

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

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Dear Dr. Hanafusa

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you have seen, the referees acknowledge that the findings are interesting and that the conclusions are overall supported by the data presented but they also raise a number of concerns and have suggestions how to further strengthen the data, including characterizing the impact of coculture itself on neurons, addition of orthogonal evidence such as genetic KD or KO for Cathepsin D's role, additional quantification of Lewy neurite phenotypes, and greater physiological or translational relevance.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO Reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (April 27th). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

I am also happy to discuss the revision further via e-mail or a video call, if you wish.

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- If the data are obtained from n {less than or equal to} 5, show the individual data points in addition to the SD or SEM.
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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Kurt Weir
Editor
EMBO Reports

Referee #1:

In this manuscript entitled "Astrocytic Cathepsin D truncates α -synuclein and promotes Lewy neurite-like aggregation in neurons", the authors utilized neuron-astrocyte co-culture system to show propagation of α -synuclein from astrocytes to neurons. The authors then find astrocytes process C terminus of α -synuclein pre-formed fibrils (α -syn PFF) via Cathepsin D, a lysosomal protease, thereby generating truncated α -synuclein which forms Lewy neurite (LN)-like aggregates in neurons. Next, the authors found inhibition of Cathepsin D in astrocytes ameliorates aggregation of α -synuclein in neurons. Finally, the authors showed α -syn PFF causes senescence-like state and lysosomal disruption in astrocytes. Overall, this study revealed active roles of astrocytes in propagation of α -synuclein in neurons using co-culture system. Following is my specific concerns.

Major comments

1. Because the authors suggested positive feedback loop of α -synuclein and Cathepsin D within astrocytes, inhibition of Cathepsin D likely ameliorates pathogenic effects of α -syn PFF also in astrocytes. It would be interesting that examining whether Cathepsin D inhibition ameliorates senescence-like state and lysosomal disruption in astrocytes.
2. Although the authors utilized a co-culture system of astrocytes and neurons, physiological aspect of the finding was barely studied. I recommend that the authors investigate physiological dysfunctions in neurons caused by α -syn PFF-treated astrocytes. At least the authors should discuss pathogenic relevance of the finding with proper citations, including following one: PMID: 38971320.

Minor comments

1. On page 6, line 106, the font size of " α -syn" looks bigger than others.

Referee #2:

This manuscript uses a physiologically relevant neuron-astrocyte co-culture system to show that astrocytic Cathepsin D (CtsD) mediates C-terminal truncation of internalized α -syn PFFs, producing seeding-competent species that are transferred to neurons and promote Lewy neurite (LN)-like aggregate elongation, while PFF exposure also induces lysosomal damage and senescence-like changes in astrocytes. The strengths include a clear mechanistic framework, strong imaging and quantitative analysis of aggregate morphology, and a co-culture model that captures tripartite synapse features. Main weaknesses are the limited mechanistic definition of the astrocyte-neuron transfer route, reliance on pharmacologic CtsD inhibition without genetic

validation, and absence of direct in vivo or human validation and functional neuronal readouts.

Comments:

- The study demonstrates astrocytic uptake of PFFs and appearance of PFF puncta on axons near synapses but does not define the transfer route (e.g., EVs, tunneling nanotubes, lysosomal exocytosis). The authors should analyze α -syn in astrocyte-conditioned medium and test its seeding activity on neuron-only cultures and assess co-localization of transferred α -syn with EV markers (e.g., CD63, CD81) and test inhibitors of exosome release (e.g., GW4869 or Rab27a/b knockdown) for effects on neuronal aggregation.
- The key conclusion that astrocytic CtsD drives pathogenic truncation relies heavily on pepstatin A, which is pharmacologic and not fully specific. The authors should perform a astrocyte-specific CtsD knockdown/knockout and show effects on α -syn truncation pattern and neuron LN-like aggregation, with rescue by wild-type versus catalytically inactive CtsD. They should also include limited tests with cysteine cathepsin inhibitors (e.g., E64d) to show that the observed truncation and seeding are predominantly CtsD-dependent.
- Truncation is inferred from antibody epitope mapping, but exact cleavage sites and fragment lengths are unknown. It would be great if the authors could Map C-terminal truncation sites by mass spectrometry (on astrocyte-derived fragments or in vitro CtsD-digested fibrils) to determine whether major species correspond to known pathogenic fragments (e.g., 1-114, 1-119).
- The authors should also compare seeding potency of defined recombinant truncated species with full-length PFFs in neurons, ideally linking a specific truncation product to enhanced LN elongation.
- The work focuses on aggregate morphology and size distribution, without direct measures of neuronal health or synaptic function. The authors should assess neuronal viability (e.g., MAP2/NeuN counts, cleaved caspase-3, LDH assay) under neuron-only vs co-culture {plus minus} PFF {plus minus} CtsD inhibition conditions. Alternatively, a basic synaptic or structural readout (e.g., VGLUT1/PSD95 puncta density or neurite length analysis) to demonstrate functional consequence of astrocyte-driven LN elongation.
- The senescence-like changes are supported by SA- β -gal and ROS but could also reflect reactive astrogliosis. It would be important to add 1-2 canonical senescence markers (e.g., p16Ink4a, p21Cip1, IL-6) by qPCR or immunostaining. The authors should also include at least one reactive astrocyte marker (e.g., GFAP, C3) or expand the Discussion to more explicitly address overlap between reactivity and senescence.
- There is lack of in vivo or human relevance. All data are from mouse primary cultures with recombinant human PFFs, and in vivo relevance is inferred from the literature. Can the authors show that human primary or iPSC-derived astrocytes exposed to PFFs recapitulate CtsD upregulation and truncation, reinforcing translational relevance.
- Please clarify blinding and sampling in aggregate quantification (number of cells/fields per condition, whether experimenters were blinded).
- Please provide an example segmentation of puncta vs LN-like structures in a supplemental figure to validate the classification criteria.

Referee #3:

This manuscript by Hanafusa et al, examines the role of astrocytes in the propagation of PFF induced alpha-synuclein pathology in neurons. The authors examine how astrocytes interact with PFF and enhance pathology in neurons using a co-culture methods and largely imaging based methods to quantify synuclein pathology. Overall, the data suggests that alpha-synuclein pathology is enhanced in neurons after co-culture with astrocytes, and suggest a possible role for astrocytes in the propagation of neuronal alpha-synuclein pathology. However, a number of methodological concerns and alternative explanations can be suggested for their data. I have listed my major concerns below.

