nature portfolio

Peer Review File

Lewy-MSA hybrid fold drives distinct neuronal a-synuclein pathology

Corresponding Author: Professor Gabor Kovacs

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Review of the manuscript “Lewy-MSA hybrid fold drives distinct neuronal a-synuclein pathology” by Masahiro Enomoto and coauthors submitted to “Communication Biology”
Synucleinopathies are a group of neurodegenerative diseases characterized by the abnormal accumulation of α-synuclein aggregates. α-Synuclein pathology can occur in neurons, as seen in Lewy body disease, or in oligodendrocytes, as in multiple system atrophy (MSA). Drug treatments for these disorders can only alleviate symptoms and do not reverse the progression of the disease. The authors present new findings suggesting the existence of a novel Lewy-MSA hybrid fold within the same brain region, which contributes to the atypical histopathological form of MSA. This is a significant area of biomedical research, and the results presented in the manuscript make a fundamental contribution to the field.
The following corrections should be made.
Abstract
The following sentence: “The ordered assembly of a-synuclein protein into filaments encoded by SNCA characterizes neurodegenerative diseases called synucleinopathies” is confusing, since it can be understood as not a monomeric a-synuclein protein, but filaments are encoded by SNCA. It is recommended to correct it for clarity indicating that it is the α-synuclein protein that is encoded by the SNCA gene, not the filaments themselves.
Introduction
-To facilitate the understanding of further text the authors should add the following introductory sentence and a reference: “α-Synuclein exists in a dynamic equilibrium between several conformational monomeric forms and the assembly of aggregates and filaments [reference: Synucleins: New Data on Misfolding, Aggregation and Role in Diseases. Biomedicines. 2022; 10(12):3241. https://doi.org/10.3390/biomedicines10123241]/
- After the sentence “LBD is traditionally considered to show neuronal predominant a-synuclein pathology” a reference or references should be added.
Results
-How the authors explain the resistance of a portion of the sarkosyl-insoluble α-synuclein in the temporal cortex of the LBD case shown on Fig. 2f?
-The text under the Figure 2 E and F is blurry and hard to read. The fonts should be increased for easy reading.
- What are the a-syn species with molecular weight of ~18 kDa and, ~12 KDa?
Discussion
-“Importantly, the large neuronal intracytoplasmic inclusions detectable on Hematoxylin and eosin staining showing a-synuclein immunoreactivity were mostly seen in the hippocampus and amygdala, while in the neocortical areas the neuronal inclusions were considerably less and mostly seen only in the a-synuclein immunostaining.” A reference or references should be added after this sentence.
-“This could also suggest that the cofactors in the Lewy core fold and in the inter-protofilament interface of the MSA Type I and Type II might be different, implying that the cofactors could be molecular determinants of α-synuclein filaments structures which drive distinct human a-synuclein pathologies.”
More specific information (at least hypothetical) about these cofactors should be added.
Clinical history
“A 68-year-old right-handed person was assessed for potential advanced therapies at the Movement Disorders Clinic of the Toronto Western Hospital.”
Was the study approved by the Institutional Review Board (IRB) for use of clinical samples from patients?

Overall, the manuscript does not contain any technical or conceptual flaws that might prohibit its publication. The conclusions are original and fact based. The outstanding finding described in the manuscript is the existence of a novel Lewy-MSA hybrid fold within the same brain region, which contributes to the atypical histopathological form of MSA.

Reviewer #2

(Remarks to the Author)
In this manuscript, Enomoto and colleagues studied a single MSA case showing atypical α-syn pathology as compared to classical MSA cases. Neuropathological examination showed brain regions with prominent neuronal cytoplasmic inclusions while others showed MSA-specific traits (GCI, neuronal nuclear inclusions). Focusing on the temporal cortex bearing this neuronal peculiar pathology, and cerebellum of this atypical MSA case, authors claim to find divergence in biochemical features of pathological α-syn (solubility, resistance to proteases or denaturation) between regions, and compared to MSA and LBD cases. The extraction and Cryo-EM structure determination of filaments from temporal cortex showed a unique type of assembly of two protofilaments with one MSA fold and one hybrid between classical MSA and Lewy pathology folds.

I find the study of an atypical MSA case showing distinct αsyn pathology between brain regions of a significant importance. The links between this “hybrid” pathology and the findings of a novel filament structure (which itself represents a considerable task), possibly at the molecular basis of the phenotype, can have significant impact in the field. However, I raised below several substantial concerns regarding the neuropathology findings, but mostly on the biochemical part of the study, which represent a strong weakness of this manuscript.

-General comments

#1 At the beginning of the results section, I strongly encourage authors to introduce the study design and aim : To mention clearly that it’s an examination of a single atypical MSA case and include here a brief summary of the clinical data before neuropath findings. In its present format, the start is a little abrupt.

#2 General comment on the figures: Globally, I strongly recommend to include within the figures the case type (MSA aMSA LBD) on IHC sections, and depict the GCIs NCIs etc with arrows, associated with a little caption directly on the picture. Same holds true for the fractions used : total homogenate, sarkosyl-insoluble fraction etc. But most of all, to write on every IHC, SDS-PAGE immunolabelling the name of the antibody used. The back-and-forth going to read the legend, and the method section makes it tough for the reader to follow.

- Major concerns :

#3 On IHC and SDS-PAGE, authors used the antibody 5G4, which is not properly specific to pathological nor aggregated α-syn. Why didn’t they include stainings/immunoblots with pS129-syn antibody such as EP1536Y (abcam) ? In order to visualize pathological α-syn on sections, and on western blotting fractions, it seems mandatory to me.

#4 On all Fig 2 SDS-PAGE, authors used Syn-1 which is an antibody directed to aa91-96/99 (and not 15-123 as mentioned in the methods and on the supplier’s datasheet). This epitope, situated in the amyloid core of the protein, makes it specific to non-amyloid α-syn! Instead, I would recommend to use EP1536Y for pSyn and MJFR-1 (abcam) for total α-syn.

