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Heterotypic Amyloid β interactions facilitate amyloid assembly and modify amyloid structure

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Dear Joost and Frederic,

Thanks for the constructive discussions today. Your proposal for how to address the concerns raised by the referees sounds good and reasonable.

I would therefore like to invite you to submit a suitably revised manuscript. You can use the link below to upload the revised version.

I have also attached a guide for helpful tips on formatting the revised version. Please make sure to pay attention to the Data Availability section and including statistical information and replicates in the figure legends

Let me know if you have any further questions.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

Konstantoulea et al investigate whether amyloid plaques attract endogenous proteins with similar properties. This is a very interesting and relevant question to understand mechanisms of fibril growth and toxicity. The authors use a wide range of biochemical, biophysical and cell biological methods, and overall the data match the conclusions. They screen the entire protein for potential sites. They identify proteins with homologous regions to the Abeta aggregation prone regions that are enriched in fibrils, and some of the even support fibril formation. Altogether, this is a very exciting study.

There are a number of issues that need to be made more clear, though.

1. The figures need to be better presented. I would strongly advice the authors to provide figures in final size with consistent line thickness and font size. At present, all figures are A4 format, but some numbers and letters are even now not readable, and some lines are rather thin while others are thick. Table 1 needs an explanatory header, now it is insufficiently clear what it reports. The supplementary figures lack informative figure legends, and some graphs have insufficient headers. E.g. it is not clear what the violin plots in Figs 5 en 6 exactly depict. It is unclear what is randomised in Fig S2, the rows and columns are not labelled, and the sequences are not adequately provided. Fig. S7 has plots in different scales on the x-axis, which makes them hard to compare. There is no adequate legend either. Numbers and letters are too small to read, and the peptide sequences should be provided.

2. When discussing the proteins having APRs, please provide a full klist, please indicate the sequences of the APR, and please discuss the impact of cellular location.

3. When discussing the fact that expression of some sequences promote aggregation but others do not, please discuss what makes the difference. When testing expression in cells, are the sequences expressed extra or intracellularly, and does this correspond to the natural localisation?

4. Also other groups have analysed fibrils interactomics before. It would be helpful for the readers if the authors would discuss such studies.

Referee #2:

The manuscript "Heterotypic Abeta interactions facilitate amyloid assembly and modify amyloid structure" by K. Konstantoulea and colleagues describes convincingly the successful identification of a series of peptides, that interact with an aggregation-prone region in the disease relate protein fragment Abeta1-42 and alter the formation of Abeta fibrils in vitro.
Expression of some of the proteins that contain these peptides is then shown to alter the aggregation behavior of an intracellular Abeta1-42-mCherry fusion in a HEK293 based system. Finally, the authors analyze a previously published dataset of the composition of Abeta rich plaques in Alzheimer patients and describe an enrichment of proteins that contain sequences with close homology to stretches in Abeta1-42 that cannot be observed in an animal model for AD or in alpha-synuclein rich inclusions in Glia cells.

The amount and quality of data generated in this manuscript is impressive, as is the diversity of techniques used. What is missing however is a deeper analysis (or at least discussion) of the specificity of the effects described. My understanding of the scrambled samples in Figure 1 is that they should be negative controls, but nevertheless at least some of them react positive with several of the later stage aggregates (Fig 1 G,H and J). It would be good to check how other aggregates/oligomers interact with the membranes used here.
For the assay described in Figure 4 it is also not clear that the effects are direct (this is even shortly mentioned for CLCN3). Here it would be helpful to test the effect of the selected constructs on other aggregation-prone proteins, meta-stable proteins, or reporters of protein folding or other parts of the quality control network to demonstrate that the Abeta-mCherry aggregation is not caused by an overall decrease of the ability of cells to maintain metastable proteins in solution.
I am also not convinced that alpha synuclein aggregates in glia cells are the optimal control for the specificity of the interactions described in Figures 5 and 6. Perhaps it is possible to perform a comparable analysis for an aggregate species that is closer related to the Abeta plaques analyzed. It will be difficult to match the extracellular localization of the Abeta plaques, but perhaps aggregates isolated in a comparable manner or a closer matched localization. Another possibility would be perhaps analysis of the protein sequestered in tau-inclusions.

