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Disease-associated tau impairs mitophagy by inhibiting Parkin translocation to mitochondria

Nadia Cummins, Andrea Tweedie, Steven Zuryn, Jesus Bertran-Gonzalez, Jürgen Götz

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29th Mar 2018

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see from the comments the referees find the analysis interesting, but they also indicate that further experiments are needed to support the key conclusions. The referees provide constructive comments on how to further strengthen the analysis. Should you be able to extend the manuscript along those lines then we would be able to consider a revised version. Let me know if we need to discuss anything further.

REFEREE COMMENTS:

Referee #1:

In the manuscript by Cummins et al., the authors examine the role of overexpression of wild type and mutant tau on mitophagy. Using fluorescent-based cell assays, the authors find that both forms of tau reduce mitophagy and that they block recruitment of GFP-Parkin to mitochondria upon depolarization. This effect appears to be direct as Parkin and Tau interact in a cell-based proximity ligation assay. Finally, the authors generate a mitophagy reporter worm and indicate that tau overexpression also impairs mitophagy in vivo.
The data in the manuscript are potentially interesting and the notion that tau prevents Parkin recruitment to damaged mitochondria could be very important and provide a crucial link between different neurodegenerative disorders. However, as it stands, the current data do not go far enough

in providing robust justification for the conclusions drawn and I feel more experiments are needed, in addition to the fluorescence microscopy already carried out.
Below are some concerns/suggestions that should be addressed to make the manuscript more convincing.

1) To monitor mitophagy, the authors use mito-QC (Fig1) and mito-Rosella (Fig7). It is my understanding that these assays work by lysosomal quenching of the GFP signal - therefore upon mitophagy, the authors should see red-only fluorescent structures. However, in the primary data shown, there are no red-only structures suggesting no mitophagy. Why is this? In addition, the general mitochondrial network is not very defined - perhaps higher resolution images would help?
2) In figure 2, as a control, it would help to see a western blot of the corresponding levels of GFP-Parkin expression in conjunction with V5 tau expression.
3) As the authors see less mitochondrial Parkin recruitment, is there also less ubiquitination of Parkin mitochondrial substrates?
4) The authors state that tau does not affect autophagosome formation (Fig 2I and J). However, from this static data, I do not think these conclusions can be drawn - and certainly autophagy may be blocked at a post formation step. The authors should perform flux style experiments (-/+ bafilomycin) and look at LC3 in the presence and absence of tau overexpression.
5) In Figure 4, I am not convinced by the PINK1 staining. Under normal conditions in most cell lines, PINK1 is undetectable and the authors should show the untreated images to confirm this. Better yet, the authors could also immunoblot for PINK1 as the fluorescent staining does not look like PINK1 on mitochondria. I think the authors also need to perform additional experiments to confirm PINK1 activity - either look at phospho-ubiquitin levels or phospho-parkin levels (both at serine 65).
6) In Figure 5, the authors should also show the Parkin clusters.
7) The PLA experiments in Figure 6 are very nice, but can the authors back these data up using a different approach, such as co-IP or in vitro binding using recombinant protein? The in vitro assay would also allow the authors to test the direct role of Tau on Parkin activity, which would be very informative.
8) As mentioned in point 1 above, in Fig7 it is hard to see mitophagy as all structures shown are positive for dsRed and GFP. As this is the first instance of the model, the authors should also perform a bit more characterization to confirm that the reporter is indeed on mitochondria and that the red-only structures (if there are any) are lysosomal.

Referee #2:

This is an interesting manuscript that has further investigated mechanisms by which tau, a protein closely associated with neurodegenerative conditions such as Alzheimer's disease, impairs mitochondria. This is an important question since it is well acknowledged that mitochondrial abnormalities give rise to several damaging downstream effects most notably a loss of neuronal ATP/energy production and neuronal dysfunction. Better understanding mitochondrial dysfunction is an area of considerable interest to the field who are urgently seeking mechanisms that may be targeted for therapeutic benefit.

The authors have sought to understand the effects of tau on mitochondrial quality control via mitophagy, specifically mitophagy mediated by the ubiquitin ligase Parkin which is normally recruited to the mitochondrial surface were it ubiquitinates outer mitochondrial membrane proteins, targeting the mitochondria for degradation by autophagolysosomal pathways. This paper extends on from several others in this area. Using N2a cells, the authors present data showing that exogenous expression of wild-type and disease-causing mutant P301L tau inhibit mitophagy by an interaction between the N-terminus of tau with Parkin preventing Parkin translocation to mitochondria by sequestering it in the cytosol. These effects were shown not to be caused by the microtubule stabilising function of tau. Tau did not affect events predicted to be upstream of Parkin translocation in mitophagy such as mitochondrial membrane depolarisation or PINK1 accumulation on the outer mitochondrial membrane. Finally, they used C. Elegans genetically altered to express wild-type or mutant tau as a means to validate their findings in an in vivo system. These are novel and important data, the paper is nicely written and the experiments appear to be well-controlled. However, in my opinion some further work is needed to substantiate some of the main claims/conclusions.

