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Loss of the m-AAA protease subunit AFG3L2 causes mitochondrial transport defects and tau hyperphosphorylation

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1st Editorial Decision 31 October 2013

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by 2 referees and their comments are provided below.

As you can see the referees find the analysis interesting and suitable for the EMBO Journal, but further analysis is also needed. In particular, more direct measurements of ROS levels should be performed, off-targets effects should be ruled out, better statistical analysis performed and some more insight into why there is a loss of mitochondria in afg3l2 neurons. Should you be able to able to address the raised concerns with the inclusion of experimental data then we would like to invite you to submit a revised manuscript for our consideration. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the concerns raised at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

This manuscript aims to explore the role of the m-AAA protease AFG3L2 in axonal mitochondrial transport. The authors use a range of mouse models to genetically manipulate processing proteases of the mitochondrial inner membrane. They find that loss of AFG3L2 leads to reduction in anterograde transport of mitochondria within neurons. By decreasing Tau levels within neurons using the antioxidant NAC, mitochondrial transport was restored. The authors conclude that dysfunctional mitochondrial transport involves ROS and cytoskeletal modifications. Although this is an interesting manuscript, in its current form, the conclusions lack significant experimental support. The ROS evidence is indirect and there is no data to mechanistically explain the reduction of mitochondrial occupancy (degradation/turnover) in afg3l2 neurons.

Main comments:

1.The authors discuss depletion of mitochondria from axons, yet the mechanism of this remains unclear. If these mitochondria are indeed turned over as suggested by the loss of mitochondrial occupancy in axons, is Parkin recruited to the mitochondrial outer membrane? Further to this, the aspect of impaired distribution machinery should be investigated. Is Miro targeted by the PINK1/Parkin pathway in this model? Could this explain the reduction in anterograde movement?

2.The title of the manuscript alludes to the fact that AFG3L2 deficient transport is a consequence of increased ROS. The authors fail to directly measure this, and instead use NAC/Vitamin E treatment to 'rescue' the anterograde transport. A direct measurement of ROS levels should be performed either using dihydroethidium (cytosolic ROS) or a dye like MitoSOX (mitochondria by FACS or confocal).

3.The authors have used RNAi to knockdown AFG3L2. Off-target effects should be ruled out by performing rescue experiments using an RNAi resistant form of the protein.

4.The statistical analysis throughout the paper should be clearer. Many of the graphs lack significance values yet significant differences are stated in the results section. This should be amended. For example Figure 1B- "The median mitochondrial length decreased from 1.4 μm in control to 1 μm in neurons with depleted levels of AFG3L2, resulting in significantly different distribution of mitochondrial length." This is true for all % mitochondrial length graphs throughout the manuscript. This aspect could also be strengthened by representative images of stained mitochondria within these neurons.

5.Can the occupancy graphs simply be depicted as standard bar graphs? The box and whisker plots make it difficult to easily tell whether the results are significant as the max and mean values are plotted rather than error bars. It would be easier to depict this as a bar graph with SEM error bars and either a Mann-Whitney U-test or an unpaired students t-test.

6.In keeping with this, the membrane potential of these mitochondria should be investigated (either by TMRM-confocal or DiO6-FACS). This relates to the above points (in terms of a reduced membrane potential to recruit parkin) and also in regards to the ROS generation. This aspect requires further investigation as treated with NAC also appears to reduce the levels of cytoskeletal components. Is this apparent when observing cells via confocal microscopy? Does NAC treatment disrupt the cytoskeletal structure of the neuron and thus affect transport? Uncoupling this aspect from reducing the levels of tau should be investigated further.

