EMBO reports

ER-mitochondria contacts and cholesterol metabolism are disrupted by disease-associated tau protein

Leonora Szabo, Nadia Cummins, Paolo Paganetti, Alex Odermatt, Andreas Papassotiropoulos, Celeste Karch, Jurgen Gotz, Anne Eckert, and Amandine Grimm
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Dear Amandine,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the original referees #1 and 3 and I am afraid that the overall decision is not a positive one.

While both referees appreciate that the analysis has been extended, referee #1 also finds that we need further biochemical analysis to support the key conclusions. Referee #3 is more positive. Please note that I didn't go back to original referee #2 as the referee found the extent of the analysis too limited for consideration in The EMBO Journal in the first place. Given the input from referee #1 that the analysis is not at a level what we would need for consideration here, I am afraid that I can't offer to consider publication here.

I have taken the opportunity to discuss the manuscript with my colleague Esther Schnapp from EMBO Reports. EMBO Reports is interested in considering the revised manuscript. If you are interested in this option, then I would suggest that you contact Esther (e.schnapp@emboreports.org) directly to discuss this option further.

For The EMBO Journal, I am very sorry that I can't be more positive on this occasion. However, I hope that you will consider the EMBO Reports option.

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Referee #1:

In this revised version of their manuscript, Szabo et al aimed to further sustain the implication of Tau in the regulation of ER mitochondria connection and cholesterol transfer. I thank the authors for their additional work and addressing most of my comments. Although the paper is much improved, there are still some specific points that are still unclear and do not reach the necessary rigor to support the authors' conclusions.

First and foremost, while the title of the manuscript is "ER-mitochondria coupling and cholesterol transfer are disrupted by disease-associated tau protein", the work does not include any assay that indeed supports a biochemical disruption in ER-mito crosstalk.
As mentioned in my previous review, it is essential that changes in organelle crosstalk are biochemically quantified. Many organelles in the cell are in close contact when imaging data is analyzed, however they do not necessarily have a functional interaction. At the moment, there are no data that consistently and rigorously link the degree of physical apposition and functional crosstalk since PLA or any other similar imaging technique do not have the resolution necessary to be a clear reporter of MAM activity or ER-mito functional communication. Therefore, the physical distance between ER-mitochondria considered to be functional in imaging data is set by the observer, and thus arbitrary.
Indeed, the controversy around the effect of MFN2 on ER-mitochondria communication, as

shown by other groups, is a great example of these limitations. Since these cells have been observed to increased ER-mitochondria apposition, as shown in this work, but a decrease in MAM activity and functional ER-mitochondria crosstalk. For these reasons, the authors' conclusions need to be supported by a functional read out of ER-mito communication.

The lack of biochemical read outs also reduces the significance of other conclusions drawn by the authors.

For example, on many occasions the steady state levels of cholesterol or pregnenolone are interpreted in a dynamic way with no biochemical data to support these interpretations. Pregnenolone levels could be reduced not only due to a lack of cholesterol transfer, but also due to its rapid conversion to steroids.

Although cholesterol esters levels were under the limit of detection in most cell models, the number of lipid droplets could be quantified significantly in Fig. 4M in their data. Given that lipid droplets are quite rich in cholesteryl esters, it is not quite clear while the authors' where not able to detect these lipids in their lipidomics analysis.

In Fig 5I reducing MFN2 expression does not affect pregnenolone synthesis in WT conditions. These data conflict with the fact that MFN2 is necessary for steroid synthesis mitochondria morphology. The authors need to show the degree of gene ablation to confirm their data. It is also puzzling that, considering the authors' conclusions, P301L-MFN2 silenced cells do not show higher levels cholesterol levels in the ER.

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Other data are unclear. For instance, when Fig 2A and Fig. 2S are compared, in the context of Tau P301L mutations, we could conclude that mitochondria morphology is independent of its degree of association with the ER?

In summary, this is a very interesting work that opens a new way of thinking about the impact of mutations in Tau in ER-mitochondria and cholesterol. However, without biochemical studies to support their conclusions, the manuscript relies on correlative studies, interpreted as causative. Alas, I believe that these limitations preclude this work to reach the thoroughness and rigor necessary for its publication in EMBO J.