My main criticism of this manuscript is however that it is not clearly enough connecting the (here clearly demonstrated) effect that some peptides can interact with aggregation-prone regions of Abeta, with the events that take place during the aggregation of Abeta in a physiological setting (as reflected here by the interactome of Abeta in patient samples). It is not clear how the interactions described in the first four figures are related to AD and e.g. how many of the interactors can be found in the proteomics data from AD patients (or in Abeta inclusions in cellular systems). The table with the investigated constructs in the biosensor assay suggests that many of the investigated proteins are not normally expressed in the cytoplasm, and therefore are most likely misfolded in this system, which puts them therefore at risk of interacting with other metastable or misfolded proteins and questions their relevance for disease.
I believe the authors have to make an effort to connect the proof of principle experiments in the first part of the manuscript with the predictions that this allows for the composition of aggregates in a patient sample, and then the analysis of these samples. If this is done and the questions regarding of the specificity mentioned above are addressed I believe that this will be a very impactful manuscript that should be published to a wide audience.

Referee #3:

The authors attempted to identify the internal sequence within Abeta peptide, which interact with monomer/oligomer/fibrillar forms of Abeta by a sliding window scan technique (Figure 1). They then explored proteins harboring the identified interacting sequences in an unbiased fashion, and tried to identify proteins that affect the Abeta fibrillization by an in vitro Abeta aggregation assay (Figures 2,3). Previous studies have searched for the internal domain of Abeta that works as an aggregation core, by systematically replacing amino acid residues spanning the entire length of Abeta by alanine or proline scanning (Williams J Mol Biol 2004, 2006). In this present study, they show that Abeta oligomers interact with the APR1 domain that overlaps with the turn region of the beta-sheet structure. Subsequently, Figures 5 and 6 show the condensation of proteins harboring the APR sequence in senile plaques. This study is interesting as the initial application of the sliding window scan method on the analysis of interaction between Abeta peptides; however, a couple of important problems remain to be addressed with the study.
Major points
(1) The data presented in Figure 4 are problematic and not relevant to the Abeta pathophysiology: the authors express Abeta peptides that should be present extracellularly, in the cytoplasm of cultured cells and examined the aggregation, which is quite artificial, making the results hard to understand. Moreover, many of the putative interactors shown in Table 2 are membrane proteins or a mitochondrial protein (6), making it quite difficult to interpret the mode or significance of the interaction with Abeta within the cytoplasm.
(2) Discussion: overall, the results of this study are not directly discussed in a straightforward manner. Importantly, the reason why monomer, oligomer and fibrillar forms of Abeta differentially intereacted with the APR is not explained (only mentioned that "mature fibrils have few interaction sites" page 7, line 11). It is unknown why various peptides that bound to APR1 showed different effects on the Abeta aggregation (e.g., inhibition of seeding, elongation or fluorescence amplitude).
(3) It is not clearly described whether there were any overlaps between the proteins identified in Figures 2,3 and those in Figures 5,6. Were the proteins detected in the former half of the study (Figures 2,3) also enriched in senile plaques?

Minor points

(1) In Figure 1f and g, Abeta were bound to Abeta 17-28/19-30 located between APR1 and 2, which is not properly discussed.

(2) Figure 3e. It should be examined if the binding peptides interfered the binding of ThT to Abeta. Importantly, figure 3e shows that the fluorescence amplitudes are reduced with p8 and p26, whereas the amount of fibrils is not reduced in 3f and 3i.

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Konstantoulea et al investigate whether amyloid plaques attract endogenous proteins with similar properties. This is a very interesting and relevant question to understand mechanisms of fibril growth and toxicity. The authors use a wide range of biochemical, biophysical and cell biological methods, and overall the data match the conclusions. They screen the entire protein for potential sites. They identify proteins with homologous regions to the Abeta aggregation prone regions that are enriched in fibrils, and some of the even support fibril formation. Altogether, this is a very exciting study.

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The tables have been provided with an explanatory header.
The Figures have been edited with these comments in mind.

2. When discussing the proteins having APRs, please provide a full list, please indicate the sequences of the APR, and please discuss the impact of cellular location.

