EMBO reports

Isotope-labeled amyloid-β does not transmit to the brain in a prion-like manner after peripheral administration

Mirjam Brackhan, Giulio Calza, Kristiina Lundgren, Pablo Bascunana, Thomas Brüning, Rabah Soliymani, Rakesh Kumar, Axel Abelein, Marc Baumann, Maciej Lalowski, and Jens Pahnke
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Prof. Pahnke,

Thank you for the submission of your manuscript to EMBO reports. I could only secure two referees for it in December, and we have now received their enclosed reports.

As you will see, the referees acknowledge that the findings are potentially very interesting. However, they do point out that it needs to be clarified whether the injected homogenized samples indeed have amyloidogenic seeding activity, and whether this activity is comparable to the one in related and published studies with opposite results. The referees also raise further points that need to be addressed.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns 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 major 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 (9th Apr 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods should be included in the main manuscript file.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also

reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at
<https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Regarding data quantification (see Figure Legends:
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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,

- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

- Please also include scale bars in all microscopy images.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision:
https://embor.msubmit.net/cgi-bin/main.plex

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

Brackhan et al investigated whether the peripheral administration of amyloid-beta (Aβ) peptides can contribute to cerebral β-amyloidosis.
The authors performed in vivo stable isotope labelling of brain-derived material (including Aβ) by feeding transgenic (APP/PS1 model) and non-transgenic mice with a 13C-lysine labelled diet. Of relevance, in initial experiments the transgenic animals were labelled for 50, 100 or 125 days; while the controls were labelled for only 10 days. However, in a second set of experiments (more informative) metabolic labelling time was similar in both groups.
Labelled brain extracts were prepared from the transgenic and control mice and injected (i.p.) into male (APP/PS1) transgenic animals. The brain, mesenteric lymph nodes, spleen and liver were harvested at different time points (1-100 days post-injection) and homogenate samples were spiked with femtomolar quantities of labelled (13C15137 N-Lys) Aβ42 prior to immunoprecipitation and analysis by multiple reaction monitoring immuno-mass spectrometry (MRM-IMS). Importantly, the authors established a sensible MS methodology for the detection of Aβ peptides, using the tryptic Aβ17-28 peptide as a surrogate measure for total Aβ.
The authors first traced intraperitoneally-injected Aβ. Their analyses showed that Aβ reaches the liver, spleen and the mesenteric lymph nodes via lymphatic drainage. Interestingly, labelled Aβ17-28 peptide was detected at 1-day post-injection in the insoluble brain fractions of both groups of mice. The analyses indicate that the transgenic mice rapidly metabolized 13C-Lys from the donor extracts and incorporated the labelled essential amino acid into the de novo generated Aβ. Therefore, in contrast to previous studies, the data do not support the notion that labelled Aβ, from peripheral injections, actually enters the brain. The authors conclude that these findings argue against the transmission of cerebral Aβ pathology via peripheral administration of Aβ aggregates.
This reviewer finds the presented data relevant and interesting. The high sensitivity of the developed methodology for the detection of labelled Aβ supports the robustness of the observations and conclusions.
Main comment:
1- As mentioned in the discussion, the results are in conflict with previous reports; including the recent study by Morales et al (2021) which uses very similar methodology in the investigation of induced cerebral Aβ pathology following peripheral administration of misfolded Aβ.
The authors indicate that "Our findings imply that accelerated cerebral β-amyloidosis previously observed after peripheral injection of Aβ-containing brain extract or blood into APPtg mouse models (Burwinkel et al.,2018; Eisele et al., 2014; Eisele et al., 2010; Morales et al., 2021; Morales et al, 2020) was caused by other factors present in the donor material rather than by seeding from the periphery."
And suggest for instance inflammatory factors.
However, this reviewer wonders whether the type of 'Aβ seeds' injected in the different studies differ and may explain the discrepancies. Have the authors tested the amyloidogenic (seeding) activity of the homogenate samples that were injected? How do they compare to other reported seeds?
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Referee #2:

This report is a tour-de-force of a very important, but challenging, question: does peripheral amyloid-beta get into the CNS or contribute to the risk of CNS amyloidosis?

The authors use a series of advanced techniques to address this question at multiple levels and should be congratulated on a thoughtful and thorough approach.

