

Appendix

Astrocytic Cathepsin D promotes α -synuclein processing and Lewy neurite-like aggregate maturation

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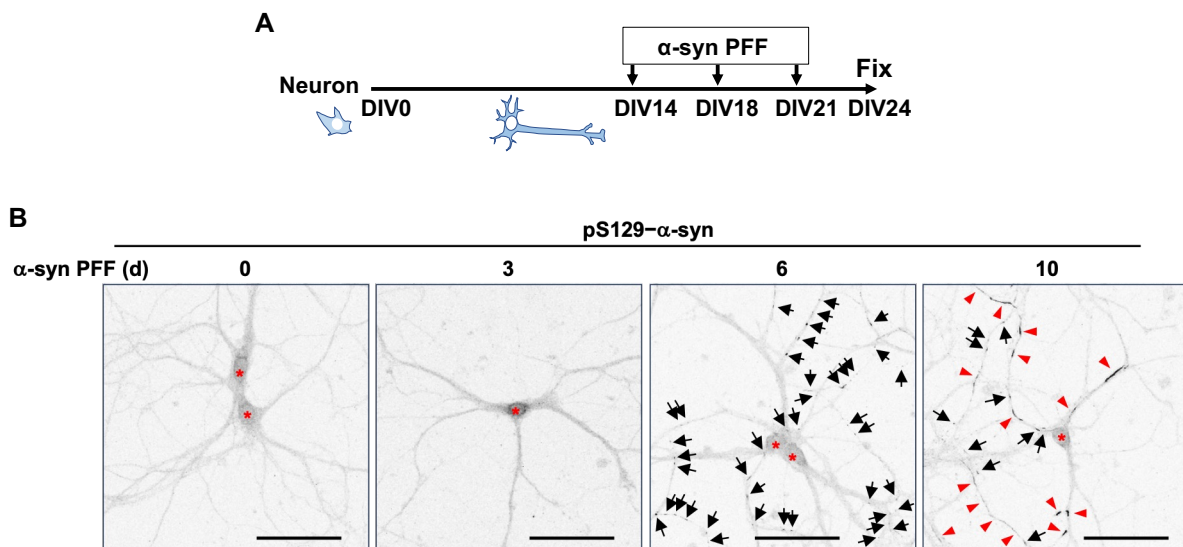
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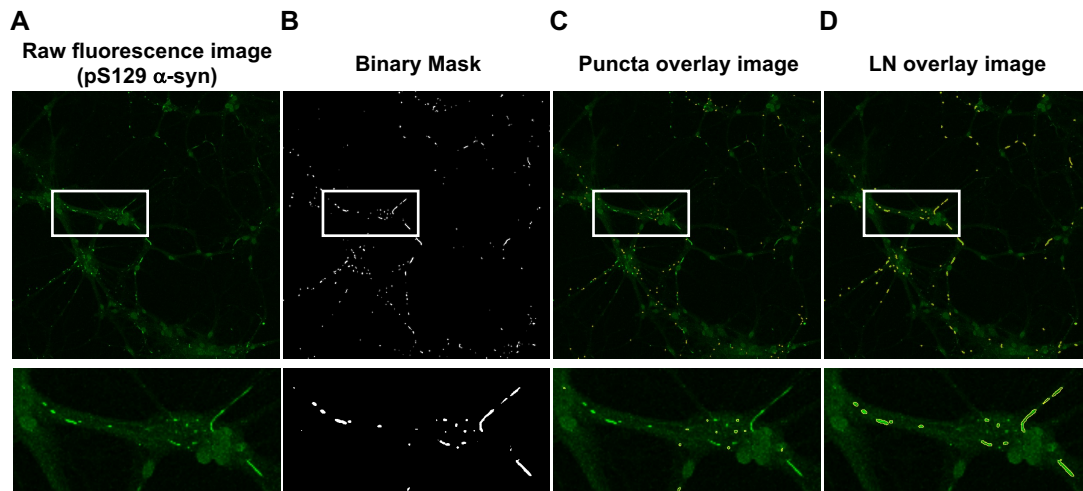
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Appendix Figure S1. α -syn PFFs induce α -syn aggregate formation in neurons.