The issue of subcellular localization is now discussed at length in the manuscript and is indicated in the tables. Importantly, the 3 hit constructs in our cellular reporter are also found in actual plaques from AD patients, suggesting that using a strict subcellular localization logic is not sound when considering plaque formation. Importantly, the majority of the proteins found in plaques do not have an extracellular annotation, suggesting plaques are formed in conjunction with cell death.

3. When discussing the fact that expression of some sequences promote aggregation but others do not, please discuss what makes the difference.

The dataset presented here is not large enough to make a meaningful analysis of the sequence determinants of the different outcomes of heterotypic amyloid interactions on Aβ aggregation. However, we have added a paragraph on this issue and the preliminary conclusions we could draw at this stage. In the longer term, we are working on an all-atom modelling pipeline to understand the impact of mismatches on amyloid fibril structure.

When testing expression in cells, are the sequences expressed extra or intracellularly, and does this correspond to the natural localisation?

Please kindly refer to our answer to comment 2.

4. Also other groups have analyzed fibrils interactomics before. It would be helpful for the readers if the authors would discuss such studies.

Indeed, other proteomics of aggregates have been previously published. We have mentioned in the text that around 35-45% of proteins identified in plaques of other MS studies have homology to Aβ APRs.

Referee #2

The manuscript "Heterotypic Abeta interactions facilitate amyloid assembly and modify amyloid structure" by K. Konstantoulea and colleagues describes convincingly the successful identification of a series of peptides, that interact with an aggregation-prone region in the disease relate protein fragment Abeta1-42 and alter the formation of Abeta fibrils in vitro.
Expression of some of the proteins that contain these peptides is then shown to alter the aggregation behavior of an intracellular Abeta1-42-mCherry fusion in a HEK293 based system. Finally, the authors analyze a previously published dataset of the composition of Abeta rich plaques in Alzheimer patients and describe an enrichment of proteins that contain sequences with close homology to stretches in Abeta1-42 that cannot be observed in an animal model for AD or in alpha-synuclein rich inclusions in Glia cells.

The amount and quality of data generated in this manuscript is impressive, as is the diversity of techniques used.

Thank you.

What is missing however is a deeper analysis (or at least discussion) of the specificity of the effects described.

We agree with this comment and thank the reviewer for pointing this out.

My understanding of the scrambled samples in Figure 1 is that they should be negative controls, but nevertheless at least some of them react positive with several of the later stage aggregates (Fig 1 G,H and J). It would be good to check how other aggregates/oligomers interact with the membranes used here.

To address this point, we have now exposed membranes to oligomers of the yeast prion sup35 as the method was originally developed for this protein (Appendix Figure S1). Further, we have now added a discussion on specificity at the end of the section on the Aβ self-interaction membranes:

“To probe the specificity of the observed interactions, we went back to the sup35NM domain originally used to develop this assay[1], and exposed a fresh membrane to reverse amyloid seeds prepared by sonication of mature sup35NM fibrils (Appendix Figure S1). Moreover, we included in the membranes control peptides consisting of single proline substitutions of peptides 12, 15, 24 and 26, respectively, as well as scrambled versions of peptides 11, 14, 25, 27 and 29. These peptides were chosen to sample the positive regions from the central and the C-terminal APRs. The sup35NM oligomers showed only very weak binding, including to spots positive for Aβ. Also, all proline substitutions were sufficient to suppress binding, as was scrambling in the central region. However, scrambling in the carboxyterminal region appears to reduce the strength of the interaction, but does not completely suppress it. This highly hydrophobic region is part of the transmembrane region of APP and interacts only with relatively late species in the aggregation pathway, which might indicate an aspecific contribution to binding in this region, perhaps via hydrophobic surfaces on the larger aggregates. On the other hand, the proline substitutions that effectively suppress interaction conserve hydrophobicity, but disrupt secondary structure propensity, suggesting that perhaps the low sequence diversity in this region renders scrambling a blunt tool.”