Major critiques are:
1) The results and discussion of peripheral amounts and locations of injected amyloid don't directly address the hypothesis framed by this manuscript, but do provide additional information on where injected amyloid goes in cellular components. The authors could consider making these descriptions supplemental or secondary to the main CNS findings.
For example, the description of Amyloid-beta 42 detected in mononuclear cells after IP injection of unlabeled tg and non-tg brain homogenates adds little to the discussion. Because endogenous, murine Amyloid-beta is produced at much lower levels than in

APP/PS1 transgenic mice, it seems surprising that "In blood plasma, Aβ42 levels were comparable between mice injected with APPtg brain extract and those injected with non-tg brain extract at all time points". This should be more directly addressed - why isn't it higher from tg tissues with much higher amyloid amounts?

Because the main hypothesis, 'does IP-injected amyloid-beta reach the brain and imply transmissibility similar to prion diseases', doesn't need the measurements in blood plasma to be complete, the authors could simply move the part of the paragraph that begins, "In blood plasma". . .) to supplemental.
Additionally, the results hypothesize about how the IP injection is cleared from the periotoneum, but don't fully describe the exact identity of the "peritoneal cells": were these mesothelial cells? Macrophages? Lymphocytes? Other? Similarly, the "mononuclear cells" in the blood plasma. In addition, we know that amyloid-beta in blood plasma can be transported via binding to soluble transport proteins such as LRP-1 (see work by zlokovic), so simply measuring the amount of the peptide in cells does not necessarily capture the entire amount in plasma. These should all be added to supplement or discussion.

2) the tg and non-tg groups were administered different durations of the stable isotope-labeled diet for the main experiments, acknowledging that the authors did, in later experiments, administer the diet for 100 d to non-tg mice to match the labeled diet duration of the original group of tg mice. The authors should move the findings of the non-tg mouse group fed the labeled diet for only 10 days to supplement clearly identifying the difference in labeling as not being comparable. Figure 4G seems to provide some of the most convincing evidence that the labeled amino acid, but not the peptide, is being taken up and incorporated (hence not supporting the hypothesis that amyloid-beta in the periphery enters the brain).

3) the hypothesis that proteases in the liver help break down amyloid-beta seems unnecessary and unproven (most protein catabolism occurs in the stomach and small intestine), and at most should be included as a speculation in discussion.

4) the absolute lack of 13C-labeled Lys in the soluble (TBS) fraction (Figure 4A) as opposed to the unlabeled 17-28 fragment seems odd when it is apparent in the insoluble fraction rather quickly (1 d). The overall proportion of soluble vs. insoluble amyloid beta is generally low - so maybe it was simply the limit of detection in this case? The authors should explain this apparent discrepancy.

Minor critiques are:
1) a parallel analysis of other proteins that would have incorporated the 13CLys would help support this claim that 13CLys is transported via bloodstream to the brain as an amino acid, where in the brain itself, it is incorporated into a variety of proteins as they are synthesized. Can the authors search their mass spec data and find labeled signatures of other proteins (e.g. apolipoprotein E, which is present in abundant amounts - see https://doi.org/10.1371/journal.pone.0038013)?
2) Consider expanding, with perhaps a Figure illustrating the phenomenon, of the finding that stable-isotope labeling in these Tg mice confirms the findings of the Michno et al 2021 study: in preparation of the labeled homogenates, the authors also noted that the earlier labeling begins, the lower the amount of insoluble Aβ that is not subject to dynamic turnover, and the higher the label incorporation into Aβ, which highlights the importance of an early start of therapies promoting Aβ clearance - showing that label incorporation into Aβ is highest when labeling starts before plaque onset.
3) Although peak intensity of the amyloid-beta 17-28 fragment was highest and therefore chosen as a surrogate for total amyloid-beta, considering choosing the second most intense peak and analyzing it in parallel (perhaps a fragment with the Lys at position 16 as well as C-terminal end at 42)

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Esther Schnapp, PhD
Senior Editor
EMBO reports

Response to Reviewers

We thank the reviewers and the editor for their thorough review of our article "Tracking peripherally administered amyloid-β by stable isotope labeling-based proteomics". The insightful comments have facilitated the revision of the manuscript.

Please find accompanying the revised manuscript, figures and tables. New insertions are shown in red. The point-by-point response to reviews follows. Text in red below indicates new insertions. Page and line refer to the revised draft.

Referee #1:

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The authors performed in vivo stable isotope labelling of brain-derived material (including Aβ) by feeding transgenic (APP/PS1 model) and non-transgenic mice with a 13C-lysine labelled diet. Of relevance, in initial experiments the transgenic animals were labelled for 50, 100 or 125 days; while the controls were labelled for only 10 days. However, in a second set of experiments (more informative) metabolic labelling time was similar in both groups.
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This reviewer finds the presented data relevant and interesting. The high sensitivity of the developed methodology for the detection of labelled Aβ supports the robustness of the observations and conclusions.