- 1) In Figure 1, the authors suggest that co-culture of neurons with astrocytes enhances specifically Lewy neurite pathology. However, implications of the astrocytes on the physical properties of neurons themselves are not considered when analyzing their data. A number of changes in neuronal properties themselves may drive the differences they see. Does the inclusion of astrocytes change the number or strength of synapses in the hippocampal cultures? Previous literature has suggested that neuronal activity can increase PFF uptake, and enhance pathological synuclein. Additionally, are the number of puncta or neurites of pathological alpha-synuclein changed? Data should indicate absolute number of puncta and neurites along side the size.
- 2) Outside of concerns for changes in neurons as a result of co-culture giving this, it could be that astrocytes induce increased kinetics of alpha-synuclein aggregation rather than changing just neurite size. Additional time points in Figure 1 are necessary to indicate if this is a change in kinetics or an actual change in pathology development.
- 3) Data in Figure 2 are critical for the interpretation of whether the pathological alpha-synuclein in neurons is derived from astrocytic or neuronal sources. With the limitations of light microscopy, it is unclear if there is sufficient resolution to determine which side of the membrane the PFFs are found on. Staining for synuclein without permeabilizing the cells would inform as to whether this is any synuclein remaining on the outside of the astrocytes that then may expose the neurons to seeding competent PFF. Because of the centrality of internalized PFF in astrocytes is to the authors argument, additional biochemical evidence of internalized PFF would be rigorous. Also, for comparison to figure 1, using some type of standardized measure to determine effect sizes across experiments is necessary.
- 4) Because of the increase of C-terminally truncated alpha-synuclein species over time in astrocytes, are astrocytes treated with PFF more seeding capable at longer time points? I.e would astrocytes pre-treated for 48 hours be more seeding competent than

astrocytes pre-treated for 6 hours.

5) This is a minor point, but the co-localization of alpha-synuclein to lysosomal markers in supplemental figure 4 needs to be quantified. By eye, the majority of alpha-synuclein appears to be outside of the lysosomal marker.

6) Pepstatin A does not solely inhibit cathepsin D, and shows activity at several other aspartic proteases. Cathepsin E, pepsin N, and renin all have been shown to be sensitive to Pepstatin A (See PMID: 18165168). Using astrocytes derived from Cathepsin D knockout mice would go a long way to boost confidence that Cathepsin D is specifically responsible for the phenotype. The authors could also alter the wording of their conclusions, discussion, and title accordingly.

7) The authors suggest that pepstatin A does not alter pathological alpha-synuclein burden in neuron-only cultures, and suggest that neurons alone do not make aggregation prone cleaved variants. The authors should directly show this in figure 4 and they did in figure 3.

8) As mentioned above, the use of Lewy neurites size as the sole marker for pathological alpha-synuclein may not be sufficient. Throughout the figures, reporting the total number of pathological alpha-synuclein neurites or puncta is also necessary. Additional supporting biochemical evidence would also boost confidence in the data. For example, if there are twice the number of Lewy neurites in one condition (neuron alone) but longer in another (astrocyte plus neuron), they may have the same amount of pathological burden

Additional comments from cross-comment session:

Referee 3:

I agree with all of the points made by reviewer 2. The manuscript needs significant revision with significant additional data

The most critical limitations of the work as highlighted in both of our reviews are: 1) Lack of characterization of how the addition of astrocytes change the primary neurons and if they are responsible for the observed changes in seeding 2) Poor and inconsistent measures of pathological alpha-synuclein 3) Their sole way of prescribing activity to Cathepsin D is through the use of a single non-specific inhibitor

Referee 2:

I agree with Referee #3 comment. Although it would be nice to have the MS analysis it may be beyond the scope to the current paper.

Dear Dr. Kurt Weir,

Thank you for your email dated January 27 regarding our manuscript entitled “Astrocytic Cathepsin D truncates α -synuclein and promotes Lewy neurite-like aggregate maturation” (EMBOR-2025-62975V1). We also appreciate the constructive feedback provided by the reviewers, which has helped us improve the quality and physiological relevance of our work.

In response to the editorial suggestions and referee comments, we have extensively revised our manuscript and performed substantial additional experiments to:

1. Characterize the impact of astrocyte co-culture on basal neuronal morphology and synaptic properties
2. Provide orthogonal support for the role of Cathepsin D using shRNA-mediated genetic knockdown
3. Expand and standardize the quantification of α -syn aggregate phenotypes
4. Further investigate the mechanism of lysosomal damage and extracellular release of damaged lysosomes

We have addressed each referee comment in detail in the accompanying point-by-point response. We are submitting the revised manuscript as a Full Article, retaining separate Results and Discussion sections to accommodate the additional data and mechanistic analyses included in this revision.

We believe that these revisions have substantially strengthened the manuscript and clarified the mechanistic framework linking astrocytic lysosomal processing to α -syn propagation.

Thank you for your continued consideration of our work for publication in *EMBO Reports*.

Sincerely,

Hiroshi Hanafusa

Our responses to the comments of Reviewer #1

Major comments

(1) Because the authors suggested positive feedback loop of α -synuclein and Cathepsin D within astrocytes, inhibition of Cathepsin D likely ameliorates pathogenic effects of α -syn PFF also in astrocytes. It would be interesting that examining whether Cathepsin D inhibition ameliorates senescence-like state and lysosomal disruption in astrocytes.

As suggested, we examined whether inhibition of Cathepsin D (CtsD) ameliorates the senescence-like state or lysosomal disruption induced by α -syn PFFs in astrocytes. To address this, we treated astrocytes with the CtsD inhibitor pepstatin A and assessed intracellular ROS accumulation and lysosomal membrane damage. We found that pepstatin A had no significant effect on the elevated intracellular ROS levels induced by α -syn PFFs (Appendix Fig. S8B). In addition, the recruitment of Galectin-3 to α -syn PFF-containing lysosomes, a marker of lysosomal membrane rupture, was unaffected by CtsD inhibition (Appendix Fig. S8A). These results indicate that CtsD activity is not required for the initial induction of lysosomal damage or the senescence-like state triggered by α -syn PFF exposure. Rather, our data support a model in which CtsD acts downstream of lysosomal stress to mediate C-terminal truncation of α -syn and the generation of seeding-competent species, consistent with the feed-forward pathway proposed in the revised manuscript.

(2) Although the authors utilized a co-culture system of astrocytes and neurons, physiological aspect of the finding was barely studied. I recommend that the authors investigate physiological dysfunctions in neurons caused by α -syn PFF-treated astrocytes. At least the authors should discuss pathogenic relevance of the finding with proper citations, including following one: PMID: 38971320.

We agree that defining downstream neuronal dysfunction induced by α -syn PFF-treated astrocytes is an important question. In the present study, our primary objective was to establish the mechanistic framework by which astrocytes process and transmit pathogenic α -syn species to promote Lewy neurite-like aggregate maturation.