So, although we cannot exclude that some aspecific interactions may occur, we think the contribution is not very large. Scrambling is often used but it is a necessary evil: The total number of (different) permutations of strings with n_i characters of type i is given by $m!/\prod_i n_i!$ (where m is the total peptide length and n the number of instances of amino acid i). So, including a low number of scrambles is the only practically feasible solution, but it is arbitrary and can only serve as a general indication. On the other hand: hand selecting scrambles is arguably biased. In a region of low sequence complexity moreover, there is always some similarity between scrambled and original sequences.

```
abeta27_scrambled      -INIGKLGGAVMG-----  12
abeta25_scrambled      -IKGALNVSIGMG-----  12
abeta29_scrambled      ------IVGAGMGGLVVI  12
abeta29                ----GAIIGLMVGGVV--  12
abeta25                GSNKGAIIGLMV------  12
abeta27                --NKGAIIGLMVGG----  12
```

For the assay described in Figure 4 it is also not clear that the effects are direct (this is even shortly mentioned for CLCN3). Here it would be helpful to test the effect of the selected constructs on other aggregation-prone proteins, meta-stable proteins, or reporters of protein folding or other parts of the quality control network to demonstrate that the Abeta-mCherry aggregation is not caused by an overall decrease of the ability of cells to maintain metastable proteins in solution.

To this end, we have performed the following experiments (described in full at the end of the paragraph on the cellular experiments):

- We have expressed our constructs in other aggregation reporter lines: the tau biosensor Hek293 line developed by Marc Diamond[2] and a sup35NM N2A line developed by Ina Vorberg[3]. (Appendix Figure S14)
- We have expressed other aggregating proteins in our Aβ reporter line, namely wild type alpha-synuclein, the repeat domain of pathogenic tau mutant P301S and the pathogenic SOD1 mutant A4V. (Appendix Figure S15)

None of these controls show effects anywhere near as strong as the effect of our hit constructs on Aβ aggregation in our sensor line, suggesting that although aspecific effects can contribute to some degree, their impact on our results and conclusions is limited.

I am also not convinced that alpha synuclein aggregates in glia cells are the optimal control for the specificity of the interactions described in Figures 5 and 6. Perhaps it is possible to perform a comparable analysis for an aggregate species that is closer related to the Abeta plaques analyzed. It will be difficult to match the extracellular localization of the Abeta plaques, but perhaps aggregates isolated in a comparable manner or a closer matched localization. Another possibility would be perhaps analysis of the protein sequestered in tau-inclusions.

Given the limitation of high-quality proteomic studies of such deposits, there are not so many alternatives. However, we did identify a suitable study on the composition of tau tangles (by Drummond et al[4]) and have added these as an additional control next to the alpha-synuclein data, which we think has its own merits. Moreover, we analyzed in similar way a proteomic dataset of amyloid plaques produced by the same group[5] (Appendix Figure S15). Those two datasets where isolated in a comparable manner. Interestingly, while we didn't find any overrepresented Aβ region in tau aggregates, in Aβ aggregates we found the c- terminus APR overrepresented.

My main criticism of this manuscript is however that it is not clearly enough connecting the (here clearly demonstrated) effect that some peptides can interact with aggregation-prone regions of Abeta, with the events that take place during the aggregation of Abeta in a physiological setting (as reflected here by the interactome of Abeta in patient samples). It is not clear how the interactions described in the first four figures are related to AD and e.g. how many of the interactors can be found in the proteomics data from AD patients (or in Abeta inclusions in cellular systems).

We thank the reviewer again for pointing this out. We have now substantially changed the order of the text to better highlight the connections between the different parts and the relevance of the constructs for AD research.

Briefly, all 9 out of 10 proteins selected for the cellular work were positive on the membrane in Figure 2 and 1 was negative. The 3 positive constructs in the reporter cell line were all positive on the membrane, and were found in patient plaques (Xiong study). Two of these peptides were studied in more detail as peptides in solution and show morphological effects by AFM and TEM.

The table with the investigated constructs in the biosensor assay suggests that many of the investigated proteins are not normally expressed in the cytoplasm, and therefore are most likely misfolded in this system, which puts them therefore at risk of interacting with other metastable or misfolded proteins and questions their relevance for disease.