#5 In Fig 2.e-f : In my opinion, the bands observed around 10KDa (and not between 15 and 20 KDa as monomeric band α-syn would be) are fragments of the protein positive with Syn1 (and I presume, negative with a Cterm antibody), putatively associated with the pathological form. It raises other questions : why didn’t authors perform a sarkosyl extraction of a control non ND sample ? Also, they should show the α-syn content, labelled with pSyn and total α-syn, of all fractions throughout extraction, including discarded pellets/supernatants. Because I foresee that other disease samples actually comprise pS129 aggregated syn that is lost during the procedure, hence the importance of comparing with a non-disease control. In summary, here it suggests that only insoluble fractions of TCx of atypical MSA contain aggregated α-syn questioning the relevance of the subsequent SAA and GdnHCl tests.

#6 In light of the former point, the SAA in figure 2.g crucially lacks two controls : seeding with extracts of a non-ND patient, and non-seeded controls.

#7 Same stands true for the GdnHCl results. Additionally, I have strong doubts about the relevance of checking on SDS-PAGE with Syn1 the resistance of aggregated α-syn to Gdn treatments. It is not like for prions, where resistance to proteases is progressively lost when aggregates are disassembled with increasing Gdn concentration, and monitored with the disappearance of the monomers that are digested. Here, I would strongly suggest to perform some filter-blot (or filter-trap) assays on the total solubilized brain extracts treated with Gdn in order to monitor the disassembly of α-syn filaments with pSyn, MJFR1 or MJFR14 antibodies.

- Comment about Cryo-EM :

#8 The structure obtained remains very interesting. Maybe authors should comment on the non-proteinaceous density ? It

could be possibly due to the extraction procedure (sarkosyl or so), thus it would have been interesting to compare with classical MSA and LBD structures obtained in parallel (however I am conscious of the difficulty to obtain these).
One question : was this Lewy-MSA fold representing 100% of the structures obtained or was there a low percentages of known forms also present in the total population of assemblies ?

Reviewer #3

(Remarks to the Author)
Comments to the Authors
In this study, Enomoto et al. investigate the structural and biochemical properties of α-synuclein filaments in synucleinopathies. The study highlights the presence of a novel hybrid filament fold, termed the "Lewy-MSA hybrid fold," in atypical MSA, a rare subtype of MSA that displays abundant neuronal α-synuclein inclusions in the limbic system. The authors demonstrate that in addition to the MSA-specific fold, this hybrid fold coexists within the same brain region, providing insights into the histopathological and cytopathological heterogeneity of synucleinopathies. Using protease-sensitivity digestion assays, seed amplification assays, and conformational stability assays, the authors link distinct filament folds to cell-specific protein pathologies. This study expands the structure-based classification of α-synucleinopathies and proposes that distinct filament folds contribute to the diverse pathological and clinical manifestations of these diseases.

Overall Recommendation
The manuscript offers valuable insights into the structural diversity of α-synuclein filaments in neurodegenerative diseases, with a novel and significant finding in the form of the Lewy-MSA hybrid fold. However, its generalizability is limited, and key claims lack experimental validation.
General and Specific Comments:
1. The identification of the Lewy-MSA hybrid protofilament as a novel structural feature combining elements of both LBD and MSA pathologies by employing Cryo-EM is a significant advancement.
2. The manuscript employs a multi-disciplinary approach, integrating clinical, neuropathological, biochemical, and structural analyses. The neuropathological description is thorough, clearly detailing the asymmetric distribution of α-synuclein pathology and its association with atypical MSA.
3. The study relies on a single case of atypical MSA, which limits the generalizability of the findings due to patient variability that can affect the pathology. Including additional cases, particularly from LBD and MSA cohorts, and performing at least neuropathological and biochemical evaluations would significantly strengthen the study's conclusions.
4. While the structural details of the Lewy-MSA hybrid protofilament are well-documented, the manuscript lacks sufficient discussion on the broader implications of this finding for disease pathogenesis or treatment strategies.
5. Additionally, I suggest that the authors clarify the reason for focusing on a single region of the brain, temporal cortex. They mention that the typical MSA doublet filaments were not identified in their study—would the results have been different if other brain regions had been examined?
6. The researchers mention that SAAs can be quantified, but more information is needed on this aspect. I suggest that they provide details on the threshold used and discuss the differences in seeding behavior between MSA cases and LBD cases. Specifically, the LBD case amplified later than the MSA cases, did not reach as high a level, and showed wider error bars. The threshold is particularly important since the overall fluorescent signal from the samples is relatively low. Additionally, I recommend that they include the positive and negative controls of the experiment, possibly in the supplementary data section, to provide insight into the signal characteristics of their controls. (Figure 2g)
7. To improve the clarity of the figure, the researchers should add labels to the blots, such as the doses of GdnCl. (Figure 2 h-k)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors corrected the manuscript as recommended by the reviewers, improving its quality.

Reviewer #2

(Remarks to the Author)
In this revised version of their manuscript, authors made substantial efforts and performed additional experiments to answer my questions and allay the concerns I raised (I thank them for that). In my opinion the manuscript gained clarity and relevance. This article represents a significant step forward and will have valuable impact in the field.

Reviewer #3

(Remarks to the Author)
The authors have adequately addressed my previous comments and revised the manuscript accordingly. The changes made to the manuscript have improved its clarity and overall quality. I find the revised version acceptable and recommend it for publication.