While a comprehensive functional analysis of neuronal dysfunction is beyond the scope of the current study, we have taken several steps to address the reviewer's concern. Specifically, we have substantially expanded the Discussion to better contextualize our findings within a physiological and disease-relevant framework. This includes incorporation of the suggested reference (PMID: 38971320), together with additional relevant studies (p. 8, lines 9–11; p. 15, lines 2–8).

In particular, we now discuss how astrocyte-mediated α -syn propagation, senescence-like state transitions, and lysosomal dysfunction may contribute to the formation of pathogenic microenvironments that facilitate disease

progression.

Minor comments

On page 6, line 106, the font size of " α -syn" looks bigger than others.

We have corrected the font size inconsistency in the revised manuscript (p. 7, line 15).

Our responses to the comments of Reviewer #2

Comments:

The study demonstrates astrocytic uptake of PFFs and appearance of PFF puncta on axons near synapses but does not define the transfer route (e.g., EVs, tunneling nanotubes, lysosomal exocytosis). The authors should analyze α -syn in astrocyte-conditioned medium and test its seeding activity on neuron-only cultures and assess co-localization of transferred α -syn with EV markers (e.g., CD63, CD81) and test inhibitors of exosome release (e.g., GW4869 or Rab27a/b knockdown) for effects on neuronal aggregation.

We agree that defining the route of astrocyte-to-neuron α -syn transfer is a key mechanistic question.

In response, we have added new experiments in the revised manuscript (Fig. 7) demonstrating that α -syn PFF-containing damaged lysosomes are engulfed by autophagosomal membranes yet evade productive lysophagy and can be detected outside astrocytic processes. These observations support a model in which extracellular release of PFF-containing lysosomal compartments contribute to astrocyte-to-neuron transfer. While these data do not exclude additional transfer routes, they provide direct evidence for a lysosome-mediated mechanism that is distinct from canonical extracellular vesicle pathways.

Regarding conditioned medium (CM) experiments, we attempted to test whether CM from PFF-treated astrocytes could induce aggregation in naïve neurons. However, under the serum-free, low-density monoculture conditions required for CM collection, neurosphere-derived astrocytes exhibited limited viability, and the levels of released α -syn species were below the threshold required to reproducibly induce detectable seeding. We therefore concluded that this approach is not technically feasible in the current system.

With respect to co-localization with EV markers and inhibition of exosome release (e.g. CD63/CD81, GW4869, Rab27a/b), we agree that these are important experiments to further distinguish potential transfer pathways. However, given that our revised data support a lysosome-mediated release mechanism, a comprehensive dissection of the relative contributions of EV-dependent and EV-independent pathways is beyond the scope of the current

study and will be an important direction for future investigation.

The key conclusion that astrocytic CtsD drives pathogenic truncation relies heavily on pepstatin A, which is pharmacologic and not fully specific. The authors should perform an astrocyte-specific CtsD knockdown/knockout and show effects on α -syn truncation pattern and neuron LN-like aggregation, with rescue by wild-type versus catalytically inactive CtsD. They should also include limited tests with cysteine cathepsin inhibitors (e.g., E64d) to show that the observed truncation and seeding are predominantly CtsD-dependent.

To provide orthogonal evidence beyond pharmacological inhibition, we performed astrocyte-specific knockdown of CtsD using AAV-mediated shRNA. As shown in the revised Fig. 4, CtsD knockdown significantly suppressed the ability of astrocytes to promote elongation of LN-like α -syn aggregates in neurons, closely phenocopying the effects observed with pepstatin A. These genetic data provide independent support that astrocytic CtsD activity is required for this process.

Regarding direct analysis of α -syn truncation patterns following CtsD knockdown, we attempted this experiment but encountered technical limitations. Efficient depletion of CtsD at the protein level required prolonged incubation, whereas neurosphere-derived astrocytes maintained under monoculture conditions for biochemical analysis exhibited limited viability over this timeframe. Consequently, we were unable to obtain samples with sufficiently sustained CtsD depletion to allow reliable detection of truncation products by immunoblotting. Nonetheless, we confirmed robust knockdown of CtsD transcripts by qPCR within the viable experimental window (Appendix Fig. S6A).

We also attempted rescue experiments using wild-type and catalytically inactive CtsD, as suggested. However, sequential AAV transduction followed by plasmid transfection resulted in substantial astrocyte toxicity, precluding reliable quantification. While we acknowledge that such rescue experiments would further strengthen mechanistic specificity, the strong concordance between pharmacological inhibition and genetic knockdown provides compelling evidence that astrocytic CtsD plays a central role in this process.

With respect to potential contributions from other cathepsins, we agree that this is an important question. Although the limited viability of astrocytes in our system precluded systematic inhibitor analysis (e.g., E64d) in the current revision, defining the relative contributions of additional lysosomal proteases will be an important direction for future investigation.

Truncation is inferred from antibody epitope mapping, but exact cleavage sites and fragment lengths are unknown. It would be great if the authors could map C-terminal truncation sites by mass spectrometry (on astrocyte-derived fragments or in vitro CtsD-digested fibrils) to determine whether major species correspond to known pathogenic fragments (e.g., 1-114, 1-119).

We agree that precise mapping of C-terminal truncation sites by mass spectrometry would provide important mechanistic insight and help determine whether the generated species correspond to previously reported pathogenic fragments such as α -syn 1–114 or 1–119.

Although this analysis is beyond the scope of the present revision, our conclusion that astrocytes generate C-terminally truncated α -syn species is supported by multiple independent lines of evidence. These include the loss of C-terminal antibody recognition, the appearance of lower-molecular-weight fragments detected by a central-region α -syn antibody, and the suppression of these fragments upon CtsD inhibition. Together, these observations consistently support C-terminal proteolytic processing of α -syn in astrocytes, even in the absence of precise cleavage site mapping.

We agree that defining the exact cleavage sites generated by astrocytic CtsD will be an important next step and plan to pursue this in future studies.

The authors should also compare seeding potency of defined recombinant truncated species with full-length PFFs in neurons, ideally linking a specific truncation product to enhanced LN elongation.

We agree that directly comparing the seeding activity of defined truncated α -syn species with full-length PFFs would be a powerful approach to link specific truncation products to enhanced LN-like aggregate elongation.

To address this, we attempted to generate recombinant truncated α -syn PFF species by in vitro digestion using His-tagged CtsD (abcam ab307480). This approach successfully produced truncation products with sizes comparable to those observed in astrocytes. However, during removal of His-tagged CtsD using affinity purification, we observed substantial loss of fibrillar α -syn due to nonspecific adsorption to the beads, resulting in marked depletion of truncated fibrils after purification. Consequently, we were unable to recover sufficient material to perform reliable downstream neuronal seeding assays.