We sincerely thank the reviewer for her/his support of our data.

Main comment:

1- As mentioned in the discussion, the results are in conflict with previous reports; including the recent study by Morales et al. (2021) which uses very similar methodology in the investigation of induced cerebral Aβ pathology following peripheral administration of misfolded Aβ.
The authors indicate that "Our findings imply that accelerated cerebral β-amyloidosis previously observed after peripheral injection of Aβ-containing brain extract or blood into APPtg mouse models (Burwinkel et

al.,2018; Eisele et al., 2014; Eisele et al., 2010; Morales et al., 2021; Morales et al, 2020) was caused by other factors present in the donor material rather than by seeding from the periphery." And suggest for instance inflammatory factors.
However, this reviewer wonders whether the type of 'Aβ seeds' injected in the different studies differ and may explain the discrepancies.
Have the authors tested the amyloidogenic (seeding) activity of the homogenate samples that were injected? How do they compare to other reported seeds?
In Morales et al (2021), samples derived from an Alzheimer's (AD) brains were used in the studies. Have the authors tested the seeding activity of the used extracts with those derived from the AD human brain. The authors should convincingly demonstrate that the injected fractions are 'biologically active' in regards to their seeding activity.

The reviewer raises a very important concern. The APPtg brain extract injected in our study was generated by homogenizing brains from 100-175-day-old APP/PS1 mice (Radde et al., 2006, https://doi.org/10.1038/sj.embor.7400784) and diluting the homogenates with 1 µl PBS per mg homogenate. Morales et al., 2021 used 18-20-month-old Tg2576 mice as brain donors, whereas Eisele et al., 2014 used 18-30-month-old APP23 and APP/PS1 mice, with brain extracts containing < 10% of extract derived from APP/PS1 mice. Both studies generated more diluted brain extracts at 10% w/v corresponding to 9 µl PBS per mg brain.
The APP/PS1 model used in our study starts depositing Aβ at 1.5 months of age, whereas Tg2576 mice and APP23 mice show first Aβ deposits at 8-10 months and 6-7 months of age, respectively. Our donor mice were sacrificed at a younger age as compared to previous studies not only because of the faster Aβ deposition in the APP/PS1 model but also because longer labeling durations would not have been feasible due to the immensely high costs of the ^{13}C-Lys-labeled diet (~8000 €/kg). To compensate for the younger age of the donor mice, we used less diluted brain extracts for injection. Those extracts had Aβ concentrations of 2.9-8.5 ng Aβ/µl corresponding to 580-1700 ng intraperitoneally injected Aβ. Eisele et al., 2014 injected brain extracts containing 10-20 ng Aβ/µl and Morales et al., 2021 estimated the amount of intraperitoneally injected Aβ to be 171.8 ng. Thus, Aβ levels in our brain extracts are comparable to previous studies. We added "or estimating the amount of intraperitoneally injected Aβ to be 171.8 ng (corresponding to an Aβ concentration of 1.7 ng/µl) (Morales et al., 2021)" (Page 12, line 264) to the discussion section.
Moreover, brain extracts generated from APP/PS1 mice have repeatedly been shown to induce Aβ seeding when injected intracerebrally into young APP/PS1 or APP23 mice (Meyer-Luehmann et al., 2006, DOI: 10.1126/science.1131864, Heilbronner et al., 2013, https://doi.org/10.1038/embor.2013.137), even when the APP/PS1 donor mice were only 3 months old (Ye et al., 2017, https://doi.org/10.15252/embr.201744067). Thus, "Aβ seeds" derived from APP/PS1 mice have demonstrated seeding activity *in vivo*.
However, the main objective of this study was to investigate whether "Aβ seeds" reached the brain from the periphery. While we were able to detect injected Aβ in peripheral organs and cells by means of not only mass spectrometry but also immunoassay and in case of the liver, immunohistochemistry, - thereby further arguing in favor of the biological activity of the injected brain extracts - our data point against *brain penetration* of Aβ. The fact that we *did not* observe accelerated Aβ deposition even at 8 months after intraperitoneal injection of APPtg brain extract is a secondary observation that is in accordance with the lack of brain penetration of Aβ. A possible explanation for the discrepancy with previous studies may be the very fast rate of Aβ deposition in APP/PS1 host mice masking any acceleration of plaque formation caused by still unknown factors in APPtg brain extracts. We added the following sentences to the discussion section: "However, we did not observe accelerated cerebral β-amyloidosis in our study. This discrepancy with previous studies may be explained by differences in the rate of Aβ deposition in the different APPtg models used as host mice. While APP23 and Tg2576 mice used in previous studies start depositing Aβ at 6-7 months