Major points
1. I have concerns about the possibility that many of the results are obtained simply as a result of protein over-expression and therefore could be non-physiological and not relevant for human disease. For example, Fig. 2G shows considerable increase in tau expression between 24 and 48 hrs that is mirrored by a decrease in Parkin clusters. Are the same effects observed upon transient transfection of other proteins? Alternatively, could siRNA be used to knockdown endogenous tau in rodent primary neurons or human neurons prior to mitophagy induction to determine if mitophagy is prevented?
2. Fig. 5. Acetylated and tyrosinated tubulin should also be examined to rule out other effects on microtubule stability.
3. Fig. 6. To show a relevance to human disease, PLA could be performed in human control/tauopathy brain.
4. The results from C. Elegans are not presented in a way that supports the conclusion that tau overexpression impairs neuronal mitophagy. For example, would it not be expected that if this was the case the GFP/DsRed ratio would be altered in untreated htau/hP301L relative to wild-type?

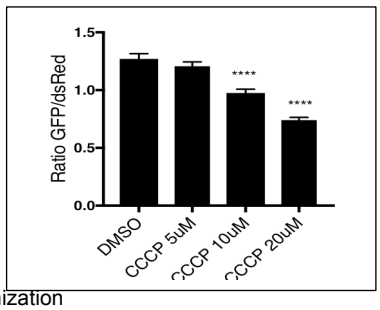
Minor points
1 Fig 1D. It would be helpful to see the images used for this analysis.
2 Fig. 2B. Please show images of untreated htau and hP301L cells to show reduced Parkin clusters 24 after transfection.
3 Fig 4 - Please show examples of PINK1 fluorescence in control, htau and hP301L cells showing examples of Parkin translocation and no Parkin translocation. It would also be useful to see PINK1 levels prior to depolarisation of these cells.
4 Fig. 4 legend is inaccurate and requires amendment.
5 No "toxicity" of these effects are actually shown (although they would be predicted), so it would be better to amend the concluding sentence of the abstract to reflect this.
6 Fig 6. The images are convincing but the data show 0.6/0.2 PLAs/V5 for htau in different experiments. It might be better to show these data as % htau and combined as a single analysis. Please also show the immunofluorescence that matched the quantification in Fig. 6F.
7 It would be useful to show htau/P301L tau phosphorylation status to rule out an effect of differential phosphorylation on Parkin sequestration/mitophagy.

1st Revision - authors' response 20th Jul 2018

Referee #1

1) To monitor mitophagy, the authors use mito-QC (Fig1) and mito-Rosella (Fig7). It is my understanding that these assays work by lysosomal quenching of the GFP signal - therefore upon mitophagy, the authors should see red-only fluorescent structures. However, in the primary data shown, there are no red-only structures suggesting no mitophagy. Why is this? In addition, the general mitochondrial network is not very defined - perhaps higher resolution images would help?

(i) It is correct that we rarely see red-only structures, and this is consistent across models, and although GFP fluorescence clearly decreases during mitophagy, the GFP quenching is incomplete. Our GFP/dsRed ratio analysis is based on Palikaras et al. (Nature, 2015), who also showed residual GFP fluorescence under conditions of mitophagy. To strengthen our finding, we validated our analysis of mito-QC by a dose-response analysis, establishing a clear relationship between CCCP concentration and GFP/dsRed ratio (see figure below, not added to the manuscript. Note: ****= p <0.0001 compared to DMSO):



(ii) We agree that the mitochondrial network could be more defined and have performed deconvolution on our representative images in Figure 1B. However, it is not an issue of resolution per se, rather appearing to be a feature of the mito-QC protein, which we find has a slightly less defined localisation in N2a cells, possibly due to the fact that mitochondrial targeting is achieved by using the Fis-1 targeting sequence. For that reason we co-stained mito-QC with a mitochondrial antibody to ATP synthase (Fig. 1A) to ensure correct localization.

2) In figure 2, as a control, it would help to see a western blot of the corresponding levels of GFP-Parkin expression in conjunction with V5 tau expression.

We have performed western blots at both the 24 and 48 h timepoints and added them to Fig. EV 3A-C.

3) As the authors see less mitochondrial Parkin recruitment, is there also less ubiquitination of Parkin mitochondrial substrates?

Yes, there is less ubiquitination. To address this question, we immunostained for ubiquitin in tau- and Parkin-expressing cells with and without CCCP treatment. Quantification of ubiquitin fluorescence intensity in mitochondrial ROIs showed a clear decrease in hP301L cells, corresponding to the impaired Parkin translocation observed at this 24h time point. This experiment is shown in Fig. EV 3 and described on p. 6-7.

4) The authors state that tau does not affect autophagosome formation (Fig 2I and J). However, from this static data, I do not think these conclusions can be drawn - and certainly autophagy may be blocked at a post formation step. The authors should perform flux style experiments (-/+ bafilomycin) and look at LC3 in the presence and absence of tau overexpression.

Thank you for pointing this out. We performed the requested experiments by starving cells in HBSS which induces autophagosomes, and compared numbers of LC3 punctae when cells were treated with or without bafilomycin A. We found that the control and the two tau groups displayed similar numbers of autophagosomes in both the DMSO and the bafilomycin conditions. We therefore conclude that general autophagy is not impaired in this cell model. These data are shown in Fig. EV 1A, B and are described on p. 6 (top paragraph).