These observations highlight a technical challenge in handling protease-processed fibrillar substrates, particularly during purification steps. Optimization of purification strategies is currently underway and will be essential for future studies aimed at defining the seeding properties of specific truncation products.

Nevertheless, our current data, combining pharmacological inhibition and astrocyte-specific CtsD knockdown, consistently demonstrate that CtsD-dependent processing promotes LN-like aggregate maturation, even though the contribution of individual truncation species remains to be resolved.

The work focuses on aggregate morphology and size distribution, without direct measures of neuronal health or synaptic function. The authors should assess neuronal viability (e.g., MAP2/NeuN counts, cleaved caspase-3, LDH

assay) under neuron-only vs co-culture {plus minus} PFF {plus minus} CtsD inhibition conditions. Alternatively, a basic synaptic or structural readout (e.g., VGLUT1/PSD95 puncta density or neurite length analysis) to demonstrate functional consequence of astrocyte-driven LN elongation.

As an alternative to direct viability assays, as suggested by the Reviewer, we performed structural analyses of neurite morphology and synaptic density to assess whether astrocyte-driven LN-like aggregate elongation is associated with early neuronal dysfunction.

Specifically, we quantified neurite length using MAP2 staining and synaptic density by co-localization of VGLUT1 and HOMER1 across all experimental conditions (Fig. EV5). We found that neurite length remained largely unchanged under all tested conditions. Although co-culture with astrocytes modestly increased baseline synaptic density, neither α -syn PFF treatment nor CtsD inhibition with pepstatin A significantly altered these parameters within a 10-day treatment window.

These results suggest that astrocyte-dependent enhancement of LN-like aggregate elongation occurs independently of overt early changes in neuronal structure or synaptic density in this primary culture model. Consistent with this interpretation, we have revised the Results to clarify that our findings likely reflect an early stage of aggregate maturation that precedes the synaptic loss or neuronal degeneration observed in more chronic disease contexts.

The senescence-like changes are supported by SA- β -gal and ROS but could also reflect reactive astrogliosis. It would be important to add 1-2 canonical senescence markers (e.g., p16^{Ink4a}, p21^{Cip1}, IL-6) by qPCR or immunostaining. The authors should also include at least one reactive astrocyte marker (e.g., GFAP, C3) or expand the Discussion to more explicitly address overlap between reactivity and senescence.

To more rigorously define the astrocytic state following α -syn PFF exposure, we performed RT-qPCR analysis of canonical senescence and reactive astrocyte markers, as suggested.

Our analysis revealed significant upregulation of senescence-associated markers (p16^{Ink4a} and p21^{Cip1}), together with reactive astrocyte markers (GFAP), in PFF-treated astrocytes (Fig. 5D). These results support the induction of a mixed astrocytic state exhibiting features of both reactive astrogliosis and cellular senescence.

We have incorporated these new data into the revised manuscript and expanded the Discussion to more explicitly address the overlap and potential progression between these states. In particular, our findings suggest that persistent proteotoxic stress may drive a transition from an initially reactive state toward a senescence-like pathogenic state, with potential implications for α -syn propagation.

There is lack of in vivo or human relevance. All data are from mouse primary cultures with recombinant human PFFs, and in vivo relevance is inferred from the literature. Can the authors show that human primary or iPSC-derived astrocytes exposed to PFFs recapitulate CtsD upregulation and truncation, reinforcing translational relevance.

We agree that validation in human primary or iPSC-derived astrocytes would further strengthen the generality and translational relevance of our findings.

Although such experiments are beyond the scope of the present revision, our study employs a physiologically relevant primary neuron–astrocyte co-culture system that preserves key neuron–glia interactions, including tripartite synaptic architecture. This system enables mechanistic interrogation of astrocyte-mediated α -syn propagation under conditions that more closely approximate the in vivo cellular environment.

In addition, the pathological relevance of our proposed mechanism is supported by prior observations that C-terminally truncated α -syn species accumulate not only in neurons but also in astrocytes in PD and DLB brains (as cited in the revised manuscript).

We have now explicitly acknowledged the absence of human astrocyte validation as a limitation and highlighted testing whether human primary or iPSC-derived astrocytes recapitulate CtsD upregulation and pathogenic truncation as an important direction for future investigation in the Discussion.

Please clarify blinding and sampling in aggregate quantification (number of cells/fields per condition, whether experimenters were blinded).

We have revised the Methods section to clarify the sampling and quantification procedures. Specifically, we now indicate the number of cells and fields analyzed per condition, the number of independent biological replicates, and that aggregate quantification was performed in a blinded manner.

Please provide an example segmentation of puncta vs LN-like structures in a supplemental figure to validate the classification criteria.

We have added a supplementary figure (Appendix Fig. S2) containing representative segmentation examples illustrating the classification of puncta and LN-like structures based on the size and ellipticity criteria used in our analysis. These examples include the original images alongside the corresponding segmentation outputs to facilitate validation of the classification approach.

Our responses to the comments of Reviewer #3

Main comments

1) In Figure 1, the authors suggest that co-culture of neurons with astrocytes enhances specifically Lewy neurite pathology. However, implications of the astrocytes on the physical properties of neurons themselves are not considered when analyzing their data. A number of changes in neuronal properties themselves may drive the differences they see. Does the inclusion of astrocytes change the number or strength of synapses in the hippocampal cultures? Previous literature has suggested that neuronal activity can increase PFF uptake, and enhance pathological synuclein. Additionally, are the number of puncta or neurites of pathological alpha-synuclein changed? Data should indicate absolute number of puncta and neurites along side the size.

As suggested, we quantified neurite morphology and synaptic density and found that astrocyte co-culture resulted in a modest increase in baseline synapse density (VGLUT1/HOMER1 co-localization), while neurite length remained unchanged (Fig. EV5). However, our data indicate that altered neuronal support alone is unlikely to account for the enhanced LN-like pathology.

Importantly, at an early time point (6 days post-PFF treatment), the total number of neuronal α -syn aggregates was significantly lower in co-cultures than in neuron-only cultures (Figs. 1 and EV2), consistent with astrocytic uptake reducing the effective availability of exogenous PFFs for neuronal seeding. If enhanced synaptic support were the principal driver of pathology, one might instead expect increased aggregation from the earliest stages. Instead, we observe an initial suppression of aggregate formation, followed by a selective shift toward elongation of LN-like structures, consistent with a distinct astrocyte-dependent mechanism that promotes aggregate maturation.

