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Novel Diffusion Barrier for Axonal Retention of Tau in Neurons and its Failure in Neurodegeneration

Xiaoyu Li, Yatender Kumar, Hans Zempel, Eva-Maria Mandelkow, Jacek Biernat and Eckhard Mandelkow

Corresponding author: Eckhard Mandelkow, Max-Planck-Society

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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.)

1st Editorial Decision 13 May 2011

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below. As you can see the referees find the analysis interesting, but also find that that some further experimental data is needed in order to consider publication here. The concerns raised are clearly outlined below. Referee #3 raises some issues with the time points that the images were taken at and some inconsistencies across the experiments. I presume that you have a reasonable explanation for the chosen time points and if so a careful explanation of this issue should be sufficient.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. 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 address the raised concerns 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

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

Yours sincerely,

Editor

The EMBO Journal

REFEREE REPORTS

Referee #1

The polarized distribution of tau has been a long enigma in the field of neuronal cytoskeleton, and is now proposed to be related to the pathology of Alzheimer disease. In this manuscript, the authors report a potentially interesting behavior of tau at the axonal initial segment (AIS). The overexpressed tau protein can move through AIS from the cell body to the axon, but not from the axon to the cell body. This rectified behavior of tau requires intact microtubules and binding affinity of tau to the microtubules, suggesting that the movement of tau would be rectified through the interaction between tau and microtubules at AIS. This would suggest a novel barrier function of AIS to maintain the neuronal polarity, and would be of potential interest to the researchers in this field. However, the results presented in this manuscript are too premature to draw conclusions on the identity of this potentially new barrier function as well as the molecular mechanism how tau moves within the axons.

1. As the authors themselves concern in the text, the labeled tau protein is expressed at extremely high levels in all the experiments, so that both authentic and exogenous tau proteins are missorted as shown in Fig 8. This problem on the experimental design poses a critical concern on the physiological relevance of the results of this study. The observed rectifying behavior could be caused by the presence of the large excess amount of tau protein, which can saturate the physiological sorting machinery and/or the physiological binding sites. The authors should demonstrate that the same diffusion barrier works at AIS even with the expression level close to the physiological level, in conditions that both labeled and authentic tau proteins are sorted correctly.

2. The authors believe that all of tau movement is caused by "diffusion" but is this really true? Some active transport mechanism might be involved to cause this microtubule-dependent rectifying behavior of AIS. It would be possible to observe what form of tau (membrane-bound or microtubule-bound) is anterogradely transported from the AIS depending on microtubules. In addition to the hypothesis by Baas and colleagues, it would be also possible that tau-bound membrane organelles are transported along with microtubule by molecular motors. Does inhibition of molecular motors by nucleotide analogues and/or kinesin antibodies also inhibit the AIS barrier?

3. Most of the results lack statistics or quantification. Some results are apparently affected by artifacts, or could be biased, as listed below. Furthermore, the authors show only one result for each experiment except for Fig 7D. Some statistics with quantitative analyses would strengthen the authors' conclusion.

4. Fig 1. The authors used confocal microscope, but the size of the axon is below the axial resolution (thickness of optical section). The bias by the different thickness of the sample can be corrected by taking the ratio between red and green channels. For example, in Fig2 A1, the cell body is brighter than ROI2, which cannot happen with simple diffusion. The inset of A1 suggests that this abnormal result would reflect the artifact caused by the differences in the thickness of the cell body and the axon. The authors should show both raw and ratio images to avoid this artifact.

5. Fig 2. The cell body shows very strong signal in C1, which might be the above mentioned artifact. Furthermore, the photoconversion area is too close to the cell body. Thus, it is almost impossible to assess whether there are similar barrier at the root of dendrites. The authors should try photoconversion at more distal dendrites. The authors should also try photoconversion only at the AIS, which would suggest some mechanistic insights of this rectifying barrier.

6. Fig 3. Does the time course correlate with the microtubule depolymerization by nocodazole? Considering the relatively slow diffusion of dendra2-tau, the barrier would have been disrupted much earlier than t=105 min. The authors should examine lower or higher concentrations of nocodazole, and compare the microtubule disruption kinetics with the barrier disruption kinetics. In some studies, application of taxol affects the axonal sorting. The authors might want to try this perturbation as well.

7. Fig 4. It is unclear whether the position of the diffusion barrier corresponds to AIS. The authors should stain AIS after photoconversion in the axon.

8. Fig 5. In nocodazole treatment, the axon was photoactivated once. Here, the second photoactivation follows the drug application. Why do the authors use different protocols? The second activation would increase the signal, so that the results can be biased.

