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Mfn2 downregulation in excitotoxicity causes mitochondrial dysfunction and delayed neuronal death

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(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.)

Editor: Andrea Leibfried

1st Editorial Decision   18 November 2013

Thank you for submitting your manuscript entitled 'Mfn2 downregulation in excitotoxicity causes mitochondrial dysfunction and sensitizes to cell death'. Please excuse the slight delay in getting back to you, based on the requirement to solicit external input form an expert advisor to the Journal.

Your analyses reveal that mitochondrial fragmentation upon excitotoxicity stimulation in neurons occurs in two phases: 1) a rapid one, that is reversible and depends on Drp1 activity and its translocation to the mitochondria; and 2) a delayed phase, that is irreversible and caused by Mfn2 downregulation. Mfn2 decrease does not occur via proteasomal degradation in this context, but because its expression is regulated by transcription factor MEF2. According to a role of MEF2 in neuronal mitochondrial dynamics, MEF2-DN transduced neurons contain fragmented mitochondria which can be rescued by Mfn2-expression.
Recognizing the potential of your results while being uncertain about their general significance, I consulted an expert in the field and trusted advisor to the journal. In essence, s/he points out that although the cell culture data indicate a role of Mfn2 in sensitizing neurons to excitotoxicity, reduced Mfn2 expression occurs only at late stages. This raises the question of cause and consequence, respective net-contribution of Mfn2 to the pathology. Based on this, we are not convinced that the paper would indeed provide the necessary pathophysiological relevance to enable

rapid proceedings at our rather demanding title. I am afraid to say that we thus have to return your manuscript at this time so that the work may be considered elsewhere without further delay.
If you were able, however, to provide an extended analysis addressing this crucial issue, we would be happy to re-engage in the discussion about possible suitability for publication in The EMBO Journal. I am very sorry to disappoint you on this occasion.

Resubmission	25 February 2014

Thank you for your email dated 19th of November 2013 regarding our manuscript titled "Mfn2 downregulation in excitotoxicity causes mitochondrial dysfunction and sensitizes to cell death" (ref: EMBOJ-2013-87367). We were pleased that you saw potential in our results.

We thank you and the consulted expert for insisting on experiments that have undoubtedly improved the paper.

You and the consulted expert requested that we provide evidence that gives support for the hypothesis that Mfn2 downregulation in excitotoxicity has a net-contribution to the pathology. In an attempt to satisfy your concerns we have performed new experiments to address the question of the active role of Mfn2 downregulation in the progress of the exicitotoxic damage. We have made a new Figure 5 that we believe provide strong evidence that Mfn2 downregulation participates in delayed cell death. In this new figure we show that:

1.- Mfn2 knock down sensitizes neurons to subtoxic doses of NMDA (former Fig. 4D)
2.- Mfn2 expression reduces to around 50% the NMDA-mediated cell death (former Fig S3D)
3.- Mfn2 protection is specific, since Mfn1 expression cannot protect against NMDAmediated cell death (new Fig. S4B).
4.- Mfn2 expression does not protect the initial NMDA-dependent cell death but completely blocks the delayed cell death (new Fig. 5C-D).

In the discussion, we insist in the known fact that evolution of excitotoxic damage is progressive, lasting from minutes to days. Neurons that do not die during or shortly after excitotoxic insult can survive or undergo delayed cell death, which is dependent on mitochondrial function. We propose that downregulation of Mfn2, by impairing mitochondrial function, can determine the fate of the neuron.

Although not directly related with the cause-consequence issue, we also include a new Fig 4B that reinforces the message that Mfn2 downregulation causes mitochondrial dysfunction.

We have changed the title to "Mfn2 downregulation in excitotoxicity causes mitochondrial dysfunction and delayed neuronal death" that fits better with the new findings.

In the electronic submission we have included the name of five potential referees.

Thank you for the opportunity to revise again our manuscript. We hope that you now find it suitable to continue with the editorial process.

2nd Editorial Decision	20 March 2014

Thank you for resubmitting your manuscript entitled 'Mfn2 downregulation in excitotoxicity causes mitochondrial dysfunction and delayed neuronal death'. I have now received all reports from the referees, which are enclosed below.

As you will see, all referees acknowledge that your conclusions are potentially interesting. However, they raise numerous concerns and are at this stage not convinced that the main claims are sufficiently supported by the presented data. Specifically, referee #3 remains unconvinced by the kinetics and level of down regulation of Drp1 and Mfn2 and the causality of this for the proposed NMDA-mediated excitotoxicity. Further, ref#2 demands significant further insights into the actual cause of neuronal cell death.

Given their constructive comments, I am willing to offer you the chance to expand and amend the paper paying particular attention to the following points:
- point 3 of referee #1
- the pathway leading to neuronal death (point 1 and 3, referee #2)
- further support for the link between MEF2 and Mfn2 in cell death (point 6, referee #2)
- the link between Drp1 reduction and excitotoxicity (point 1 and 4, referee #3)
- necessary data quantification as of point 3, referee #3

If you address these crucial comments and pay attention to all other issues raised, I would present a revised manuscript to some of the original referees. I like to stress already at this point that I would need strong support especially from referee #3, to consider publication in The EMBO Journal. Please do not hesitate to contact me if you have any questions regarding your revision.

P.S. I will also send you an annotated pdf version of your manuscript that referee #1 provided and that includes some more important comments partially along the same lines as outlined by referee #3 and which should be helpful in revising your manuscript.

REFEREE COMMENTS

Referee #1:

This is a very interesting study. Deserves publication in EMBO after addressing the following, mostly minor, comments.

The study by Martorell et al. investigated the downregulation of Mfn2 during NMDA-mediated excitotoxicity in cultured neurons and in an animal model of cerebral focal ischemia. Using a well-designed experimental approach, the authors elegantly demonstrate a biphasic mitochondrial response to NMDA-mediated toxicity, in which Drp1 activation increases mitochondrial fragmentation in the early phase and transcriptional regulation of Mfn2 sustains the fragmented morphology in the late phase and propagates neuronal cell death. The deleterious consequences of Mfn2 downregulation were reversed by treatment of the cells with exogenous Mfn2, suggesting that Mfn2 plays a distinct and clinically-relevant role in minimizing excitotoxic neuronal damage. Though clearly written and experimentally solid, the study has several minor issues that need to be addressed.