Referee #2

In their manuscript, Kondadi et al. address the patho-physiological mechanism of neurodegenerative disorders caused by mutations in AFG3L2, a subunit of the m-AAA protease of the inner mitochondrial membrane. They examine constitutive and neuronal depletion models of Afg3l2, showing that anterograde transport of mitochondria from the cell body to the growth cone of neurons is decreased, which is not a consequence of mitochondrial fragmentation due to activation of the protease OMA1. Furthermore, the authors show that the microtubule-binding protein tau is hyperphosphorylated upon neuronal Afg3l2 depletion in vivo. Tau depletion can partially rescue the observed transport defect, as can anti-oxidative treatment. In conclusion, the authors propose a model where increased ROS levels due to mitochondrial dysfunction lead to cytoskeletal remodeling and tau hyperphosphorylation, which subsequently impairs mitochondrial transport and causes axonal degeneration.

As the authors pointed out, evidence linking mutations in m-AAA protease subunits and the resulting axonal degeneration has been lacking so far. Therefore, the proposed explanation, supported by experimental data, could represent an attractive model not only for m-AAA associated mitochondrial disorders, but also for the role of mitochondrial dysfunction in neurodegenerative disease in general. Although some minor points (see below) should be addressed, overall the paper is of high quality.

Points that should be addressed:

1. The authors claim that anterograde transport of mitochondria is selectively impaired in Afg3l2 depletion models (pp. 5/6). However, even though not statistically significant, also retrograde transport seems to be decreased. The authors should therefore comment on the possibility that transport in either direction is affected by the depletion. In addition, it would be important to know whether the speed of mitochondrial transport is altered in the Afg3l2-deficient neurons.

2. Please comment also on the fact that anterograde transport seems to be affected in Oma1 knockout cells compared to the control, irrespectively of further impairment by Afg3l2 knock-down (compare Fig. 1D and 2D). Can the authors completely rule out that fragmentation contributes to the transport defects they observed?

3. The tau phosphorylation pattern seems to be different in constitutive and neuronal Afg3l2 knockout mice. In particular, there is no difference between WT and KO regarding S396 phosphorylation (Fig. 3F), whereas tau seems to be hyperphosphorylated on that residue in the NKO model (Fig. 4D). In addition, what about the phosphorylation of T181 and S199 in the NKO model?

4. On p. 9, the authors state that no evidence could be obtained for the activation of MARK. However, no western blot is shown in Fig. S2B.

5. The treatment with NAC seems to increase motion of mitochondria, including retrograde transport (Fig. 6D). Although the authors state that other mechanisms in addition to the anti-oxidant function of NAC could be involved, it should be commented on this specific difference.

6. Related to 5., the protein levels of cytoskeletal components should be shown after Vit. E treatment (Fig.7), as done in Fig. 6E. In particular, can the authors show that Vit. E treatment acts more exclusively as an antioxidant than NAC?

7. Fig.6E shows decrease levels of a number of proteins in NAC-treated neurons. Could NAC be toxic to neurons, especially at high concentrations. How long can neurons survive in the presence of NAC? What is the evidence that NAC displays antioxidant activity at the concentrations used by the authors (200 microM and 1 mM)?

8. In this respect, it would also be very interesting if anti-oxidant treatment of either constitutive or tissue-specific knockout mouse models can increase neuronal survival and/or prevent neurodegeneration. Even though I concede that this question is beyond the scope of the current paper, I encourage the authors to address this possibility in the discussion section.

Response to the Referees

Referee #1

1. The authors discuss depletion of mitochondria from axons, yet the mechanism of this remains unclear. If these mitochondria are indeed turned over as suggested by the loss of mitochondrial occupancy in axons, is Parkin recruited to the mitochondrial outer membrane? Further to this, the aspect of impaired distribution machinery should be investigated. Is Miro targeted by the PINK1/Parkin pathway in this model? Could this explain the reduction in anterograde movement?