Toronto, 29th April, 2025

Re: Manuscript Number: COMMSBIO-25-0002-T

Title: **Lewy-MSA hybrid fold drives distinct neuronal a-synuclein pathology**

Dear Editor,

Thank you for inviting us to revise and resubmit our manuscript. We appreciate the helpful comments of the reviewers and provide a point-by-point response below. Please find below our response and corresponding changes in the manuscript. In our response we refer to the highlighted version of the manuscript to show the changes. In summary, we performed further **immunoblotting and immunohistochemistry studies using further anti-α-synuclein antibodies**. In addition, we had the opportunity to perform **cryo-EM from a further anatomical brain region (i.e., frontal cortex)** that showed similar neuronal inclusions as the hippocampus /temporal cortex but in lower density and we were able to demonstrate the new fold in the frontal cortex sample as well (see added description rows 231-242. We reduced the word count of the Abstract to 150 words per Journal requirement. We believe that the manuscript has been significantly improved, and it is an impactful contribution to the literature.

Kind regards,

Gabor G. Kovacs on behalf of all authors.

REVIEWERS' COMMENTS:

REVIEWER #1

The authors present new findings suggesting the existence of a novel Lewy-MSA hybrid fold within the same brain region, which contributes to the atypical histopathological form of MSA. This is a significant area of biomedical research, and the results presented in the manuscript make a fundamental contribution to the field. The following corrections should be made.

COMMENT #1: Abstract/The following sentence: "The ordered assembly of a-synuclein protein into filaments encoded by SNCA characterizes neurodegenerative diseases called synucleinopathies" is confusing, since it can be understood as not a monomeric a-synuclein protein, but filaments are encoded by *SNCA*. It is recommended to correct it for clarity indicating that it is the α-synuclein protein that is encoded by the SNCA gene, not the filaments themselves.

Our response and changes in the manuscript:

Thank you. We corrected as follows: "The ordered assembly of α-synuclein protein encoded by *SNCA* into filaments characterizes neurodegenerative diseases called synucleinopathies."

COMMENT #2: Introduction/To facilitate the understanding of further text the authors should add the following introductory sentence and a reference: "α-Synuclein exists in a dynamic equilibrium between several conformational monomeric forms and the assembly of aggregates and filaments [reference: Synucleins: New Data on Misfolding, Aggregation and Role in Diseases. Biomedicines. 2022; 10(12):3241. https://doi.org/10.3390/biomedicines10123241]/.

Our response and changes in the manuscript:

Thank you: we added this important sentence.

COMMENT #3: Introduction/After the sentence "LBD is traditionally considered to show neuronal predominant a-synuclein pathology" a reference or references should be added.

Our response and changes in the manuscript:

We added two references:

Kovacs, G. G. *et al.* Biomarker-Based Approach to alpha-Synucleinopathies: Lessons from Neuropathology. *Mov Disord*, (2024).

Attems, J. *et al.* Neuropathological consensus criteria for the evaluation of Lewy pathology in post-mortem brains: a multi-centre study. *Acta Neuropathol* **141**, 159-172, (2021).

COMMENT #4: Results/How the authors explain the resistance of a portion of the sarkosyl-insoluble α-synuclein in the temporal cortex of the LBD case shown on Fig. 2f?

Our response:

Thank you for your comment. The partial resistance of sarkosyl-insoluble α-synuclein observed in the temporal cortex of the LBD case in Fig. 2f likely reflects the presence of highly aggregated, compact conformers with a tightly packed β-sheet core that are more resistant to PK digestion. In contrast, this resistance is not observed in the typical MSA case as well as in the cerebellum of our atypical MSA case, due to fundamental differences in α-synuclein conformation and aggregation state. MSA-derived α-synuclein tends to form more structurally homogeneous filaments with distinct strain-specific folding properties, resulting in increased sensitivity to denaturation and less stable sarkosyl-insoluble species under the same assay conditions (please see Schweighauser M. *et al*, Nature, 2020).

COMMENT #5: The text under the Figure 2 E and F is blurry and hard to read. The fonts should be increased for easy reading.

Our response and changes in the manuscript:

Thank you. We have edited Figure 2 and based on comments from reviewers #2 and #3 we added new images moved panels **h-k** from the former Figure 2 to the supplemental extended file. In the revised version of the manuscript and the fonts are now bigger for easy reading.

COMMENT #6: What are the a-syn species with molecular weight of ~18 kDa and, ~12 KDa?

Our response:

Thank you for your question. After preforming a conformational stability assay using the detergent-soluble fraction from postmortem human brain tissue and probed with the Syn-1 antibody, we believe that the ~18 kDa band likely represents post-translationally modified or conformationally altered monomeric alpha-synuclein, while the ~12 kDa band likely corresponds to C-terminally truncated forms such as 1–120/1–122, which retain the Syn-1 epitope and are commonly associated with disease. In contrast, when using the sarkosyl-insoluble fraction, different alpha-synuclein species are observed due to the presence of aggregated, misfolded, and protease-resistant forms, which expose different truncation or cleavage patterns, reflecting the biochemical heterogeneity of pathological alpha-synuclein.

COMMENT #7: Discussion/*"Importantly, the large neuronal intracytoplasmic inclusions detectable on Hematoxylin and eosin staining showing a-synuclein immunoreactivity were mostly seen in the hippocampus and amygdala, while in the neocortical areas the neuronal inclusions*

were considerably less and mostly seen only in the a-synuclein immunostaining." A reference or references should be added after this sentence.

Our response and changes in the manuscript:

Thank you. This is the summary of our neuropathological observation for the present case and therefore no reference can be added. We clarified this in the paragraph.

COMMENT #8: Discussion/*"This could also suggest that the cofactors in the Lewy core fold and in the inter-protofilament interface of the MSA Type I and Type II might be different, implying that the cofactors could be molecular determinants of α-synuclein filaments structures which drive distinct human a-synuclein pathologies*." More specific information (at least hypothetical) about these cofactors should be added.

Our response and changes in the manuscript:

Previous studies suggest polyphosphates, phosphatidylinositol phosphates and inositol phosphates are potential candidates. We have added these sentences with new citations as below. See Discussion rows 309-316 and new references 30 and 31.