1. In Figure 9, a clear schematic is presented demonstrating the impact of NMDA receptor activation on the induction of excitotoxicity. The figure suggests that it is the increase in cytosolic Ca2+ that leads to transcriptional inactivation of MEF2A, thereby leading to reduced Mfn2 expression. It is unclear where this link between Ca2+ and MEF2A is demonstrated within the results. Are there specific MEF2A transcriptional elements that are repressed by increased Ca2+? Within the electrophoretic mobility shift assays, could the Ca2+ responsiveness of these transcriptional activators been demonstrated by titrating Ca2+ or treating with Ca2+ chelators? This concept should be clarified in the Discussion, as it is unclear how Ca2+ signaling affects the MEF2A activity.

2. With further regard to Ca2+ signaling, it is clearly shown that NMDA increases cytosolic Ca2+, leading to mitochondrial depolarization and reduced ATP synthesis capacity. Knowing that cytosolic Ca2+ was increased, what can be hypothesized about mitochondrial matrix Ca2+ during excitotoxicity? A recent review (Court FA and Coleman MP, Trends Neurosci 35(6): 364-372, 2012) suggests that neuronal mitochondrial Ca2+ can induce opening of the permeability transition pore (PTP), which might be responsible for the depolarization observed in the present study. Was PTP Ca2+ sensitivity and/or PTP opening addressed in these experiments? If not, it might be worth pursuing, as this may provide another potential target for the post-ischemic therapeutic window.

3. In relation to issue #2 discussed above, what is the relationship between the increase in cytosolic Ca2+, mitochondrial fragmentation, and inner membrane depolarization? Clearly, the rise in intracellular Ca2+ is the activating signal that leads to Drp1 activation and transcriptional downregulation of Mfn2. However, does the fragmentation precede depolarization, or vice versa? This is an important point to address, as mitochondrial depolarization can lead to Opa1 degradation, thereby inducing fragmentation. That being said, no change in Opa1 was observed either in the cultured neurons or following clamp removal in the MCA occlusion model, so this pathway of mitochondrial fragmentation is unlikely. However, it is critical to address which event occurs first, the change in morphology or the impact on membrane potential.

4. Though the authors demonstrate nicely that Mfn2 downregulation is not due to proteasomal degradation, they neglect to comment on overall mitophagic flux. Could the neuronal death induced by excitotoxicity be related to impaired autophagy, leading to a stagnant pool of depolarized mitochondria that potentially have undergone permeability transition? This does not necessarily need to be addressed experimentally, but it would be worth mentioning in the Discussion. Clearly, impaired mitochondrial turnover could impact the excitotoxic neuronal phenotype.

5. It is surprising to this reviewer that reactive oxygen species were not addressed in this study. Though somewhat refreshing to see a mechanism not involving ROS, it is well-established that ischemic episodes in the brain, heart, and other tissues, can lead to alterations in ROS production and scavenging. Is there potential for ROS-mediated transcriptional inactivation of Mfn2 in excitotoxicity?

6. The authors mention that the findings are directly applicable to neurodegenerative diseases and in clinical situations of traumatic brain injury/stroke. From these findings, what are the molecular entities that would be pharmacologically targeted in the human patient? How would mitochondrial morphology and/or Mfn2 expression be manipulated? In the MCA occlusion model, were attempts taken to preserve Mfn2 expression and reduce the post-ischemic damage?

MINOR ISSUES

1. In the last paragraph of the Introduction on page 5, "fussion" and "fision" are misspellings. In addition, in the second paragraph on page 19 of the Discussion, "OXPOS" should be changed to "OXPHOS."

2. On page 16 of the Results section, it is mentioned that "We found that under basal conditions, MEF2 was bound to this region but after NMDA stimulation there was no enrichment of this region with the chromatin immunoprecipitate (Fig. 6G)." This figure reference is incorrect and should be Fig. 8G.

Referee #2:

The manuscript by Martorell-Riera et al reports that during delayed excitotoxic injury, mitochondrial fragmentation is triggered by a MEF2-dependent downregulation of the mitochondrial fusion protein Mfn2, and that this eventually leads to excitotoxic neuron death.

Criticisms:

1. The authors did not sufficiently address the question what processes eventually will kill the neuron? Is it ATP depletion? If so, the authors need to determine ATP levels in response to mfn2 gene silencing and mfn2 overexpression during the NMDA exposure. If not, is it calpain activation? Again, the authors would need to determine calpain activity in these scenarios.

2. The authors should repeat the key findings of this study in a model of cerebral ischemia in vitro such as oxygen/glucose deprivation.

3. The mode of cell death induced is very poorly characterised. Previous studies have indicated a role for mitochondrial outer membrane permeablisation and Bcl-2 family proteins in delayed

excitotoxcity. The authors need to determine whether Bcl-2 overexpression or Bcl-xL overexpression protects against Mfn2 depletion, delayed mitochondrial fragmentation, as well as cell death. Does the fission machinery require bax as well? Is cytochrome c released?

4. There is significant evidence that elevated cytoplasmic Ca2+ levels are not sufficient to increase excitotoxic cell death - in fact Reynolds, Stout and co-workers have shown that protection (by mitochondrial resp chain inhibition) occurred in the presence of elevated cytosolic Ca2+ (Stout et al, Nat Neurosci, 1998). The authors should comment. Do mitochondria take up more Ca2+ during excitotoxic cell death when mfn2 is silenced?

5. TMRM measurements are volume-sensitive measurements, and it appears that the mitochondrial volume is decreasing. The authors should provide an alternative measure of mitochondrial respiratory activity and its modulation by mfn2 o.e. or gene silencing, e.g. detection of alterations in TMRM fluorescence via single-cell imaging in response to mitochondrial complex inhibition (or Seahorse-based detection of mitochondrial activity).