5) In Figure 4, I am not convinced by the PINK1 staining. Under normal conditions in most cell lines, PINK1 is undetectable and the authors should show the untreated images to confirm this. Better yet, the authors could also immunoblot for PINK1 as the fluorescent staining does not look like PINK1 on mitochondria. I think the authors also need to perform additional experiments to confirm PINK1 activity - either look at phospho-ubiquitin levels or phospho-parkin levels (both at serine 65).

Thank you for bringing this to our attention. We therefore firstly performed further experiments using three different PINK1 antibodies to address this. However, none of the antibodies yielded conclusive results after CCCP treatment (see figure below, A-C) and we are not certain that the antibody we used for the immunostaining in original Figure 4 (Novus, BC100-494) is working appropriately in this cell line. We have therefore decided not to include the PINK1 staining results in this manuscript. However, given that we have already shown that mitochondrial membrane potential was not altered by tau expression and because we demonstrate tau-Parkin interaction as a pathomechanism, the PINK1 data is peripheral to the main conclusion of this manuscript, and will be examined by us more thoroughly in a separate study.

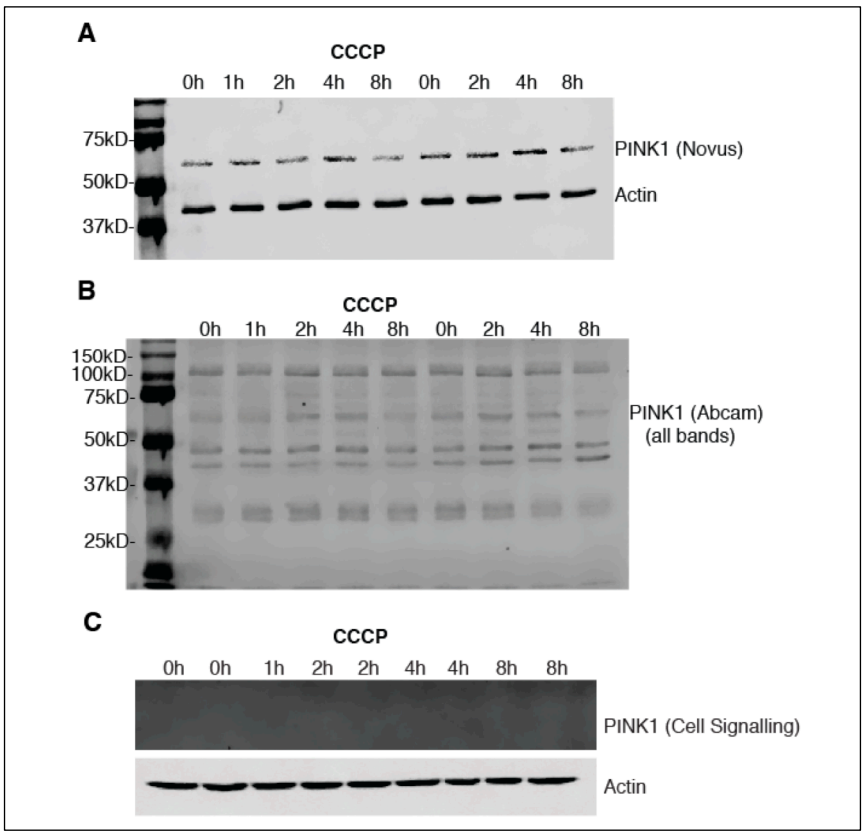


Fig. 1 PINK1 antibody tests.
N2a cells transfected with GFP-Parkin were treated with CCCP (10uM) for indicated time points (0 h = DMSO control) and whole cell lysates were immunoblotted for PINK1 using different antibodies. ***A****. Novus antibody and actin loading control.* ***B****. Abcam antibody (same membrane as in A, stripped and reprobed in a different fluorescence channel).* ***C.*** *Cell Signalling antibody and actin loading control.*

Next, as per this reviewer's suggestion, we also tried immunoblotting for phospho-Parkin (S65) using the Abcam antibody ab154995. However, we did not detect any signal under multiple conditions, and we just learned that this antibody has in fact been discontinued. Importantly, however, as we identified reduced ubiquitination of mitochondrial substrates (Fig EV 2) and it is well established that Parkin ubiquitinates mitochondrial proteins after translocation, it follows that we should also see less phospho-Parkin/ubiquitin, as there is less overall Parkin and ubiquitin on mitochondria under conditions of pathological tau accumulation. As such, we would conclude that assessing phospho-ubiquitin or phospho-Parkin is not critical to the conclusions of our manuscript.

6) In Figure 5, the authors should also show the Parkin clusters.

We agree with the reviewer and have added representative images of Parkin clusters with and without Taxol/JPL treatment to Fig. 4C, F (previously Fig. 5).

7) The PLA experiments in Figure 6 are very nice, but can the authors back these data up using a different approach, such as co-IP or in vitro binding using recombinant protein? The in vitro assay

would also allow the authors to test the direct role of Tau on Parkin activity, which would be very informative.

