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## Proper synaptic vesicle formation and neuronal network activity critically rely on syndapin I

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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.)

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1st Editorial Decision

27 March 2011

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Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see all three referees are positive and support publication after appropriate revision. I would thus like to invite you to prepare a revised manuscript in which you need to address the points raised by the referees in an adequate manner. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your revisions included in the next, final version of the manuscript. Please do not hesitate to contact me at any time in case you would like to discuss any aspect of the revision further.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer 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.

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

Yours sincerely,

Editor  
The EMBO Journal

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REFEREE COMMENTS

Referee #1

This is an interesting study by Britta Qualmann and colleagues on the characterization of the syndapin 1 knockout mouse. Syndapin 1 is an accessory factor that belongs to the F-BAR family of proteins and has been implicated in the regulation of actin and endocytosis, including synaptic vesicle recycling. Syndapin 1 has also been implicated in the recruitment and regulation of fission factor, dynamin. This comprehensive characterization of the syndapin-1 KO mouse is important, as it provides genetic evidence for a role of an F-BAR protein in synaptic vesicle trafficking, neurotransmission and control of excitability. This mutant shares several features in common with other mouse mutants, such as the dynamin 1 and amphiphysin 1 KO. It also confirms the tight partnership between syndapin-1 and dynamin isoforms. Altogether, I have no major criticisms. The authors have done a great job with the characterization of the KO mouse and the manuscript is generally well written. I only recommend that the authors should write a shorter, more focused discussion, because currently, there are several aspects that appear tangential to the main message of the MS and some repetition.

Referee #2

Qualmann and colleagues report here on the role of syndapin 1, an FBAR protein family member, in synaptic vesicle formation and network activity. Syndapin 1 KO mice are viable and fertile but suffer from epileptic seizures. Furthermore, evoked transmission is reduced at both excitatory and inhibitory synapses. Ultrastructural analysis of the hippocampus and in particular of retinal ribbon synapses reveals increased numbers of endosomal and endocytic intermediates and enlarged SVs, a phenotype somewhat reminiscent of what is seen in dynamin 1 KO mice. Consistent with such a functional partnership between syndapin and dynamin the authors show that dynamins 1-3 display reduced membrane association *in vitro*. Finally, slice preparations from KO animals show altered hippocampal network activity, which likely explains the observed seizures. The authors thus suggest an important function of syndapin 1 in regulating dynamin recruitment to membranes, thereby regulating presynaptic vesicle cycling under conditions of high level activity.

This is a high-quality Ms presenting a near complete analysis of the synaptic phenotypes observed in syndapin 1 KO mice. The finding that syndapin recruits dynamin to membranes is novel and of general interest as are the general implications of this work for the neuroscience community. I thus recommend publication of this work in The EMBO J provided a few points have been addressed.

1. In fig. 3 it is shown that activity-induced SV cycling causes the accumulation of omega-shaped endocytic intermediates. Based on the recruitment assays one might expect that these structures contain clathrin coats (as seen in Dyn 1 KO mice) but (partially) lack dynamin. Such analysis would greatly improve the paper.

2. Syndapin has previously been implicated in bulk endocytosis by Cousin, Robinson & colleagues. The data presented here seem to argue against this idea. What is the effect of syndapin 1 loss on exo-endocytic cycling of FM dyes such as FM2-10 (excluded from bulk endosomes) vs. FM1-43? Alternatively, one could analyze uptake of HRP.

3. As syndapin has been claimed to regulate ARP2/3-mediated actin polymerization. Do KO neurons show defects in spine architecture? Do nerve terminals from KO mice display alterations with respect to F actin filament organization?

Referee #3

This paper analyzes knockout mice lacking syndapin-1. The phenotype is very interesting, and the paper is overall highly suitable for EMBO J. However, there are many overall rather minor problems that need to be addressed.