We agree with the reviewer that our cellular reporter system is artificial and e.g. does not accurately incorporate elements such as subcellular localization. However, with all the additional controls we have now provided (above), we do believe that our data cannot be put down to aspecific effects resulting from overexpression of misfolded proteins. Moreover, we would like to point out that the proteomic data on plaque composition such as we used in our manuscript by no means exclusively contains extracellular proteins. Rather on the contrary, there are numerous proteins from different subcellular compartments present, notably from the endolysosomal system, but also proteins traditionally annotated as cytoplasmic. This shows that these plaques are scars of a pathology and likely contain proteins released from cells dying in their immediate vicinity. Therefore, a carefully constructed cellular model that tries to closely mimic healthy subcellular protein localization may ultimately be no better model than our crude setup. Importantly, we do not claim that we have found key modulators of Aβ aggregation in this study, but we do think our work shows proof of concept that such modulators likely exist and should be identified using more sophisticated models, likely *in vivo*.
We have added a sentence in the description of the cellular model to make the readers aware of the subcellular localization of the proteins found in plaques and have added a Supplementary Figure to show this (Appendix Figure S15A).

I believe the authors have to make an effort to connect the proof of principle experiments in the first part of the manuscript with the predictions that this allows for the composition of aggregates in a patient sample, and then the analysis of these samples. If this is done and the questions regarding of the specificity mentioned above are addressed I believe that this will be a very impactful manuscript that should be published to a wide audience.

We thank the reviewer for pointing out that we had not presented the data in a way that made clear the connection between the parts, we have now changed the order of the text and explicitly explained how each protein occurs in the different experiments to improve this as mentioned above. We think the end result is compelling.

Referee #3

The authors attempted to identify the internal sequence within Abeta peptide, which interact with monomer/oligomer/fibrillar forms of Abeta by a sliding window scan technique (Figure 1). They then explored proteins harboring the identified interacting sequences in an unbiased fashion, and tried to identify proteins that affect the Abeta fibrillization by an in vitro Abeta aggregation assay (Figures 2,3). Previous studies have searched for the internal domain of Abeta that works as an aggregation core, by systematically replacing amino acid residues spanning the entire length of Abeta by alanine or proline scanning (Williams J Mol Biol 2004, 2006). In this present study, they show that Abeta oligomers interact with the APR1 domain that overlaps with the turn region of the beta-sheet structure. Subsequently, Figures 5 and 6 show the condensation of proteins harboring the APR sequence in senile plaques. This study is interesting as the initial application of the sliding window scan method on the analysis of interaction between Abeta peptides; however, a couple of important problems remain to be addressed with the study.

Major points
(1) The data presented in Figure 4 are problematic and not relevant to the Abeta pathophysiology: the authors express Abeta peptides that should be present extracellularly, in the cytoplasm of cultured cells and examined the aggregation, which is quite artificial, making the results hard to understand. Moreover, many of the putative interactors shown in Table 2 are membrane proteins or a mitochondrial protein (6), making it quite difficult to interpret the mode or significance of the interaction with Abeta within the cytoplasm.

Aβ aggregates are indeed found extracellularly. However, accumulation of intracellular Aβ has been found to occur early in disease. Aβ produced intracellularly (eg Endoplasmic Reticulum) or internalized through endocytosis results in accumulation of Aβ in different compartments including mitochondria and possibly cytosol [6]. Indeed, the proteins are overexpressed, so they are found in cytoplasm and at their physiological location. Example is CLCN3 where you can see it localizing in the membrane (Appendix S12C). However, it is an artificial model not perfectly mimicking Aβ aggregation. But it serves as a model to show that proteins harboring those homologous regions can interact and affect Aβ aggregation in a highly complex environment. Moreover, although there is no clear mechanism to explain the interaction of intracellular proteins to Aβ at this time, many intracellular proteins have been found in extracellular aggregates (see also response to reviewer 2). Finally, 4/10 (CACNB1, CLCN3, ANXA1, CSPG5) of the proteins we tested can be mapped in SynGO (Synaptic Gene Ontologies) for localizing at synapses. PACN1 has been also found in synapses and is required for synaptic vesicle endocytosis (uniprot). iPLA2 (PLA2G6) is implicated in neurodegeneration and in mitochondria dysfunction induced by Aβ[7]. So, although our model is not ideal for studying Aβ pathophysiology, the potential interactors studied here are proteins that can be found in highly relevant for the disease locations.

(2) Discussion: overall, the results of this study are not directly discussed in a straightforward manner. Importantly, the reason why monomer, oligomer and fibrillar forms of Abeta differentially interacted with the APR is not explained (only mentioned that "mature fibrils have few interaction sites" page 7, line 11). It is unknown why various peptides that bound to APR1 showed different effects on the Abeta aggregation (e.g., inhibition of seeding, elongation or fluorescence amplitude).