We have now also performed co-IPs to detect any interaction between Parkin and hTau or hP301L tau. Using this alternative approach, we have confirmed our findings from the PLA experiments that Parkin and each of the tau proteins physically interact. The data are shown in Fig. 5A and described in the bottom paragraph of p. 10. We have also referred to two earlier papers that also used co-IP to demonstrate physical interaction between these proteins, which further support our findings (Moore et al, J Neurochem, 2008; Petrucelli et al, HMG, 2004).

8) As mentioned in point 1 above, in Fig7 it is hard to see mitophagy as all structures shown are positive for dsRed and GFP. As this is the first instance of the model, the authors should also perform a bit more characterization to confirm that the reporter is indeed on mitochondria and that the red-only structures (if there are any) are lysosomal.

As described above, we believe that GFP quenching of the Rosella reporter is incomplete during mitophagy. This is a normal feature of the Rosella reporter and the detectable level of GFP signal remaining is dependent upon fluorescence imaging settings used during microscopy. Importantly, our mito-Rosella *C. elegans* strains are derived from those used by Palikaras et al. (Nature, 2015), who performed an extensive characterisation and validation of this biosensor in living *C. elegans*. The only modification introduced in our manuscript was to express the identical mito-Rosella protein in neurons under the control of neuronal-specific promoter. We have added a sentence in the top paragraph of p. 11 to clarify this point. It should also be noted that we observed similar changes in the GFP to dsRed fluorescence ratio under mitophagy conditions to those reported by Palikaras et al.

Furthermore, we confirmed that mito-Rosella localised to mitochondria in neurons of live *C. elegans* animals by staining them with Mitotracker Deep Red, which emits a fluorescence signal in the far-red channel. This showed that under native conditions, mito-Rosella-positive particles colocalised with Mitotracker as expected (new Fig. EV 4A). Please note that the Mitotracker dye stains all mitochondria, whereas the mito-Rosella is only expressed in neuronal mitochondria.

Referee #2:

Major points

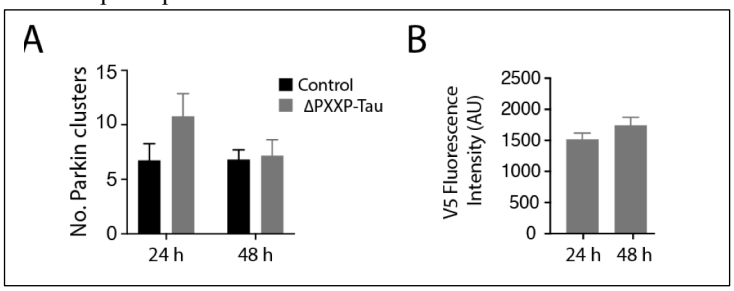
1. I have concerns about the possibility that many of the results are obtained simply as a result of protein over-expression and therefore could be non-physiological and not relevant for human disease. For example, Fig. 2G shows considerable increase in tau expression between 24 and 48 hrs that is mirrored by a decrease in Parkin clusters. Are the same effects observed upon transient transfection of other proteins? Alternatively, could siRNA be used to knockdown endogenous tau in rodent primary neurons or human neurons prior to mitophagy induction to determine if mitophagy is prevented?

For multiple reasons, we are confident that our results are not caused by a general and unspecific protein overexpression artefact:

1. Regarding Fig. 2G: The difference on the Y axis in Fig. 2G is because the 24 h images were taken at 8 bit, while the 48 h images were taken at 12 bit, and the values do not reflect any real difference in expression level. However, this is unintentionally misleading and we thank the reviewer for picking this up. To avoid confusion, we have now normalised the

fluorescence intensity to hTau in each graph as the fluorescence intensity units are simply not comparable across these experiments. These graphs are now shown in Fig. EV 3D, E.

2. We performed western blots against Tau at 24 h and 48 h, which we have now added to Fig. EV 3A-C. From these blots it is evident that there is only a small increase in hTau expression level over time, and no increase in hP301L levels. This indicates that there is no major increase in the expression of Tau at 48 h, as originally suggested by the differences in the Y-axes of the original Fig. 2G. Therefore, the phenotypic consequences of tau expression at 48 h are not primarily due to tau expression levels, but specifically due to the longer timeframe during which the cells were burdened by this pathology.
3. Importantly, overexpression of other proteins did not have the same effect as those of hTau and hP301L. To prove this experimentally, we expressed a form of Tau lacking the PXXP domain (which cannot interact with Parkin) and assessed Parkin translocation at both 24 h and 48 h. Importantly, this form of tau did not inhibit Parkin translocation at either time point post-transfection:



4. We also point out that at 24 h, neither hTau (see Fig. 2C), nor MTBD-Tau (new Fig. 5K) inhibited Parkin translocation, illustrating a functional specificity to the tau species that is independent of expression levels (i.e. hP301L and ΔTau).

As overexpressing Tau inhibits mitophagy, we do not hypothesise that knocking down endogenous tau would have the same effect. Importantly, mitophagy can be induced in cells irrespective of whether they have endogenous tau (as N2a cells do) or not (as most other cell lines used in similar experiments by others such as HELA cells). We also do not suggest that tau has any physiological role in the mitophagy process, as would be tested by the suggested experiment. Our study demonstrates that pathological tau inhibits mitophagy.