COMMENT #9: Clinical history "A 68-year-old right-handed person was assessed for potential advanced therapies at the Movement Disorders Clinic of the Toronto Western Hospital." Was the study approved by the Institutional Review Board (IRB) for use of clinical samples from patients?

Our response:

Yes, we confirm that the study was approved, and this information is found in the ***METHODS section/Neuropathology***.

"Brain tissue was examined in the frame of an ongoing Brain Donation program of the Toronto Western Hospital following the approval of the local ethics committee (University Health Network Research Ethics Board, Nr. 20-5258) and adherence to appropriate consent protocols."

GENERAL COMMENT: Overall, the manuscript does not contain any technical or conceptual flaws that might prohibit its publication. The conclusions are original, and fact based. The outstanding finding described in the manuscript is the existence of a novel Lewy-MSA hybrid fold within the same brain region, which contributes to the atypical histopathological form of MSA.

Our response:

Thank you for the constructive and helpful comments.

REVIEWER #2

I find the study of an atypical MSA case showing distinct αsyn pathology between brain regions of a significant importance. The links between this "hybrid" pathology and the findings of a novel filament structure (which itself represents a considerable task), possibly at the molecular basis of the phenotype, can have significant impact in the field. However, I raised below several substantial concerns regarding the neuropathology findings, but mostly on the biochemical part of the study, which represent a strong weakness of this manuscript.

COMMENT #1: At the beginning of the results section, I strongly encourage authors to introduce the study design and aim. To mention clearly that it's an examination of a single atypical MSA case and include here a brief summary of the clinical data before neuropath findings. In its present format, the start is a little abrupt.

Our response and changes in the manuscript:

Thank you. We added that we examine one patient and added further information on clinical data here also in addition to the section in the Methods part. See highlighted version of the manuscript.

COMMENT #2 General comment on the figures: I strongly recommend including within the figures the case type (MSA aMSA LBD) on IHC sections, and depict the GCIs NCIs etc with arrows, associated with a little caption directly on the picture. Same holds true for the fractions used: total homogenate, sarkosyl-insoluble fraction etc. But most of all, to write on every IHC, SDS-PAGE immunolabelling the name of the antibody used. The back-and-forth going to read the legend, and the method section makes it tough for the reader to follow.

Our response and changes in the manuscript:

We thank the reviewer for this valuable suggestion. We have revised the figures to include the case type (e.g., MSA, aMSA, LBD) directly on the IHC images, and we now indicate GCIs, NCIs, and other relevant inclusions with arrows. We have also labeled the fractions used (sarkosyl-insoluble fraction) on each relevant panel. We have also included the name of the antibody used on every immunoblot panel, as suggested, to eliminate the need for the reader to refer to the figure legend or methods section.

COMMENT #3: On IHC and SDS-PAGE, authors used the antibody 5G4, which is not properly specific to pathological nor aggregated α-syn. Why didn't they include stainings/immunoblots with pS129-syn antibody such as EP1536Y (abcam)? In order to visualize pathological α-syn on sections, and on western blotting fractions, it seems mandatory to me.

Our response and changes in the manuscript:

In the original version we used 5G4 antibody for immunohistochemistry only. We respectfully disagree with the comments of the 5G4 antibody used for IHC. This antibody has been well-characterized (see: PMID: 22370907 and PMID: 24878508) and used widely for neuropathology studies. Comparisons with other antibodies including the phos129Ser antibody has been reported; see a list of references including those independent from the authors of the present paper:

Kumar, S.T., et al. *Neurobiol. Dis.* **146**, 105086 (2020). https://doi.org/10.1016/j.nbd.2020.105086 PMID: 32971232

van Diggelen, F., et al. Two conformationally distinct α-synuclein oligomers share common epitopes and the ability to impair long-term potentiation. *PLoS ONE* **14**, e0213663 (2019). https://doi.org/10.1371/journal.pone.0213663PLOS PMID: 30901378

Sorrentino, Z.A., *et al.* Unique α-synuclein pathology within the amygdala in Lewy body dementia: implications for disease initiation and progression. *Acta Neuropathol. Commun.* **7**, 142 (2019). Https://doi.org/10.1186/s40478-019-0787-2 PMID: 31477175

Altay, M.F., et al.. Prominent astrocytic alpha-synuclein pathology with unique post-translational modification signatures unveiled across Lewy body disorders. *Acta Neuropathol. Commun.* **10**, 163 (2022). Https://doi.org/10.1186/s40478-022-01468-8 PMID: 36371251

And please find below the paper here we compared 5G4 with other synuclein antibodies:

Martinez-Valbuena, I., Visanji, N.P., Kim, A. *et al.* Alpha-synuclein seeding shows a wide heterogeneity in multiple system atrophy. *Transl. Neurodegener.* **11**, 7 (2022). https://doi.org/10.1186/s40035-022-00283-4 PMID: 35125105

For the sake of completeness, we added images using IHC and antibodies for phosphorylated α-synuclein and the MJFR-1 antibody to extended supplemental file that *confirm the findings* with the 5G4 antibody: see Extended file Suppl-Fig.2.

For SDS-PAGE we used the α-synuclein antibody Syn-1 clone, ref: 610786, BD Biosciences). Following the reviewer's suggestion, we have now provided the Western blots in Figure 2 using the EP1536Y antibody for phosphorylated α-synuclein and the MJFR-1 antibody (Abcam) for total α-synuclein. These changes are reflected in the updated version of the manuscript and in the revised figure 2 (see panels e-h).

COMMENT #4: On all Fig 2 SDS-PAGE, authors used Syn-1 which is an antibody directed to aa91-96/99 (and not 15-123 as mentioned in the methods and on the supplier's datasheet). This epitope, situated in the amyloid core of the protein, makes it specific to non-amyloid α-syn! Instead, I would recommend using EP1536Y for pSyn and MJFR-1 (abcam) for total α-syn.