1. Data: the EMs do not look sharp, but that may be due to the file size restrictions of the review copy. For the biochemistry, it would be of interest to perform a more in-depth analysis of protein complexes involved in endocytosis. For the electrophysiology, the authors should show the mini amplitude results, and should remove Figure 6E and 6F, which is meaningless - one can't do that kind of thing in slices, that is what input/output curves are for .... Figure 8A should be put into Figure 7, and the rest of Figure 8 fits better into the SOMs since there is no tangible result. Interpreting Figure 7 as a change in excitatory/inhibitory balance (whatever that term means - it was fashionable for a while, but is pretty much being abandoned now, since there is no single 'excitation' and 'inhibition') is too simplistic, although the data are interesting. What one would like to see in the slice physiology is the response of synapses to stimulus trains. Short-term plasticity analyses would be very informative given the EM phenotype.

2. Presentation. The title is inappropriate - this is a paper about syndapin-1, not about 'networks', and pandering to the current fashion of emphasizing networks in what is essentially a cell biology paper is misleading. After all, if synapses don't work well, networks won't either, so the conclusion that mice with a deficit in synaptic function have network dysfunction is trivial. Much of the writing needs to be changed. For example, for a scientific article the sentence in the abstract "'F-BAR proteins are predestined to serve as membrane curvature sensors and/or inducers and may thereby support membrane remodeling processes, yet, their in vivo functions urgently await disclosure.'" 'Predestination' is a religious term, and really doesn't belong here, and it is hard to imagine that proteins are just there waiting for us to study them ....

All of these problems can be easily fixed, and overall this is a very interesting study that would fit into EMBO J.

1st Revision - authors' response

19 July 2011

Our detailed responses to the referee comments.

Referee #1

(...) Altogether, I have no major criticisms. The authors have done a great job with the characterization of the KO mouse and the manuscript is generally well written. I only recommend that the authors should write a shorter, more focused discussion, because currently, there are several aspects that appear tangential to the main message of the MS and some repetition.

*We thank the referee for her/his positive evaluation of our work. The revised manuscript now includes a discussion that has i) been streamlined and shortened and ii) liberated from as many redundancies as possible.*

*We hope that the referee will agree with us that the new discussion reads more smoothly and thus is more enjoyable.*

Referee #2

(...) This is a high-quality Ms presenting a near complete analysis of the synaptic phenotypes observed in syndapin 1 KO mice. The finding that syndapin recruits dynamin to membranes is novel and of general interest as are the general implications of this work for the neuroscience community. I thus recommend publication of this work in The EMBO J provided a few points have been addressed.

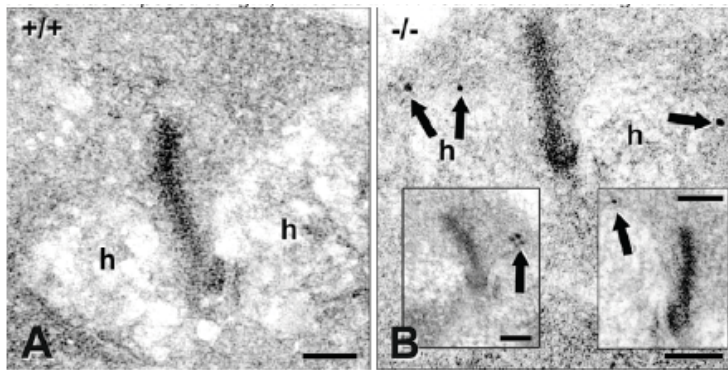
*We thank the referee for her/his positive evaluation of our work. Please see below for our point-to-point responses to the further suggestions and questions of the referee.*

1. In Fig. 3 it is shown that activity-induced SV cycling causes the accumulation of omega-shaped endocytic intermediates. Based on the recruitment assays one might expect that these structures contain clathrin coats (as seen in Dyn 1 KO mice) but (partially) lack dynamin. Such analysis would greatly improve the paper.