(A) Experimental design. Primary hippocampal neurons were treated once with α -syn PFFs (0.5 μ g/ml) at DIV14, DIV18, or DIV21. All cultures were fixed at DIV24.

(B) Aggregate formation in neurons following α -syn PFF treatment. After incubation with α -syn PFFs for the indicated durations, neurons were fixed and immunostained for MAP2 (neurons) and pS129- α -syn (aggregates, inverted grayscale). Arrows indicate punctate aggregates, arrowheads indicate LN-like aggregates, and asterisks indicate nuclei. Scale bar, 50 μ m.



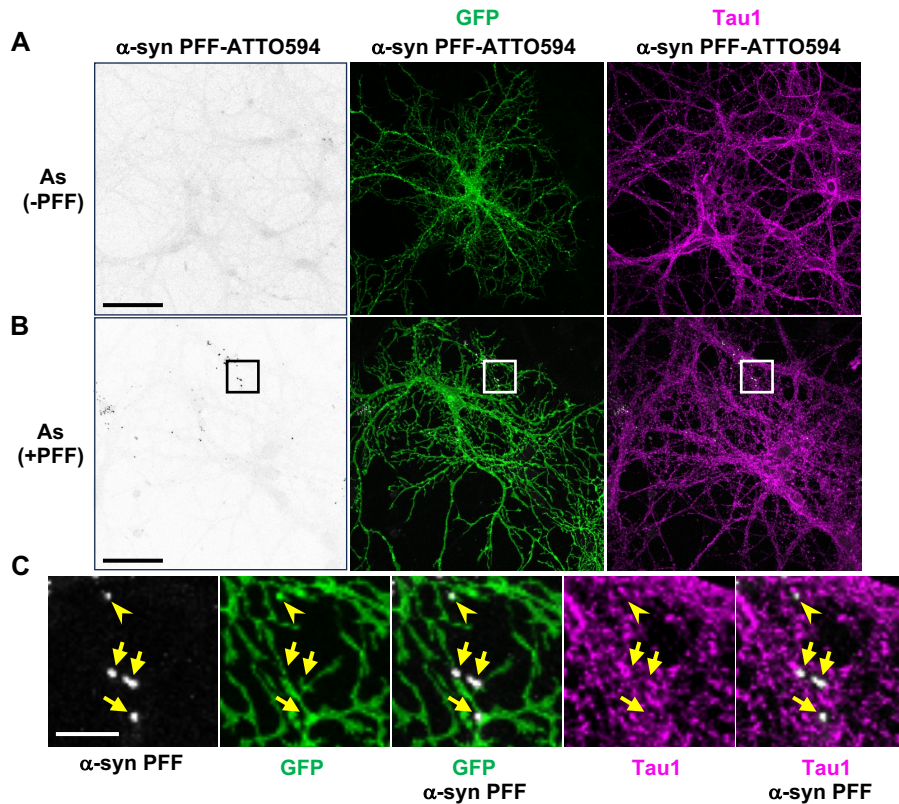
Appendix Figure S2. Example segmentation and classification of α -syn aggregates.

(A) Raw fluorescence image of pS129- α -syn immunostaining. Higher-magnification views of the boxed regions are shown in lower panels.

(B) Corresponding binary mask generated following background subtraction (rolling ball radius), Gaussian smoothing, and automatic thresholding using the MaxEntropy or Otsu method.

(C,D) Overlay images showing detected aggregates superimposed on the original fluorescence image.