In response to the Reviewer's request, we have also revised Figure EV2 to include the absolute numbers of both puncta and LN-like aggregates alongside size distribution analyses, providing a more comprehensive quantitative description of these effects.

2) Outside of concerns for changes in neurons as a result of co-culture riving this, it could be that astrocytes induce increased kinetics of alpha-synuclein aggregation rather than changing just neurite size. Additional time points in Figure 1 are necessary to indicate if this is a change in kinetics or an actual change in pathology development.

We thank the Reviewer for this important suggestion to distinguish whether the observed effect reflects accelerated aggregation kinetics or a qualitative change in aggregate maturation.

To address this, we analyzed additional time points as requested. At 6 days post-PFF treatment, co-culture with astrocytes significantly reduced the total number of neuronal α -syn aggregates relative to neuron-only cultures (Fig.

EV2). By day 10, although total aggregate numbers were no longer significantly different between conditions, the length of LN-like aggregates were markedly increased in the presence of astrocytes (Fig. 1B–E).

These findings indicate that astrocytes do not simply accelerate the overall kinetics of aggregation, which would be expected to increase aggregate burden at earlier time points. Instead, our data support a selective effect on aggregate maturation, whereby astrocytes promote the elongation of α -syn aggregates into LN-like structures over time.

3) Data in Figure 2 are critical for the interpretation of whether the pathological alpha-synuclein in neurons is derived from astrocytic or neuronal sources. With the limitations of light microscopy, it is unclear if there is sufficient resolution to determine which side of the membrane the PFFs are found on. Staining for synuclein without permeabilizing the cells would inform as to whether this is any synuclein remaining on the outside of the astrocytes that then may expose the neurons to seeding competent PFF. Because of the centrality of internalized PFF in astrocytes is to the authors argument, additional biochemical evidence of internalized PFF would be rigorous. Also, for comparison to figure 1, using some type of standardized measure to determine effect sizes across experiments is necessary.

We thank the Reviewer for this important comment regarding the evidence for astrocytic internalization of PFFs and the interpretation of their transfer to neurons.

While we acknowledge the spatial resolution limitations of light microscopy, multiple independent lines of evidence support the conclusion that the relevant α -syn PFF species are internalized and processed within astrocytes, rather than remaining surface-associated.

1. Intracellular lysosomal localization.

We show that astrocyte-associated PFFs co-localize with lysosomal markers, including LAMP1, as well as with Gal-3- and LC3-positive damaged lysosomal structures (Fig. 7; Appendix Fig. S4), supporting intracellular trafficking of PFFs into the lysosomal compartment.

2. Functional dependence on lysosomal CtsD.

Both pharmacological inhibition (pepstatin A) and astrocyte-specific CtsD knockdown significantly suppressed astrocyte-dependent promotion of neuronal LN-like aggregation (Fig. 4). Because CtsD functions within lysosomes, these results provide functional evidence that processing of internalized PFF, rather than extracellularly associated fibrils, underlies the observed effect.

3. Biochemical evidence for astrocyte-specific processing.

We have added data showing that neurons generate negligible levels of soluble C-terminally truncated α -syn species compared with astrocytes (Fig. EV4), further supporting astrocytes as the principal source of the transferred pathogenic species.

In addition, our new data indicating extracellular release of PFF-containing damaged lysosomes provide a potential mechanistic basis by which processed intracellular material may subsequently reach neurons.

Regarding quantification, we agree that standardized measurements are important. We have revised the analyses to apply consistent aggregate classification and quantification criteria across experiments, including standardized reporting of aggregate number and LN-like morphology metrics (Appendix Fig. S2). Because Figs. 1 and 2 involve distinct experimental paradigms (direct PFF exposure versus astrocyte preloading), we interpret effect sizes within each paradigm rather than making direct quantitative comparisons across experiments.

4) Because of the increase of C-terminally truncated alpha-synuclein species over time in astrocytes, are astrocytes treated with PFF more seeding capable at longer time points? I.e would astrocytes pre-treated for 48 hours be more seeding competent than astrocytes pre-treated for 6 hours.

As suggested, we examined whether the seeding capacity of astrocytes increases as a function of PFF pretreatment duration.

Specifically, we compared astrocytes pre-treated with α -syn PFFs for 8, 24, or 48 hours for their ability to induce neuronal aggregation. Astrocytes pre-treated for 8 hours exhibited little detectable seeding activity, whereas those pre-treated for 48 hours showed significantly greater seeding activity than astrocytes pre-treated for 24 hours (Fig. EV3).

These results demonstrate a time-dependent increase in astrocyte-derived seeding competence and are consistent with the progressive accumulation of C-terminally truncated α -syn species in astrocytes over time. Together, these findings further support our model that astrocytic CtsD-dependent processing contributes to the maturation and transmission of seeding-competent α -syn species.

5) This is a minor point, but the co-localization of alpha-synuclein to lysosomal markers in supplemental figure 4 needs to be quantified. By eye, the majority of alpha-synuclein appears to be outside of the lysosomal marker.

As requested, we quantified the co-localization between α -syn PFFs and the lysosomal marker LAMP1 and have included these analyses in the revised Appendix Fig. S4B.

This analysis showed that the fraction of internalized α -syn PFF puncta co-localizing with LAMP1-positive compartments significantly increased in a time-dependent manner following PFF treatment, consistent with progressive trafficking of internalized PFFs to lysosomes.

These quantification data complement the qualitative observations and

support the conclusion that a substantial fraction of internalized α -syn is associated with lysosomal compartments, particularly at later time points.

6) Pepstatin A does not solely inhibit cathepsin D, and shows activity at several other aspartic proteases. Cathepsin E, pepsin N, and renin all have been shown to be sensitive to Pepstatin A (See PMID: 18165168). Using astrocytes derived from Cathepsin D knockout mice would go a long way to boost confidence that Cathepsin D is specifically responsible for the phenotype. The authors could also alter the wording of their conclusions, discussion, and title accordingly.

To address this concern, we included independent genetic validation using AAV-mediated CtsD shRNA in astrocytes. As shown in the revised Fig. 4, astrocyte-specific CtsD knockdown significantly suppressed the promotion of neuronal LN-like aggregation, closely phenocopying the effects observed with pepstatin A. These orthogonal genetic data support the conclusion that astrocytic CtsD plays a central role in this process, beyond the effects of pharmacological inhibition alone.

While astrocytes derived from CtsD knockout mice would provide an additional complementary approach, this was beyond the scope of the present revision. In line with the Reviewer's suggestion, we have also refined the wording throughout the Results and Discussion to more precisely reflect a CtsD-dependent mechanism supported by both pharmacological and genetic evidence.