6. The authors should provide evidence that MEF2 modulates excitotoxic cell death via mfn2 (and not other targets).

Minor criticisms:

7. In Fig 1A, it would be good to add data on cell death at the various time points. When does cell death set in?

8. Some of the axis labels read e.g. 1,25 when they should read 1.25

Referee #3:

The paper by Soriano and coworkers describes the role of Mfn2 in neuronal excitoxicity. The start-point is the down-regulation of the expression of Mfn2 and Drp1 at different time frames in vivo and in vitro during neural exocitotoxicity. The authors show also that MEF2 controls the expression of Mfn2 in a neuron-specific manner.
Some of the data appear weak. Indeed, there is not a clear downregulation of Drp1 and Mfn2 at the time points indicated in the paper. Moreover, a clear upregulation of Mfn1 is observed in vitro, which is not commented in the text and correlates with the data published (Wappler et al, 2013). Overall, critical data are missing that would allow to safely draw the conclusions made by the authors.

Major points:

1. The authors claim to see a reduction of the protein levels of Mfn2 after exposure of primary cortical cells to NMDA for 4 hours (Figure 1A). It is hard to see such a reduction of the levels of Opa1 (Figure 1A). The same holds true for the levels of Drp1 that are unchanged at all the time points considered. Rather an overexpression of Mfn1 is observed after 8 hours of NMDA treatment, which is not discussed in the text. Moreover, given the variability of the in vivo experiment it would be useful to add a second control to verify the expression of Mfn2, Mfn1, Opa1 and Drp1 (panel C of figure 1), as performed for the other samples. This criticism applies to all the other blots. In the discussion sections, the authors cite the paper by Wappler et al, 2013 but in this case the authors show a dramatic reduction in Drp1 expression, a smaller reduction in Mfn2 expression and an increase in Mfn1 expression.

2. The authors claim that they observe a 20% reduction of the protein levels of Drp1 in vivo (panel C of figure 1), which indeed is very hard to see in the data presented.

3. Regarding figure 3, it would be preferable to show the images of cells transfected also with Drp1-K38A and treated with 7-Ni and mdivi-1. The quantification is not sufficient. The same consideration applies to panel A of figure 3 and other quantifications. The authors should show representative images in the text and the quantification as supplementary figures.

4. The authors state that excitotoxicity promotes mitochondrial fragmentation by a fast mechanism that relies on Drp1 activation by showing that Drp1 expression decreases during the first hour in vitro and a partial recovery of tubular mitochondria after expression of Drp1-K38A. This is not fully convincing: it is hard to appreciate a significant reduction in the levels of expression of Drp1 after treatment with NMDA and, secondly, the recovery of mitochondrial morphology might be an unrelated effect of Drp1. The authors do not show a direct correlation between NMDA activity and Drp1. The authors do not show a cause-effect mechanism but rather a correlation. Other experiments should be designed in order to demonstrate the connection between Drp1 reduction and excitotoxicity.

5. The authors claim to detect a slight increase in cytosolic basal [Ca2+] in Mfn2 KD neurons. They also show that the application of NMDA causes an increase in cytosolic [Ca2+] that is much higher in Mfn2 KD cells and correlates with greater spectrin cleavage (Fig. S3). One would expect a slight increase in spectrin cleavage in Mfn2 KD cells and a higher cleavage in Mfn2 KD cells treated with NMDA. Why is this not the case? Overall, the quality of Ca2+ traces is very poor: most labs have replaced indo-1 with new-generation probe, and I suggest that the authors do the same (although the low quality is not only due to the probe itself).

6. In panel C of figure 6 the authors should show the expression levels of Drp1 and SESN2 1 and 2 hours after NMDA application.

7. In panel C of figure 7 key data are missing, such as control samples treated with NMDA and cells infected with MEF2-DN plus NMDA as quantified in panel F of figure 7.

Minor points:

1. It is useless to repeat the densitometry of Western blots of panels A and of figure 1 in the main figure (panels B and D of figure 1) and in the supplementary figure 1.

2. Pag. 16 mislabelled figure 6G instead of 8G.

1st Revision - authors' response   18 June 2014

Referee #1:

This is a very interesting study. Deserves publication in EMBO after addressing the following, mostly minor, comments.

The study by Martorell et al. investigated the downregulation of Mfn2 during NMDA-mediated excitotoxicity in cultured neurons and in an animal model of cerebral focal ischemia. Using a well-designed experimental approach, the authors elegantly demonstrate a biphasic mitochondrial response to NMDA-mediated toxicity, in which Drp1 activation increases mitochondrial fragmentation in the early phase and transcriptional regulation of Mfn2 sustains the fragmented morphology in the late phase and propagates neuronal cell death. The deleterious consequences of Mfn2 downregulation were reversed by treatment of the cells with exogenous Mfn2, suggesting that Mfn2 plays a distinct and clinically-relevant role in minimizing excitotoxic neuronal damage. Though clearly written and experimentally solid, the study has several minor issues that need to be addressed.

We are pleased that the reviewer finds the manuscript interesting, clearly written, experimentally solid and that deserves publication in EMBO J.

1. In Figure 9, a clear schematic is presented demonstrating the impact of NMDA receptor activation on the induction of excitotoxicity. The figure suggests that it is the increase in cytosolic Ca2+ that leads to transcriptional inactivation of MEF2A, thereby leading to reduced Mfn2

expression. It is unclear where this link between Ca2+ and MEF2A is demonstrated within the results. Are there specific MEF2A transcriptional elements that are repressed by increased Ca2+? Within the electrophoretic mobility shift assays, could the Ca2+ responsiveness of these transcriptional activators been demonstrated by titrating Ca2+ or treating with Ca2+ chelators? This concept should be clarified in the Discussion, as it is unclear how Ca2+ signaling affects the MEF2A activity.