In all our experiments, we noticed a correlation between mitochondrial fragmentation and reduced mitochondrial occupancy in axons. As the Referee suggests, one possible explanation could be increased turnover of mitochondria by mitophagy. We have now tested if parkin is recruited to mitochondria in *Afg3l2* downregulated neurons. However, we could not find evidence of parkin recruitment to mitochondria in our experimental conditions. In contrast, parkin recruitment was observed when mitochondria were depolarized using antimycin A, as previously described (Wang et al., Cell 2011). These data have now been included in the supplementary Figure S2A. Consistently, we do not observe a significant overall reduction of the membrane potential of mitochondria in *Afg3l2*-depleted neurons. In Figure S2B, C, we show the data obtained in several neurons in two independent experiments as boxplots, as widely recommended for imaging experiments to provide as much descriptive statistic data as possible. There is no statistically significant difference, but it is clear that in the *Afg3l2*-downregulated samples we can find a few neurons with a reduced membrane potential. We have included a description of these experiments in the Results section (pages 5-6). We also discuss that our results leave open the possibility that mitophagy occurs with a parkin-independent mechanism (see Discussion, page 13, first paragraph).
The related Referee question about targeting of Miro by the parkin-PINK pathway (or any other mechanism) is a very interesting point. However, this is very difficult to address in primary neurons, and it has been so far studied only in cultured cells (Wang et al., Cell 2011). We think that investigating this aspect is beyond the purpose of our current study.

2. The title of the manuscript alludes to the fact that AFG3L2 deficient transport is a consequence of increased ROS. The authors fail to directly measure this, and instead use NAC/Vitamin E treatment to 'rescue' the anterograde transport. A direct measurement of ROS levels should be performed either using dihydroethidium (cytosolic ROS) or a dye like MitoSOX (mitochondria by FACS or confocal).

We agree that this is an important point and have tried several dyes as suggested by the Referee. In our experimental conditions, we obtained the best results using CellRox green, a dye that fluoresces upon oxidation, labeling both nuclear and mitochondrial DNA. This dye shows a clear increase in fluorescence when primary neurons are treated with menadione, which generates intracellular ROS through futile redox cycling (Figure S7C, D). Moreover, we could clearly observe that both NAC and vitamin E at the concentration used in our study are efficient to reduce the fluorescence of CellRox green (Figure S7C, D). However, we could not obtain convincing data showing that AFG3L2-depleted neurons have increased ROS levels. We have repeated this experiment three times and quantified several neurons in each experiment. Only in one experiment, we could measure a significant difference in the fluorescence intensity (Experiment 1, Figure S7B). We show in Figure S7B all the data obtained in the 3 experiments as boxplots, to show the variability of the data. It is possible that the sensitivity of the dye is not sufficient to detect small changes in ROS production and differences can depend on the extent of downregulation in different experiments. Moreover, it should be pointed out that the use of redox-sensitive

probes has several caveats, since these dyes are very susceptible to artifactual reactions, and the relations between a given dye and ROS levels is often nonlinear (for an extensive review see Murphy et al. Cell metab, 2011). Despite the fact that the data obtained with antioxidants are very striking, we agree with the Referee that this is an indirect measure. Therefore, we decided to change the title of the manuscript to avoid any overstatement of our data. The new title reads: "Loss of AFG3L2 causes axonal transport defects of mitochondria rescued by tau reduction and antioxidants".
We have also modified the text of the last chapter of the Results to describe these data and discuss this problem more extensively on page 15.

3.The authors have used RNAi to knockdown AFG3L2. Off-target effects should be ruled out by performing rescue experiments using an RNAi resistant form of the protein.

We have now used two independent siRNA oligonucleotides to repeat the experiments shown in Figure 1, and found comparable results. This data is shown in the new supplementary Figure S1. We have preferred this approach over a rescue with an RNAi resistant form of the gene, since we know that overexpression of AFG3L2 causes mitochondrial fragmentation in cell lines.

