Supplementary Information

Table S1
Figures S1-S9

Internalized a-synuclein fibrils are rapidly truncated and resists degradation in neurons while glial cells rapidly degrade α-synuclein fibrils.

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Loading Controls	Host	Source	Reference	Use
GAPDH (D16H11)	Rbt	Cell Signaling	5174	WB
α-tubulin	Rbt	Abcam	4074	WB
α-Synuclein Species		**Company**	**Reference**	**Use**
α-Synuclein (total, Syn1)	Rt	BD Transduction	610787	WB
HuαS	Rbt	In House	[1]	WB/ICC
LB509	Ms	Abcam	27766	WB/ICC
NAC-2 αS/N-2 αS	Rbt	Pekka Jäkälä, Kupio University	[1]	WB
Pan-Syn	Rbt	Abcam	ab53726	WB
Glial and Neuronal Markers		**Company**	**Reference**	**Use**
Iba1	Rbt	Wako Chemical	019-19741	ICC
GFAP	Rbt	Dako Cytomation	Z0334	ICC
NeuN	Ms	Millipore	MAB377	ICC
O4	Ms	R&D System	MAB1326	ICC
Autophagy/Lysosome Function/Organelle		**Company**	**Reference**	**Use**
LC3	Rbt	Cell Signaling	2775	WB
p62	Rbt	Cell Signaling	5114	WB
pS6	Rbt	Cell Signaling	2211	WB
S6 (total)	Ms	Cell Signaling	2317	WB
4EBP	Rbt	Cell Signaling	9644	WB
CTSD	Rbt	Abcam	ab75852	WB/ICC
Gal3	Ms	Abcam	Ab2785	ICC
Poly-Ubq	Rbt	Dako	Z0458	WB/ICC
Grp78	Rbt	Novus	NB300-520	ICC
Lamp1	Rbt	LS-Bio	LS-B4246	WB, ICC
Lamp2	Rbt	LS-Bio	LS-B581	ICC
EEA1	Rbt	Gene Tex	GTX 109638	WB, ICC
GM-130	Rbt	Novus	NBP2-53420	ICC
p62	Ms	Abcam	Ab56416	ICC
LC3	Rbt	Cell Signaling	3868	ICC

Table S1. List of primary antibodies utilized in experiments. Ms, Mouse; Rt, Rat; Rbt, Rabbit; WB, western blot; ICC, immunocytochemistry.

1. Li, W., et al., *Aggregation promoting C-terminal truncation of alpha-synuclein is a normal cellular process and is enhanced by the familial Parkinson's disease-linked mutations.* Proc Natl Acad Sci U S A, 2005. **102**(6): p. 2162-7.