We apologize because obviously the schematic cartoon was not clear enough. Synaptic calcium influx is a strong activator of MEF2 in large part by calcineurin-dependent dephosphorylation of MEF2 (Cohen & Greenberg, 2008).However, as we discus massive influx of calcium through the NMDA receptor causes MEF2 degradation. In figure 7 we reproduce previous results showing that NMDAR activation causes degradation of MEF2A in vitro and, to our knowledge, we also show for the first time in vivo degradation of MEF2A in a MCAO model. In the revised version of the manuscript, in Figure E7B we also include a representative western blot showing partial degradation of MEF2 in primary cortical neurons subjected to oxygen and glucose deprivation. The NMDA-mediated MEF2A degradation is calcium dependent since MEF2A degradation triggered by NMDA application does not occur in absence of extracellular calcium (new Figure E7C). Concomitantly, with the degradation of MEF2A, in Fig 8G we show that MEF2A does not bind to the Mfn2 promoter after NMDA application. We have amended the figure 9 stating clearly that excessive calcium influx lead to MEF2A degradation and clarified it in the discussion. In the new version of the figure 9 we have also summarized the new results about the mechanism by which Mfn2 downregulation enhances delayed cell death.

2. With further regard to Ca2+ signaling, it is clearly shown that NMDA increases cytosolic Ca2+, leading to mitochondrial depolarization and reduced ATP synthesis capacity. Knowing that cytosolic Ca2+ was increased, what can be hypothesized about mitochondrial matrix Ca2+ during excitotoxicity? A recent review (Court FA and Coleman MP, Trends Neurosci 35(6): 364-372, 2012) suggests that neuronal mitochondrial Ca2+ can induce opening of the permeability transition pore (PTP), which might be responsible for the depolarization observed in the present study. Was PTP Ca2+ sensitivity and/or PTP opening addressed in these experiments? If not, it might be worth pursuing, as this may provide another potential target for the post-ischemic therapeutic window.

The referee is right. There are a number of studies showing the opening of the mPTP during excitotoxicity. Although the precise composition of the mPTP is not known, cyclophilin D (Cyp D) appears to be a central component of the mPTP. Because several mechanisms can participate in excitotoxic cell death, to clarify the role of Mfn2 downregulation we have used doses of NMDA (15 µM) that are only toxic when Mfn2 is knocked down. Under these conditions we have pre-treated cortical neurons with cyclosporine A (CsA), a commonly used CypD (and mPTP) inhibitor, before NMDA application and found that CsA does not protect them (new Fig E5D). CypD KO cells are resistant to necrosis but not to apoptotic cell death. And it is generally accepted that necrosis occurs during early phase of excitotoxicity and apoptosis during the delayed one. Thus, the lack of protection by CsA goes in line with our results that Mfn2 intervenes in delayed excitotoxicity and the new results obtained at the request of Referee 2 indicating that reduction of Mfn2 facilitates Bax translocation to mitochondria and cytochrome c release (new Fig. 5 D-I).

Regarding to the mitochondrial calcium. We have performed cell live imaging to determine mitochondrial calcium in control or Mfn2 KD neurons. We found reduced calcium uptake by mitochondria (new Fig 4E, F and Fig E4B) that correlates with increased cytoplasmic calcium. The levels of mitochondrial calcium after stimulation with NMDA keep steady for at least 15 minutes (not shown). Thus, all these data seem to indicate that at the conditions used in the present study downregulation of Mfn2 does not facilitate the opening of the mPTP. In support of our results in neurons, recently it has been shown that mPTP function is not affected in hearts of Mfn2 KO mice and inhibition of mPTP did not alter mitochondrial or cardiac degeneration of these animals (Song et al, 2014).

3. In relation to issue #2 discussed above, what is the relationship between the increase in cytosolic Ca2+, mitochondrial fragmentation, and inner membrane depolarization? Clearly, the rise in intracellular Ca2+ is the activating signal that leads to Drp1 activation and transcriptional

downregulation of Mfn2. However, does the fragmentation precede depolarization, or vice versa? This is an important point to address, as mitochondrial depolarization can lead to Opa1 degradation, thereby inducing fragmentation. That being said, no change in Opa1 was observed either in the cultured neurons or following clamp removal in the MCA occlusion model, so this pathway of mitochondrial fragmentation is unlikely. However, it is critical to address which event occurs first, the change in morphology or the impact on membrane potential.

This is an interesting point. We have now quantified mitochondrial membrane potential (mMP) and monitored mitochondrial morphology along a time-lapse and found that just before fission the mMP is a 57% lower than before NMDA application. An additional 20% of depolarization takes place 15 minutes after fission (new Fig E2B, C). Interestingly, Shirihai's lab has observed very recently the same phenomenon in brown adipocytes.

4. Though the authors demonstrate nicely that Mfn2 downregulation is not due to proteasomal degradation, they neglect to comment on overall mitophagic flux. Could the neuronal death induced by excitotoxicity be related to impaired autophagy, leading to a stagnant pool of depolarized mitochondria that potentially have undergone permeability transition? This does not necessarily need to be addressed experimentally, but it would be worth mentioning in the Discussion. Clearly, impaired mitochondrial turnover could impact the excitotoxic neuronal phenotype.

This is an important question that we believe needs to be addressed in an independent study. Although autophagy was first described in the sixties during the last years there has been a blossoming in the understanding of the mechanisms and physiological relevance of autophagy. The relation between autophagy and cell death is controversial and in most of the cases autophagy constitutes a cytoprotective response. Previous studies have shown that Mfn2 KO cells show reduced autophagy in response of ER stress. Interestingly, recently it has been shown that Mfn2 acts as a receptor for parkin, which is recruited to mitochondria to promote mitophagy. In cardiomyocites Mfn2 KD prevented autophagosome-lysosome fusion (Zhao et al, 2012). All these evidences would indicate that the referee's hypothesis that Mfn2 downregulation would impair mitochondrial turnover in excitotoxicity may be right, but future experiments are required to verify it.