4.The statistical analysis throughout the paper should be clearer. Many of the graphs lack significance values yet significant differences are stated in the results section. This should be amended. For example Figure 1B- "The median mitochondrial length decreased from 1.4 μm in control to 1 μm in neurons with depleted levels of AFG3L2, resulting in significantly different distribution of mitochondrial length." This is true for all % mitochondrial length graphs throughout the manuscript. This aspect could also be strengthened by representative images of stained mitochondria within these neurons.

We carefully reconsidered how to statistically evaluate our data, taking into consideration the experimental variation between neurons and between different experiments. For mitochondrial length, we measured at least 120-150 mitochondria per experiment in at least three independent experiments. Data were binned, averaged across experiments, and a statistical evaluation was performed using the χ^2 test. We have described accurately the statistical analysis in the material and methods (page 20), and included *p* values in the figures or figure legends, when < 0.05. We added representative pictures of mitochondria in *Afg3l2*-downregulated neurons in Figure S1.

5.Can the occupancy graphs simply be depicted as standard bar graphs? The box and whisker plots make it difficult to easily tell whether the results are significant as the max and mean values are plotted rather than error bars. It would be easier to depict this as a bar graph with SEM error bars and either a Mann-Whitney U-test or an unpaired students t-test.

The reason of showing the boxplot was to present the variation between neurons, providing several parameters of descriptive statistics. However, we have now depicted the occupancy graphs as bar graphs with SEM error bars, as recommended by the Reviewer, and performed statistical analysis using the Student's t test. The corresponding figures now show average of three independent experiments, with each experiment comprising data obtained from at least 8 axons. We have changed Figures 1, 2, 5-7, S1, S3 accordingly.

6.In keeping with this, the membrane potential of these mitochondria should be investigated (either by TMRM-confocal or DiO6-FACS). This relates to the above points (in terms of a reduced membrane potential to recruit parkin) and also in regards to the ROS generation. This aspect requires further investigation as treated with NAC also appears to reduce the levels of cytoskeletal components. Is this apparent when observing cells via confocal microscopy? Does NAC treatment disrupt the cytoskeletal structure of

the neuron and thus affect transport? Uncoupling this aspect from reducing the levels of tau should be investigated further.

We have measured the membrane potential with TMRM and found no major differences (see response to point 1 and Figure S2). We cannot detect alterations in the cytoskeletal component by immunofluorescence, indicating that the differences we detect by western blot do not reflect a general disruption of the cytoskeletal structure. Furthermore, we have explored in more depth the effect of vitamin E on cytoskeletal components. Remarkably, we found that vitamin E specifically reduces tau phosphorylation, without affecting the total levels of tau, tubulin, or actin. This is consistent with the function of vitamin E as an antioxidant. These data have been now included in Figure S7F. We have also modified the text of the last chapter of the Results to describe these data.

Referee #2:

1. The authors claim that anterograde transport of mitochondria is selectively impaired in Afg3l2 depletion models (pp. 5/6). However, even though not statistically significant, also retrograde transport seems to be decreased. The authors should therefore comment on the possibility that transport in either direction is affected by the depletion. In addition, it would be important to know whether the speed of mitochondrial transport is altered in the Afg3l2-deficient neurons.

We have carefully examined our data, by considering all experiments in which we have compared control versus *Afg3l2* downregulation (Data from Figures 1, 5, and 6). Even after putting together all these data (n= 10), we do not see a statistically significant reduction of retrograde transport of mitochondria. Instead, we confirmed that *Afg3l2* downregulation increased fragmentation and decreased occupancy. As suggested by the Referee, we measured the average velocity of mitochondrial transport in both anterograde and retrograde directions and did not find any difference caused by depletion of AFG3L2. Notably, we observed a significant increase in the speed of anterograde mitochondrial transport in NAC-treated control axons. These data have been now added in Figure 1E, 5D-E, 6E-F, 7D-E.

2. Please comment also on the fact that anterograde transport seems to be affected in Oma1 knockout cells compared to the control, irrespectively of further impairment by Afg3l2 knock-down (compare Fig. 1D and 2D). Can the authors completely rule out that fragmentation contributes to the transport defects they observed?