*This is a very interesting question, albeit technically not so easy to address. We conducted several rounds of postembedding immunoelectron microscopy of mouse retinae.*

*Fixation, embedding, and further handling of mouse retinae immunoelectron microscopy were performed according to Wolfrum and Schmitt (2000), nanogold labeling being silver-enhanced (Danscher 1981). We used purified mouse anti-clathrin heavy chain (1:400; BD Biosciences, Franklin Lakes, N.J., USA) as a primary antibody and a goat anti-mouse Fab conjugated to nanogold™ (Nanoprobes, Stony Brook, NY) as secondary antibody. Counterstained ultrathin sections were analyzed with a LEO transmission electron microscope (TEM) 906E (Zeiss, Oberkochen, Germany).*

*In line with impressions that some of the endocytic structures in light-exposed syndapin 1 KO retinae seen in our more highly contrasted images in former Figure 3 (now Fig. 4) have coats, a higher extent of clathrin staining was observed in syndapin 1 KO mice. The figure below shows examples of immunogold-labeled clathrin gold in the vicinity the plasma membrane in syndapin 1 KO retinae exposed to light, whereas in WT retinae such labeling was not observable.*



*Fig. X Postembedding immunogold labeling for the endocytotic protein clathrin in retinae of wildtype and syndapin 1 KO mice after long exposure to light.*

*The electron micrographs show that clathrin labeling is almost absent in WT ribbon synapses (A). In contrast, in syndapin 1 KO mice (B and further examples as insets), silver-enhanced immunogold-labelling of clathrin in the vicinity of the plasma membrane can frequently be observed (arrows). h, horizontal cell process.*

*We are happy to include these analyses in the Supplementary Material of the manuscript, if the referee feels we should do so. For now, we did not include them in the revised manuscript because we are unsure whether - because the plasma membrane is hard to be seen under the conditions allowing for immunolabelling - these data sets really will be appreciated by the readers.*

2. Syndapin has previously been implicated in bulk endocytosis by Cousin, Robinson & colleagues. The data presented here seem to argue against this idea. What is the effect of syndapin 1 loss on exo-endocytic cycling of FM dyes such as FM2-10 (excluded from bulk endosomes) vs. FM1-43? Alternatively, one could analyze uptake of HRP.

*The referee is referring to Clayton et al (2009). It is true that the phenotypes of the syndapin I KO mice, which are somewhat similar but much weaker than the dynamin-loss-of-function phenotypes, argue against the simplified view that syndapin I is the sole phosphosensor of dynamin and that this function is crucial for dynamin function, as suggested by work with dissociated neuronal cultures (Cousin & Robinson lab papers). However, we do not think that our results argue against a role of syndapin I in bulk endocytosis. Our data clearly demonstrate a role of syndapin I in activity-dependent high-capacity retrieval.*

*To explicitly address a role of syndapin I in bulk endocytosis, as suggested by the referee, we established primary cultures of hippocampal neurons of WT and syndapin I KO mice and examined FM1-43 recycling in nerve terminals after moderate and strong stimulation according to procedures reported by Clayton et al (2008; 2009). The results of these additional series of experiments are presented in the newly inserted Figure 3 of the revised manuscript. Already upon 300 stimuli at 10 Hz, small differences in the performance of syndapin I KO neurons in comparison to WT neurons were observed. These data correspond to  $\Delta F1$  shown in the newly inserted Figure 3, panels B-D. Dye turnover was significantly reduced upon the following, much stronger stimulation ( $\Delta F2$ ; shown in the newly inserted Figure 3, panels B,C and E. Also the  $\Delta F2/\Delta F1$  ratio determined according to Clayton et al (2008) showed a significant reduction in syndapin I KO neurons (wt,  $1.71 \pm 0.15$  vs. KO,  $1.36 \pm 0.07$ ;  $p=0.028$ ). Thus, also these analyses show that syndapin I-deficiency particularly disturbs high capacity retrieval of SVs.*

3. As syndapin has been claimed to regulate ARP2/3-mediated actin polymerization. Do KO neurons show defects in spine architecture? Do nerve terminals from KO mice display alterations with respect to F actin filament organization?