Other

EV2. FACL4 is an acyl-CoA ligase. Given its role in lipid metabolism, it is quite expected that this enzyme affects cholesterol metabolism as well as other lipid species. However, this is not

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The revised study by Szabo and colleagues provides strong evidences demonstrating the implication of wild type Tau protein and mutated Tau form (P103L) in the regulation of MAMs structure and function and in the transfer of cholesterol from the endoplasmic reticulum to mitochondria and in its conversion to pregnenolone. The revised manuscript includes new significant data that reinforce the original study through the validation of the observed effects in human derived iPSC holding P103L Tau mutation and most importantly by using targeted metabolomics studies. Several controls were included to strengthen the conclusions.
Authors answers satisfactory to all comments. Thus, the revised manuscript merits publication in EMBO Journal.

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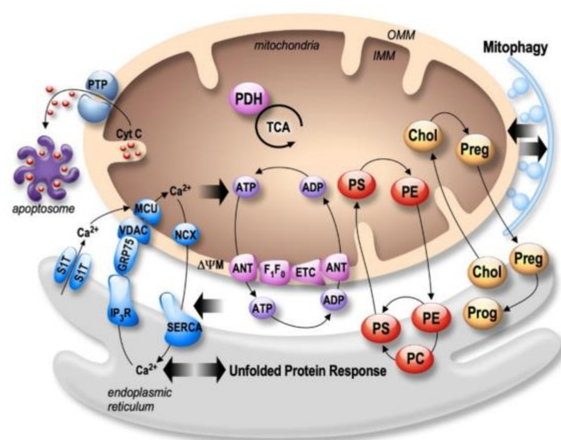

Figure from Eysert F et al, 2020 (doi:10.3390/ijms21249521) showing different MAMs function, including transport and metabolism of cholesterol (Chol).

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   Answer: We thank the referee for raising this important point. In a later study, we have shown that it is pregnenolone synthesis that is impaired in the P301L cells (Grimm et al., 2019b, doi:10.1111/jne.12796). Indeed, in this study, the downstream conversion of pregnenolone was blocked by the addition of trilostane and abiraterone, which completely inhibits the conversion of pregnenolone into progesterone or dehydroepiandrosterone. Therefore, we know that in our P301L cells, the decrease in pregnenolone level is due to a decrease in pregnenolone synthesis and not a rapid conversion to other steroids.
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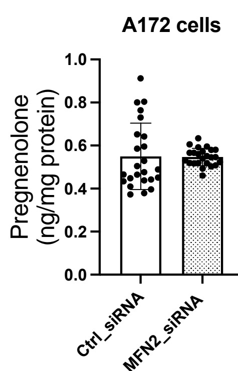
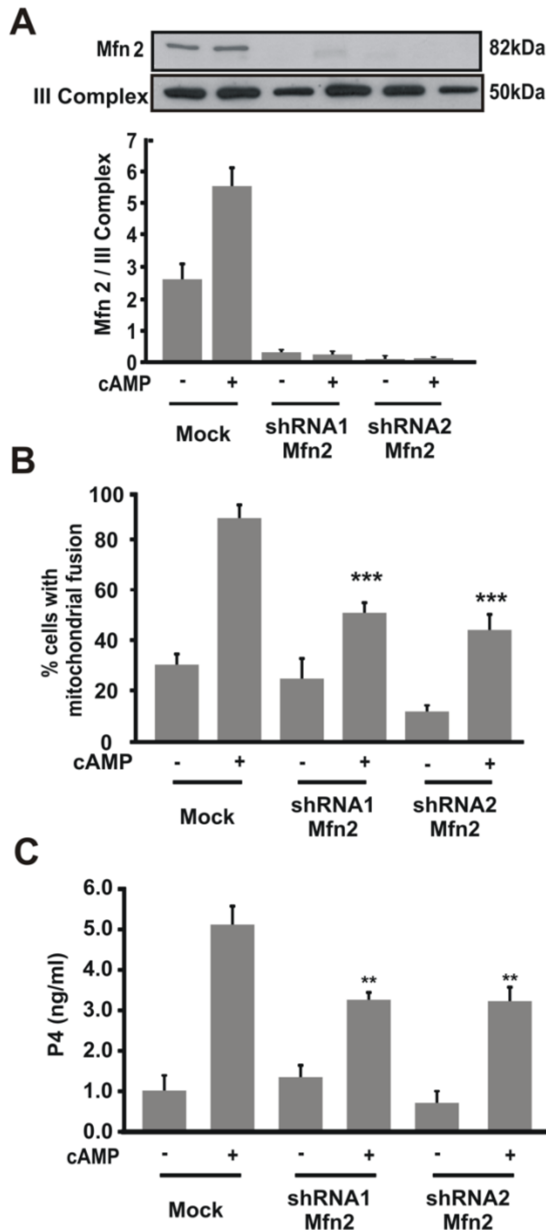
   Answer: We thank the referee for this comment. Indeed, MFN2 KD does not affect pregnenolone synthesis in WT condition. We obtained similar data in a previous study in which we investigated the link between the clock-controlled mitochondrial dynamics and cyclic pregnenolone synthesis in A172 glioma cells (Witzig et al, Cells 2020, doi:10.3390/cells9102323). In this study, MFN2 KD affected the circadian variations of pregnenolone synthesis, by did not affect pregnenolone level per se (see opposite graph). We rather showed that it is the dynamic process of mitochondrial fusion that plays a role in the clock-controlled mitochondrial steroidogenesis.

