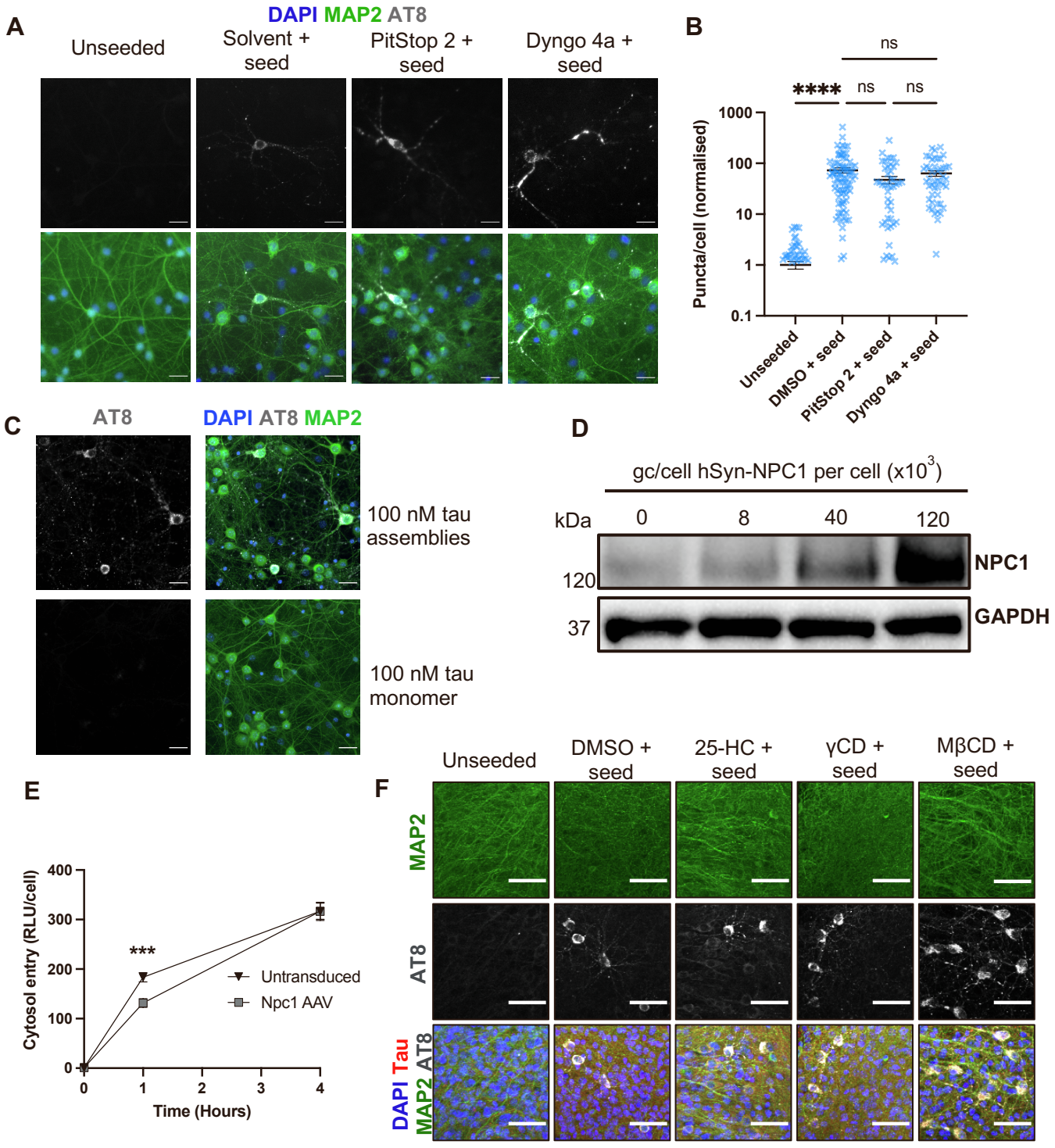


Figure S7 (related to Figure 6 and 7): Endocytosis inhibition does not change seeding and cholesterol manipulation affects neuron entry and slice seeding.

A and B) Fluorescence microscope images and quantification of seeded P301S neurons after pre-treatment with PitStop 2 (20 μM), Dyngo 4a (2 μM) or solvent (DMSO) for 1 h prior to a 7 day seeding assay with 100 nM tau assemblies. AT8 positive puncta were quantified as shown in (**B**); n=3, N=3 independent experiments. Scale bars, 25 μm. Error denotes SEM. **** $p < 0.0001$ by Kruskal-Wallis test with Dunn's multiple comparisons.

C) Fluorescence microscope images of P301S neurons seeded with 100 nM tau assemblies or monomer for 7 days prior to fixation and immunostaining. Scale bars, 30 μm.

D) Western blotting of DIV 7 WT neuron lysates after transduction with AAV1/2 hSyn-NPC1 at depicted multiplicity 96 h prior.

E) Entry of 50 nM tau-HiBiT assemblies to GPLN-neurons transduced with 100,000 genome copies/cell of AAV1/2-hSyn-NPC1 for 96 h prior to assay on DIV 7; n=3, N=3 independent experiments. Error denotes SEM. *** $p < 0.001$ unpaired t test.

F) Fluorescence microscope images of OHSCs quantified in **Fig. 7** (main text). Scale bars, 50 μm.