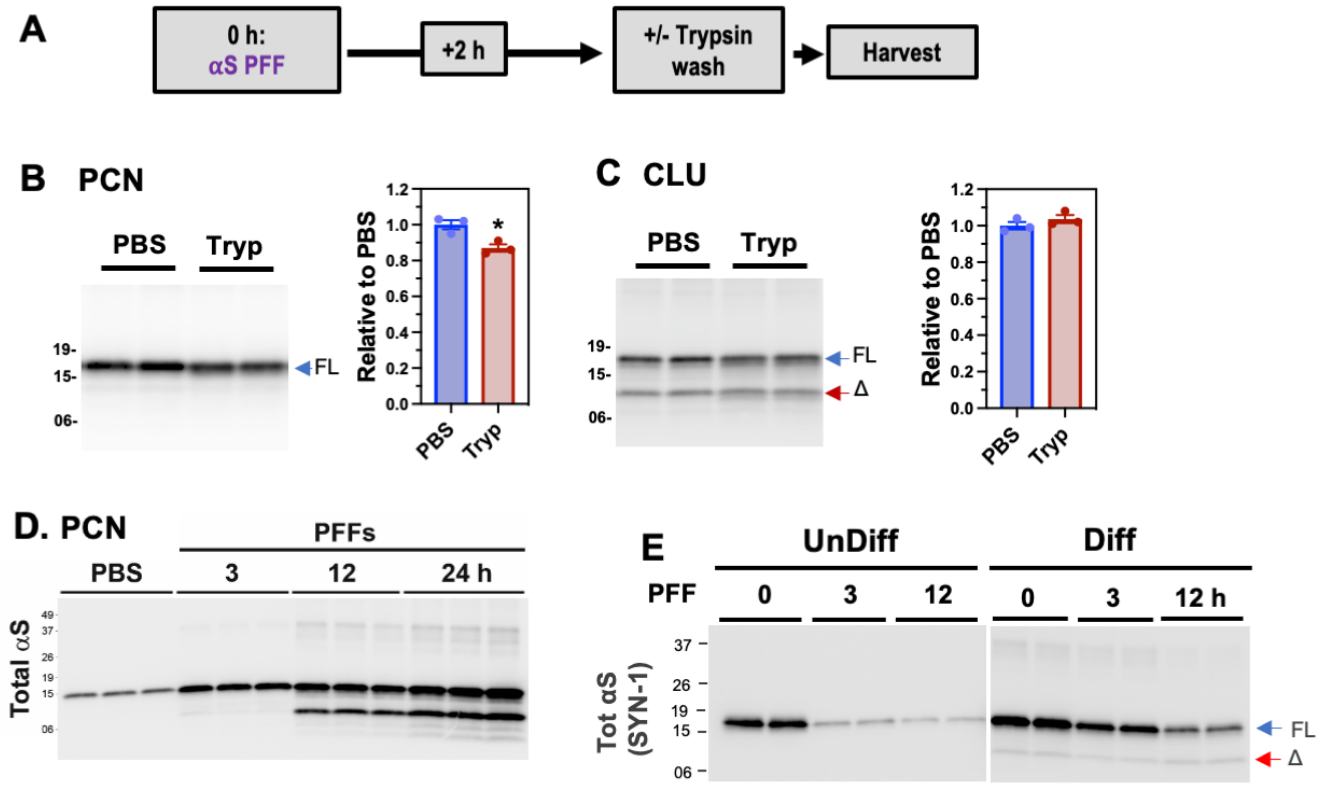

Figure S1. Comparison of PBS wash and Trypsin wash in removing uninternalized αS PFF (A-D) and metabolism of αS PFF in SH-SY5Y cells (E). Scheme **(A)** showing cultured primary cortical neuron **(PCN, B)** or CLU198 cells **(CLU, C)** incubated with 4 µg/ml αS PFF for 2 hours and washed with PBS or trypsin before the harvest. The levels of Tot αS were detected by immunoblot analysis. **B)** In PCN, approximately ~85-90% residual αS following PBS wash is accounted by trypsin resistant fraction. **C)** In CLU cells, the amount of residual αS is not different between PBS and trypsin-washed cells. These results indicate that the bulk of residual αS following PBS wash is internalized αS (*$p<0.05$, t-test, Mean±SEM; n=3). **D)** Analysis αS PFF uptake in PCN without washing shows that αS continues to increase past the 24 hours following PFF treatment. Thus, uptake of new αS PFF occurs for prolong periods without washing. **E)** Uptake and metabolism of αS PFF in an undifferentiated **(UnDiff)** and neuronally differentiated **(Diff)** human neuroblastoma cell line (SH-SY5Y cells). Immunoblot analysis of total lysates for αS shows that, similar to the CLU cells (Fig. 2), internalized αS is rapidly degraded in undifferentiated SH-SY5Y cells. In differentiated SH-SH5Y cells, internalized αS is more stable with the presence of the truncated αS (Δ).