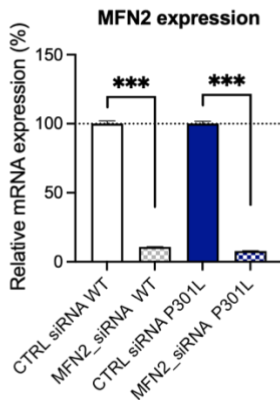
   In line, others have shown that MFN2 KD impairs mitochondrial fusion and steroid synthesis (with progesterone P4 level used as readout of steroid synthesis) upon cAMP stimulation, but not under basal condition (see Figure 7C below).



From Duarte A, et al PlosONE 2012
https://doi.org/10.1371/journal.pone.0045829

Figure 7. Mfn2 protein is necessary for steroid synthesis.
MA-10 cells were transfected with a plasmid containing different shRNA Mfn2 (shRNA1 or shRNA2). After 48 h, cells were stimulated with 8Br-cAMP (0.5 mM) for 1 h. **A.**Isolated mitochondrial proteins were obtained and western blotting was performed. Membranes were sequentially blotted with anti-Mfn2 and anti-III Complex antibodies. An image of a representative western blot is shown. For each band, the OD of the expression levels of Mfn2 protein was quantified and normalized to the corresponding III Complex protein. The relative levels of Mfn2 protein are shown. **B.** Cells were fixed and scored as previously described. Quantitative analysis of mitochondrial fusion is shown. The results are expressed as the means ± SEM of three independent experiments. ***P*<0.01 vs. cAMP mock. **C.** P4 levels were determined by RIA in the incubation media. Data represent the means ± SEM of three independent experiments and expressed as ng/ml. ***P*<0.01 vs. 8Br-cAMP mock.

Regarding the degree of gene ablation, we can of course provide the corresponding data (see graph below (WT cells: Ctrl siRNA vs MFN2 siRNA = -89.2%; P301L cells: Ctrl siRNA vs MFN2 siRNA= -92.2%)



Regarding our conclusion, we indeed state that "we observed a slight but significant increase in pregnenolone in P301L cells + MFN2 siRNA (Fig 5J), indicating that the increase of ER-mitochondria association via MFN2 KD can partially restore pregnenolone synthesis in P301L

cells". Even if cholesterol staining was increased in mitochondria and decreased in the ER of P301L, we cannot exclude that other factors may influence pregnenolone synthesis: As stated before, we previously showed that the dynamic process of mitochondrial fusion plays a role in the mitochondrial steroidogenesis (Witzig et al, Cells 2020, doi:10.3390/cells9102323).

Statements to clarify these different points can be added in the manuscript.