7) The authors suggest that pepstatin A does not alter pathological alpha-synuclein burden in neuron-only cultures, and suggest that neurons alone do not make aggregation prone cleaved variants. The authors should directly show this in figure 4 and they did in figure 3.

As requested, we directly examined whether C-terminal truncation of α -syn PFFs occurs in neuron-only cultures.

We found that α -syn PFF treatment of neuron-only cultures produced negligible amounts of C-terminally truncated α -syn species in the soluble fraction, even 48 hours after exposure (Fig. EV4). In contrast, astrocyte-only cultures generated robust levels of these truncated fragments within the same timeframe (Fig. 3), supporting a specific contribution of astrocytes to this processing step.

These findings are consistent with our observation that pepstatin A does not alter α -syn aggregate burden in neuron-only cultures, further indicating that neurons alone do not efficiently generate aggregation-prone truncated species under these conditions.

To address the Reviewer's concern, we have included these comparative data in the revised Fig. EV4 and cross-referenced them in the Fig. 3.

8) As mentioned above, the use of Lewy neurites size as the sole marker for

pathological alpha-synuclein may not be sufficient. Throughout the figures, reporting the total number of pathological alpha-synuclein neurites or puncta is also necessary. Additional supporting biochemical evidence would also boost confidence in the data. For example, if there are twice the number of Lewy neurites in one condition (neuron alone) but longer in another (astrocyte plus neuron), they may have the same amount of pathological burden

To address this concern, we have revised our analyses to report both the absolute numbers and size distributions of puncta and LN-like aggregates. This combined quantification provides a more complete assessment of aggregate burden in addition to morphology.

These analyses indicate that the astrocyte-dependent phenotype is not simply explained by redistribution of a similar aggregate burden into longer structures. Although total aggregate numbers are reduced in co-cultures at early time points, by day 10 they become comparable between conditions, while LN-like aggregates are significantly elongated in the presence of astrocytes. Thus, astrocytes preferentially promote the qualitative maturation of α -syn pathology rather than merely altering the partitioning of a fixed aggregate burden.

We have incorporated these expanded quantitative analyses into the revised figures and text.

Additional comments from cross-comment session:

Referee 3:

I agree with all of the points made by reviewer 2. The manuscript needs significant revision with significant additional data

The most critical limitations of the work as highlighted in both of our reviews are: 1) Lack of characterization of how the addition of astrocytes change the primary neurons and if they are responsible for the observed changes in seeding 2) Poor and inconsistent measures of pathological alpha-synuclein 3) Their sole way of prescribing activity to Cathepsin D is through the use of a single non-specific inhibitor

We thank Referee 3 for these additional comments and for highlighting the key issues requiring substantial revision. In response to these concerns, we have extensively revised the manuscript and incorporated significant new experimental data addressing each of the three major points raised:

1. Characterization of neuronal changes in co-culture.

We have quantified neuronal structural properties, including neurite morphology and synaptic density, in the presence and absence of astrocytes. While astrocytes provide modest baseline synaptic support, our time-course analyses (6 versus 10 days) indicate that astrocytes do not simply enhance overall seeding through altered neuronal state, but instead preferentially

promote maturation of LN-like pathology.

2. Standardized and rigorous quantification of pathological α -syn.

We have expanded our analyses to include both absolute aggregate numbers and size distributions for puncta and LN-like aggregates, rather than relying on morphology alone. In addition, we provide representative segmentation examples, clarify sampling and blinding procedures, and apply consistent image analysis criteria across experiments to improve rigor and reproducibility.

3. Specificity of Cathepsin D involvement.

To address the concern that our conclusions relied heavily on a single pharmacological inhibitor, we have incorporated independent genetic evidence using shRNA-mediated knockdown of CtsD in astrocytes. This orthogonal approach supports a central role for astrocytic CtsD in promoting neuronal LN-like aggregate maturation.

Together, these revisions address the principal concerns raised during the cross-comment discussion and strengthen the mechanistic framework and overall rigor of the study.

Referee 2:

I agree with Referee #3 comment. Although it would be nice to have the MS analysis it may be beyond the scope to the current paper.

We thank Referee 2 for this thoughtful perspective and for concurring with Referee 3 regarding the mass spectrometry (MS) analysis. We agree that mapping the precise C-terminal truncation sites by MS would provide valuable insight; however, this analysis is beyond the scope of the present study.

While we did not include MS analysis in this work, the combined genetic, biochemical, and functional evidence presented here provides a robust mechanistic framework supporting our conclusions. We consider detailed characterization of the truncation sites by MS to be an important direction for future investigations.

Dear Dr. Hanafusa

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you can see, the referees find that the study is significantly improved during revision and retain mostly concerns on presentation, clarity, and quantification, which we will request you address in a final round of revision. Referee 3 also has a remaining concern about the use of neurite length as a measure of pathological α -synuclein which we request you address in your Discussion section and requests soluble/insoluble blots for mouse synuclein which we do not ask you to include.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed:

- Please rename the "Competing Financial Interests" section to "Disclosure and Competing Interests Statement", in accordance with our updated Guide to Authors (<https://www.embopress.org/competing-interests>)
- CRediT has replaced the traditional author contributions section because it offers a systematic machine readable author contributions format that allows for more effective research assessment. Please remove the Author Contributions section from the manuscript and use the free text boxes beneath each contributing author's name in our online systems to add specific details on the author's contribution. More information is available in our guide to authors.
- Please remove the instructions and example table from the Reagents and Tools table.
- Please rename the file Dataset EV1 to Table EV1 in our system.
- Please note that EMBO press papers are accompanied online by:
 - A) a short (2 sentences) summary of the findings and their significance,
 - B) 2-5 short bullet points highlighting the key results, and
 - C) a synopsis image in .jpg or .png format that is exactly 550 pixels wide and 300 pixels high. Please note that the text needs to be legible at the final size. Please upload this information along with your revised manuscript (the text for A and B should be provided in one separate Word file uploaded as Cover Art/Synopsis Image).
- Please describe housing conditions for the mice used in this study.
- Our production/data editors have asked you to clarify several points in the figure legends (see below). Please incorporate these changes in the manuscript and return the revised file with tracked changes with your final manuscript submission.
- Please note that the exact p values are not provided in the legends of 1D; 4B; 5C.
- Please note that information related to n is missing in the legends 5D.
- Please note that the error bars are not defined in the legends of 5D.
- Please note that the measure of center for the error bars needs to be defined in the legends of figure EV-3B.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Kurt Weir
Editor
EMBO Reports

Referee #1:

The authors addressed my concerns successfully.