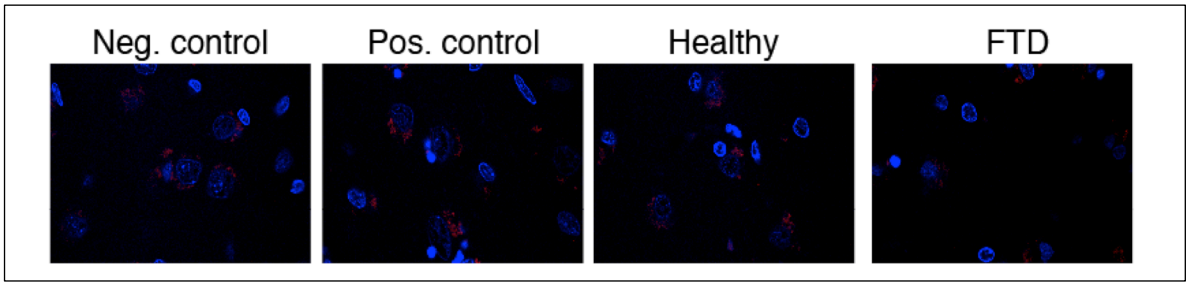
2. Fig. 5. Acetylated and tyrosinated tubulin should also be examined to rule out other effects on microtubule stability.

We have now performed western blots for acetylated and tyrosinated tubulin in cells transfected with hTau and hP301L. These are now included in Fig. 4D, E. We found no differences in these tubulin modifications between control cells and tau cells. These results are described in the text on p. 9.

3. Fig. 6. To show a relevance to human disease, PLA could be performed in human control/tauopathy brain.

We agree that showing the tau-Parkin interaction in human brain would be informative: however, we had only limited human tissue available. We performed a preliminary PLA reaction using a section from a healthy control subject, one from an FTD patient, including several controls (negative control = PLA for Tau and H3, positive control = PLA for Parkin and tubulin). However, we were unable to obtain a PLA signal in the positive control group and did not detect any PLA signal in the

FTD tissue, as shown in the figure below. As the tissue was paraffin-embedded, which in our experience is not optimal for PLA, we believe this lack of a signal is due to technical issues.



(The red fluorescence faintly visible is autofluorescence from lysosomes, also visible in the green channel and larger than PLA punctae).

4. The results from C. Elegans are not presented in a way that supports the conclusion that tau overexpression impairs neuronal mitophagy. For example, would it not be expected that if this was the case the GFP/DsRed ratio would be altered in untreated htau/hP301L relative to wild-type?

There are two points to this. Firstly, the baseline ratios are not comparable across strains, because the tau strains had GFP co-injection markers in the pharynx/ coelomocytes. As we performed imaging on an epifluorescence microscope, this meant that the background GFP intensity was slightly different between strains. For this reason, we cannot compare the absolute values between the strains directly, which is why we have displayed them as separate graphs even though they were all treated equally as one experiment. We discuss this issue on p. 23 in the Methods section. Secondly, it is currently an area of intense debate to what extent neurons undergo basal mitophagy (as opposed to acute stress-induced mitophagy as assessed e.g. with drugs). We believe that basal mitophagy is probably too low or too dynamic to be detected in our model without an added stimulus, and we therefore make no claims about this process. Our experiments specifically show that tau inhibits mitophagy in response to acute stress. To clarify this, we have changed the wording in the corresponding Results section on p. 12.

Minor points

1 Fig 1D. It would be helpful to see the images used for this analysis.

Representative images have been added to Fig. 1D.

2 Fig. 2B. Please show images of untreated htau and hP301L cells to show reduced Parkin clusters 24 after transfection.

We have added the DMSO treated images to Fig. 2B.

3 Fig 4 - Please show examples of PINK1 fluorescence in control, htau and hP301L cells showing examples of Parkin translocation and no Parkin translocation. It would also be useful to see PINK1 levels prior to depolarisation of these cells.

We have removed the PINK1 data. Please see our response to Reviewer 1.

4 Fig. 4 legend is inaccurate and requires amendment.

This figure has been removed.

5 No "toxicity" of these effects are actually shown (although they would be predicted), so it would be better to amend the concluding sentence of the abstract to reflect this.

We have changed this sentence to “As mitochondria are dysfunctional in neurodegenerative conditions, these data suggest a vicious cycle, with tau also inhibiting the degradation of damaged mitochondria.” This better reflects our point that mitochondria are well known to be damaged in disease and models of tauopathy, even though we have not addressed this separately in our study.

6 Fig 6. The images are convincing but the data show 0.6/0.2 PLAs/V5 for htau in different experiments. It might be better to show these data as % htau and combined as a single analysis. Please also show the immunofluorescence that matched the quantification in Fig. 6F.

We agree and have normalised the Y axis values to 1 for hTau to allow for an easy interpretation. However, we cannot combine these graphs, as they constitute separate experiments/imaging sessions. We have added the images corresponding to original Fig. 6F. These images now comprise Fig. 5J and the quantification is Fig. 5K.

7 It would be useful to show htau/P301L tau phosphorylation status to rule out an effect of differential phosphorylation on Parkin sequestration/mitophagy.