*Syndapin I indeed can modulate N-WASP- and Arp2/3 complex-mediated actin nucleation and thus both of these further questions of referee 2 are certainly interesting. Studying aspects of postsynaptic organisation of dendritic spines in syndapin I KO mice in detail however reaches far beyond the topic of the current study.*

*In presynapses, the organization of actin filaments is difficult to visualize and to quantitatively evaluate. Changes of the F- to G-actin ratio, however, can be measured biochemically and immunohistochemically. During our revision work we did both types of experimental series to address the referee's question.*

*Panels A and B of the newly added Supplementary Figure S3 of the revised manuscript shows that hippocampal slices from syndapin I KO incubated for 24 h with Alexa Fluor®488-conjugated DNase I and Alexa Fluor®568-conjugated phalloidin did not differ from WT slices in quantitative immunofluorescence measurements.*

*For biochemical F-actin/G-actin quantification, synaptosomes prepared as described previously (Wyneken et al, 2001) were subjected to F-actin separation from G-actin by a 20 min centrifugation at 100,000 g according to Boyle et al (2007). Quantitative Western blotting using fluorescently labeled secondary antibodies, detected by a LI-COR Odyssey system showed that also with this independent method F-/G-actin ratios in synapses of syndapin I KO mice did not differ from WT mice. These additional data sets are shown in panels C and D of the newly added Supplementary Figure S3.*

Referee #3

This paper analyzes knockout mice lacking syndapin-1. The phenotype is very interesting, and the paper is overall highly suitable for EMBO J. However, there are many overall rather minor problems that need to be addressed.

*We thank the referee for her/his positive evaluation of our work. Please see below for our point-to-point responses to your criticism.*

1. Data: the EMs do not look sharp, but that may be due to the file size restrictions of the review copy. For the biochemistry, it would be of interest to perform a more in-depth analysis of protein complexes involved in endocytosis.

*The revised manuscript now includes data on further endocytic proteins besides dynamin I, dynamin II, dynamin III, synapsin Ia/Ib and endophilin. Figure 5 (= revised Fig. 6G,H) now additionally includes quantitative examinations of the membrane recruitment of clathrin, amphiphysin and EHD protein(s) from WT and syndapin I KO cytosol. The recruitment of none of these three additional components was dependent on syndapin I.*

*Importantly, further examinations revealed that none of these additional components showed any alterations in membrane-association that were syndapin-specific. In contrast to dynamin I, II and III, whose membrane-association were all rescued by syndapin I readdition to KO cytosol, the protein amounts of clathrin, amphiphysin and EHD protein(s) did not differ in syndapin I KO samples compared to KO material supplemented with recombinant syndapin I (revised Fig. 6G,H).*

For the electrophysiology, the authors should show the mini amplitude results

*The revised manuscript now shows the amplitudes of mEPSCs of wildtype and syndapin I KO (new Figure 7C of the revised manuscript).*

*Furthermore, a comparison of the amplitudes of mIPSCs of WT vs. syndapin I KO has been inserted into the revised manuscript (Figure 7F).*

(the authors) should remove Figure 6E and 6F, which is meaningless

*The referee is right. The fact that eEPSC and eIPSC amplitudes are diminished in CA1 pyramidal neurons of syndapin I KO mice compared to WT cells (panels E and F of the former Fig. 6 = Fig. 7H and J in the revised manuscript) indeed can also be extracted from the data sets shown in the panels G,I.*

*We added these panels only for one reason, the shown comparisons between the two data points directly mirror panels shown in the publication of Ferguson et al (2007) studying dynamin I KO mice (Fig. 2C). The panels thereby allow for a direct comparison of syndapin I and dynamin I KO phenotypes. We hope that the referee agrees with us that offering the readers a possibility for direct comparison improves the accessibility of our results, as it becomes obvious that syndapin I KO phenocopies dynamin I deficiency in eEPSC and eIPSC amplitude examinations.*

*We hope that the referee finds our explanation for the inclusion of these two panels in Figure 7 plausible and acceptable. In this case, we would like to leave them in the manuscript. In our eyes, the possibility to more easily compare data from related publications is worth the minimal space used for panels H and J of the revised Figure 7.*

Figure 8A should be put into Figure 7, and the rest of Figure 8 fits better into the SOMs since there is no tangible result. Interpreting Figure 7 as a change in excitatory/inhibitory balance (whatever that term means - it was fashionable for a while, but is pretty much being abandoned now, since there is no single 'excitation' and 'inhibition') is too simplistic, although the data are interesting.