Here, we want to make the point that the mitochondrial transport phenotype of *Afg3l2*-deficient neurons is not caused by mitochondrial fragmentation (due to OMA1 activation and degradation of L-OPA1; Ehses et al., 2009; Baker et al., 2014). In OMA1 knock-out neurons, depletion of *Afg3l2* does not lead to extensive fragmentation or reduced occupancy, but continues to affect transport. We have revised the Abstract, Results and Discussion to make this point clearer. We would also like to point out that it is misleading to directly compare data across experiments, due to experimental variations in different imaging sessions.

3. The tau phosphorylation pattern seems to be different in constitutive and neuronal Afg3l2 knockout mice. In particular, there is no difference between WT and KO regarding S396 phosphorylation (Fig. 3F), whereas tau seems to be hyperphosphorylated on that residue in the NKO model (Fig. 4D). In addition, what about the phosphorylation of T181 and S199 in the NKO model?

We have repeated the western blot using anti-tauS396 with a lower amount of lysate and we could detect a slight increase also in the constitutive knockout (Figure 3F). We also checked the phosphorylation of T181 and S199 in the NKO model. S199 was only slightly affected, while phosphorylation at T181 was clearly enhanced (Figure 4C). Given the different age at which we analyze the two models, we are not entirely surprised of these differences in the extent of phosphorylation at different sites. We have amended the text accordingly.

4. On p. 9, the authors state that no evidence could be obtained for the activation of MARK. However, no western blot is shown in Fig. S2B.

The blot has now been added (Figure S4).

5. The treatment with NAC seems to increase motion of mitochondria, including retrograde transport (Fig. 6D). Although the authors state that other mechanisms in addition to the anti-oxidant function of NAC could be involved, it should be commented on this specific difference.

We have revised the discussion to further comment on this issue (see Discussion page 15).

6. Related to 5., the protein levels of cytoskeletal components should be shown after Vit. E treatment (Fig.7), as done in Fig. 6E. In particular, can the authors show that Vit. E treatment acts more exclusively as an antioxidant than NAC?

We have checked the effect of vitamin E on phospho-tau, total tau, and levels of other cytoskeletal components and found that vitamin E only reduces the amount of phospho-tau, consistent with its role as antioxidant. These data have been added in Figure S7F (see also response to Referee 1, point 6).

7. Fig.6E shows decrease levels of a number of proteins in NAC-treated neurons. Could NAC be toxic to neurons, especially at high concentrations. How long can neurons survive in the presence of NAC? What is the evidence that NAC displays antioxidant activity at the concentrations used by the authors (200 microM and 1 mM)?

We did not observe toxicity at the concentration of NAC and vitamin E used. Furthermore, neuronal damage impairs mitochondrial transport, while here we observe the opposite. Importantly, we can now show that at the concentration used NAC and vitamin E efficiently reduce menadione-induced ROS increase in primary neurons (Figure S7C, D).

8. In this respect, it would also be very interesting if anti-oxidant treatment of either constitutive or tissue-specific knockout mouse models can increase neuronal survival and/or prevent neurodegeneration. Even though I concede that this question is beyond the scope of the current paper, I encourage the authors to address this possibility in the discussion section.

This is indeed a very interesting question, but requires a long-term experimental paradigm. As suggested, we commented on this possibility in the Discussion (last sentence).

Accepted 05 March 2014

Thank you for submitting your revised manuscript to The EMBO Journal. Your manuscript has now been re-reviewed by referee #1 who is happy with the introduced changes. Given this I am therefore very pleased to accept the paper for publication here.

Please see below for important information. If you have any questions just contact me, and most importantly congratulation on a very nice paper!!

REFEREE REPORT

Referee #1:

This is a revised manuscript and the authors have adequately addressed the issues raised. This is a very nice and important study and I believe it is now suitable for publication.