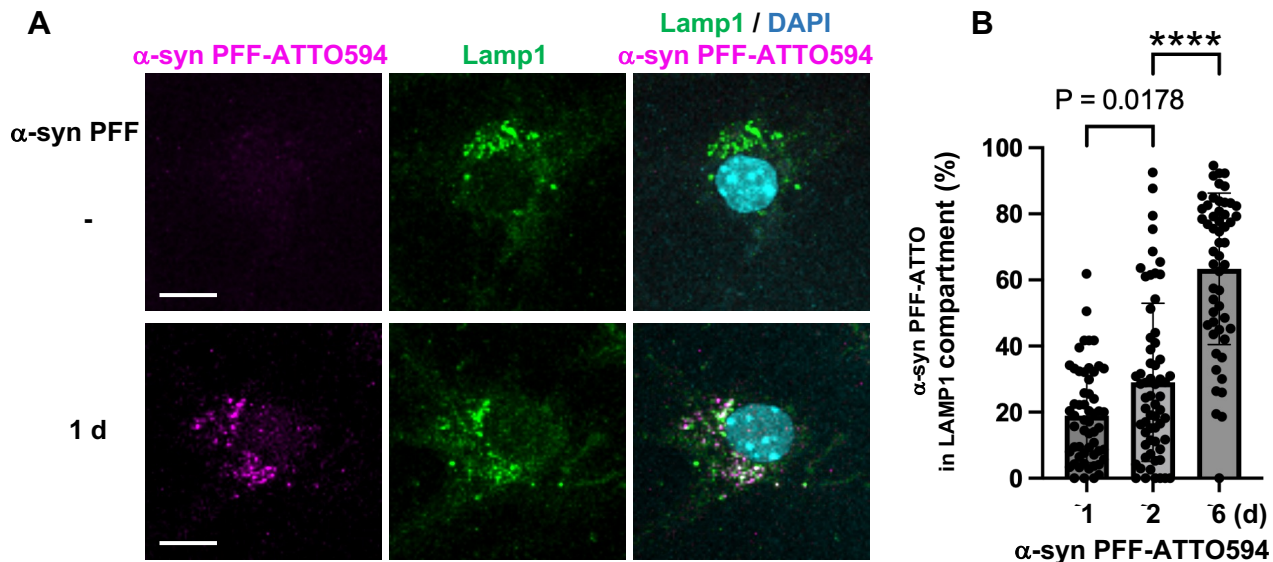
Puncta **(C)** were defined as small aggregates ($1\text{--}5\ \mu\text{m}^2$), whereas LN-like structures **(D)** were defined as larger ($\geq 5\ \mu\text{m}^2$) elongated aggregates with low ellipticity.



Appendix Figure S3. Astrocyte-to-neuron transfer of α -syn PFFs.

(A,B) Transfer of α -syn PFFs from astrocytes to neurons. Astrocytes expressing Mem-GFP were treated with or without α -syn PFF-ATTO594 (0.5 μ g/ml) for 24 h, passaged, and subsequently co-cultured with naïve neurons for 10 days. Cultures were fixed and immunostained for Tau1 (axons, magenta). GFP labels astrocytes. As (-PFF) indicates untreated astrocytes, whereas As (+PFF) indicates α -syn PFF-ATTO594-preloaded astrocytes. Scale bar, 50 μ m.