5. It is surprising to this reviewer that reactive oxygen species were not addressed in this study. Though somewhat refreshing to see a mechanism not involving ROS, it is well-established that ischemic episodes in the brain, heart, and other tissues, can lead to alterations in ROS production and scavenging. Is there potential for ROS-mediated transcriptional inactivation of Mfn2 in excitotoxicity?

To test this possibility we have pre-treated cortical neurons before NMDA application with Trolox (general scavenger) and apocynin (a NADPH oxidase inhibitor which is the major source of ROS in excitotoxicity) and determined Mfn2 mRNA expression. The use of antioxidants did not prevent NMDA-mediated Mfn2 downregulation (new Fig E7A).

6. The authors mention that the findings are directly applicable to neurodegenerative diseases and in clinical situations of traumatic brain injury/stroke. From these findings, what are the molecular entities that would be pharmacologically targeted in the human patient? How would mitochondrial morphology and/or Mfn2 expression be manipulated? In the MCA occlusion model, were attempts taken to preserve Mfn2 expression and reduce the post-ischemic damage?

As the referee will understand, protection to MCAO by preventing Mfn2 downregulation is beyond the scope of the current study. Here we have opened the possibility to use Mfn2 as therapeutic target. The finding that Mfn2 is downregulated mainly at transcriptional level and that Mfn2 reduction facilitates Bax translocation to mitochondria suggests therapeutic strategies. For instance, to use a chemical library for high-throughput screening assay for Mfn2 promoter activation could provide us a molecule that prevents Mfn2 mRNA downregulation in excitotoxicity. In the same line, synthetic modified RNA (modRNA) has emerged as new therapeutic strategy to control the spatial and temporal delivery of gene products (Zangi et al, 2013). A third possibility would depend on a better understanding of how Mfn2 deficiency facilitates mitochondrial Bax translocation. If it depends on changes on Mfn2 interaction with Bax or other Bcl-2 family protein members, a fine mapping of the domain implicated in these interactions would allow us to generate TAT fused

peptides to interfere or keep these interactions. We have not included this in the discussion, mainly for space limitations, but if the editor and the referee agree we would be happy to do so.

MINOR ISSUES

1. In the last paragraph of the Introduction on page 5, "fussion" and "fision" are misspellings. In addition, in the second paragraph on page 19 of the Discussion, "OXPOS" should be changed to "OXPHOS."

We apology for this typo. Now it has been corrected. Note that we have modified slightly the discussion to fit the new findings and the OXPHOS section has been removed.

2. On page 16 of the Results section, it is mentioned that "We found that under basal conditions, MEF2 was bound to this region but after NMDA stimulation there was no enrichment of this region with the chromatin immunoprecipitate (Fig. 6G)." This figure reference is incorrect and should be Fig. 8G.

We apology for this oversight. The figure is now well labeled.

Referee #2:

The manuscriopt by Martorell-Riera et al reports that during delayed excitotoxic injury, mitochondrial fragmentation is triggered by a MEF2-dependent downregulation of the mitochondrial fusion protein Mfn2, and that this eventually leads to excitotoxic neuron death.

Criticisms:

1. The authors did not sufficiently address the question what processes eventually will kill the neuron? Is it ATP depletion? If so, the authors need to determine ATP levels in response to mfn2 gene silencing and mfn2 overexpression during the NMDA exposure. If not, is it calpain activation? Again, the authors would need to determine calpain activity in these scenarios.

To answer the referee's question we have opted for the independent determination of NAD^+ and NADH levels in control or Mfn2 KD neurons that are a better read-out of the metabolic status of the cell than single ATP determination (ATP/ADP ratio is more relevant). We have found that there are no significant changes through the time (new Fig E5C), thus energetic crisis does not seem to be what causes the cell death.

We have determined calpain activation indirectly by analyzing spectrin cleavage along a time course and found that when stimulated with 15 μM NMDA, Mfn2 KD neurons show enhanced calpain activity (new Fig E5E). This is in line with the new data where we show that Mfn2 reduction facilitates mitochondrial Bax translocation (see answer to point 3) given that calpain has been shown to be downstream effectors of Bax in delayed excitotoxicity (D'Orsi et al, 2012).
Neurons transfect poorly, thus for this kind of experiment it is necessary to transduce neurons with virus. Overexpression of Mfn2 experiments have not been possible because excessive Mfn2 expression causes perinuclear clusters of mitochondria that are noxius (Huang et al, 2007). In transfection experiments it is possible to modulate Mfn2 expression by modifying the ratios of the different plasmids transfected but reduction in the MOI used results in a diminution of the transduced neurons rather that the diminution of the expression of Mfn2 in the transduced neurons.

2. The authors should repeat the key findings of this study in a model of cerebral ischemia in vitro such as oxygen/glucose deprivation.

We have performed OGD experiments and found that similarly to in vitro NMDA bath application or in vivo MCAO:
 1) Mfn2 is the only protein of the fusion/fission machinery that is reduced (new Fig E1B, C).

2) Mfn2 is downregulated at mRNA level (new Fig E6C).
3) MEF2A is degraded (new Fig E7B).

3. The mode of cell death induced is very poorly characterised. Previous studies have indicated a role for mitochondrial outer membrane permeablisation and Bcl-2 family proteins in delayed excitotoxcity. The authors need to determine whether Bcl-2 overexpression or Bcl-xL overexpression protects against Mfn2 depletion, delayed mitochondrial fragmentation, as well as cell death. Does the fission machinery require bax as well? Is cytochrome c released?

We thank the referee for suggesting those experiments that we believe have improved notably our study.

Because several mechanisms can participate in excitotoxic cell death, to clarify the role of Mfn2 downregulation we have used doses of NMDA (15 µM) that are only toxic when Mfn2 is knocked down. Under these conditions both Bcl-xL and a pool of 4 commercial siRNAs targeting Bax conferred total protection (new Fig 5D, E). This suggested a mechanism dependent of Bax, that is consistent with the protection of Mfn2 to delayed excitotoxicity we showed in the original version of the manuscript (Fig 5C). Next, we have studied how Mfn2 affects Bax translocation to the mitochondria after application of 30 µM NMDA and found that Mfn2 expression reduces it. We have analyzed cytochrome c release and we have found that, correlating with lower mitochondrial Bax translocation, Mfn2 expression was able to reduce the NMDA mediated cytochrome c release as well (new Fig 5H, J).