(Sturchler-Pierrat et al, 1997) and 8-10 months (Kawarabayashi et al, 2001), respectively, APP/PS1 mice used in our study already show first Aβ deposits at 1.5 months (Radde et al., 2006), potentially masking any accelerated plaque formation caused by still unknown factors in the donor material." (Page 13, line 276)

Regarding the use of extracts derived from human AD brains, we did not include this option in our study as it precludes stable isotope labeling and tracking of Aβ. Morales et al., 2021 used brain extracts derived from Tg2576 mice for all intraperitoneal injection experiments. They merely used brain extracts derived from an AD patient for oral administration and did not demonstrate any effect on cerebral Aβ pathology via this application route. Additionally, Meyer-Luehmann et al., 2006 (DOI: 10.1126/science.1131864) reported comparable intracerebral seeding activity between brain extracts from APPtg mice and AD patients.

To provide further evidence for the seeding activity of our APPtg brain extracts, we performed an *in vitro* seeding assay, more specifically the thioflavin T (ThT) fluorescence assay. We first tested various dilutions of the crude APPtg and non-tg brain extracts and detected very high initial fluorescence masking any increase of fluorescence over time associated with Aβ aggregation. Thus, we isolated Aβ42 fibrils from APPtg extract and subjected non-tg extract to the same isolation protocol. Aβ42 fibrils extracted from APPtg extract exhibited seeding activity in a concentration-dependent manner while isolate from non-tg brain extract had a delaying effect on Aβ aggregation. Thereby, we present *in vitro* evidence for the presence of seeding-competent Aβ aggregates in our APPtg extract.

We added an additional Expanded View figure (Figure EV1) as well as the following sentences to the results section: "Additionally, we isolated Aβ42 fibrils from unlabeled APPtg extract and tested their *in vitro* seeding activity by thioflavin T (ThT) fluorescence assay. Aβ42 fibrils extracted from APPtg brain extract exerted a concentration-dependent seeding effect on Aβ42 aggregation (Figure EV1A) whereas isolate generated from non-tg brain extract delayed Aβ42 aggregation kinetics (Figure EV1B). Seeded Aβ42 fibrils presented a heterogenous morphorlogy (Figure EV1C,D) while Aβ42 fibrils aggregated without the presence of seeds displayed a more homogenous morphology (Figure EV1E)." (Page 5, line 102). We also provided additional information in the materials and methods section: "

Fibril extraction from brain extracts

Fibrils were extracted using a modified method from a recent study (Kollmer et al., 2019). Unlabeled APPtg brain extract (300 µl) was diluted with an equal volume of Tris calcium buffer (20mM Tris, 138mM NaCl, 2mM $CaCl_2$, 0.1% NaN_3, pH 8) and centrifuged at 21,000 x g for 30 minutes. The pellet was re-suspended in 1 ml Tris calcium buffer and 50 µl of Collagenase (5 mg/ml) and 10 µL of DNAse I were added. The solution was vortexed, incubated overnight at 37 °C and centrifuged at 21,000 x g for 30 minutes. The pellet was dissolved in 1 ml wash buffer (50 mM Tris, 10mM EDTA, pH 8) and 40 µl of 10 % SDS was added. The solution was incubated at 37°C for 30 minutes and centrifuged. This step was repeated twice and two further washing steps of the pellet were performed in wash buffer without SDS. Finally, the pellet containing fibrils was dissolved in 50 µl of 20 mM sodium phosphate buffer, 0.2 mM EDTA pH 8.0, and used for further experiments. Unlabeled non-tg brain extract was processed identically as control.

Dot blot assay

The concentration of Aβ in fibril extract was estimated from dot blot using Aβ42 monomer as reference. Fibril extract generated from APPtg brain extract was sonicated and spotted on a nitrocellulose membrane in different volumes (1,2,3, and 4 µl, respectively). 4 µl of Aβ42 in different concentrations (1,2,3,6, and 8 µM, respectively) was spotted as standard. The membrane was air-dried, blocked with blocking buffer (intercept® blocking buffer, LI-COR, Lincoln, USA) and incubated overnight at 4 °C with primary anti-Aβ antibody (6E10, anti-

mouse, 1:5,000, BioLegend™). The membrane was washed thrice with PBS-T, followed by incubation with secondary anti-mouse antibody (IRDye 800 CW, 1:20,000, LI-COR) for 60 minutes at 25 °C. The membrane was washed thrice with PBS-T and developed using Odyssey® DLx Imaging System. Blot analysis was performed for estimation of Aβ concentration using Image J.