9. Fig 6. Does the OA treatment affect the diffusion of tau-4KXGA? This would serve as a good control experiment.

Referee #2

In this manuscript, Li and colleagues of the Mandelkow's laboratory address a long-standing and crucial cell biological/ neurobiology question: spatial restriction of cytoskeletal proteins (tau in this case). This question acquired, in more recent times, relevance to brain pathology, due to the miss-distribution of tau in the neurons of individuals affected by AD. While here authors do not even attempt to investigate to which extent miss-distribution is cause or consequence in this pathology, they do use the AD to justify the notion that mechanisms of retention are affected and therefore ought to exist. Since there is no mention to the disease, it is my recommendation that any reference to it is reduced. The subject of tau restriction to axons is most interesting per se and so there is little need to justify its study based on the miss-localization in a conditions for which there is no clear view of cause-consequence relationship. In addition to the relevance of the question, authors make use of relevant and appropriate and state-of-the-art experimental tools. Thus, Tau diffusion in given axonal segments is not analyzed after bleaching but after activation of photoconvertible GFP. Trhough this, appropriate quantification procedures and the use of pharmacological treatments and tau mutants it is reached the conclusion that tau restriction to axons is due to the existence of a retrograde diffusion barrier. Although most of the results are consistent with this view, some conceptual and experimental clarification and additions are needed before I can recommend acceptance in The EMBO Journal.

1) Authors seem to be concerned about the presence of tau in dendrites after transfections. My recommendation is that they should point out that axonal sorting is tight and precise and therefore that any excess of a cargo, whether a membrane protein or a cytoskeletal, may result in the saturation of the cells' sorting capacity. In fact, authors are expressing high levels of a "cargo" but not the natural complement of molecules involved in the tightness of sorting. This has been observed in many occasions, especially with axonal proteins. The sentences spent to explain this result (missorting into dendrites) reduces the flow of the text in the "right" direction.

2) This is a matter of discussion and I am willing to accept authors explanations but, to me, the term barrier has a "physical" connotation (like that made from the clustering of ankyrins and sodium channels on the plasma membrane of the AIS). Therefore, given that no hints that a post-translational modification are a true physical barrier, capable of preventing the axonal accumulation of other cytoskeletal or other proteins (see below) I would suggest to authors to reconsider the title and final message of the paper, perhaps orienting it towards a more simple still very relevant concept, such as the mechanisms governing the axonal retention of tau.

3) Authors need to define the limit of the mechanism behind tau's impossibility to move retrogradely: is it restricted to the first 50 micometers or is also operative at more distant places? I would like to see data in this regard.

4) The experiment with OA is not very informative because of lack of specificity. The use of mutants make the OA experiment un-necessary. The use of mutants can be easily justified ,independently from any OA result (that type of experiment is something one does in the lab but there is no need to show in a final publication).

5) The paper would benefit by showing the relationship between breaking down the barrier for tau retrograde flow and membrane carrier transport specificity (i.e. does it increase dendritic cargoes missorting?).

Conclusion: excellent piece of work, of high conceptual impact. I oreover, paper very nicely written, unconventional and logical. A few clarifications and however required before I can recommend final acceptance.

Referee #3

This paper claims to identify a barrier restricting the retrograde trafficking and/or diffusion of axonal Tau. This is an interesting idea, and potentially important as a way to help explain how Tau becomes missorted. The concept that the AIS functions as a regulator of polarity, and that this can be disrupted in disease situations like Alzheimer's, makes this study important and builds on other studies showing AIS dysfunction after disease or injury. It is not completely novel though, as the existence of a diffusion barrier at the AIS has been reported by many different labs. What is new, is that this is claimed to be a retrograde diffusion barrier. Although I'm generally fairly enthusiastic about the concepts and ideas, I find myself unconvinced by the data and have several suggestions for improvement. I list these in no specific order of significance or importance.

1. In figure 2, the authors should show what Dendra staining looks like at t=-1 min. Also, the figures included show dendra staining at 5 min - I see no staining in the region of the AIS, and the noise in panel A4 is very high.
2. All the times analyzed should be the same. For example Tau-dendra axonal photoconversion in panel 2B3 is shown at t=30 min, but analysis in Fig. 3 claiming to show the dependence on microtubules is shown for t=75 and 105 minutes. The control Tau-dendra should also be shown at t=75 and 105 minutes. Similarly, the experiments with the mutant Tau proteins (Fig. 6) should be analyzed at 75 and 105 minutes, not 30 minutes.
3. Furthermore, the length of axon that is photoconverted is not equivalent in all of these experiments. This should be held constant.
4. Figure 1E has been modified to 'mask' out another neuron. This should be stated in the text - or more correctly the original rather than the 'doctored' image should be shown.
5. The experiments with LatA do not address the dependence of this process on F-actin since the authors do not demonstrate that their treatment disrupts the F-actin at the AIS. There are pools of F-actin not susceptible to disruption. Similarly, the authors state in the results and discussion that the assembly/maintenance of all other AIS components is F-actin dependent. This is not true. Indeed, Brachet et al. (2010) demonstrate that the diffusion barrier (actin-dependent) forms after the clustering of ankG and all other membrane proteins.
6. experiments with the mutant Tau constructs should have half-times measured both proximal and distal to the region of photoconversion (and the proximal region should be outside of where the AIS would be expected to be found).
7. What are the times waited after photoconversion in Figs. 6E and 6F.