Regarding the question of whether Bcl-2 family members regulate mitochondrial morphology after NMDA treatment. We have performed these experiments and found that both, Bcl-xL and Bax KD protects against delayed mitochondrial fragmentation (new Fig E5F, G), in agreement with the known role of Bcl-2 family members modulating mitochondrial dynamics.

4. There is significant evidence that elevated cytoplasmic Ca2+ levels are not sufficient to increase excitotoxic cell death - in fact Reynolds, Stout and co-workers have shown that protection (by mitochondrial resp chain inhibition) occurred in the presence of elevated cytosolic Ca2+ (Stout et al, Nat Neurosci, 1998). The authors should comment. Do mitochondria take up more Ca2+ during excitotoxic cell death when mfn2 is silenced?

We have analyzed mitochondrial calcium uptake in control and Mfn2 KD neurons after 15 µM NMDA application. We found that mitochondria in Mfn2 KD neurons uptake less calcium than in control neurons (new Fig 4E, F). We have also performed experiments to show that increased cytoplasmic calcium in Mfn2 KD neurons after NMDA application is due to reduced mitochondrial calcium uptake because depolarization with CCCP equals the levels of cytoplasmic calcium in control and Mfn2 KD neurons (new Fig E4B). In the study cited by the referee the authors transiently depolarize the mitochondria to avoid mitochondrial calcium uptake after short glutamate stimulation. Although Stout et al did not show mitochondrial calcium uptake in this study (they showed rise in intracellular calcium), it is known that mitochondrial calcium uptake depends on mitochondrial membrane potential, and that depolarization prevents completely mitochondrial calcium uptake. Actually, we observed release of stored mitochondrial calcium after depolarization (new Fig E4B). Therefore, one difference between Stout et al and our study is that they prevented completely mitochondrial calcium uptake (or it is supposed so) and in our study, although diminished, still there is mitochondrial calcium uptake. Another consideration is that there are a number of evidences indicating that excessive mitochondrial calcium uptake produces mitochondrial dysfunction that leads to cell death and in our study we show that Mfn2 KD have dysfunctional mitochondria (new Fig 4A-D) which may sensitize to lower calcium uptake.

5. TMRM measurements are volume-sensitive measurements, and it appears that the mitochondrial volume is decreasing. The authors should provide an alternative measure of mitochondrial respiratory activity and its modulation by mfn2 o.e. or gene silencing, e.g. detection of alterations in TMRM fluorescence via single-cell imaging in response to mitochondrial complex inhibition (or Seahorse-based detection of mitochondrial activity).

We have performed Seahorse respirometric analysis and observed that Mfn2 KD has a reduced respiratory control ratio, an excellent marker of mitochondrial dysfunction (Brand & Nicholls, 2011), and reduced spare respiratory capacity (new Fig 4A, B), which would explain the bigger reduction in mitochondrial membrane potential when there is a bigger energetic demand such as dealing with increased intracellular calcium (Fig 4C).

6. The authors should provide evidence that MEF2 modulates excitotoxic cell death via mfn2 (and not other targets).

Now, in the new figure 7G we show that neurons expressing dominant negative MEF2 are sensitized to doses of NMDA at the threshold of toxicity (15 μM) and that Mfn2 expression completely blocks it.

Minor criticisms:

7. In Fig 1A, it would be good to add data on cell death at the various time points. When does cell death set in?

In the Figure 5C we show data on cell death at various time points. For the sake of linearity we would prefer to keep the order of the figures, but we would be willing to change it if the reviewer and editor find it more appropriate.

8. Some of the axis labels read e.g. 1,25 when they should read 1.25

We apologize for this oversight. Now it has been amended.

Referee #3:

The paper by Soriano and coworkers describes the role of Mfn2 in neuronal excitoxicity. The start-point is the down-regulation of the expression of Mfn2 and Drp1 at different time frames in vivo and in vitro during neural exocitotoxicity. The authors show also that MEF2 controls the expression of Mfn2 in a neuron-specific manner.

Some of the data appear weak. Indeed, there is not a clear downregulation of Drp1 and Mfn2 at the time points indicated in the paper. Moreover, a clear upregulation of Mfn1 is observed in vitro, which is not commented in the text and correlates with the data published (Wappler et al, 2013). Overall, critical data are missing that would allow to safely draw the conclusions made by the authors.

Major points:

1. The authors claim to see a reduction of the protein levels of Mfn2 after exposure of primary cortical cells to NMDA for 4 hours (Figure 1A). It is hard to see such a reduction of the levels of Opa1 (Figure 1A). The same holds true for the levels of Drp1 that are unchanged at all the time points considered. Rather an overexpression of Mfn1 is observed after 8 hours of NMDA treatment, which is not discussed in the text. Moreover, given the variability of the in vivo experiment it would be useful to add a second control to verify the expression of Mfn2, Mfn1, Opa1 and Drp1 (panel C of figure 1), as performed for the other samples. This criticism applies to all the other blots. In the discussion sections, the authors cite the paper by Wappler et al, 2013 but in this case the authors show a dramatic reduction in Drp1 expression, a smaller reduction in Mfn2 expression and an increase in Mfn1 expression.

We assume that when the referee says that it is hard to see reduction levels in Opa1, s/he means Mfn2, because Opa1 does not change indeed.