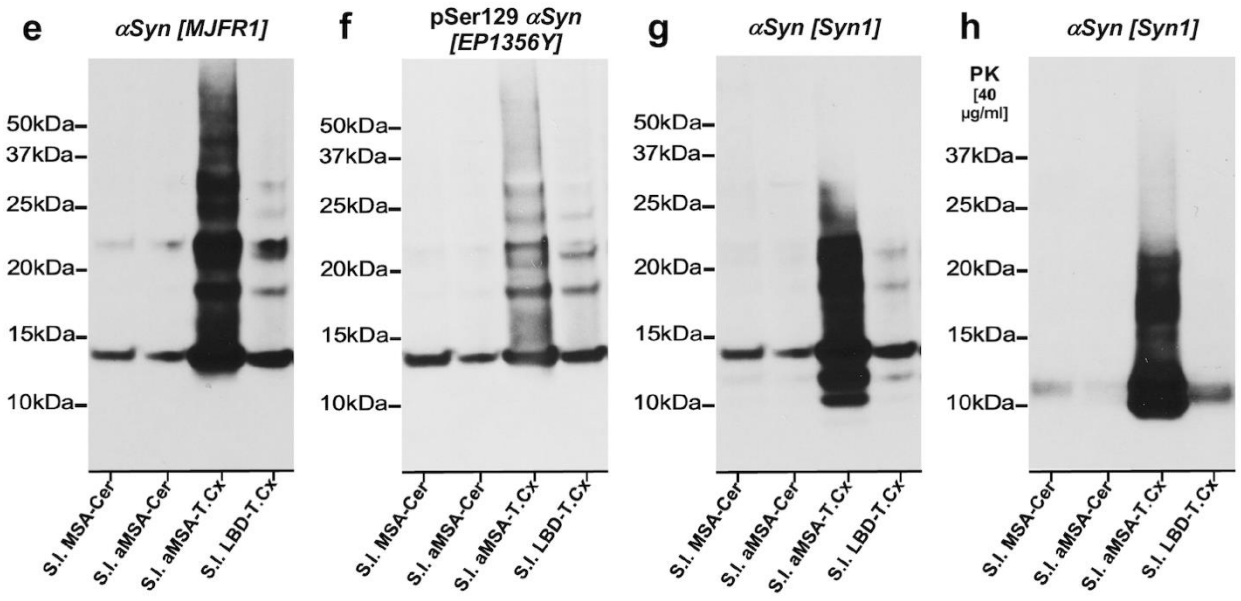
Our response and changes in the manuscript:

Following the reviewer's suggestion, we have now amended the Syn1 epitope and provided new western blots in Figure 2 using the EP1536Y antibody for phosphorylated α-synuclein and the MJFR-1 antibody (Abcam) for total α-synuclein. These changes are reflected in the updated version of the manuscript and in the revised figure 2 (see panels e-h).

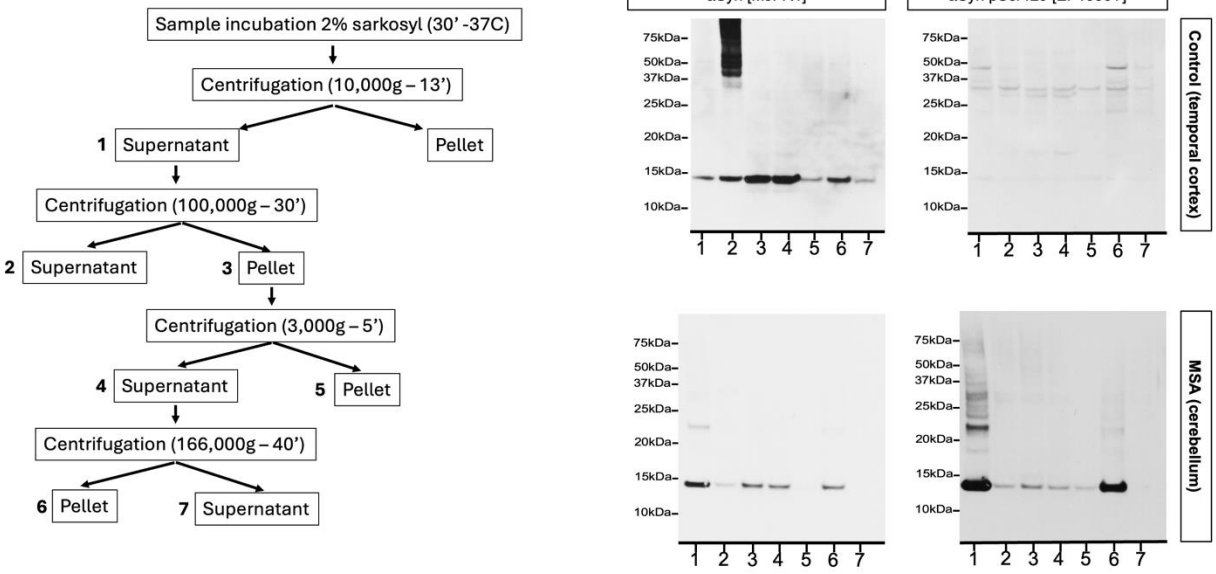
COMMENT #5 In Fig 2.e-f : In my opinion, the bands observed around 10KDa (and not between 15 and 20 KDa as monomeric band α-syn would be) are fragments of the protein positive with Syn1 (and I presume, negative with a Cterm antibody), putatively associated with the pathological form. It raises other questions: why didn't authors perform a sarkosyl extraction of a control non-ND sample? Also, they should show the α-syn content, labelled with pSyn and total α-syn, of all fractions throughout extraction, including discarded pellets/supernatants. Because I foresee that other disease samples actually comprise pS129 aggregated syn that is lost during the procedure, hence the importance of comparing with a non-disease control. In summary, here it suggests that only insoluble fractions of TCx of atypical MSA contain aggregated α-syn questioning the relevance of the subsequent SAA and GdnHCl tests.