We probed for two phosphorylation sites by western blot, using the phospho-Tau-specific AT8 and AT180 antibodies. This preliminary analysis suggested that there was no major role for differential phosphorylation, as we were unable to detect AT8 and AT180 was either undetectable or very low. Furthermore, as evident from the western blots in Fig. EV 3A, there was no size shift of hP301L compared to hTau, also suggesting that there were no big differences in gross phosphorylation. However, to completely rule out an effect of differential phosphorylation would involve extensive experiments, as there are many phospho-sites on tau, not all of which have antibodies available to them. There is also the possibility of additive effects of individual phospho-sites, complicating this matter further. For these reasons, this question, although interesting, is outside the scope of our current study.

We hope that with these extensive revisions, our manuscript is suitable for publication in EMBO Journal.

2nd Editorial Decision 6th Aug 2018

Thanks for sending us your revised manuscript. Your study has now been re-reviewed by the two referees.

Both referees appreciate the introduced changes. Referee #1 has some remaining good points that I would like to ask you to address in a last round of revision. Let me know if we need to discuss any points specifically - happy to discuss further.

REFEREE REPORTS:

Referee #1:

This a re-review of the revised manuscript by Cummins et al., concerning the effects of Tau expression on mitophagy and Parkin recruitment to mitochondria. The authors have done more work and addressed a lot of my comments, however there are still some concerns. I do think this manuscript has potentially important results, but as this is for EMBO journal I feel more is needed to

rule out ambiguity. I'm a little disappointed the authors did not carry out some of the experiments I requested (looking directly at Parkin substrate ubiquitination/activity - these would have been a more convincing than the IF data showing increased mitochondrial ubiquitination. Western blot analysis may be more sensitive and allow assessment under endogenous Parkin conditions (pertinent given the other Reviewer's concerns with overexpression artefacts). However, my main concern with the in vivo data remains.
Here are my comments:
1) I'm still not convinced at all that the authors are monitoring mitophagy with their worm model. The primary data shown is approx. 9 dots per condition and for the mitophagy stimulation, all these dots appear to reduce their green intensity slightly - does this mean that all the mitochondria are undergoing mitophagy? I have looked at the Palikaras et al. paper that the authors reference, and here there are much clearer differences - some of the dots maintain their green intensity, while some lose almost all, with the red signal strongly increasing. Much more convincing than the data here. Perhaps if the authors also included larger images to see more of the neurons, this would help. Regardless, they need to demonstrate that the structures with an increased RFP/GFP signal are autolysosomal. This means co-staining with lysotracker or other lysosomal markers to prove this. Also, I have looked in more detail at the treatment times - 1 h treatment to induce mitophagy does not seem long enough to me - even HeLa cells over expressing vast amounts of Parkin take longer than this! A time course may be needed if possible (though I'm sure these compounds are quite toxic).
2) I think the authors need to be cautious in assuming that the mechanism of mitophagy is conserved in cells not overexpressing Parkin. Work has shown that mitochondrial depolarisation can induce mitophagy independently of Parkin (PMID 26266977, 24176932), so it is entirely feasible that the mitophagy observed in Figures 1 and 6 do not depend on Parkin. Thus the mechanism of Tau action could be different. Ideally the authors would knock out Parkin and show that mitophagy is blocked in these instances. They should at least discuss these possibilities in their conclusions.
3) Related to the above point, in the discussion on page 14 the authors state that "in vivo evidence for mitophagy remains sparse". There have been two publications recently that demonstrate in vivo mitophagy in neurons, both in mice and flies (PMID 29337137, 29500189). The authors may want to reference these, especially as the mitophagy observed appears independent of the Parkin pathway.
4) A minor point relates to the use of AO in inducing mitophagy on Page 5. The authors state that AO "can induce mitophagy by a different means to CCCP". They may want to check/rephrase this, as while I agree they target mitochondria in different ways, they both lead to mitochondrial depolarisation and it is this that likely induces mitophagy.

Referee #2:

The authors have revised the manuscript to address the issues that I had previously raised. In my opinion the manuscript is now suitable for publication and will be of significant interest to the field.

2nd Revision - authors' response 1st Nov 2018

Our reply to ***Referee 1*** is below.

I do think this manuscript has potentially important results, but as this is for EMBO journal I feel more is needed to rule out ambiguity. I'm a little disappointed the authors did not carry out some of the experiments I requested (looking directly at Parkin substrate ubiquitination/activity - these would have been a more convincing than the IF data showing increased mitochondrial ubiquitination.

The experiment requested in the previous revision round (July 2018) was "*As the authors see less mitochondrial Parkin recruitment, is there also less ubiquitination of Parkin mitochondrial substrates?"* We had addressed this question by performing an analysis of endogenous ubiquitin specifically on mitochondria, reflecting mitochondrial substrates. This analysis showed unambiguously that, as expected, there was indeed decreased mitochondrial ubiquitination in hP301L-Tau cells (Fig. EV3).

Western blot analysis may be more sensitive and allow assessment under endogenous Parkin conditions (pertinent given the other Reviewer's concerns with overexpression artefacts).

N2a cells have little endogenous Parkin (as we know from both immunostaining and western blot of these cells), which is why we used a Parkin overexpression paradigm, a standard in the field. This reviewer refers to the other reviewer's previous comments about overexpression artefacts; however, these specifically regarded the overexpression of Tau and were addressed by us to reviewer 2's satisfaction, using four separate lines of argumentation (please see our previous response to reviewers, July 2018). Finally, we would like to point out that our PLA studies were performed in conditions of endogenous Parkin.