Thioflavin T fluorescence assay

To test the seeding activity of fibrils extracted from APPtg brain extract, a Thioflavin T (ThT) fluorescence assay was performed. 20 µl of sodium phosphate buffer (20 mM, 0.2 mM EDTA, pH 8.0) containing 3 µM Aβ42 and 10 µM ThT was added to 384-well plates along with different volumes (1,2,3,5,8,10, and 20 µl, respectively) of isolate generated form APPtg or non-tg brain extracts. Excitation filter at 440 nm and emission filter at 480 nm were selected and fluorescence was recorded at quiescent conditions at 37 °C using a fluorimeter (FLUOStar Galaxy from BMG Labtech, Offenberg, Germany). The fluorescence data was plotted with respect to time after subtracting the minimum fluorescence value for each sample from all data points.

Transmission electron microscopy

To visualize the morphology of seeded Aβ42 fibrils at the aggregation kinetic end points, transmission electron microscopy (TEM) was performed. 5 µl of aggregated samples from 384-well plates were spotted on a 400 mesh formvar/carbon coated copper grid. Samples were washed twice with 10 µl of MQ water and stained with 1% uranyl formate. TEM (FEI Tecnai 12 Spirit BioTWIN, operated at 100 kV) images were recorded using a 2 k × 2 k Veleta CCD camera (Olympus Soft Imaging Solutions, GmbH, Münster, Germany). For each sample, 10-15 images were taken at a magnification of 20,000 x – 43,000 x.

” (Page 20, line 446)

Referee #2:

This report is a tour-de-force of a very important, but challenging, question: does peripheral amyloid-beta get into the CNS or contribute to the risk of CNS amyloidosis?

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Major critiques are:

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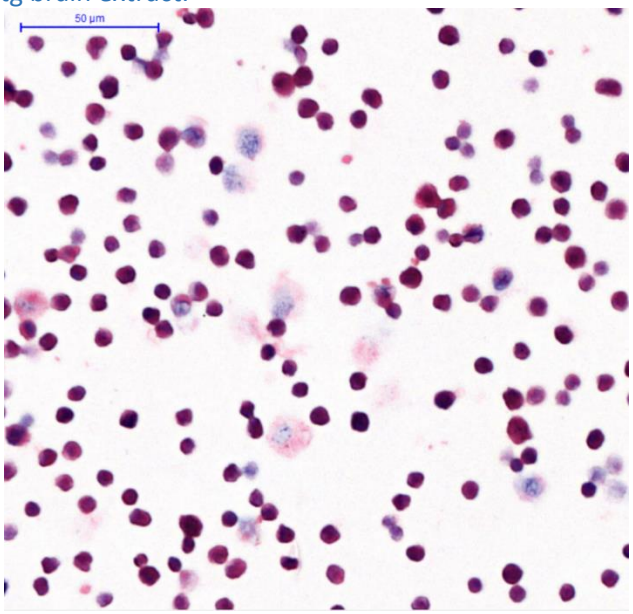
Additionally, the results hypothesize about how the IP injection is cleared from the peritoneum, but don't fully describe the exact identity of the "peritoneal cells": were these mesothelial cells? Macrophages? Lymphocytes? Other? Similarly, the "mononuclear cells" in the blood plasma. In addition, we know that amyloid-beta in blood plasma can be transported via binding to soluble transport proteins such as LRP-1 (see work by zlokovic), so simply measuring the amount of the peptide in cells does not necessarily capture the entire amount in plasma. These should all be added to supplement or discussion.

The reviewer makes a valid point by suggesting that we could consider making the peripheral data supplementary or secondary. However, we believe that these data support the validity of our experimental approach, showing that injected Aβ can be tracked even at very low amounts in peripheral tissues, and therefore should remain in the main text. Moreover, we decided to describe the main CNS findings at the end of the results section as they represent the key element and highlight of the work.