Our response and changes in the manuscript:

We thank the reviewer for this comment and for raising important points regarding the interpretation of the ~10 kDa bands, the extraction workflow, and the need for appropriate controls. In response, we have now performed a panel using three different α-synuclein antibodies. Across all three antibodies, we consistently observed a band around ~14 kDa. In addition, using the Syn-1 antibody, we detected lower molecular weight fragments of approximately ~10 kDa. These fragments were most prominent in the temporal cortex of the aMSA case but were also present, to a lesser extent, in the three other samples (see new Figure 2 e-h):



In response to the reviewer's suggestion, we have performed one control (non-ND) case and one typical MSA case processed in parallel using the same sarkosyl extraction protocol. Due to limited availability of tissue from the atypical MSA case, we were unfortunately unable to perform this analysis in additional samples. We have included only in this rebuttal letter the immunoblots showing the α-synuclein content (using both EP1536Y and MJFR-1 antibodies) across all major fractions throughout the extraction procedure, including previously discarded supernatants and pellets. Please see below:



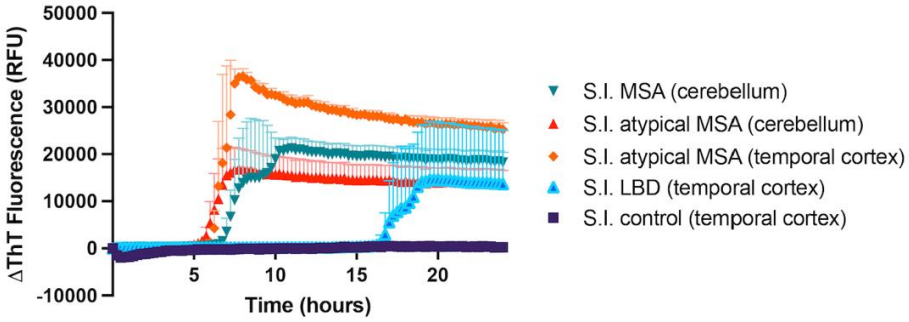
We also wish to clarify that the Western blot proteinase K digestion and SAA experiments were performed specifically on the sarkosyl-insoluble fraction, as this was the fraction used for the cryo-EM analysis. We have clarified this in the revised version of the legend of Figure 2.

COMMENT #6 In light of the former point, the SAA in figure 2.g crucially lacks two controls: seeding with extracts of a non-ND patient, and non-seeded controls.

Our response and changes in the manuscript:

In the revised version of the manuscript, we now include the recommended control in the SAA experiment shown in the new Figure 2 (i): seeding with extracts from a non-neurodegenerative (non-ND) control case. This controls confirm the specificity of the assay and support the interpretation of the seeding results. Additionally, we note that this assay has been extensively validated in our previous work (*Martinez-Valbuena et al., Transl Neurodegener, 2022*), where we demonstrated its robustness and specificity across a large set of disease and control samples under the same reaction conditions.

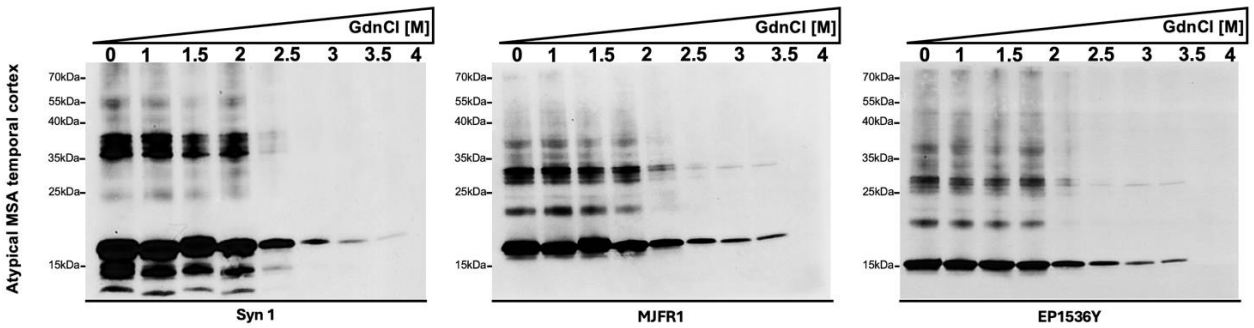
i



COMMENT #7: Same stands true for the GdnHCl results. Additionally, I have strong doubts about the relevance of checking on SDS-PAGE with Syn1 the resistance of aggregated α-syn to Gdn treatments. It is not like for prions, where resistance to proteases is progressively lost when aggregates are disassembled with increasing Gdn concentration, and monitored with the disappearance of the monomers that are digested. Here, I would strongly suggest to perform some filter-blot (or filter-trap) assays on the total solubilized brain extracts treated with Gdn in order to monitor the disassembly of α-syn filaments with pSyn, MJFR1 or MJFR14 antibodies.

Our response and changes in the manuscript:

We thank the reviewer for these insightful comments regarding the interpretation and methodology of the GdnHCl conformational stability assay. We agree that filter-trap assays would provide a more direct measure of filament disassembly and appreciate the suggestion to use pSyn and total α-syn antibodies such as EP1536Y, MJFR1, or MJFR14. Unfortunately, due to limited availability of tissue from the temporal cortex of the atypical MSA case, we are unable to perform additional experiments, including the recommended filter-trap assays. Nonetheless, in response to the reviewer's concern, we have moved the CSA analysis to the supplementary material (Supplementary Fig. 3) in the revised version of the manuscript. To highlight the relevance and specificity of the detection, we re-blotted the CSA membranes using two additional antibodies, EP1536Y and MJFR-1. In the temporal cortex of the atypical MSA patient, we tested all three antibodies—Syn-1, EP1536Y, and MJFR-1—and observed consistent denaturation patterns, confirming the robustness of the results. We hope this adjustment addresses the reviewer's concerns within the limitations of the available material.