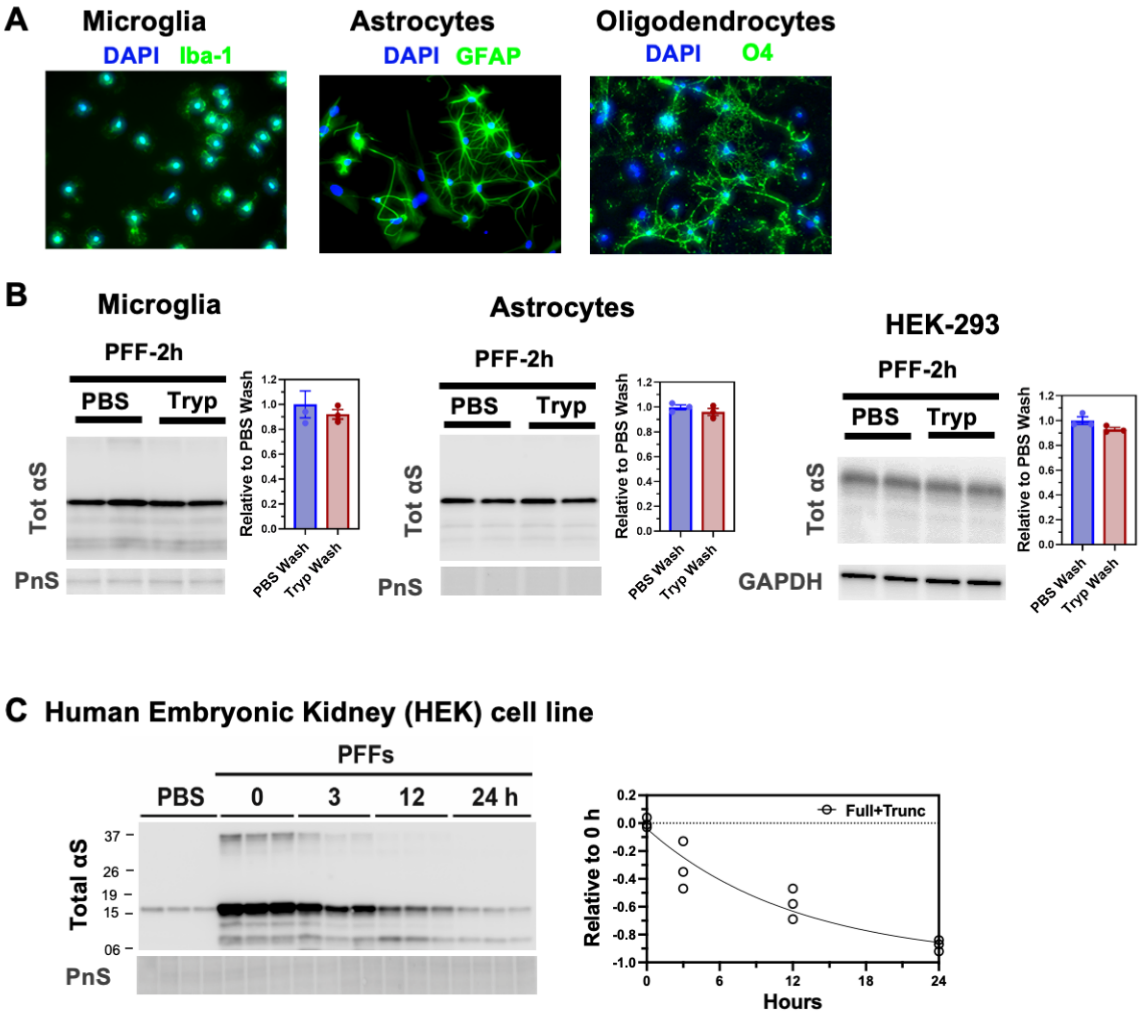

Figure S2. (A, B) Uptake of αS pre-formed fibrils (PFFs) in glial cells. (A) primary glial cells were isolated and cultured from newborn mouse pup's cortex (See Methods and Materials) and the purity was confirmed by staining with specific cellular marker Iba1, GFAP, and O4 antibodies for microglia, astrocytes, and oligodendrocytes, respectively. **(B)** Indicated cells were also incubated with 4 µg/ml αS PFF for 2 hours followed by PBS or trypsin wash before harvesting. The levels of Tot αS were detected, (Mean±SEM; n=3). **(C) Degradation of αS PFFs in HEK-293 cells.** Cells were pre-incubated for 2h with 4 µg/ml αS PFF followed by trypsin wash. After washing, cells were replenished with full media and incubated for the indicated time and levels of Tot αS were detected. The graph shows the amount of residual αS remaining from the 0 h time point. Ponceau S (PnS) protein stain or GAPDH were used to confirm loading.

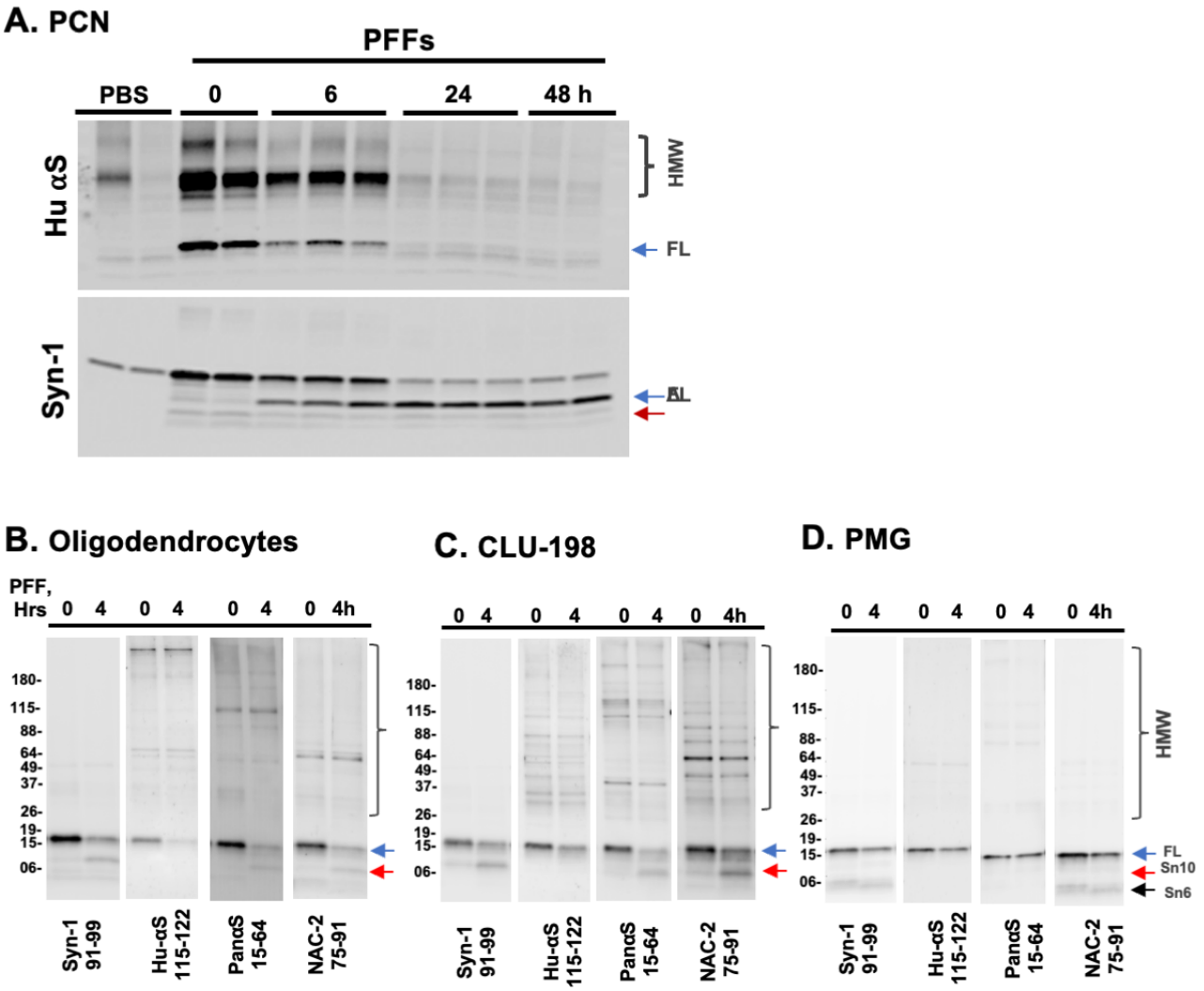

Figure S3. Metabolism of the C-terminal αS epitope in neurons and glial cells. (A) Primary cortical neurons (PCN) were pre-incubated for 2h with 4 µg/ml αS PFF. After washing off excess PFF, cells were replenished with full media and incubated for the indicated time. Total lysates were used for αS Immunoblot analysis using HuαS specific (top) and Syn-1 (bottom) antibodies. Since the epitope for HuαS antibody is located within the C-terminal region (amino acids 115-122), the results show that internalized αS is C-terminally truncated. **(B)** Primary oligodendrocytes, **(C)** neuronally differentiated CLU-198 cells, and **(D)** Primary microglia (PMG) were pre-incubated for 2h with 4 µg/ml αS PFF followed by trypsin wash. The cells were replenished with full media and incubated for 4 hours before harvesting. Total lysates were used for epitope mapping of the truncated αS. In all cells, the C-terminal HuαS epitope is missing in the truncated variants. While the N-terminal epitope recognized by the Pan-αS antibody is present in FL and Sn10 variants, the N-terminal region is missing in lower MW variant(s) (e.g.Sn6).