Overall, the concept is interesting, but the experiments to show it is F-actin independent are not convincing, and the images demonstrating exclusion from the AIS are not convincing since they are all done at different time points.

1st Revision - authors' response 26 August 2011

Referee #1 (Remarks to the Author):

The polarized distribution of tau has been a long enigma in the field of neuronal cytoskeleton, and is now proposed to be related to the pathology of Alzheimer disease. In this manuscript, the authors report a potentially interesting behavior of tau at the axonal initial segment (AIS). The overexpressed tau protein can move through AIS from the cell body to the axon, but not from the axon to the cell body. This rectified behavior of tau requires intact microtubules and binding affinity of tau to the microtubules, suggesting that the movement of tau would be rectified through the interaction between tau and microtubules at AIS. This would suggest a novel barrier function of AIS to maintain the neuronal polarity, and would be of potential interest to the researchers in this field. However, the results presented in this manuscript are too premature to draw conclusions on the

identity of this potentially new barrier function as well as the molecular mechanism how tau moves within the axons.

1. As the authors themselves concern in the text, the labeled tau protein is expressed at extremely high levels in all the experiments, so that both authentic and exogenous tau proteins are missorted as shown in Fig. 8. This problem on the experimental design poses a critical concern on the physiological relevance of the results of this study. The observed rectifying behavior could be caused by the presence of the large excess amount of tau protein, which can saturate the physiological sorting machinery and/or the physiological binding sites. The authors should demonstrate that the same diffusion barrier works at AIS even with the expression level close to the physiological level, in conditions that both labeled and authentic tau proteins are sorted correctly.

>Answer< We agree with the referee that high expression of proteins can cause artefacts. But we would argue that in our case the expression of tau is not extremely high, as the referee assumes. We have now performed further tests to lower the expression of tau by reducing the amount of transfected DNA and the period before observation. This was done down to the point where the fluorophore Dendra2 was no longer visible by fluorescence and could only be detected by antibodies against Dendra2, making the concentration roughly comparable with that of endogenous tau (Fig. S8). Even in these conditions we find that exogenous and endogenous tau become missorted into soma and dendrites, in addition to its axonal spreading. This supports the view that the sorting machinery is operating near its capacity, and even small amounts of overloading cause a "spillover" into the wrong compartments. Nevertheless, once tau becomes axonal it is restricted by the retrograde barrier, in contrast to dendritic tau which is not restricted.

2. The authors believe that all of tau movement is caused by "diffusion" but is this really true? Some active transport mechanism might be involved to cause this microtubule-dependent rectifying behavior of AIS. It would be possible to observe what form of tau (membrane-bound or microtubule-bound) is anterogradely transported from the AIS depending on microtubules. In addition to the hypothesis by Baas and colleagues, it would be also possible that tau-bound membrane organelles are transported along with microtubule by molecular motors. Does inhibition of molecular motors by nucleotide analogues and/or kinesin antibodies also inhibit the AIS barrier?

>Answer< On the basis of FRAP experiments it was found earlier that Tau propagation over short and intermediate distances is dominated by diffusion (Samsonov and Popov 2004, Konzack et al. 2007), and in this study we confirmed that the propagation of exogenous Tau is also mainly diffusion dependent (new Fig. S8b). Active transport is superimposed on diffusion and is likely caused by the cotransport of tubulin oligomers with Tau (Baas et al. 2006). However it is unlikely that membrane bound organelles are responsible for the transport of Tau (Myers et al., Baas 2006). We added an analysis of different Tau constructs and investigated whether there is a difference in anterograde or retrograde movement, and found that strong microtubule binding of Tau modulates diffusion, which reflects the slow transport of a diffusible protein (Tau) on a carrier structure (tubulin oligomer) (see new Fig. S7, and the analogous case for neurofilament proteins, Scott et al., Roy 2011).