We apologize for not having chosen the best blots to convey the densitometric analysis of 3 to 6 independent westerns in Fig 1B. Now we have re-run some of the samples and showed new representative western blots (Fig 1A). We would like to add that western blot is a semiquantitative technique and unless the changes are massive it is necessary to perform densitometric analysis and normalize to at least one housekeeping protein. We show reduction of Mfn2 protein levels after 4

hours of NMDA (30 μM) application in another 4 panels in the new version of the manuscript (Figs 3B, 6A, 6D and 7D) from a total of 23 independent experiments. In Fig 1C and D we show reduction of Mfn2 protein levels 6 h after clamp release of an in vivo MCAO model (six animals). To request of referee 2 we have also subjected in vitro cortical neurons to oxygen and glucose deprivation (OGD) and observed Mfn2 reduction 3 h after OGD. As we discussed, these results are supported by other 2 independent studies. Although between these studies there are some discrepancies about the behavior of other proteins of the fusion/fission machinery in all of them consistently there is a reduction in Mfn2 levels (Table R). We have studied the mechanism by which Mfn2 is reduced in excitotoxicity and found that it was at transcriptional level, measured this time by quantitative real time RT-PCR (Fig 6C and E6C). This correlates with the results of an unbiased microarray analysis where it is found a 50% reduction of Mfn2 mRNA expression in the penumbra of rats subjected to MCAO (Ramos-Cejudo et al, 2012). Thus, our data and the data from other 3 independent laboratories clearly indicate that Mfn2 expression is reduced in excitotoxicity.

Study	model	Mfn1	Mfn2	Opa1	Drp1
Wappler et al. (2013)	In vitro OGD	↑	↓ (40%)	=	↓
Kumari et al (2012)	In vivo MCAO	N.D.	↓ (30%)	↓	↑or =
Martorell-Riera et al. (unpublished)	In vitro NMDA bath application	=	↓ (40%)	=	=
	In vitro OGD	=	↓ (50%)	=	= or ↓
	In vivo MCAO	=	↓ (50%)	=	=

Table R. Summary of the changes in mitochondrial fission/fusion proteins in three independent studies.

2. The authors claim that they observe a 20% reduction of the protein levels of Drp1 in vivo (panel C of figure 1), which indeed is very hard to see in the data presented.

Drp1 is a mitochondrial fission protein. Because excitotoxicity causes mitochondrial fission, it would be expected an increase in Drp1 protein levels. To our surprise, we found, both in vitro and in vivo, a tendency to decrease the protein levels that only reached significance at 2 hours after clamp release in vivo and normalized to actin (not porin) or 4 h of NMDA treatment in vitro and 6h after clamp release in vivo when normalized to porin. Thus, significance was reached only at some points probably due to the number of repeats (6 animals for in vivo and 4 independent experiments for the in vitro westerns) but clearly in any case there was not the expected increase in Drp1 levels, and certainly increasing the number of repeats would not change it. More information about the role of Drp1 in excitotoxicity is addressed in point 4.

3. Regarding figure 3, it would be preferable to show the images of cells transfected also with Drp1-K38A and treated with 7-Ni and mdivi-1. The quantification is not sufficient. The same consideration applies to panel A of figure 3 and other quantifications. The authors should show representative images in the text and the quantification as supplementary figures.

Now we provided images through all the figures. We have opted to keep the analysis as well in the main text, but if the referee and editor insist we have no problem to include them in the supplementary figures.

4. The authors state that excitotoxicity promotes mitochondrial fragmentation by a fast mechanism that relies on Drp1 activation by showing that Drp1 expression decreases during the first hour in vitro and a partial recovery of tubular mitochondria after expression of Drp1-K38A. This is not fully convincing: it is hard to appreciate a significant reduction in the levels of expression of Drp1 after treatment with NMDA and, secondly, the recovery of mitochondrial morphology might be an unrelated effect of Drp1. The authors do not show a direct correlation between NMDA activity and Drp1. The authors do not show a cause-effect mechanism but rather a correlation. Other experiments should be designed in order to demonstrate the connection between Drp1 reduction and excitotoxicity.

With all due respect, the reviewer has misread this section. What we literally state is: "We observed a tendency for a decline in Drp1 in excitotoxicity (Fig. 1). Nonetheless, what determines Drp1 activity is its subcellular localization. Drp1 is mainly cytosolic and by posttranslational modifications it is recruited to the mitochondria by Mff and/or Fis1 where it promotes fission" (page 8, first paragraph). Therefore, we determined the GFP-Drp1 location in control and NMDA treated cells and showed mitochondrial recruitment in the NMDA treated cells (Fig 2C). Next, we used a widely used and accepted dominant negative form of Drp1 (Drp1-K38A) which Lys 38 within the GTPase domain is muted to Ala. This mutant Drp1 protected against NMDA-mediated mitochondrial fragmentation, as should be expected if Drp1 intervenes in NMDA-mediated fragmentation. Moreover, we used a known pharmacological inhibitor of Drp1 (Mdivi-1) with similar results. Because Bossy-Wetzel's lab have already established that nitrosylation of Drp1 mediates the NMDA-dependent mitochondrial fragmentation we used 7-Nitroindazole, an inhibitor of the NO synthase, which prevented the NMDA-mediated mitochondrial fragmentation in the same extent than Drp1-K38A and Mdivi-1. In summary, we showed 1) increased Drp1 recruitment to mitochondria after NMDA application; 2) prevention of NMDA-mediated mitochondrial fragmentation by dominant negative Drp1 mutant; 3) prevention of NMDA-mediated mitochondrial fragmentation by pharmacological inhibition of Drp1 and 4) prevention of NMDA-mediated mitochondrial fragmentation by inhibiting formation of NO.

In the new version, at request of referee 1 we have monitored which event occurs first, mitochondrial depolarization of fission. We have found that just before fission the mMP is a 57% lower than before NMDA application. Additional 20% depolarization takes place 15 minutes after fission (new Fig E2B, C). Because release of mitochondrial calcium after mitochondrial depolarization has been reported to activate calcineurin which de-phosphorylates and activates Drp1 we have inhibited calcineurin with CsA and found that this did not protect against NMDA-mediated mitochondrial fragmentation (new Fig E2D). We have also inhibited the calcium dependent CaMKs which activates Drp1 but it did not prevent either NMDA-mediated mitochondrial fragmentation (new Fig E2D).