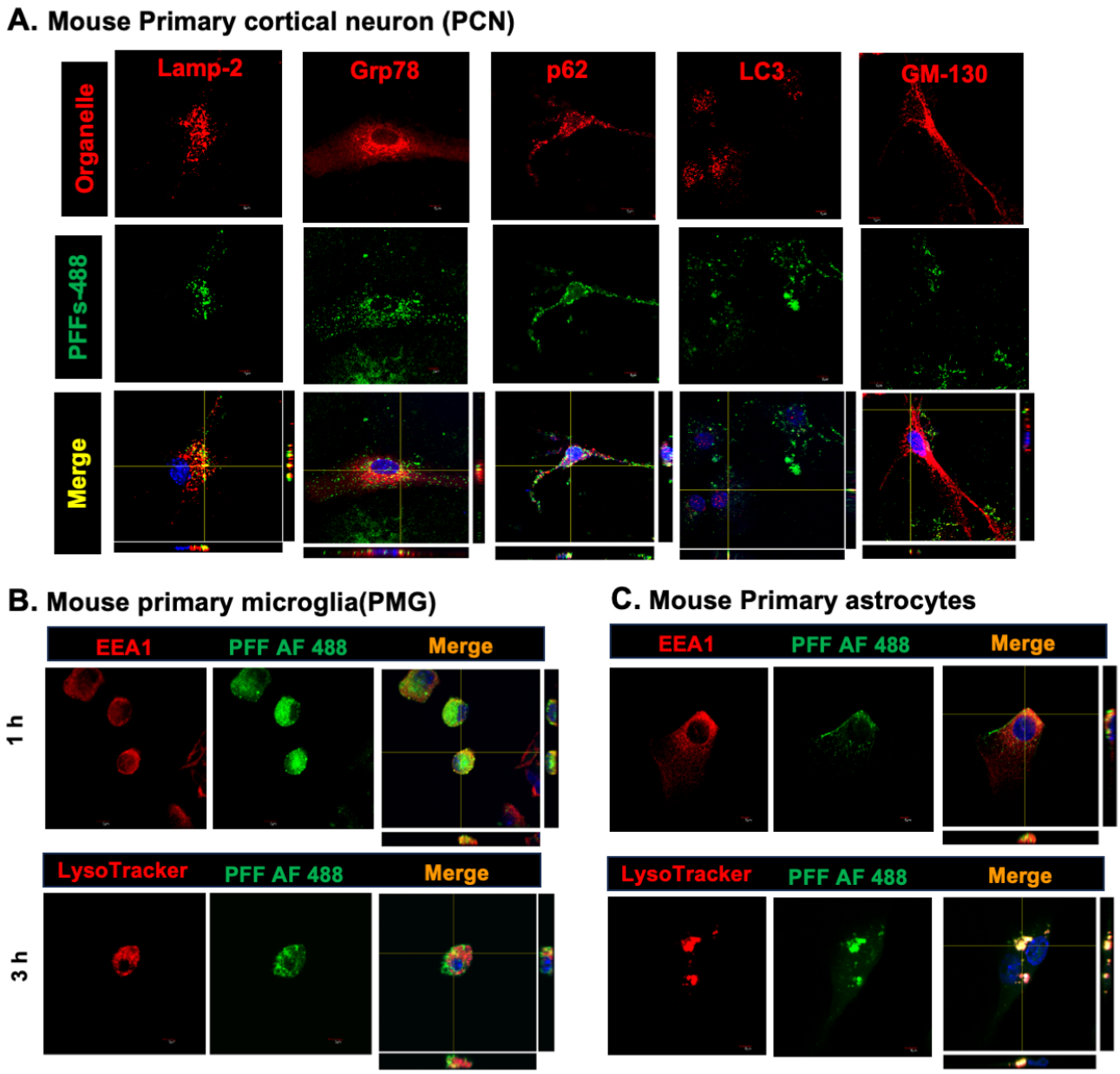

Figure S4. Localization of internalized αS pre-formed fibrils (PFFs) with intracellular organelles. (A) Primary cortical neurons (PCN) were treated with PFFs/PFFs-AF488 for 3 hours and the cells were washed with trypsin to remove excess PFFs before fixing cells. PFFsAF488 (green) were colocalized with various organelle markers: Lysosome, Lamp-2; Endoplasmic Reticulum, Grp-78; Lysosomal substrate, p62; Autophagosome, LC3; Golgi, GM130. **(B,C)** Internalized αS PFFs traffics to endosome and Lysosomes in glial cells. Primary microglia (PMG) **(B)** and astrocytes **(C)** were treated with PFFs-AF488 and colocalized with the early endosome marker (EEA1) (red) at 1 h and Lysosomes (LysoTracker) at 3 h.