We have improved the explanation of why different Aβ species interact may differentially with the peptide arrays and we have added a comment on why different peptide may interact differently with Aβ aggregates.

(3) It is not clearly described whether there were any overlaps between the proteins identified in Figures 2,3 and those in Figures 5,6. Were the proteins detected in the former half of the study (Figures 2,3) also enriched in senile plaques?

We agree with the reviewer that this part was unclear. We have now changed the order of the text and have explicitly linked the results sections, including in the data tables. Briefly, the 3 hits found to affect Aβ aggregation in the cell line are also found in patient plaques (in the Xiong dataset) and were found to interact *in vitro*.

Minor points
(1) In Figure 1f and g, Abeta were bound to Abeta 17-28/19-30 located between APR1 and 2, which is not properly discussed.

Our membranes have Aβ sequence as a sliding window. In figure 1f Aβ binds to the spot B7 which correspond to LVFFAEDVGSNK. This sequence still corresponds to middle APR. In G similarly, the positions that bind to Aβ are LVFFAEDVGSNK, VFFAEDVGSNKG, FFAEDVGSNKGG. Those sequences have half of the middle APR as well as correspond to the regions found up in amyloid plaques.

(2) Figure 3e. It should be examined if the binding peptides interfered the binding of ThT to Abeta. Importantly,

figure 3e shows that the fluorescence amplitudes are reduced with p8 and p26, whereas the amount of fibrils is not reduced in 3f and 3i.

Indeed, the fluorescence amplitude is reduced for a number of peptides. However, figure 3f and i, do not represent an analysis of the number of fibrils, since deposition of the resulting fibrils maybe different and not corresponding to the actual number. TEM and AFM analysis was done to identify differences in length or width of the fibrils, which is independent of and orthogonal to Tht binding.

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1 Tessier, P. M. & Lindquist, S. Prion recognition elements govern nucleation, strain specificity and species barriers. *Nature* **447**, 556-561, doi:10.1038/nature05848 (2007).

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3 Hofmann, J. P. *et al.* Cell-to-cell propagation of infectious cytosolic protein aggregates. *Proc Natl Acad Sci U S A* **110**, 5951-5956, doi:10.1073/pnas.1217321110 (2013).

4 Drummond, E. *et al.* Phosphorylated tau interactome in the human Alzheimer's disease brain. *Brain* **143**, 2803-2817, doi:10.1093/brain/awaa223 (2020).

5 Drummond, E. *et al.* Proteomic differences in amyloid plaques in rapidly progressive and sporadic Alzheimer's disease. *Acta Neuropathol* **133**, 933-954, doi:10.1007/s00401-017-1691-0 (2017).

6 LaFerla, F. M., Green, K. N. & Oddo, S. Intracellular amyloid-beta in Alzheimer's disease. *Nat Rev Neurosci* **8**, 499-509, doi:10.1038/nrn2168 (2007).

7 Zhu, D. *et al.* Phospholipases A2 mediate amyloid-beta peptide-induced mitochondrial dysfunction. *J Neurosci* **26**, 11111-11119, doi:10.1523/JNEUROSCI.3505-06.2006 (2006).

Dear Joost and Frederic,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referee #2 who appreciates the introduced changes. I am therefore very pleased to let you know that we will accept the manuscript for publication here.

Before sending you the formal acceptance letter there are just a few issues to sort out. Referee #2 has a few remaining points that should be easy enough to address. Regarding Table 1 (point #1) - I also find this table very informative. I don't have any good suggestions for how to improve the layout, but I think the symbols should be better clarified. Like what is the difference between X and no marking. Also, what is the difference between - and --?

In addition will you also take care of the following editorial points:

- we are missing 3-5 keywords

- please make sure that the funding information matches between the online submission system and what is provided in the manuscript.

- We need an author contribution section

- Please upload the synopsis image as a separate file

- Regarding the Suppl. Tables: More complex tables that are best displayed in excel format and not as a PDF should be uploaded as datasets, in excel format, one file per table. Their legends should be added as a separate tab. The tables should be labelled as "Dataset EV1" etc. Simpler tables such as S1 and S5 can be made appendix tables and merged with appendix figures in one PDF.