Our finding that mice injected with APPtg brain extract do not have higher blood plasma Aβ42 levels than mice injected with non-tg brain extract is consistent with previous work by Eisele et al., 2014 (https://doi.org/10.1523/JNEUROSCI.1608-14.2014) who did not find differences in blood plasma Aβ levels after intraperitoneal injection of APPtg brain extract but detected Aβ in the cellular blood fraction in monocytes. This implies that injected Aβ is not present in appreciable amounts in blood plasma. Therefore, we performed a separate experiment isolating mononuclear cells from whole blood by Ficoll gradient. To make it clear that these cells were isolated from whole blood and not from plasma, we modified the following sentence in the method section: "In a ~~subset of animals~~ separate experiment ~~(n = 2-3 per time point at 5 h – 7 d after APPtg extract injection, n = 1 per time point at 5 h – 7 d after non-tg extract injection)~~, EDTA-anticoagulated blood was taken by intra-cardiac puncture (n = 2-3 mice per time point at 5 h – 7 d after APPtg extract injection, n = 1 per time point at 5 h – 7 d after non-tg extract injection) and mononuclear cells were isolated from blood samples using Ficoll-Paque PREMIUM 1.084 (GE Healthcare) according to the manufacturer's instructions." (Page 24, line 537). We did not confirm the identity of the mononuclear blood cells but according to the manufacturer's handbook (https://us.vwr.com/assetsvc/asset/en_US/id/16286835/contents), these cells consist of lymphocytes and monocytes. We added "i.e. lymphocytes and monocytes" (Page 8, line 166) to the results section. We detected more Aβ42 in mononuclear cells isolated from mice injected with APPtg extract than in mononuclear cells isolated from mice injected with non-tg extract during the first 24 h after injection. Thus, we concluded that injected Aβ was transported by mononuclear blood cells. However, we cannot rule out that injected Aβ is present in blood plasma at levels below the detection limit of our immunoassay or that binding of Aβ to soluble transport proteins reduces Aβ detection in the immunoassay. We added the following sentences to the discussion section: "In further concordance with this previous study, our data imply that injected Aβ is present in mononuclear blood cells but not in blood plasma during the first day after injection, suggesting that Aβ is initially transported to peripheral organs by mononuclear blood cells. However, we cannot rule out that injected Aβ is present in blood plasma at levels below the detection limit of the immunoassay or that binding of Aβ to soluble transport proteins such as low-density lipoprotein receptor–related protein-1 (Sagare et al, 2007) leads to epitope masking, thereby reducing Aβ detection in the immunoassay." (Page 13, line 292)

We inspected Giemsa-stained peritoneal cells and identified predominantly lymphocytes and to a lesser extent macrophages and mesothelial cells. We did not see any obvious

differences in the cell composition or amount between mice injected with APPtg extract and mice injected with non-tg extract. Nor did the cell composition or amount change over time at later time points after injection. However, at 5 h and occasionally at 1 d after injection, the injected brain material was still visible. We added "In the peritoneal cells consisting predominantly of lymphocytes and to a lesser extent of macrophages and mesothelial cells" (Page 8, line 161) to the results section. Furthermore, we added the following information to the methods section: "Ten microliters of resuspended peritoneal cells were air-dried overnight on Superfrost® Plus slides at room temperature. Slides were fixed in methanol for 5 minutes and subjected to Giemsa staining to identify the composition of the peritoneal cells. " (Page 24, line 533). We decided not to add an additional Figure with images of the peritoneal cells to the manuscript as it would not add anything to our findings. However, please find here a representative image of the peritoneal cells of an animal at 3 d after injection of APPtg brain extract.



2) the tg and non-tg groups were administered different durations of the stable isotope-labeled diet for the main experiments, acknowledging that the authors did, in later experiments, administer the diet for 100 d to non-tg mice to match the labeled diet duration of the original group of tg mice. The authors should move the findings of the non-tg mouse group fed the labeled diet for only 10 days to supplement clearly identifying the difference in labeling as not being comparable. Figure 4G seems to provide some of the most convincing evidence that the labeled amino acid, but not the peptide, is being taken up and incorporated (hence not supporting the hypothesis that amyloid-beta in the periphery enters the brain).

We agree with the reviewer that our experimental setups first using brain donors with different labeling durations and later brain donors with equal labeling durations is rather confusing. When first generating the labeled negative controls, we tried to plan our feeding durations in the most economical way possible due to the immensely high costs of the ^{13}C-Lys-labeled diet (~8000 €/kg) and decided on a labeling duration of 10 days because the average life time of brain proteins had been reported to be 9 days. This labeling duration of 10 d resulted in 83% overall labeling of the brain proteome. We were very surprised by the results of the injection experiments, as we had not expected such fast label incorporation into Aβ, nor had we anticipated that an overall labeling difference of 97% in APPtg extract vs 83% in non-tg extract would result in a significant difference in the abundance of labeled Aβ. We added "Unequal labeling duration of APPtg and non-tg donors" to the graph titles of Fig. 4B and "Equal labeling duration of APPtg and non-tg donors" to the graph title of Fig. 4G to point out differences in experimental setups in a clearer manner. However, we would like to

leave the data of the main injection experiments in Fig. 4 for the following reasons. 1) Fig. 4B includes a variety of time points from 1-100 d after injection, pointing to incorporation of the labeled amino acid from day 1 after injection with a slow but steady increase until 100 d after injection. 2) Fig. 4B argues in favor of a "dose-dependent" incorporation of the labeled amino acid, i.e. the higher the percentage of labeled brain proteins in the donor extract, the higher the abundance of labeled Aβ in the injected mice. 3) Figure 4 as a whole shows the entire evolution of the experiments leading to the main conclusion of the manuscript that peripherally injected Aβ does not reach the brain while the mice incorporate labeled amino acids from the injected brain extracts.