COMMENT #8 The structure obtained remains very interesting.

Maybe authors should comment on the non-proteinaceous density? It could be possibly due to the extraction procedure (sarkosyl or so), thus it would have been interesting to compare with classical MSA and LBD structures obtained in parallel (however I am conscious of the difficulty to obtain these).

Our response and changes in the manuscript:

Thank you. Regarding the possibility that the non-proteinaceous density is due to the sarkosyl used in the extraction procedure, we added these sentences in the Results section (see highlighted version rows 218-226). We also compared the Lewy-MSA hybrid fold with the classical MSA and LBD folds as shown in Extended data and suppl-Fig. 5. The superimposition of Lewy-L (K32-K45) of the Lewy-MSA hybrid fold with that of the classical LBD fold including electron density maps revealed that the relative position of non-proteinaceous density of Lewy-L to K32, K34, K43 and K45 is nearly identical with that of the Lewy fold of LBD. As opposed, the coordination patterns of the non-proteinaceous densities of MSA Type I and Type II folds were notably distinct from that of the Lewy-L. This indicates that that the coordination pattern of a non-proteinaceous material of Lewy-L is identical with that of the Lewy fold from LBD. We have added these sentences to the Results section (see highlighted version rows 218-226): We have also further discussed about a molecular identity of the non-proteinaceous material in Discussion section (see highlighted version rows 309-316):

COMMENT #9: Was this Lewy-MSA fold representing 100% of the structures obtained or was there a low percentage of known forms also present in the total population of assemblies?

Our response:

As shown in Extended data and suppl-Fig. 4, in the temporal cortex nearly 100% of filaments picked were Lewy-MSA hybrid filaments. We have also performed cryo-EM for the frontal cortex and found that ~90% of filaments were Lewy-MSA hybrid filaments and the rest of ~10% filaments exhibited a distinct 2D image, which were suboptimal for structure determination by cryo-EM. Thus, no classical MSA or LBD folds were present in the temporal cortex and the frontal cortex.

REVIEWER #3

This study expands the structure-based classification of α-synucleinopathies and proposes that distinct filament folds contribute to the diverse pathological and clinical manifestations of these diseases. The manuscript offers valuable insights into the structural diversity of α-synuclein filaments in neurodegenerative diseases, with a novel and significant finding in the form of the Lewy-MSA hybrid fold. However, its generalizability is limited, and key claims lack experimental validation.

COMMENT #1: The identification of the Lewy-MSA hybrid protofilament as a novel structural feature combining elements of both LBD and MSA pathologies by employing Cryo-EM is a significant advancement.

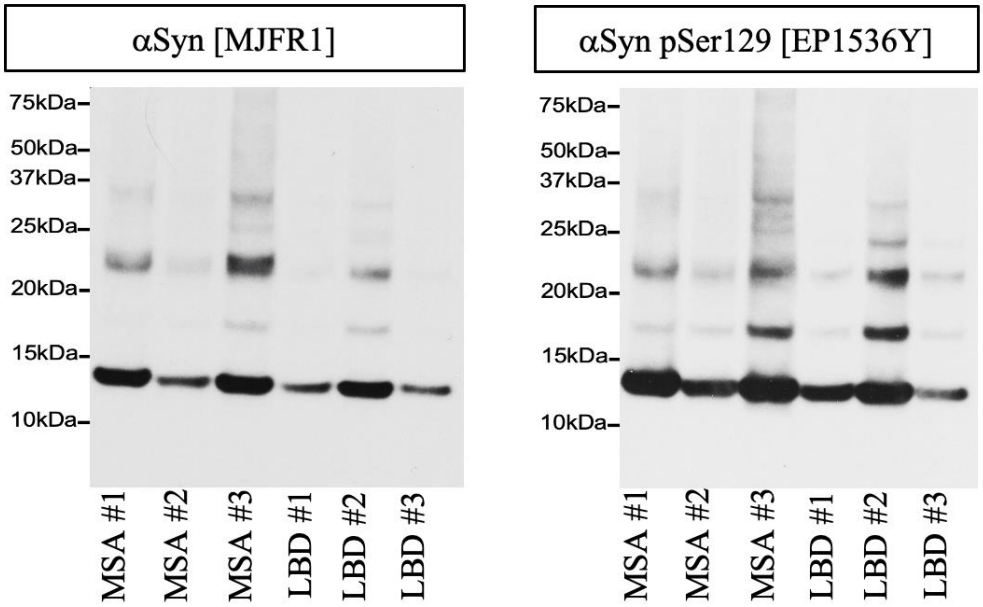
COMMENT #2: The manuscript employs a multi-disciplinary approach, integrating clinical, neuropathological, biochemical, and structural analyses. The neuropathological description is thorough, clearly detailing the asymmetric distribution of α-synuclein pathology and its association with atypical MSA.

COMMENT #3: The study relies on a single case of atypical MSA, which limits the generalizability of the findings due to patient variability that can affect the pathology. Including additional cases, particularly from LBD and MSA cohorts, and performing at least neuropathological and biochemical evaluations would significantly strengthen the study's conclusions.

Our response and changes in the manuscript (Comments#1-3):

We thank the reviewer for the supportive comments. We acknowledge that the use of a single atypical MSA case limits the generalizability of the findings. However, the primary aim of this study was to determine whether this specific case—characterized by atypical clinical and pathological features—harbored a distinct α-synuclein fold. The inclusion of typical MSA and LBD cases in our biochemical and histological analyses was not intended to establish group comparisons, but rather to provide context and support for the uniqueness of the newly identified fold by showing consistency with the accompanying biochemical and neuropathological profiles. We have clarified these points in the revised manuscript to better reflect the rationale and interpretation of these findings (please see lines 136-141). We believe that adding additional typical MSA and LBD cases to the main biochemical and histological sections could dilute the main message of the paper and shift the focus away from the central discovery—namely, the identification of a distinct α-synuclein filament fold in an atypical MSA case.