Referee #2:

EMBOR-2025-62975V2

This is a revised manuscript.

The orthogonal AAV-shRNA knockdown of astrocytic Cathepsin D (Fig. 4E-F) directly addresses the principal concern from the first round regarding sole reliance on pepstatin A, and it phenocopies the pharmacological result. The new neuron-only versus astrocyte comparison of soluble truncated α -syn (Fig. EV4), the time-resolved seeding competence of PFF-preloaded astrocytes

(Fig. EV3), the expanded aggregate quantification with absolute counts and segmentation examples (Fig. EV2; Appendix Fig. S2), the senescence and reactivity marker panel (Fig. 5D), and the lysophagy-evasion and extracellular-lysosome data (Fig. 7; Appendix Fig. S9) collectively strengthen the mechanistic framework.

I recommend acceptance after minor revision. Before publication, the authors should: (1) add a concise limitations statement covering the absent cleavage-site identification, the lack of WT versus catalytically dead CtsD rescue, the residual pepstatin A off-target activity at Cathepsin E and other aspartic proteases, and the lack of formal exclusion of EV-mediated transfer; (2) provide a quantitative measure of the externalized PFF-laden lysosome phenotype in Fig. 7D and Appendix Fig. S9 rather than relying on representative reconstructions; (3) confirm that co-localization metrics, sample sizes, units of replication, statistical tests, and multiple-comparison corrections are stated in every figure legend; and (4) soften the *in vivo* implications of the feed-forward model in the Abstract and Discussion to reflect that the evidence is from a co-culture system.

Referee #3:

In this revision, the authors added significant new data and re-analysis to help boost confidence in the overall findings of the paper that astrocytic cathepsin D alters PFF processing and leads to significantly more pathological alpha-synuclein in a co-culture of primary neurons and astrocytes. My main major concern remains my point originally raised in major concern 8 in my review of the initial submission. While the authors standardized measures of Lewy Body and Lewy Neurite like structures throughout their manuscript, the finding of increased Lewy neurite size alone by IHC I do not believe is sufficient to conclude that there is an increase in pathological alpha-synuclein in neurons (either through changes in processing, maturation, or total amount) without supporting biochemical evidence. This is especially true as puncta do not significantly differ, and the meaning of longer neurites is therefore hard to attribute to a specific physiological process. Added western blots in revision help to prove that processing of the exogenous PFF is altered in astrocytes and cathepsin D is mechanistically responsible for PFF processing, but must be quantified. The authors could add similar soluble/insoluble blots for mouse synuclein in their culture experiments to prove that altered processing of exogenous human PFF leads to altered propagation/amount/maturation of endogenous mouse synuclein in neurons.

Response to the Editor and Reviewers

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

Manuscript ID: EMBOR-2025-62975V2

Dear Dr. Weir,

We thank you and the referees for the careful evaluation of our revised manuscript and for the constructive comments. We are pleased that the referees found that the manuscript was significantly improved during revision. We have carefully considered all remaining comments and have revised the manuscript accordingly.

In particular, we have further clarified the limitations of our study, quantified the relevant biochemical and imaging data, clarified the statistical information in the figure legends, and moderated statements concerning the pathological implications of our findings. We have also made all of the editorial and formatting changes requested by the editorial office.

Below, we provide a point-by-point response to the comments.

Response to the Editor

Editorial comment 1

Please rename the "Competing Financial Interests" section to "Disclosure and Competing Interests Statement", in accordance with our updated Guide to Authors.

Response:

We have renamed the section "Competing Financial Interests" to "Disclosure and Competing Interests Statement" in accordance with the updated EMBO Reports guidelines.

Editorial comment 2

CRedit has replaced the traditional author contributions section... Please remove the Author Contributions section from the manuscript and use the free text boxes beneath each contributing author's name in our online systems...

Response:

We have removed the Author Contributions section from the manuscript. The specific contributions of each author will be entered in the CRediT fields in the online submission system as requested.

Editorial comment 3

Please remove the instructions and example table from the Reagents and Tools table.

Response:

We have removed the instructions and example table from the Reagents and Tools section.

Editorial comment 4

Please rename the file Dataset EV1 to Table EV1 in our system.

Response:

We have renamed Dataset EV1 to Table EV1 as requested.

Editorial comment 5

Please provide a short summary, 2–5 bullet points highlighting the key results, and a synopsis image...

Response:

We have prepared the requested two-sentence summary, key findings in bullet-point format, and synopsis image, and will upload them as separate files in the submission system. The synopsis image has been prepared at the requested dimensions of 550×300 pixels.

Editorial comment 6

Please describe housing conditions for the mice used in this study.

Response:

We have revised the Methods section to clarify that pregnant mice were purchased at embryonic day 17 (E17) and used approximately 24 h after arrival for preparation of primary hippocampal cultures. We have also added the conditions under which the animals were maintained during this interval.

Editorial comment 7

Please note that the exact p-values are not provided in the legends of 1D, 4B, and 5C.

Response:

We have revised Figs. 1D, 4B, and 5C to include the exact *P* values.

Editorial comment 8

Please note that information related to n is missing in the legends 5D.

Response:

We have added the sample size information (*n*) to the legend of Fig. 5D, including the experimental unit and number of independent experiments, as appropriate.

Editorial comment 9

Please note that the error bars are not defined in the legend of 5D.

Response:

We have clarified the definition of the error bars in the legend of Fig. 5D.

Editorial comment 10

Please note that the measure of center for the error bars needs to be defined in the legend of figure EV-3B.

Response:

We have revised the legend of Fig. EV3B to specify the measure of center represented by the error bars.

Response to Referee #2

Comment 1

Add a concise limitations statement covering the absent cleavage-site identification, the lack of WT versus catalytically dead CtsD rescue, the residual pepstatin A off-target activity at Cathepsin E and other aspartic proteases, and the lack of formal exclusion of EV-mediated transfer.

Response:

We have added a dedicated “Limitations of the study” section to the Discussion that explicitly addresses these points.

Specifically, we now state that the precise cleavage site(s) of the C-terminally truncated α -syn species were not determined; that rescue experiments comparing wild-type and catalytically inactive CtsD were not performed; that pepstatin A may inhibit other aspartic proteases, including Cathepsin E; and that extracellular vesicle-mediated transfer or other secretory mechanisms cannot be formally excluded.

We have also clarified that whether the CtsD-dependent truncated α -syn species identified in astrocytes are themselves transferred to neurons remains unresolved.

Comment 2

Provide a quantitative measure of the externalized PFF-laden lysosome phenotype in Fig. 7D and Appendix Fig. S9 rather than relying on representative reconstructions.