In Fig. 6 and EV3, treating with GSK3b inhibitors reduced cholesterol in the ER in P301L cells, but it shows to increase cholesterol and cholesterol metabolites levels as measured by metabolomics. Also, GSK3b inhibitors in P301L cells restores cholesterol levels in the ER and mitochondria to WT, but not pregnenolone levels. These data are unclear. It is also puzzling that incubation with GSK3b inhibitors, which induce the phosphorylation of DRP1, do not affect mitochondrial morphology.
Answer: We thank the referee for this comment. Because GSK3b is involved various major signal transduction pathways (see discussion part lines 477-496), it is still unknown whether/how it influences cholesterol homeostasis. We agree that this important point deserves to be investigated in future studies, as the fact that GSK3b inhibition does not completely restores pregnenolone levels.
Regarding the effects of GSK3b on DRP1 phosphorylation, previous studies indeed showed that inhibition of GSK3b (using LiCl in a millimolar concentration range) reduces mitochondrial fission (Wu JH et al, Neurosci letter 2013 doi:10.1016/j.neulet.2013.08.057; Chou CH et al, PlosOne 2012 doi:10.1371/journal.pone.0049112; Huang et al, Diabetes 2015 doi: 10.2337/db14-0758).
In our study, with used the selective GSK3b inhibitor CHIR99021 at 100 nM which might explain why we do not see a clear effect of mitochondrial morphology (at least in WT cells).

Discussion points can be added in the manuscript.

Other data are unclear. For instance, when Fig 2A and Fig. 2S are compared, in the context of Tau P301L mutations, we could conclude that mitochondria morphology is independent of its degree of association with the ER?
Answer : We thank the referee for this comment. Data obtained with the iPSC-P301L were indeed very exciting as they show for the first time the pure effect of the P301Ltau mutation on mitochondrial morphology (not in an overexpression model).
Therefore we wrote lines 161-164:
"Data obtained in iPSCs suggest that it is the P301L overexpression in SH-SY5Y cells (mimicking abnormal tau accumulation in tauopathy) that would be responsible for mitochondrial elongation, while the P301L mutation per se (in iPSC-P301L) induces mitochondrial fragmentation and disruption of the ER-mitochondria association."
And lines 357-363:
"On the contrary, Tau KO cells and iPSC-P301L showed a more fragmented mitochondrial network, suggesting that the absence of tau or the presence of pathological tau at endogenous levels differently impact mitochondrial shape. Indeed, P301L-tau overexpression rather mimics the accumulation of pathological tau, which may lead to the disruption of the physical association of mitochondria and the mitochondrial fusion protein dynamin-related protein 1 (DRP1), leading to mitochondrial elongation, as previously described (DuBoff et al., 2012)."

In an ongoing study, we are currently comparing the effect of different tau mutations, namely P301L, R406W and IVS10+16, on key mitochondrial parameters, such as bioenergetics, dynamics and ER-mitochondria association. This new study is conducted on patient-derived IPSCs bearing the different tau mutations versus the CRISPR-generated wild-type tau controls. Thus, we will shed light on the effect of different tau mutation (express at endogenous level) on mitochondrial function, including bioenergetics and dynamics.

Of note, MAMs are indeed important for mitochondrial dynamics, however, to our knowledge, there are no data showing that mitochondria morphology is strictly dependent on its degree of association with the ER.

In summary, this is a very interesting work that opens a new way of thinking about the impact of mutations in Tau in ER-mitochondria and cholesterol. However, without biochemical studies to support their conclusions, the manuscript relies on correlative studies, interpreted as causative. Alas, I believe that these limitations preclude this work to reach the thoroughness and rigor necessary for its publication in EMBO J.
Answer: We thank the referee for all the constructive comments that helped to increase the quality of your manuscript, and we agree that our data raise additional questions that need to be answered in future studies. Nevertheless, we addressed all comments raised by the referee with extensive experimentation, resulting in 11 new figure panels significantly substantiating our claims. We strongly believe that our data nicely support your hypothesis stating that abnormal tau protein disturbs the physical interaction between the ER and mitochondria, leading to impairments in cholesterol transport from the ER to mitochondria, and that artificially increasing the ER-mitochondria coupling partially alleviate tau-induced defects in intramitochondrial cholesterol transport and metabolism.