However, my main concern with the in vivo data remains.
1) I'm still not convinced at all that the authors are monitoring mitophagy with their worm model. The primary data shown is approx. 9 dots per condition…

We like to point out that the mitochondrial dots shown in Figure 6 are simply high magnification representative images. Our analysis was in fact extensive, with as many as 50 individual mitochondria analysed per animal ventral cord, and 16 - 39 animals analysed per group, in one experiment representative of 2-3 independent replications.

…and for the mitophagy stimulation, all these dots appear to reduce their green intensity slightly - does this mean that all the mitochondria are undergoing mitophagy?

We understand that the high magnification images shown in Figure 6 may give the impression that all mitochondria undergo mitophagy. However, images of whole animals reveal that, whereas most mitochondria exhibited a decreased mito-Rosella ratio, some mitochondria remained at basal levels, demonstrating significant inter-mitochondrial variation. To better illustrate this point, we have now included images of whole worms in the Appendix Fig. S1. This figure also shows examples of single mitochondria in vehicle conditions undergoing basal mitophagy, providing further evidence that the biosensor works as expected.

I have looked at the Palikaras et al. paper that the authors reference, and here there are much clearer differences - some of the dots maintain their green intensity, while some lose almost all, with the red signal strongly increasing. Much more convincing than the data here. Perhaps if the authors also included larger images to see more of the neurons, this would help.

We acknowledge that there are differences between the responses we observed and those reported by Palikaras et al. (2015) *Nature*. A possible explanation may be that Palikaras et al. measured mitophagy in body wall muscle, a *C. elegans* tissue which is packed with mitochondria. This is in contrast to neurons (the tissue we analysed), which have significantly fewer mitochondria per cell. The difference in how widespread mitophagy is induced across the network may therefore be in part due to the tissue-dependent differences in the number and density of organelles. For instance, it is possible that the mitophagy machinery becomes saturated in muscle cells treated with a mitochondrial stressor such as sodium azide or CCCP. As a result, fewer organelles may undergo mitophagy at any given point. In neurons, due to the lower number of organelles in each cell, the same treatments may induce more widespread mitophagy. As mentioned above, examples of larger images are now shown in the Appendix Figure S1. We have also added statement to the legend of Figure 6 referring to these larger images for clarity.

Regardless, they need to demonstrate that the structures with an increased RFP/GFP signal are autolysosomal. This means co-staining with lysotracker or other lysosomal markers to prove this.

Immunhistochemistry using certain antibodies is a common issue in *C. elegans,* as staining of whole animals is hindered by the poor diffusibility of antibodies across the cuticle and tissues. We have nonetheless attempted to prove colocalisation of mitochondria to lysosomes following sodium azide treatment using different techniques, but encountered a range of technical difficulties. Firstly, immunostaining of worms for the endogenous LMP-1 (a marker of lysosomes) with a monoclonal antibody (available from the Developmental Studies Hybridoma Bank; Hadwiger et al. (2010) *PloS One*) proved unsuccessful in producing any staining in any cell-type. Secondly, we applied

Lysotracker as also suggested by this reviewer. Although this marker has previously been used to stain gut granules in the *C. elegans* intestine through feeding (e.g. Roh et al. (2012*) Cell Metabolism*); this was not an option for neurons, and the dye was unable to penetrate into the nervous system of *C. elegans* trialling a number of protocols. In support, there are no reports in the literature of Lysotracker being employed to label lysosomes in *C. elegans* neurons, as these dyes are not developed for staining of whole animals.

Also, I have looked in more detail at the treatment times - 1 h treatment to induce mitophagy does not seem long enough to me - even HeLa cells over expressing vast amounts of Parkin take longer than this! A time course may be needed if possible (though I'm sure these compounds are quite toxic).

Regarding the time course of mitophagy, it was shown by Ashrafi et al. (2014) *J. Cell Biol.*, that mitochondria can localise to autophagosomes after 20 minutes of antimycin treatment and to lysosomes after 50 minutes of antimycin treatment in primary neuron axons. It is therefore not unreasonable for mitophagy to proceed within 1 hour *in vivo* in *C. elegans*. The Palikaras et al. study used 2 hour treatments of CCCP to observe mitophagy in muscle cells in *C. elegans*. We found that in neurons, sodium azide is more potent at inducing mitophagy and it is therefore not unexpected that it would induce mitophagy in this tissue after 1 hour.