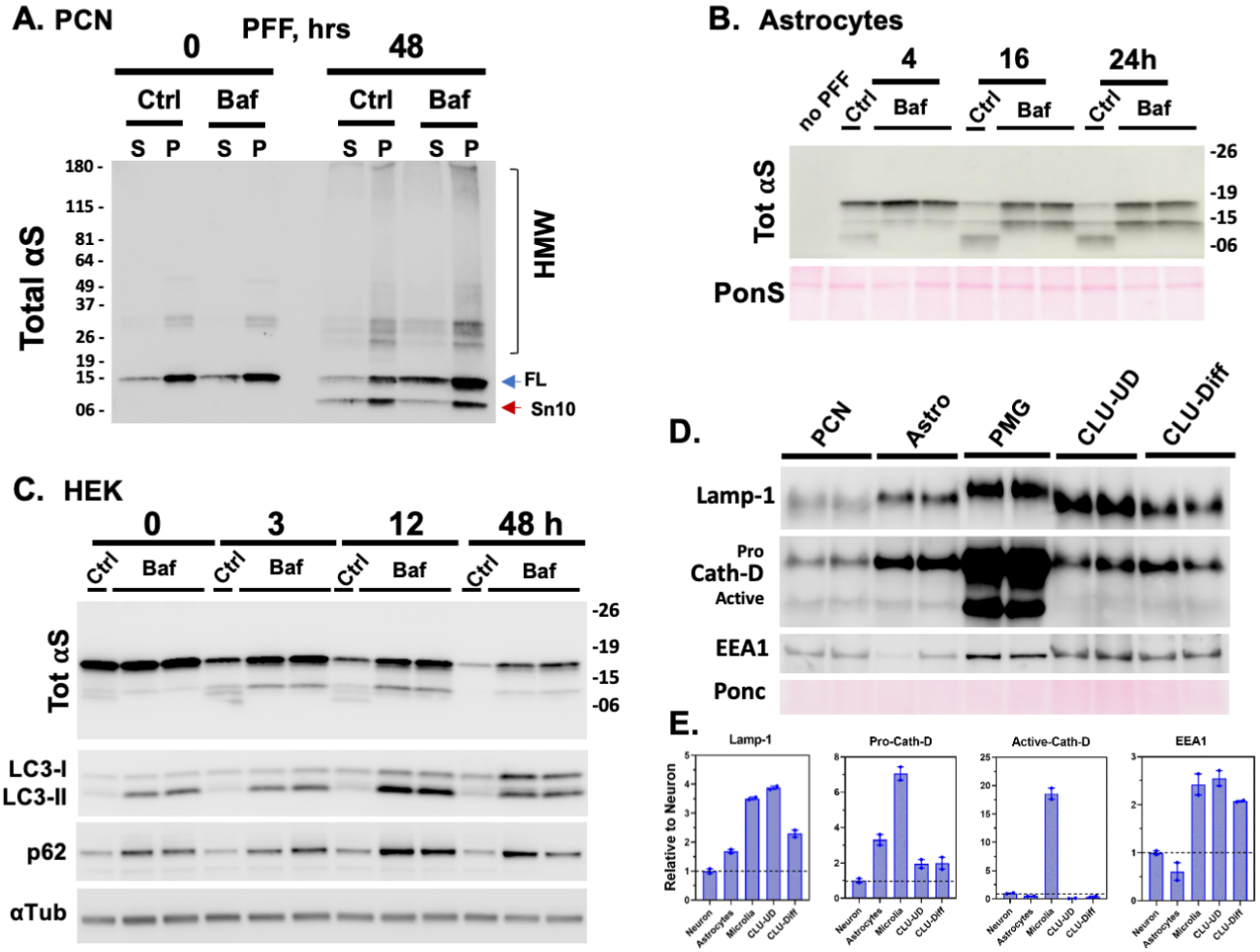

Figure S5. (A) Lysosomal inhibition stabilizes both low and high molecular weight αS. Mouse primary cortical neurons (PCN) were pre-incubated for 2h with 100 ηM bafilomycin A1 prior addition of 4 μg/ml pre-formed fibrils αS (PFF) for 2h. After trypsin-wash, cells were replenished with full media and Baf for the indicated time. Triton X-100 soluble (S) and insoluble (P) fractions were separated and analyzed for Tot αS (Syn-1) and Pan-S by immunoblotting. (**B, C) Lysosome inhibition stabilizes internalized αS PFF in astrocytes (B) and HEK293 cells (C).** Cells were pre-incubated for 100 nM Baf and 4 μg/ml αS-PFF and lysates were collected at indicated times. Immunoblot analysis of αS shows that while most of αS is metabolized by 12-16 hours in astrocytes (**B**) and HEK293 cells (**C**). Baf treatment leads to continued accumulation of αS, even at 24-48 h. Also shown is Ponceau-S total protein stain or α-tubulin immunoblot to control for equal loading. Immunoblot analysis of LC3-I/-II and p62 verify the inhibition of lysosomes. (**D, E) Neuronal cells have lower lysosomal markers. D)** Primary cortical neuron (PCN), primary astrocytes, primary microglia (PMG), and differentiated or undifferentiated mouse hippocampal CLU cells were harvested, and lysosomal markers (Lamp-1, Cathepsin D, and EEA1) were evaluated by immunoblotting. (**E)** Quantitative analysis of immunoblot shown in D. While statistical analysis was not done, the duplicate samples clearly show that lysosomal markers are lower in neurons than in non-neuronal cells.