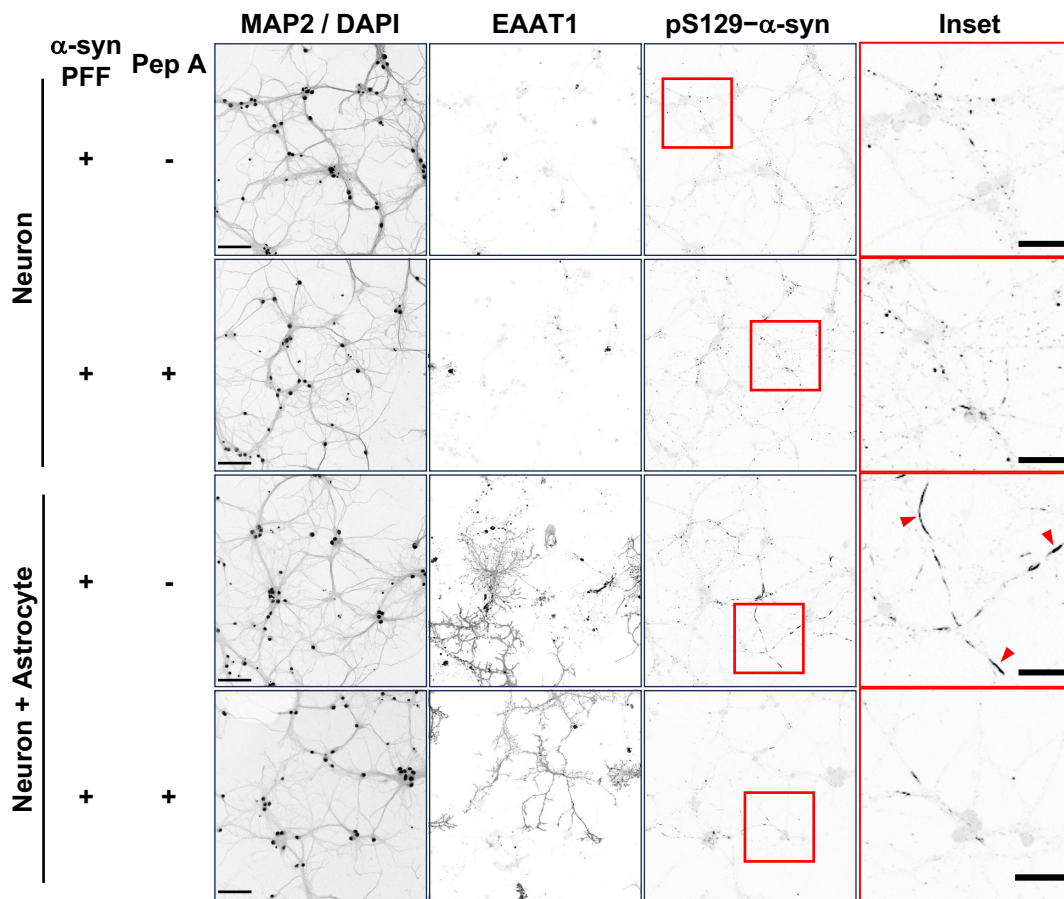
(C) Higher-magnification views of the boxed regions in **B**. Arrowheads indicate α -syn PFF-ATTO594 puncta on GFP-positive astrocytic processes, whereas arrows indicate puncta along neuronal axons. Scale bar, 10 μ m.



Appendix Figure S4. Localization of internalized α -syn PFFs in lysosomes.

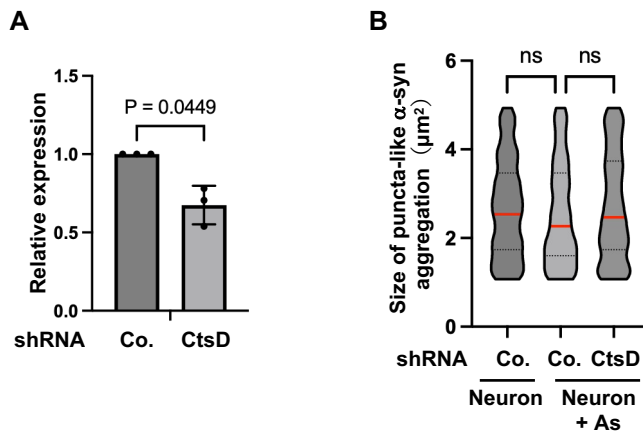
(A) Astrocytes differentiated from neurospheres were treated with α -syn PFF-ATTO594 (0.5 μ g/ml) for 1 day and immunostained for LAMP1 (lysosomes, green). DAPI labels nuclei (cyan). Scale bar, 10 μ m.

(B) Quantification of the co-localization between α -syn PFFs and LAMP1. The percentage of PFF signals localized within LAMP1-positive compartments was calculated ($n = 50$ cells per condition). Data are representative of three independent experiments. Statistical significance was assessed using the Kruskal–Wallis test followed by Dunn’s multiple-comparison test. **** $p < 0.0001$.



Appendix Figure S5. Effect of Pep A on neuronal α -syn aggregation.