Because all these results are in agreement with previous studies. From then on, as the referee will understand, we focused our study in the novel finding of delayed Mfn2 donwregulation and its implication in the excitotoxic process.

5. The authors claim to detect a slight increase in cytosolic basal [Ca2+] in Mfn2 KD neurons. They also show that the application of NMDA causes an increase in cytosolic [Ca2+] that is much higher in Mfn2 KD cells and correlates with greater spectrin cleavage (Fig. S3). One would expect a slight increase in spectrin cleavage in Mfn2 KD cells and a higher cleavage in Mfn2 KD cells treated with NMDA. Why is this not the case? Overall, the quality of Ca2+ traces is very poor: most labs have replaced indo-1 with new-generation probe, and I suggest that the authors do the same (although the low quality is not only due to the probe itself).

We appreciate the suggestion of the referee to use Fluo-4. We have repeated the experiments using this probe and although in line with the observed with Indo-1, we believe the quality has improved enormously. These results are in the new Fig 4G and H. Indo-1 results are in the new Fig E4A.

In the initial version of the manuscript we showed spectrin cleavage in control and Mfn2 KD neurons after only 30 minutes stimulation. Because this short time, it was necessary to over-expose the films to observe spectrin cleavage. Now we have stimulated for 4 and 6 hours with 15 μM NMDA with much clearer results as we show in new Fig E5E.

6. In panel C of figure 6 the authors should show the expression levels of Drp1 and SESN2 1 and 2 hours after NMDA application.

We believe the referee meant to complete former Fig 6C with data of Mfn1 and SESN2 expression after 1 and 2 h after NMDA application. Now we show the requested data.

7. In panel C of figure 7 key data are missing, such as control samples treated with NMDA and cells infected with MEF2-DN plus NMDA as quantified in panel F of figure 7.

Now we show the westerns with all the requested conditions in new Figure 7C

Minor points:

1. It is useless to repeat the densitometry of Western blots of panels A and of figure 1 in the main figure (panels B and D of figure 1) and in the supplementary figure 1.

We are not repeating the densitometries of figure 1 in the supplementary figure 1. In figure 1 we normalize the densitometry to actin, a common total cell loading control, and in supplementary figure 1 we normalize to porin, a common mitochondrial loading control. This way we show that Mfn2 reduction in excitotoxic conditions is not due to a reduction of mitochondrial mass. Although the result looks practically identical normalinzing to both, actin and porin, we believe it strengths the finding of the reduction in Mfn2 protein levels in excitotoxic conditions. Nonetheless, we would not object its removal if the reviewer and editor consider it necessary since that would not affect the general message of our study.

2. Pag. 16 mislabelled figure 6G instead of 8G.

We apologize for this oversight. Now it has been corrected.

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3rd Editorial Decision   16 July 2014

Thank you for submitting a revised version of your manuscript to The EMBO Journal. I have now received comments from two of the original referees of your manuscript who are both satisfied with the amount of revisions. I attach their comments below.

Please revise your manuscript once more to discuss the use of Rhod-2 for mitochondrial Ca2+ measurements as outlined by referee #2. I would also appreciate if you could carefully check the grammar throughout your manuscript once more.

Further, we have noticed a few additional issues that we have to ask you to address before we can proceed with your manuscript.
During our routine control for figure quality and integrity we came upon a potential image irregularity in figure 8F and figure 1. We would therefore ask you to provide us with source data for all panels in these figures. For the sake of clarity and comparison we would prefer to have the source data presented with the same exposure as that depicted in the final figure if possible. Please contact me if you have any questions concerning these points.

I would also be grateful at this stage if you were to provide original source data, particularly uncropped/-processed electrophoretic blots for all main figures of your manuscript. This is in accord with our policy to make original results better accessible for the community and thus increase reliability of published data. We would welcome one PDF-file per figure for this information. These will be linked
online as supplementary "Source Data" files.

Finally, we will also publish in the HTML version of the paper a synopsis to complement the abstract and to allow an easy access to the main findings. I would be grateful if you were to provide 3-5 one sentence bullet points that highlight your findings and that are non-redundant with your abstract.

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

Referee #2:

The authors addressed all major concerns in a very detailed response.

The authors need to add a sentence on the limitations of using Rhod-2 for mitochondrial Ca2+ measurements. Like TMRM it is also voltage sensitive, hence a depolarisation of mitochondria will lead to reduced Rhod2 fluorescence.

Referee #3:

The paper has been accurately revised also through the addition of new experimental evidence.

Authors' response   17 July 2014

To make the figure 8F I used a powerpoint from my PhD with the figure already assembled. Figure 8F is a ChIP performed on HeLa cells overexpressing MEF2A. Since we have in Figure 8G a much more relevant ChIP of the endogenous MEF2A on primary cortical neurons, the data of Fig 8F is not crucial for the conclusions or robustness of the study at all. We propose either to pass it to supplementary (where it could have perfectly been from the beginning) or to remove it from the manuscript. Please let me know how we can proceed. If you want a copy of my thesis or anything else to check that it effectively is an old data I'm more than happy to send it to you.

Regarding the source of data for Figure 1, I have it ready to upload to the submission system.

2nd Revision - authors' response   21 July 2014

We submit what we hope is the final and acceptable version of our manuscript "Mfn2 downregulation in excitotoxicity causes mitochondrial dysfunction and delayed neuronal death".
We thank you and the referees for insisting on experiments that have undoubtedly improved the paper.
The new sentence suggested by referee 2 is marked in yellow on page 11 of the manuscript.
As we agreed, former figure 8F has been removed as well as any reference throughout the text.
The rest of source data for the main figures have been uploaded in the submission system.
The highlights of this study are:

-   MEF2 regulates Mfn2 basal transcription in neurons.

-   MEF2 degradation in excitotoxicity causes Mfn2 downregulation.

-   Reduced Mfn2 expression causes mitochondrial dysfunction and altered calcium homeostasis.

-   Mfn2 downregulation in excitotoxicity participates in delayed cell death by facilitating Bax recruitment to mitochondria.