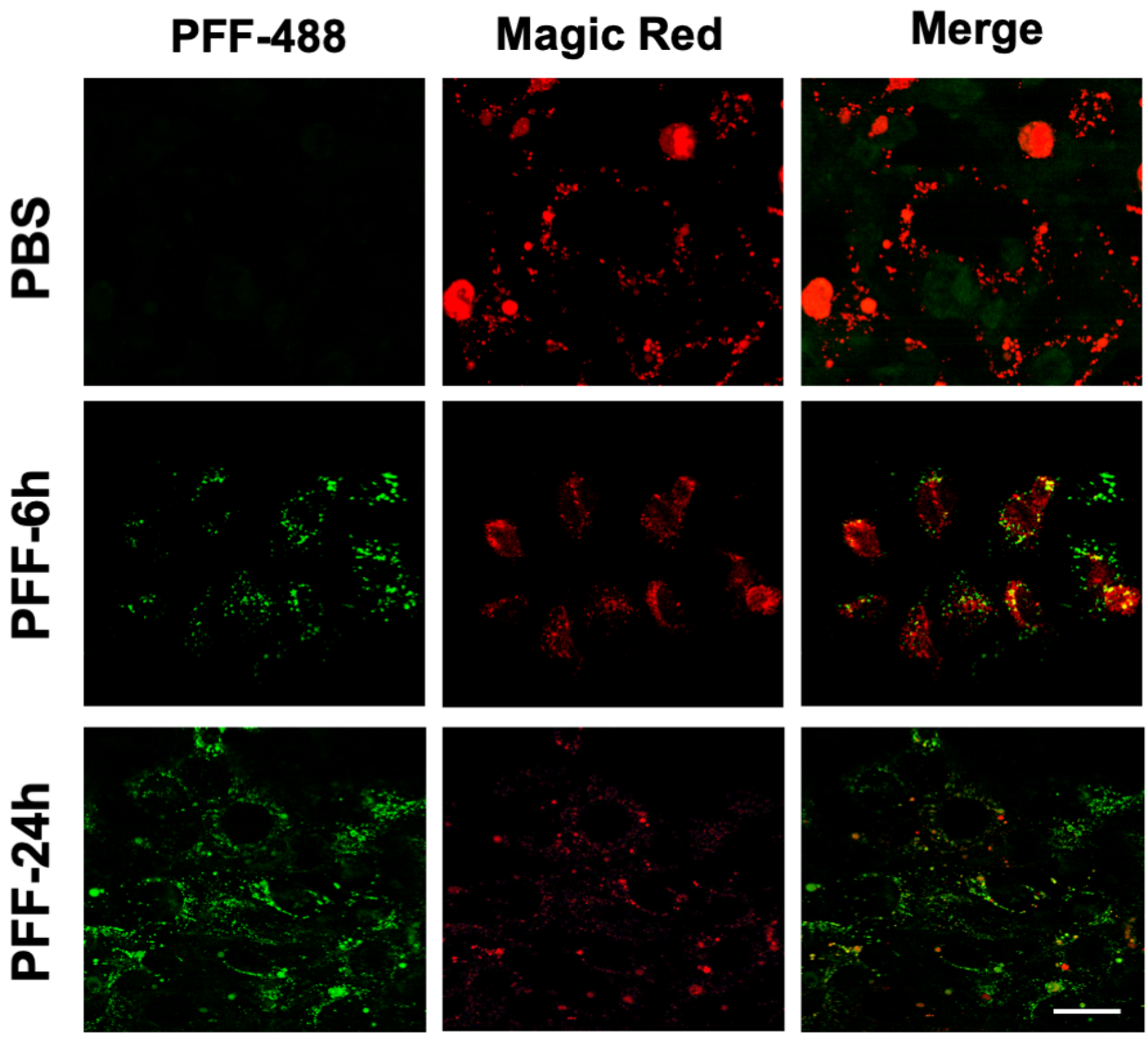

Figure S6. Internalized a**S PFFs inhibit lysosomal function in neuronal cells.** Neuronally differentiated CLU198 cells were treated with PFF-AF488, washed, and incubated for 6- or 24-h before confocal live cell imaging. The cells were also treated with Magic Red Cathepsin-B assay reagent for the last hour before imaging. The overall Magic Red signal appears reduced in PFF-treated cells, even at 6-h post-PFF treatment. Quantitative analyses of 24 h time point is shown in Fig. 8. Bar=10 μm.