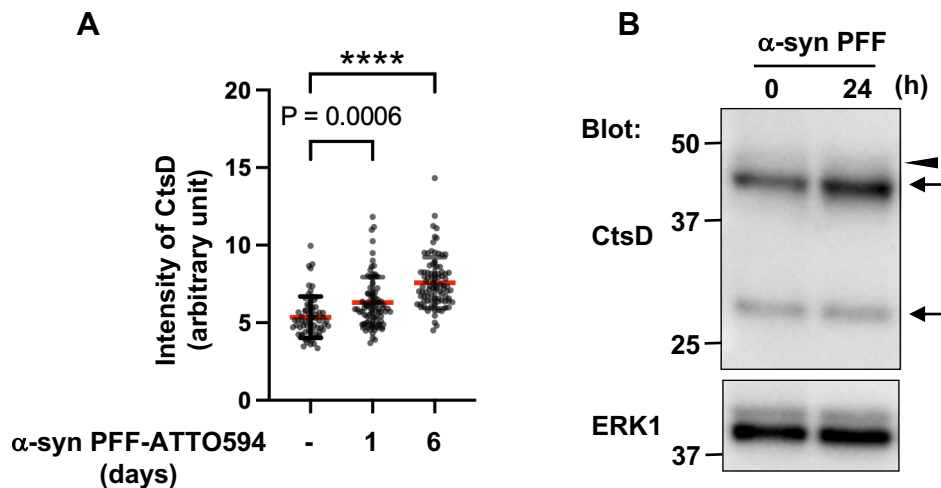
Neuron-only cultures and neuron–astrocyte co-cultures were treated with α -syn PFFs (0.5 μ g/ml) in the presence or absence of Pep A (10 μ M) for 10 days, with Pep A replenished every 3–4 days. Cells were immunostained for MAP2 (neurons), EAAT1 (astrocytes), and pS129- α -syn (aggregates). DAPI labels nuclei. Insets show higher-magnification views of the boxed regions. Arrowheads indicate large LN-like α -syn aggregates (>30 μ m²) in neurons. Scale bars, 50 μ m (left) and 25 μ m (insets).



Appendix Figure S6. Effect of CtsD knockdown on puncta-like α -syn aggregation in neurons.

(A) Efficiency of CtsD knockdown by shRNA in astrocytes. Astrocytes differentiated from neurospheres were infected with AAV2 vectors expressing control (Co.) shRNA or CtsD-targeting shRNA for 4 days, followed by RT-qPCR analysis. Relative CtsD mRNA expression levels were normalized to GAPDH. Statistical significance was assessed using an unpaired two-tailed Student's *t*-test.

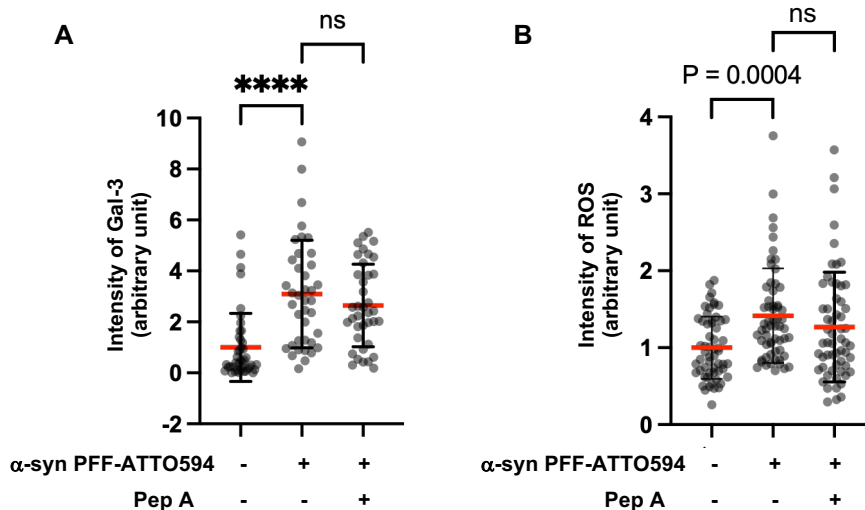
(B) Quantification of puncta-like α -syn aggregates. The size of pS129- α -syn-positive signals was measured across 10 fields of view per condition. Data are representative of three independent experiments. Red bars in violin plots indicate medians. Statistical significance was assessed using the Kruskal-Wallis test followed by Dunn's multiple-comparison test. ns, not significant.