Other

EV2. FACL4 is an acyl-CoA ligase. Given its role in lipid metabolism, it is quite expected that this enzyme affects cholesterol metabolism as well as other lipid species. However, this is not direct effect, nor it means that FACL4 is a cholesterol enzyme.
Answer : We thank the referee. We agree that FACL4 is not a cholesterol enzyme. Nevertheless, several studies showed that it is involved in cholesterol transport into mitochondria. Please see Duarte A, et al PlosONE 2012, Doi:10.1371/journal.pone.0045829; Fan J & Papadopoulos V PlosOne 2013 doi :10.1371/journal.pone.0076701

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Authors answers satisfactory to all comments. Thus, the revised manuscript merits publication in EMBO Journal.

Answer : We thank the referee for all the comments.

1st Editorial Decision **23rd May 2023**

Dear Amandine,

Thank you for the transfer of your revised manuscript to EMBO reports, and for your proposed point-by-point response. We agree with your suggestions for how to revise your manuscript and invite you to do so, and to respond to all referee comments in the manuscript text and to remove all overstatements regarding a functional ER-mitochondria coupling, including in the ms title and abstract.

A few editorial requests will also need to be addressed before we can proceed with the official acceptance of your paper:

- The FUNDING INFO in your manuscript and in our online submission system do not match, please correct.

- Please describe your novel findings in the abstract in present tense.

- Please correct the conflict of interest subheading to "Disclosure and Competing Interest Statement"

- Please remove the Author Contributions from the ms file. We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions, if you wish. See also guide to authors https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines.

- Please remove "data not shown" (page 26) as per journal policy. Either show the data or re-write.

- Table EV2 should be renamed to Dataset EV1 and the legend should be included in the excel file and removed from the ms file. Please also correct the callouts to Dataset EV1.

- Table EV1 needs to be uploaded as an individual Table EV1 file with its legend included.

- I attach to this email a related ms file with comments by our data editors. Please address all comments in the final ms.

- The movie legends should be zipped with their respective movie file and uploaded as one file per movie.

I look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
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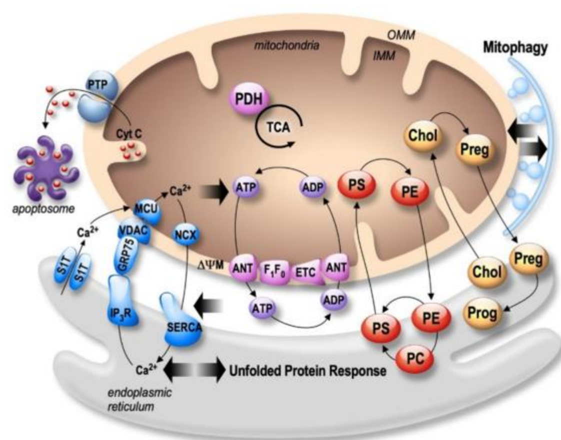

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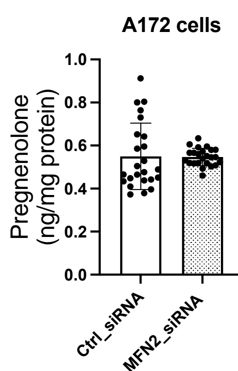