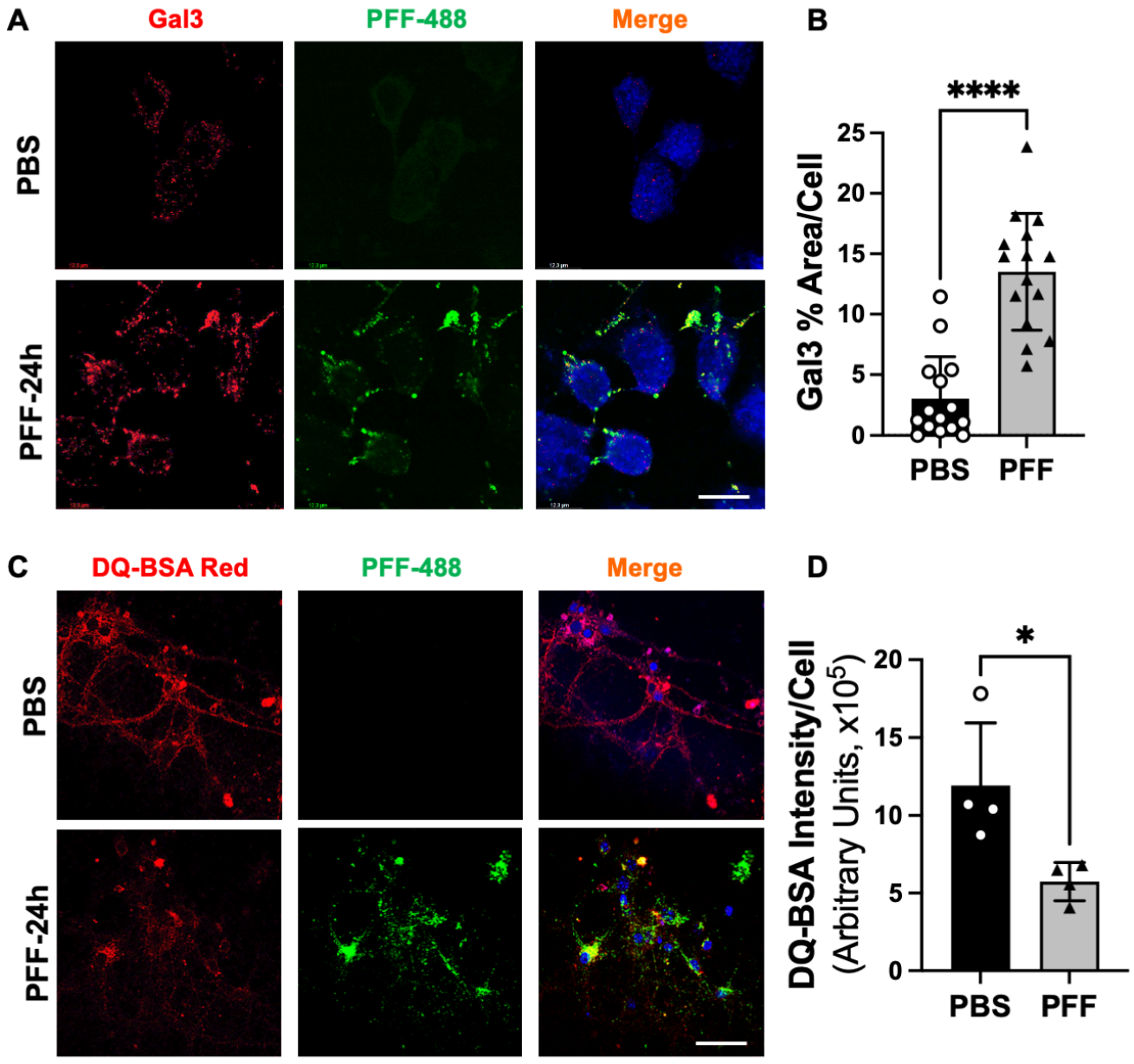

Figure S7. Internalization of a**S PFFs leads to lysosomal damage and lysosomal dysfunction in neuronal cells. A, B)** Neuronally differentiated CLU198 cells were treated with PFF-AF488, washed, and incubated for 24-h, fixed in 4% PFA, and stained for Gal3, an indicator of lysosomal damage. The cells were imaged using confocal microscopy. Representative confocal images show increased Gal3 staining in PFF-treated cells **(A)**. **C, D)** Primary cortical neurons were treated with PFF-AF488, washed, and incubated for 24-h and treated with DQ-BSA Red, which is hydrolyzed in functional lysosomes to produce red fluorescence. Confocal images show that PFF treatment is associated with reduced DQ-BSA Red signal **(C)**. Quantitative analysis of total DQBSA Red signal intensity, normalized to the number of cells, confirms that PFF treatment leads to lysosomal dysfunction in neurons **(D)**. *p<0.05, unpaired t-test, n=4 independent cultures. Bar=10 μm (A), 50 μm (C).