3) the hypothesis that proteases in the liver help break down amyloid-beta seems unnecessary and unproven (most protein catabolism occurs in the stomach and small intestine), and at most should be included as a speculation in discussion.

We agree with the reviewer that this point is rather a speculation than a hypothesis, for which we do not have proof. We revised the wording as follows: "we provide evidence that peripherally injected Aβ does not penetrate the brain but ~~is~~ may be metabolized along with other labeled proteins" (Page 10, line 226). "We ~~hypothesize~~ speculate that ^{13}C-Lys-containing proteins and peptides from the injected donor extract ~~is~~ may be metabolized ~~by proteases in the liver~~ in the periphery, resulting in release of ^{13}C-Lys – an essential amino acid – into the bloodstream. From there, ^{13}C-Lys ~~is~~ may be transported into the brain and used for *de novo* production of Aβ." (Page 11, line 246).

4) the absolute lack of 13C-labeled Lys in the soluble (TBS) fraction (Figure 4A) as opposed to the unlabeled 17-28 fragment seems odd when it is apparent in the insoluble fraction rather quickly
(1 d). The overall proportion of soluble vs. insoluble amyloid beta is generally low - so maybe it was simply the limit of detection in this case? The authors should explain this apparent discrepancy.

We agree with the reviewer that the most likely explanation for this discrepancy is that soluble 13C-Lys Aβ is below the limit of detection as the abundance of soluble unlabeled Aβ is already very low. We added the following sentences to the discussion section: "While we observed incorporation of ^{13}C-Lys into insoluble Aβ, we did not detect ^{13}C-Lys Aβ in the soluble brain fraction at any time point. This discrepancy is most likely attributable to the proportion of soluble to insoluble Aβ in our brain samples, with soluble unlabeled Aβ being detected with an abundance 1,000-10,000-fold lower than insoluble unlabeled Aβ. Given that this low abundance of soluble unlabeled Aβ was already close to the limit of detection and that insoluble ^{13}C-Lys Aβ was detected with an abundance 1,000-fold lower than insoluble unlabeled Aβ, the abundance of soluble ^{13}C-Lys Aβ would be below the limit of detection in our study." (Page 11, line 250).

Minor critiques are:

1) a parallel analysis of other proteins that would have incorporated the 13CLys would help support this claim that 13CLys is transported via bloodstream to the brain as an amino acid, where in the brain itself, it is incorporated into a variety of proteins as they are synthesized. Can the authors search their mass spec data and find labeled signatures of other proteins (e.g. apolipoprotein E, which is present in abundant amounts - see https://doi.org/10.1371/journal.pone.0038013)?

We thank the reviewer for this helpful suggestion. However, our MS methodology was designed to specifically detect ^{13}C-Lys Aβ17-28 *at very low levels*. Our strategy includes Aβ enrichment by immunoprecipitation and specific detection of the four most intensive transitions of Aβ17-28 by MRM. Thus, there are no signatures of other proteins in our MS

data. To follow the reviewer's suggestion, we measured the insoluble fraction without previous immunoprecipitation but with trypsin digestion on Orbitrap QExactive HF. While we were able to sequence over 4700 proteins and quantify over 3000 of them with FDR<0.01, we did not detect ^{13}C-Lys Aβ fragments or any other ^{13}C-Lys-containing peptides. This is attributable to the fact that ^{13}C-Lys Aβ and possibly other proteins that may have incorporated ^{13}C-Lys are present in extremely low amounts. To detect labeled signatures of other proteins at very low levels, we would have to enrich these proteins specifically by immunoprecipitation, digest them, identify the most easily detectable fragments that would have to include ^{13}C-Lys, and design a new MRM-based strategy detecting the most intensive transitions. This task would take a long time to complete and would be beyond the scope of the study.

2) Consider expanding, with perhaps a Figure illustrating the phenomenon, of the finding that stable-isotope labeling in these Tg mice confirms the findings of the Michno et al 2021 study: in preparation of the labeled homogenates, the authors also noted that the earlier labeling begins, the lower the amount of insoluble Aβ that is not subject to dynamic turnover, and the higher the label incorporation into Aβ, which highlights the importance of an early start of therapies promoting Aβ clearance - showing that label incorporation into Aβ is highest when labeling starts before plaque onset.