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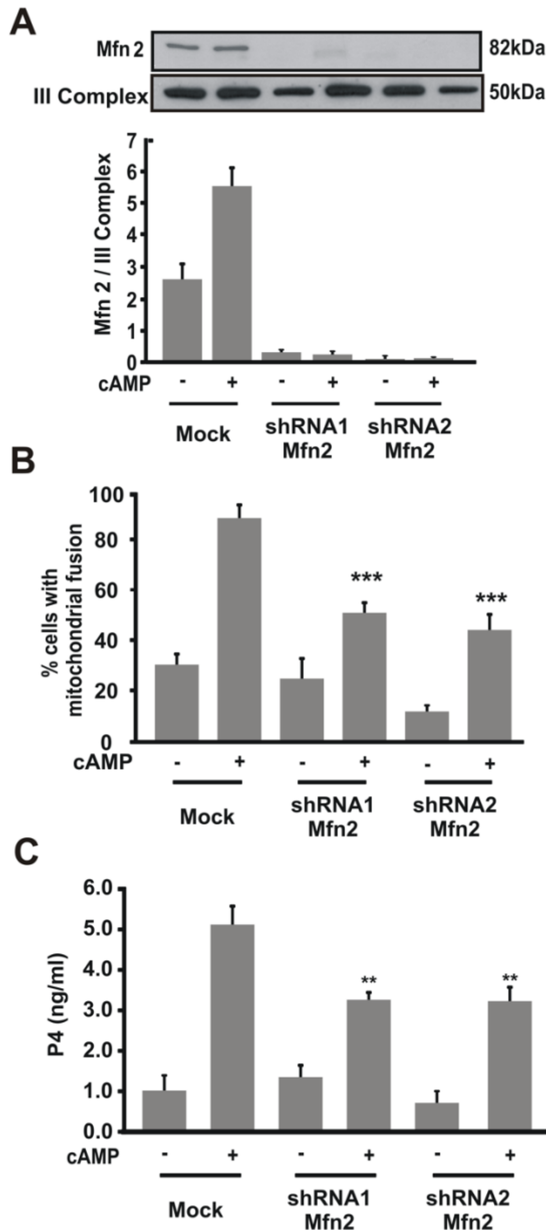

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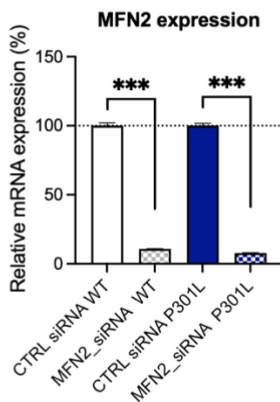

Regarding our conclusion, we indeed state that “we observed a slight but significant increase in pregnenolone in P301L cells + MFN2 siRNA (Fig 5J), indicating that the increase of ER-mitochondria association via MFN2 KD can partially restore pregnenolone synthesis in P301L

cells". Even if cholesterol staining was increased in mitochondria and decreased in the ER of P301L, we cannot exclude that other factors may influence pregnenolone synthesis: As stated before, we previously showed that the dynamic process of mitochondrial fusion plays a role in the mitochondrial steroidogenesis (Witzig et al, Cells 2020, doi:10.3390/cells9102323).

Statements to clarify these different points can be added in the manuscript.

In Fig. 6 and EV3, treating with GSK3b inhibitors reduced cholesterol in the ER in P301L cells, but it shows to increase cholesterol and cholesterol metabolites levels as measured by metabolomics. Also, GSK3b inhibitors in P301L cells restores cholesterol levels in the ER and mitochondria to WT, but not pregnenolone levels. These data are unclear. It is also puzzling that incubation with GSK3b inhibitors, which induce the phosphorylation of DRP1, do not affect mitochondrial morphology.
Answer: We thank the referee for this comment. Because GSK3b is involved various major signal transduction pathways (see discussion part lines 477-496), it is still unknown whether/how it influences cholesterol homeostasis. We agree that this important point deserves to be investigated in future studies, as the fact that GSK3b inhibition does not completely restores pregnenolone levels.
Regarding the effects of GSK3b on DRP1 phosphorylation, previous studies indeed showed that inhibition of GSK3b (using LiCl in a millimolar concentration range) reduces mitochondrial fission (Wu JH et al, Neurosci letter 2013 doi:10.1016/j.neulet.2013.08.057; Chou CH et al, PlosOne 2012 doi:10.1371/journal.pone.0049112; Huang et al, Diabetes 2015 doi: 10.2337/db14-0758).
In our study, with used the selective GSK3b inhibitor CHIR99021 at 100 nM which might explain why we do not see a clear effect of mitochondrial morphology (at least in WT cells).

Discussion points can be added in the manuscript.