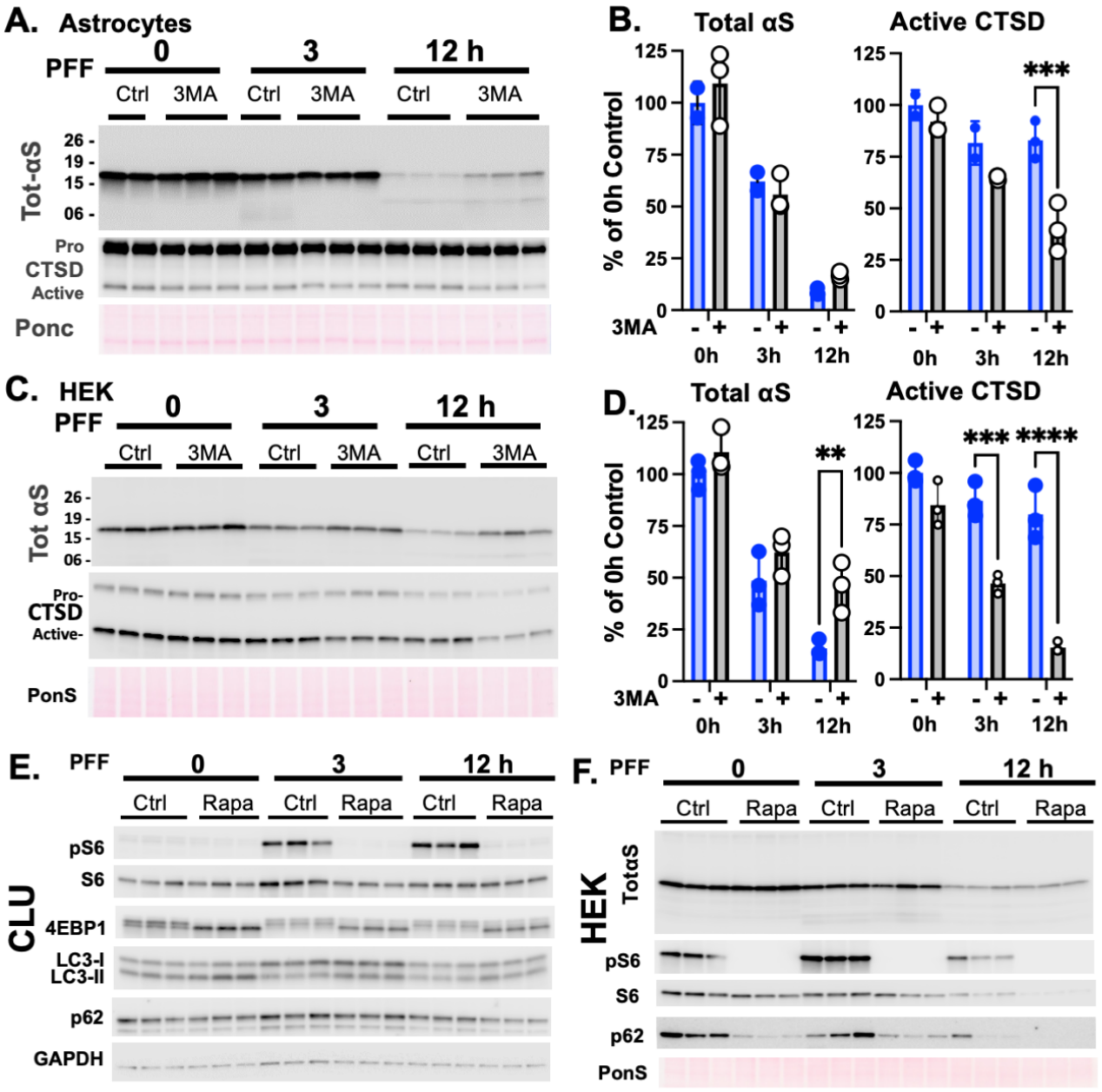

Figure S8. (A-D) Inhibition of autophagy leads to modest lysosomal inhibition with a modest impact on αS accumulation in astrocytes and HEK293 cells. Primary cultures of astrocytes (**A, B**) and HEK293 cell line (**C, D**) treated with 3MA to inhibit autophagy as described in Figure 8. The levels of internalized αS PFF and the lysosomal protease, Cathepsin-D (CTSD), determined by immunoblot analysis, show that 3MA treatment leads to a modest but significant increase in αS levels in HEK293 cells (**C, D**) and decreased active CTSD levels in both Astrocytes and HEK293 cells (**B, D**). ***p<0.01, ***p<0.001, ****p<0.0001*, Two-way ANOVA. **(E, F) Rapamycin increases autophagy but does not affect αS PFF metabolism. (E**) CLU198 shown in Fig. 9B analyzed for mTOR inhibition (pS6, 4EBP) and autophagy (LC3-II and p62). In addition to inhibition of mTOR, Rapa treatment leads to increased LC3-II and reduced p62, indicating to enhanced autophagy. **(F)** In HEK293 cells, Rapa treatment does not affect the metabolism of internalized αS.

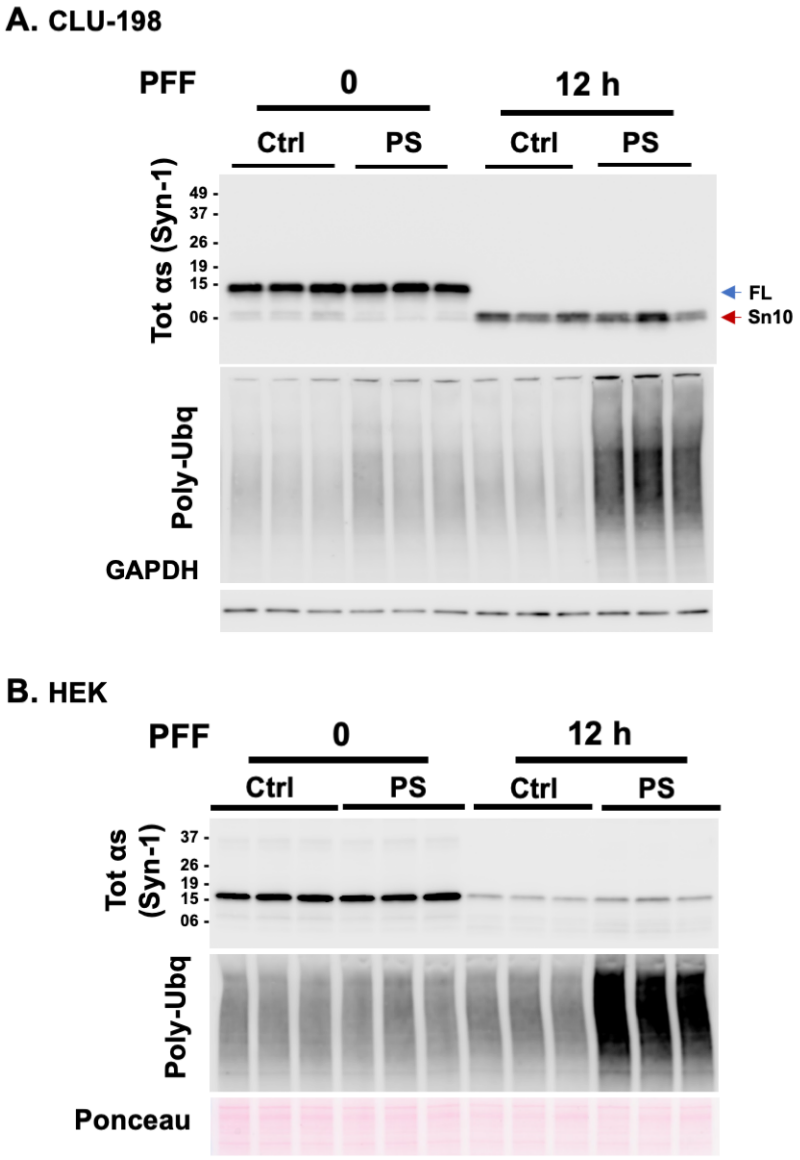

Figure S9. Proteosome inhibition does not impact αS PFF processing/metabolism. (A) Differentiated mouse hippocampal cell CLU-198 and **(B)** Human embryonic kidney HEK cells were pre-incubated with 15 nM PS-341 (PS; 4h) and 4 µg/ml pre-formed fibrils αS (PFFs) for 2h before the trypsin-wash. After the trypsin wash, cells were replenished with full media and PS for the indicated time. Tot αS (Syn-1), Poly-Ubiquitin (Poly-Ubq), and GAPDH were detected by immunoblotting analysis. Ponceau S was used in **B**. PS-341 treatment do not impact αS truncation in CLU-198 cells (A) and does not impact αS metabolism in HEK293 cells (**B**).