Response:

We agree that representative three-dimensional reconstructions alone are insufficient to establish the frequency of this phenotype quantitatively.

We have therefore added a quantitative analysis of the PFF-positive/LAMP1-positive puncta detected beyond the apparent astrocytic membrane boundary in Fig. 7D. Quantification of 10 astrocytes revealed an average 3.2 PFF-positive/LAMP1-positive puncta beyond the apparent astrocytic membrane boundary per cell. The corresponding quantitative information has been included in the figure legend for Fig. 7D.

Comment 3

Confirm that co-localization metrics, sample sizes, units of replication, statistical tests, and multiple-comparison corrections are stated in every figure legend.

Response:

We have reviewed all figure legends and revised them to clarify, where applicable, the co-localization metrics, sample sizes, experimental units of replication, statistical tests, and multiple-comparison correction procedures.

We have also ensured that the definitions of error bars and the measures of center are explicitly stated where required.

Comment 4

Soften the in vivo implications of the feed-forward model in the Abstract and Discussion to reflect that the evidence is from a co-culture system.

Response:

We agree with the referee and have moderated the implications of our findings in both the Abstract and Discussion. In particular, we now frame our findings as supporting a model of astrocytic lysosomal remodeling based on a primary neuron–astrocyte co-culture system, rather than implying that this mechanism has been established in vivo. We also explicitly acknowledge in the Discussion that whether this pathway operates in vivo or contributes to PD pathogenesis remains to be determined.

Response to Referee #3

We have considered the remaining concern regarding the interpretation of LN-like aggregate size and have revised the manuscript accordingly.

Comment

While the authors standardized measures of Lewy Body and Lewy Neurite like structures throughout their manuscript, the finding of increased Lewy neurite size alone by IHC I do not believe is sufficient to conclude that there is an increase in pathological alpha-synuclein in neurons (either through changes in processing, maturation, or total amount) without supporting biochemical evidence. This is especially true as puncta do not significantly differ, and the meaning of longer neurites is therefore hard to attribute to a specific physiological process.

Response:

We agree with the referee that an increase in the size of pS129- α -synuclein-positive LN-like structures alone should not be interpreted as evidence for an increase in the total amount of pathological α -synuclein in neurons.

We have therefore revised the manuscript throughout to distinguish morphological aggregate maturation from an overall increase in neuronal α -synuclein pathology or aggregate burden.

In particular, we now emphasize that astrocyte co-culture selectively increases the size of LN-like pS129- α -synuclein-positive structures without significantly increasing the number of punctate or LN-like aggregates. We have accordingly revised our interpretation to state that astrocytes promote the enlargement and

morphological maturation of LN-like aggregates, rather than increasing overall α -synuclein pathology.

We have further added an explicit limitation to the Discussion stating:
“Although these measurements provide evidence for morphological aggregate maturation, increased LN-like aggregate size alone does not establish an increase in the total amount of pathological α -syn or changes in endogenous neuronal α -syn levels.”

We now conclude that our findings support a contribution of astrocytic CtsD to the morphological maturation of neuronal α -synuclein aggregates in this co-culture system, rather than an increase in overall α -synuclein pathology.

Comment

Added western blots in revision help to prove that processing of the exogenous PFF is altered in astrocytes and cathepsin D is mechanistically responsible for PFF processing, but must be quantified.

Response:

We have quantified the immunoblot data examining C-terminally truncated and full-length α -synuclein species in PFF-exposed astrocytes. The quantitative analyses have been incorporated into the revised Fig. 3A and 3C, and the corresponding statistical analyses and sample sizes are now provided.

These analyses strengthen the conclusion that PFF uptake by astrocytes is accompanied by time-dependent generation of C-terminally truncated α -synuclein species and that this processing is reduced by inhibition of aspartic protease activity.

Comment

The authors could add similar soluble/insoluble blots for mouse synuclein in their culture experiments to prove that altered processing of exogenous human PFF leads to altered propagation/amount/maturation of endogenous mouse synuclein in neurons.

Response:

As noted by the Editor, these additional experiments are not required for the present revision. We have instead revised the manuscript to clearly distinguish morphological maturation of LN-like aggregates from an increase in the total burden of pathological α -synuclein. We also explicitly acknowledge in the Discussion that increased LN-like aggregate size alone does not establish changes in the total amount or biochemical state of endogenous neuronal α -synuclein. Future biochemical studies will be required to address this question directly.

We thank the Editor and all three referees for their thoughtful and constructive comments. We believe that these revisions have improved the clarity and rigor of the manuscript and have more clearly defined the scope and limitations of our conclusions.

Sincerely,

Hiroshi Hanafusa
on behalf of all authors

Dr. Hiroshi Hanafusa
Nagoya University
Division of Biological Science
Japan

Dear Dr. Hanafusa,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

Your manuscript will be processed for publication by EMBO Press. It will be copy edited and you will receive page proofs prior to publication. Please note that you will be contacted by Springer Nature Author Services to complete licensing and payment information.

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Should you be planning a Press Release on your article, please notify the production team via this form in order to coordinate publication and release dates: <https://forms.cloud.microsoft/e/eH4A8JVP6g>

If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to EMBO Reports.

Yours sincerely,

Esther Schnapp, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: Hiroshi Hanafusa
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2025-62975V2

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Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: [10.31222/osf.io/9sm4x](#)). Please follow the journal's guidelines in preparing your

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.

Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	
Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For antibodies provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Reagents and Tools Table
DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Reagents and Tools Table
Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository OR supplier name, catalog number, clone number, and/OR RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods
Report if the cell lines were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	
Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository OR supplier name, catalog number, clone number, OR RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail housing and husbandry conditions .	Not Applicable	
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Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	
Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Design

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Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if external detailed step-by-step protocols are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about sample size estimate even if no statistical methods were used.	Yes	Materials and Methods, Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, have they been described?	Yes	Materials and Methods
Include a statement about blinding even if no blinding was done.	Yes	Materials and Methods
Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not Applicable	
If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.		
For every figure, are statistical tests justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods, Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was replicated in laboratory.	Yes	Figures
In the figure legends: define whether data describe technical or biological replicates .	Yes	Figures

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving human participants : State details of authority granting ethics approval (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving human participants : Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving human participants : For publication of patient photos , include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental animals : State details of authority granting ethics approval (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving specimen and field samples : State if relevant permits obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of select agents and toxins (CDC): https://www.selectagents.gov/sat/list.htm .	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the authority granting approval and reference number for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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For tumor marker prognostic studies , we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For phase II and III randomized controlled trials , please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have primary datasets been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section
Were human clinical and genomic datasets deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective data citations in the reference list .	Not Applicable	