To address the reviewer’s concern, however, we have now performed sarkosyl-insoluble (SI) extractions from three typical MSA and three typical LBD cases and immunoblotted these samples using the Syn-1 antibody. These results are included only in the rebuttal letter (please see below), and they demonstrate that the α-synuclein profile observed in the temporal cortex of the atypical MSA case is unique and not seen in typical MSA or LBD cases. We hope this additional data further supports the reviewer’s point while maintaining the focus of the manuscript.



COMMENT #4: While the structural details of the Lewy-MSA hybrid protofilament are well-documented, the manuscript lacks sufficient discussion on the broader implications of this finding for disease pathogenesis or treatment strategies.

Our response and changes in the manuscript:

Thank you, we have now expanded on this issue, see highlighted version (Discussion rows 301-316).

COMMENT #5: Additionally, I suggest that the authors clarify the reason for focusing on a single region of the brain, temporal cortex. They mention that the typical MSA doublet filaments were not identified in their study—would the results have been different if other brain regions had been examined?

Our response and changes in the manuscript:

In the initial manuscript, we focused on the temporal cortex, which exhibited the most severe atypical MSA pathology. Subsequently, we further examined the cerebellum and the frontal cortex using cryo-EM. The small amount of sample from the cerebellum was suboptimal for structure determination by cryo-EM primarily due to the low contrast of the filament images surrounded by a high amount of other materials that were extracted together with the filaments. We had the opportunity to examine the frontal cortex for cryo-EM. From the frontal cortex, we also identified the same Lewy-MSA hybrid fold, which is included in the revised manuscript. Indeed, the neuropathology was similar to the temporal cortex but less severe. In this sample we were able to generate a higher resolution image of the newly discovered fold. We have now added information on the frontal cortex sample.

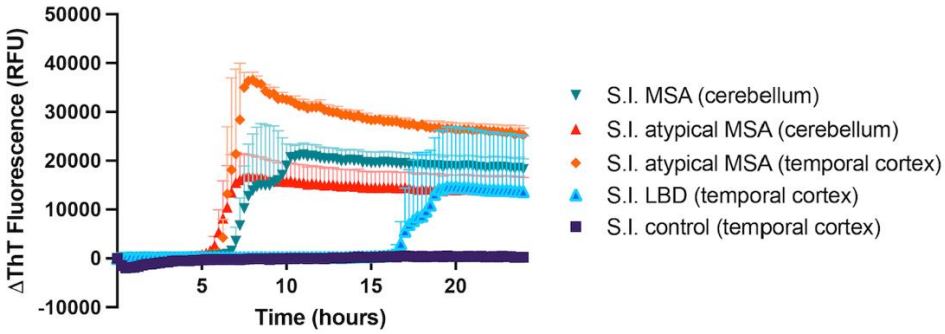
COMMENT #6. The researchers mention that SAAs can be quantified, but more information is needed on this aspect. I suggest that they provide details on the threshold used and discuss the differences in seeding behavior between MSA cases and LBD cases. Specifically, the LBD case amplified later than the MSA cases, did not reach as high a level, and showed wider error bars. The threshold is particularly important since the overall fluorescent signal from the samples is

relatively low. Additionally, I recommend that they include the positive and negative controls of the experiment, possibly in the supplementary data section, to provide insight into the signal characteristics of their controls. (Figure 2g).

Our response and changes in the manuscript:

We thank the reviewer for these comments. In this SAA, we did not apply a specific fluorescence threshold for defining positive amplification, as both the non-neurodegenerative (non-ND) control and the unseeded negative control consistently show no increase in fluorescence signal over the 24-hour assay period (please see revised Figure 2i):

i



This absence of background amplification provides a clear baseline, eliminating the need for a defined threshold. As previously established in our assay protocol (Martinez-Valbuena *et al.*, Translational Neurodegeneration, 2022; Martinez-Valbuena *et al.*, Acta Neuropathologica, 2022), this approach has proven robust and reliable for distinguishing disease-specific seeding activity.

Regarding the observed differences in seeding kinetics between MSA and LBD cases, we would like to clarify that the LBD sample was amplified using a reaction buffer specifically optimized for MSA-derived seeds, as detailed in our prior work (Martinez-Valbuena et al., Translational Neurodegeneration, 2021). This condition enables us to effectively distinguish between MSA and LBD conformers based on their seeding profiles—MSA typically seeds rapidly and with higher fluorescence, while LBD seeds more slowly and reaches a lower plateau. The broader variability and slower kinetics observed in the LBD case here are consistent with this optimized condition.

We included the LBD temporal cortex sample for comparison because the atypical MSA case showed predominantly neuronal inclusions in the temporal cortex—histopathologically resembling patterns more typical of cortical Lewy body pathology rather than classic oligodendroglial MSA pathology. We therefore sought to assess whether the seeding behavior of this region was more consistent with LBD or MSA. The results of the SAA, together with the cryo-EM findings, now support that the atypical MSA case harbors a distinct, hybrid α-synuclein filament conformation with a unique seeding signature that does not fully align with either MSA or LBD.

COMMENT #7. To improve the clarity of the figure, the researchers should add labels to the blots, such as the doses of GdnCl. (Figure 2 h-k).

Our response and changes in the manuscript:

We thank the reviewer for this helpful suggestion. We have now added the GdnCl concentration labels to the blots to improve clarity. As recommended by Reviewer #2, this figure has been moved to the supplementary material (Extended Data -Supplementary Figure 3) in the revised version of the manuscript.