*As the referee suggested, the seizure data from the former Figure 8 have been combined with the data of the former Figure 7. Please see revised Figure 8A-H.*

*In further compliance with the referee's suggestions, we moved the Manganese-enhanced magnetic resonance imaging of WT and syndapin I KO mice and corresponding quantitative volumetric analyses revealing an increase of the hippocampal volume in syndapin I KO mice into the Supplementary Material (new Supplementary Figure S2).*

*As far as the interpretation of the data shown in Figure 8 (former Figure 7) is concerned, it is obvious that the excitability of syndapin I KO networks is increased and that the threshold for epileptiform activity is much lower. As we observed that both excitatory and inhibitory synaptic transmission is negatively affected by syndapin I deficiency, it is clear that mono-causal explanations do not apply but that the observed gross alterations of the network activity are a sum of changes in both the inhibitory and the excitatory transmission. Since the referee did not like the term "balance" to describe the delicate fine-tuning of brain activity, we reworded the headline of Figure 8 and the text of the revised manuscript. It now simply describes the altered hippocampal network activity and the resulting generalized seizures with tonic-clonic convulsion of syndapin I KO mice.*

What one would like to see in the slice physiology is the response of synapses to stimulus trains. Short-term plasticity analyses would be very informative given the EM phenotype.

*In order to comply with this suggestion of the referee, we conducted an additional series of experiments and measured EPSC amplitudes (recorded in the whole-cell configuration from pyramidal neurons in the CA1-region) to stimulus trains of 600 stimuli at 40 Hz as described by Clayton et al (2010).*

*As shown by the newly inserted Figure 7K of the revised manuscript, syndapin I KO mice displayed a significantly reduced depression of EPSCs amplitudes during high-frequency stimulation ( $P < 0.05$ , two-way RM-ANOVA). In our opinion, this difference to WT is most likely due to syndapin's involvement in the integration of recently endocytosed vesicles into one of the major vesicle pools (e.g. reserve pool). Disturbed exchange dynamics of SVs between reserve, recycling and readily-releasable (RR) pools may become overt during strong stimulation as slower decay, in particular when an initial stimulus train of the same type is used for priming as in our study.*

*2. Presentation. The title is inappropriate - this is a paper about syndapin-1, not about 'networks', and pandering to the current fashion of emphasizing networks in what is essentially a cell biology paper is misleading. After all, if synapses don't work well, networks won't either, so the conclusion that mice with a deficit in synaptic function have network dysfunction is trivial.*

*We appreciate the interest of the referee in mechanistical and cell biological aspects of syndapin function. Also the focus of our own previous work was mainly on these aspects. With the analyses of a knockout mouse, however, more systemic and physiological aspects come into play additionally. The only readily observable macroscopic phenotype of syndapin I KO mice is the occurrence of seizures with tonic-clonic convulsions without loss of consciousness. The way by which synapses adapt and respond to changes in network activity level varies not only between different regions of the brain (Virmani et al., 2006) but apparently also between excitatory and inhibitory synapses (Ertunc et al., 2007). In agreement with this, it has been reported that inhibitory neurons are very sensitive to impaired endocytic activity (Ferguson et al., 2007; Hayashi et al., 2008; Boumil et al., 2010). As a result, alterations in network activity will change the proportion to which particular mechanisms of vesicle replenishment are involved in excitatory versus inhibitory synapses. This will in turn lead to a shift in net excitatory drive once particular mechanisms are impaired by a certain mutation. We don't think that this is trivial. Our whole-cell recordings show a decrease of both, EPSCs and IPSCs amplitudes in syndapin I KO mice. The drop in IPSC amplitudes seem to be slightly larger than the drop in EPSC amplitudes. It thus seems plausible to expect a subtle shift in the fine-tuning of the mechanisms mentioned above during higher network activity, but this hypothesis would have to be proven. We did this and indeed we found alterations in network properties that resembled those of slices from epileptic mice and importantly also in the context of the whole animal, we observed increased seizure susceptibility.*

*Also, it should be noted that loss of effector proteins in SV recycling does not necessarily lead to altered network activities and seizures. To our knowledge, in this context, seizures were thus far only observed for bassoon, amphiphysin, SV2A, AP3B and synapsin KO mice.*

Much of the writing needs to be changed. For example, for a scientific article the sentence in the abstract ""F-BAR proteins are predestined to serve as membrane curvature sensors and/or inducers and may thereby support membrane remodeling processes, yet, their in vivo functions urgently await disclosure." 'Predestincation' is a religious term, and really doesn't belong here, and it is hard to imagine that proteins are just there waiting for us to study them ....