Other data are unclear. For instance, when Fig 2A and Fig. 2S are compared, in the context of Tau P301L mutations, we could conclude that mitochondria morphology is independent of its degree of association with the ER?
Answer : We thank the referee for this comment. Data obtained with the iPSC-P301L were indeed very exciting as they show for the first time the pure effect of the P301Ltau mutation on mitochondrial morphology (not in an overexpression model).
Therefore we wrote lines 161-164:
"Data obtained in iPSCs suggest that it is the P301L overexpression in SH-SY5Y cells (mimicking abnormal tau accumulation in tauopathy) that would be responsible for mitochondrial elongation, while the P301L mutation per se (in iPSC-P301L) induces mitochondrial fragmentation and disruption of the ER-mitochondria association."
And lines 357-363:
"On the contrary, Tau KO cells and iPSC-P301L showed a more fragmented mitochondrial network, suggesting that the absence of tau or the presence of pathological tau at endogenous levels differently impact mitochondrial shape. Indeed, P301L-tau overexpression rather mimics the accumulation of pathological tau, which may lead to the disruption of the physical association of mitochondria and the mitochondrial fusion protein dynamin-related protein 1 (DRP1), leading to mitochondrial elongation, as previously described (DuBoff et al., 2012)."

In an ongoing study, we are currently comparing the effect of different tau mutations, namely P301L, R406W and IVS10+16, on key mitochondrial parameters, such as bioenergetics, dynamics and ER-mitochondria association. This new study is conducted on patient-derived IPSCs bearing the different tau mutations versus the CRISPR-generated wild-type tau controls. Thus, we will shed light on the effect of different tau mutation (express at endogenous level) on mitochondrial function, including bioenergetics and dynamics.

Of note, MAMs are indeed important for mitochondrial dynamics, however, to our knowledge, there are no data showing that mitochondria morphology is strictly dependent on its degree of association with the ER.

In summary, this is a very interesting work that opens a new way of thinking about the impact of mutations in Tau in ER-mitochondria and cholesterol. However, without biochemical studies to support their conclusions, the manuscript relies on correlative studies, interpreted as causative. Alas, I believe that these limitations preclude this work to reach the thoroughness and rigor necessary for its publication in EMBO J.
Answer: We thank the referee for all the constructive comments that helped to increase the quality of your manuscript, and we agree that our data raise additional questions that need to be answered in future studies. Nevertherless, we addressed all comments raised by the referee with extensive experimentation, resulting in 11 new figure panels significantly substantiating our claims. We strongly believe that our data nicely support your hypothesis stating that abnormal tau protein disturbs the physical interaction between the ER and mitochondria, leading to impairments in cholesterol transport from the ER to mitochondria, and that artificially increasing the ER-mitochondria coupling partially alleviate tau-induced defects in intramitochondrial cholesterol transport and metabolism.

Other

EV2. FACL4 is an acyl-CoA ligase. Given its role in lipid metabolism, it is quite expected that this enzyme affects cholesterol metabolism as well as other lipid species. However, this is not direct effect, nor it means that FACL4 is a cholesterol enzyme.
Answer : We thank the referee. We agree that FACL4 is not a cholesterol enzyme. Nevertheless, several studies showed that it is involved in cholesterol transport into mitochondria. Please see Duarte A, et al PlosONE 2012, Doi:10.1371/journal.pone.0045829; Fan J & Papadopoulos V PlosOne 2013 doi :10.1371/journal.pone.0076701

Referee #3:

The revised study by Szabo and colleagues provides strong evidences demonstrating the implication of wild type Tau protein and mutated Tau form (P103L) in the regulation of MAMs structure and function and in the transfer of cholesterol from the endoplamsmic reticulum to mitochondria and in its conversion to pregnenolone. The revised manuscript includes new significant data that reinforce the original study through the validation of the observed effects in human derived iPSC holding P103L Tau mutation and most importantly by using targeted metabolomics studies. Several controls were included to strengthen the conclusions.

Authors answers satisfactory to all comments. Thus, the revised manuscript merits publication in EMBO Journal.

Answer : We thank the referee for all the comments.

1st Revision - Editorial Decision 16th Jun 2023

Dr. Amandine Grimm
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Switzerland

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