Appendix Figure S7. α -syn PFF uptake induces CtsD expression in astrocytes.

(A) Quantification of CtsD expression. Astrocytes differentiated from neurospheres were treated with α -syn PFF-ATTO594 (0.5 μ g/ml) for the indicated durations and immunostained for CtsD. Data are presented as mean $\pm$ s.d. ($n = 80$ cells) and are representative of three independent experiments. Statistical significance was assessed using the Kruskal–Wallis test followed by Dunn’s multiple-comparison test. **** $p < 0.0001$.

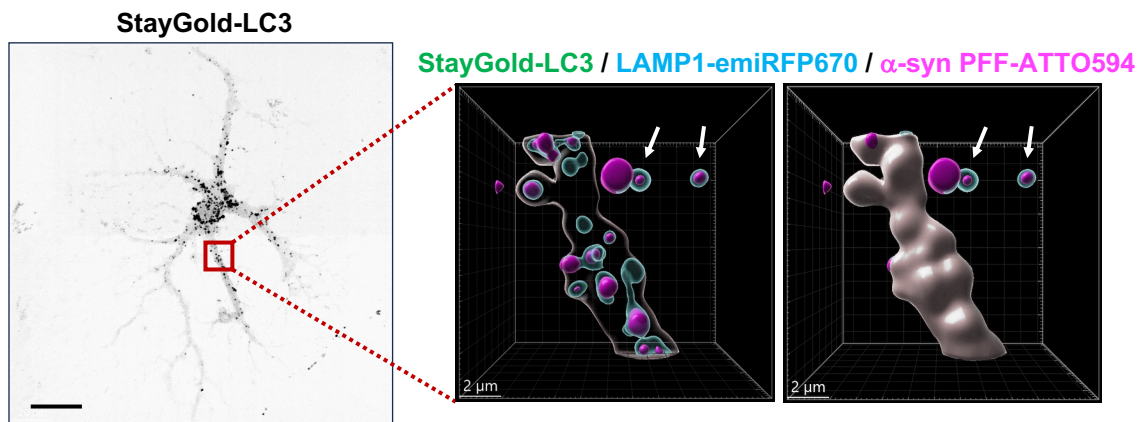
(B) Effect of α -syn PFFs on CtsD protein levels. Astrocytes were treated with α -syn PFFs (0.5 μ g/ml) for 24 h, and cell lysates were subjected to immunoblot analysis for CtsD. Arrows indicate the active forms (48 kDa and 33 kDa) of CtsD, and the arrowhead indicates pro-CtsD. ERK1 serves as a loading control.



Appendix Figure S8. Effect of Pep A on PFF-induced lysosomal damage and ROS accumulation.

(A) Quantification of Gal-3 signals. Astrocytes differentiated from neurospheres were treated with or without α -syn PFFs (0.5 μ g/ml) for 1 day in the presence or absence of Pep A, followed by immunostaining for Gal-3. Data are presented as mean $\pm$ s.d. ($n = 40$ cells per condition). Statistical significance was assessed using the Kruskal–Wallis test followed by Dunn’s multiple-comparison test. ns, not significant; **** $p < 0.0001$.

(B) Quantification of total cellular ROS levels. Astrocytes differentiated from neurospheres were treated with or without α -syn PFF (0.5 μ g/ml) for 4 days in the presence or absence of Pep A, followed by staining with CellROX Deep Red to assess ROS levels. Data are presented as mean $\pm$ s.d. ($n = 60$ cells per condition). Statistical significance was assessed using the Kruskal–Wallis test followed by Dunn’s multiple-comparison test. ns, not significant.



Appendix Figure S9. Localization of PFF-containing LAMP1-positive puncta beyond the astrocytic membrane boundary.

The left panel shows the mStayGold-LC3 signal in Fig. 7B. The right panels show three-dimensional reconstructions of the boxed regions generated using Imaris software. Arrows indicate PFF-containing, LAMP1-positive puncta located beyond the astrocytic membrane boundary. Scale bars, 20 μ m (left) and 2 μ m (insets).