All of these problems can be easily fixed, and overall this is a very interesting study that would fit into EMBO J.

*We thank the referee for his/her suggestion on the manuscript style. The word "predestined" has been deleted from the abstract of the revised manuscript. The sentence now simply reads "FBAR proteins may serve as membrane curvature sensors and/or inducers...". We hope that the referee appreciates the more revised version of the manuscript.*

2nd Editorial Decision

01 August 2011

Thank you for sending us your revised manuscript. Our original referees 2 and 3 have now seen it again, and you will be pleased to learn that in their view you have addressed all criticisms in a satisfactory manner and that the manuscript will ultimately be publishable in The EMBO Journal.

Still, there are a number of editorial issues that need further attention before we can formally accept the manuscript:

\* Please include an author contributions section as well as a conflict of interest statement into the main body of the manuscript text after the acknowledgements section.

\* Please include the number of independent repeats into the legends of figures 1F, 3D, 3E, 3G, 7B, 7C, 7E, 7F, 7H, 7J.

\* Please include scale bars together with explanations for figures 2A, 2B, S2A. Could you also provide us with an explanation of the scale bars in the figure for referee 2, please, as this will be needed for the Peer Review Process File?

\* Prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication and explanation in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) and/or explanation in the figure legend to ask for the original scans. In the case of the present submission there are a number of panels that do not fully meet these requirements: figure 1D (do all lanes come from the same gel?), 6B (do all lanes come from the same gel?), S7 (bottom panel). I therefore like to kindly ask you to include suitably amended versions of these figures and/or figure legends in the final version of this manuscript. Please be reminded that according to our editorial policies we also need to see the original scans for the figures in question.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final version of the paper.

Thank you very much for your cooperation.

Yours sincerely,

Editor  
The EMBO Journal



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REFeree COMMENTS

Referee #2

This is a complete analysis of the phenotype of syndapin 1 KO mice with interesting implications for presynaptic physiology. The data are of excellent quality. I thus enthusiastically support publication of this work in the EMBO J.

Referee #3

The paper is now acceptable for publication, although I still believe the title is misleading and does not serve the purpose of the paper well.

2nd Revision - authors' response

19 August 2011

Thank you for the very positive response on our submission of the revised manuscript "*Proper synaptic vesicle formation and neuronal network activity critically rely on syndapin I*". We are very pleased to learn that the reviewers are fully content with our work.

We shall be happy to herewith address the few remaining editorial issues raised in your decision mail from August 1st.

1. *Author contributions* and a *conflict of interest statement* have now been added. Sorry that we forgot this.
2. The number of independent experiments in Fig. 1, 3 and 7 have now been added directly to the respective panels of figures. As we have documented the numbers of individual experiments in all other figures in a similar manner, we hope that you will agree with us that having this additionally information directly in the figure is preferable over inserting the requested information into the legends.
3. Scale bars have been added to the brain images shown in Fig. 2A and B and to the Manganese-enhanced magnetic resonance imaging of mouse brains shown in Fig. S2A. The scale bars in the figure for referee 2 correspond to 100 nm. We apologize for the missing information.
4. The data shown in the panels 1D, 6B and S7 are of course from the same gels. In part, the order of samples had been changed and/or intercalating lanes were omitted. Therefore, we inserted the white lines into these panels. We apologize that we did not explain this in the legends. This information has now been added to the legends of figure 1, 6 and S7 of the revised manuscript.
5. We have compiled the original data that was used for the three panels 1D, 6B and S7. Please see the merged PDF "*Koch\_Suppl2\_Original\_Data\_Fig1D\_6B\_S7.pdf*". The data is labeled and we herewith submit it as additional Supplementary Data File (publishable). We hope that our study will now be found acceptable for publication in *The EMBO Journal* and are looking forward to hearing from you,