2*) I think the authors need to be cautious in assuming that the mechanism of mitophagy is conserved in cells not overexpressing Parkin. Work has shown that mitochondrial depolarisation can induce mitophagy independently of Parkin (PMID 26266977, 24176932), so it is entirely feasible that the mitophagy observed in Figures 1 and 6 do not depend on Parkin. Thus the mechanism of Tau action could be different. Ideally the authors would knock out Parkin and show that mitophagy is blocked in these instances. They should at least discuss these possibilities in their conclusions.*

We agree that Parkin-independent mechanisms could be involved in mitophagy and have added this to our discussion; although the canonical pathway to mitophagy, in particular when CCCP is used to depolarise mitochondria in cells, is still considered to be Parkin-dependent (e.g. as reviewed in Nguyen et al. (2016) *Trends Cell Biol*). We also addressed this point experimentally in our *in vivo* model. To assess whether the *C. elegans* homologue of Parkin, PDR-1, was required for the mitophagy we observed, we crossed the mito-Rosella worms to PDR-1 mutant worms, which lack a functional PDR-1/Parkin protein. These worms were unable to undergo mitophagy in response to sodium azide treatment (new Fig. EV 4B), indicating that mitophagy was indeed PDR-1/Parkin-dependent. This experiment also provides evidence in relation to this reviewer's point 1, as it indicates that the mito-Rosella worms do reflect mitophagy, which is prevented when PDR-1 is knocked out.

Furthermore, as our cell culture experiments (Figure 1) were performed in the presence of exogenous Parkin, and Tau inhibited the translocation of this Parkin, we consider this to be an important, although not exclusive, mechanism by which Tau impairs mitophagy. We have added this point to our discussion (p.14) as suggested by the reviewer:

"One limitation of our experiments in N2a cells is that Parkin was overexpressed, possibly exaggerating the importance of Parkin-dependent mitophagy in this system. This does not rule out the possibility that mitophagy can also be achieved by alternative pathways that operate in a Parkin-independent manner [14,51]. However, the mitophagy we observed in C. elegans was dependent on the Parkin homologue PDR-1, so it is likely that inhibition of Parkin/PDR-1 translocation was one of the contributing mechanisms, as determined in the cell culture model."

3) Related to the above point, in the discussion on page 14 the authors state that "in vivo evidence for mitophagy remains sparse". There have been two publications recently that demonstrate in vivo mitophagy in neurons, both in mice and flies (PMID 29337137, 29500189). The authors may want to reference these, especially as the mitophagy observed appears independent of the Parkin pathway.

Thank you for bringing this to our attention. We have now included these 2018 references in our discussion on p. 14, together with another very recent relevant publication by Cornelissen et al. in *eLife*.

4) A minor point relates to the use of AO in inducing mitophagy on Page 5. The authors state that AO "can induce mitophagy by a different means to CCCP". They may want to check/rephrase this, as while I agree they target mitochondria in different ways, they both lead to mitochondrial depolarisation and it is this that likely induces mitophagy.

Thank you for raising this point. This sentence now reads "…can induce mitochondrial depolarisation by a different means…".

We hope that with these revisions, our manuscript is now suitable for publication in EMBO Journal.

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Corresponding Author Name: Jürgen Götz
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2018-99360

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?	Effect size was not pre-specified.
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?	Cells/ worms were excluded from the analysis if the fluorescent signal was saturated. This was pre-established.
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.	Samples (wells for cell culture or C.elegans) were allocated randomely to treatment.
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.	Investigators were blinded for all image analyses requiring subjective decisions.
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.	Data were formally tested for normality using the D'Agostino-Pearson omnibus test and for homogeneity of variance with the Brown-Forsythe test. Non-parametric tests were used for data that failed the normality test.
Is there an estimate of variation within each group of data?	Yes. We include error bars of SEM.
Is the variance similar between the groups that are being statistically compared?	Yes, this was tested with the Brown-Forsythe test.

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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).	For immunostaining and proximity ligation assays (PLAs), the following antibodies were used: alpha-Tubulin mouse (Sigma, T6074, 1:500), ATP synthase beta subunit mouse (Abcam, Ab14730, 1:700), Parkin rabbit (Abcam, Ab15954, 1:500), SOD2 rabbit (Abcam, Ab13533, 1:500), Tau 5 mouse (Millipore, 577801, 1:500), 4R-Tau (1E1) mouse (Millipore, 05-804, 1:500), phospho-H3 rabbit (Millipore, 06-570), Ubiquitin mouse (Enzo Life Sciences, BML-PW8810, 1:100), V5 rabbit (Sigma, V8137, 1:500), V5 mouse (Invitrogen, R960-25, 1:500), and V5 chicken (Abcam, Ab9113, 1:500-700). For immunoblotting, antibodies used were: β-Actin mouse (Abcam, Ab8226, 1:3,000), GFP rabbit (Millipore, 3080P, 1:2,000), acetylated Tubulin mouse (Sigma, T6074, 1:2,000), tyrosinated Tubulin mouse (Sigma, T9028, 1:2,000), alpha-Tubulin mouse (Sigma, T6074, 1:2,000), and V5 rabbit (Sigma, V8137, 1:2,500-5,000).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N2a: ATCC CCL-131. Not recently autheticated. Mycoplasma testing was performed every 12 months and always showed no contamination .

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Male Tau transgenic pR5 mice (Götz et al, 2001) carrying the P301L mutation found in familial cases of FTD and wild-type littermates were used at 14 -15 months of age. Mice were housed under standard 12h light-dark cycle, with ad libitum access to food.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experimentation was approved by the Animal Ethics Committee of the University of Queensland (approval numbers QBI/412/14/NHMRC and QBI/312/14/NHMRC).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Our reporting has been informed by the ARRIVE checklist.

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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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	NA
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