- Is Fig 3A-C reused in S3? If so please state that in the figure legend

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Take a look at the word file and the comments regarding the figure legends and respond to the issues.

That should be all. You can use the link below to upload the revised version

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 10th Jan 2022.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #2:

I believe that this version of the manuscript is greatly improved and like the reorganization of the manuscript. The authors have addressed my questions sufficiently.

I only have a few minor points:

- I believe that the information in Table 1 is very important, but for me it was quite hard to extract all the information present. Perhaps a different design or a color code would be helpful here.
- I think that some additional information about the amyloid-plaques from no AD brains would be helpful, e.g. what kind of

disease these samples represent
- the manuscript does not contain figure EV2 (it instead contains a second version of figure S13)
- On page 17 the authors state that "However, it is also important to note that the majority of the proteins found in patient plaques discussed above are annotated as extracellular, and thus include many proteins that in healthy cells reside in various intracellular localisations". I don't understand that statement, do the authors suggest that these proteins are annotated wrongly as extracellular because they were found in extracellular plaques? Perhaps this can be clarified.

Dear Editor,
Dear Karin,

Thank you very much for allowing us to address these final points. We have listed our responses below.

We also handled all the publisher's comments and requests.

Best Regards
Joost and Frederic

Before sending you the formal acceptance letter there are just a few issues to sort out. Referee #2 has a few remaining points that should be easy enough to address. Regarding Table 1 (point #1) - I also find this table very informative. I don't have any good suggestions for how to improve the layout, but I think the symbols should be better clarified. Like what is the difference between X and no marking. Also, what is the difference between - and --?
The table was changes. We added the significance and the effect. Moreover we clarified the symbols.

In addition will you also take care of the following editorial points:

- we are missing 3-5 keywords
Amyloid beta, heterotypic aggregation, Alzheimer's disease, toxicity

- please make sure that the funding information matches between the online submission system and what is provided in the manuscript.
This was taken care of, except for the Stichting Alzheimer Onderzoek, which is not listed (only the international one).

- We need an author contribution section
Author contribution added.

- Please upload the synopsis image as a separate file
done

- Regarding the Suppl. Tables: More complex tables that are best displayed in excel format and not as a PDF should be uploaded as datasets, in excel format, one file per table. Their legends should be added as a separate tab. The tables should be labelled as "Dataset EV1" etc. Simpler tables such as S1 and S5 can be made appendix tables and merged with appendix figures in one PDF.
Done

- Is Fig 3A-C reused in S3? If so please state that in the figure legend
Yes. Also 3H in S9. Added in the figure legend

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Table was changed.
- I think that some additional information about the amyloid-plaques from no AD brains would be helpful, e.g. what kind of disease these samples represent

We added at page 13, second paragraph the “To investigate if a similar overrepresentation is seen in amyloid plaques from brains of cognitively healthy elderly people (non-AD)

- the manuscript does not contain figure EV2 (it instead contains a second version of figure S13)

The title on figure EV2 was corrected.

- On page 17 the authors state that "However, it is also important to note that the majority of the proteins found in patient plaques discussed above are annotated as extracellular, and thus include many proteins that in healthy cells reside in various intracellular localisations". I don't understand that statement, do the authors suggest that these proteins are annotated wrongly as extracellular because they were found in extracellular plaques? Perhaps this can be clarified.

The above statement was changed to “However, it is also important to note that the majority of the proteins found in patient plaques discussed above reside in various intracellular localisations in healthy cells”

2nd Revision - Editorial Decision **29th Oct 2021**

Dear Joost and Frederic,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a look at it and all looks good!

I am therefore very pleased to accept the manuscript for publication here.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2021-108591R1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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 $\chi 2$ 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method was used to predetermine sample size
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusion of samples
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	When normality was not obsereved , appropriate tests were used.
Is there an estimate of variation within each group of data?	Variation is shown in graphs.

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Is the variance similar between the groups that are being statistically compared?	When no similar variance was observed, analysis was adjusted accordingly

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The antibodies used, their clone and catalog number are indicated in the material and methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were authenticated and tested for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

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E- Human Subjects

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13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	No data produced require deposition
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