We thank the reviewer for this excellent suggestion. We added an illustration to Figure 1 (referred to as Figure 1E while Figure 1E was changed to Figure 1F) and the following description to the figure legend: "E Illustration of ^{13}C-Lys incorporation into Aβ depending on the starting point of labeling in APPtg mice. The later labeling starts, the higher is the amount of already generated, insoluble Aβ that is not metabolized anymore and consequently, not available for label incorporation. The earlier labeling starts, the higher is the fraction of newly produced Aβ incorporating the label. " (Page 34, line 829).

3) Although peak intensity of the amyloid-beta 17-28 fragment was highest and therefore chosen as a surrogate for total amyloid-beta, considering choosing the second most intense peak and analyzing it in parallel (perhaps a fragment with the Lys at position 16 as well as C-terminal end at 42).

To reduce the oligomerization/aggregation propensities of hydrophobic full-length Aβ peptides and their known "tailing effects" in reversed phase chromatography (doi.org/10.1021/acs.biochem.8b00393), we chose to perform the trypsin digestion experiments in order to generate shorter, more soluble Aβ fragments. In our initial experiments, we utilized both synthetic Aβ1-40 and Aβ1-42 peptides, found as components of Aβ deposits in the APP/PS1 brain (see compendium of tested conditions at: http://urn.fi/URN:NBN:fi:aalto-201712187965). Digestion of Aβ1-40 and Aβ1-42 peptides resulted in similar digestion pattern, yielding fragments of various length corresponding mostly to expected *m/z* Aβ17-28 and Aβ1-16 peptides. The very hydrophobic C-terminal fragments of Aβ, ending at position 42 were detected in MALDI MS tryptic digestion experiments with synthetic Aβ1-42 peptide with very low intensities. Based on these observations, the ion with the highest intensity, corresponding to Aβ17-28 $[MH]^+$ (*m/z* 1325.818), with good solubility and high recovery following several steps of IP-based MS procedure was selected as a surrogate to measure the total Aβ content. This strategy to utilize this peptide has been independently described by others (DOI: 10.1016/j.aca.2014.06.024 and patented patent/EP2575596B1). We have not tested the less intensive Aβ1-16 peptide since unlike Aβ 17-28 it could also not be detected in TBS-soluble brain fractions (S1) from APP/PS1 animals, in Orbitrap QExactive HF Plus MS/MS measurements. Consequently, we specifically designed the MRM-based strategy allowing detection and quantification of the four most intensive transitions of easily detectable Aβ17-28 peptide to be followed in all experiments. Due to this selective, Aβ17-28-targeted

strategy, it is not possible to follow the reviewer's suggestion and analyze Aβ1-16 fragments retrospectively.

Dear Prof. Pahnke

Thank you for the submission of your revised manuscript to EMBO reports. As my colleague Esther Schnapp is currently traveling, I have temporarily taken over the handling of your manuscript. We have now received the full set of referee reports (copied below) and both referees are very positive about the study and support publication without further revision.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the official acceptance of your study.

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- Please move the data availability section to the end of Materials and Methods.

- Data availability: The current link resolves to a data sharing platform, OSF. We generally ask authors to deposit data in one of the publicly accessible databases, such as Pride or Peptide Atlas for mass spectrometry data. Please deposit your data in one of these databases and add this link and the name of the database to the Data availability section. If the data on OSF contain more raw data than the proteomics, it could remain part of the section too. Please contact us, if you want to discuss this further.

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- We noticed that Table EV2 is called out before Table EV1 and recommend renaming them.

- Tables EV1 and EV2 need to be removed from the manuscript and uploaded as separate files

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

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

The authors have addressed satisfactorily the point raised by this reviewer and revised the manuscript accordingly.

Referee #2:

The authors have done a good job to address the questions the reviewers had, as well as the additional experiment they did to address the Referee #1 concern in the text and with additional data in Figure legends 3 and 4 about exactly how many mice at

which time points were used. We have no other major concerns.

The authors have addressed all minor editorial requests.

Prof. Jens Pahnke
University of Oslo
Department of Pathology
Sognsvannsveien 20
Oslo 0318
Norway

Dear Prof. Pahnke,

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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 replicates.
- 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 Presentation.

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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.
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  - are tests one-sided or two-sided?
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  - definition of error bars as s.d. or s.e.m.

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Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	No data were excluded
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

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