With regard to the nature of the motor proteins we believe that this question cannot be addressed with the given experimental model, (a) because nucleotide analogues do not penetrate into the cell, and they have multiple cellular targets, not just motors, (b) a number of different motors contribute to movements of cytoskeletal elements in the axon (dynein, myosin, different kinesins (Myers et al., Baas 2006)). Clarification of this issue would therefore far beyond the scope of this paper.

3. Most of the results lack statistics or quantification. Some results are apparently affected by artifacts, or could be biased, as listed below. Furthermore, the authors show only one result for each experiment except for Fig 7D. Some statistics with quantitative analyses would strengthen the authors' conclusion.

>Answer< We added statistical analysis in Figure 7D. A movie for the nocodazole experiment was added (Movie 6). Other representative experiments were also added (Movies 1-5) to address the points listed below (see supplement). Additionally, we quoted in the text and in the figure legends

that the experiments shown are representations of several experiments (at least 3). Further statistics was added in Fig. S7.

4. Fig 1. The authors used confocal microscope, but the size of the axon is below the axial resolution (thickness of optical section). The bias by the different thickness of the sample can be corrected by taking the ratio between red and green channels. For example, in Fig2 A1, the cell body is brighter than ROI2, which cannot happen with simple diffusion. The inset of A1 suggests that this abnormal result would reflect the artifact caused by the differences in the thickness of the cell body and the axon. The authors should show both raw and ratio images to avoid this artifact.

>Answer< We chose the confocal setup because it makes analysis with high axial and lateral resolution possible. In addition, we can define the regions of photoactivation with high precision. Since the volume of the cell body is by far bigger than the volume of the axon, ratiometric analysis would only strengthen our argument that Tau gets enriched in the axon, and is retained there. To convince readers of the efficiency of our photoconversion and the usability of our analysis system, we appended Fig. S1, with an example of a ratiometric analysis and quantification of the average efficiency of photoconversion.

5. Fig 2. The cell body shows very strong signal in C1, which might be the above mentioned artifact. Furthermore, the photoconversion area is too close to the cell body. Thus, it is almost impossible to assess whether there are similar barrier at the root of dendrites. The authors should try photoconversion at more distal dendrites. The authors should also try photoconversion only at the AIS, which would suggest some mechanistic insights of this rectifying barrier.

>Answer< We examined photoconversion in distal dendrites and found that photoconverted Tau moves rapidly from the dendrites into the cell body and other dendrites (see new Movie 5), and gets sorted into the axon as well (new Figure S4). Photoactivation in the AIS directly showed that Tau moves into the axon and is retained there (new Fig. S2).

6. Fig 3. Does the time course correlate with the microtubule depolymerization by nocodazole? Considering the relatively slow diffusion of dendra2-tau, the barrier would have been disrupted much earlier than t=105 min. The authors should examine lower or higher concentrations of nocodazole, and compare the microtubule disruption kinetics with the barrier disruption kinetics. In some studies, application of taxol affects the axonal sorting. The authors might want to try this perturbation as well.

>Answer< The barrier remains stable as long as microtubules remain intact, but breaks down within 3-5min minutes after nocodazol treatment, emphasizing the requirement for intact microtubules to maintain the barrier. This correlates well with the rate of microtubule breakdown (~3 min; as shown previously by Baas et al. 1991) after addition of nocodazol in our conditions. To maintain consistency, we have now replaced the previous Fig. 3 (observation time from t=75 to t=105 min after nocodazol treatment) with a new figure (observation time from t=30 to t=45 min after nocodazol). We also appended the movie of the experiment as Movie 6.
Regarding taxol, we did not include this in our current study since its effects on Tau and microtubules are too diverse. Apart from stabilizing microtubules taxol has a number of other targets in the cell and strongly affect sorting of cell components (Mattson et al. 1992, Witte et al., Bradke 2008).

7. Fig 4. It is unclear whether the position of the diffusion barrier corresponds to AIS. The authors should stain AIS after photoconversion in the axon.

>Answer< We added an experiment (supplement) where we photoactivated, conducted an AnkyrinG staining, reidentified the photoactivated cells and imaged. It shows that the diffusion barrier is efficient within the region of the canonical AIS defined by AnkyrinG staining (Fig. S6).

8. Fig 5. In nocodazole treatment, the axon was photoactivated once. Here, the second photoactivation follows the drug application. Why do the authors use different protocols? The second activation would increase the signal, so that the results can be biased.

>Answer< A second photoactivation was initially used in order to increase the signal. However, in controls, repeated activations did not change the outcome of the experiment, i.e. Tau was retained in the axon (as can also be seen in the new Fig. S3). Still, for consistency, we replaced the earlier nocodazole experiment by a new one and appended the experiment as a movie (Movie 6, supplement).

9. Fig 6. Does the OA treatment affect the diffusion of tau-4KXGA? This would serve as a good control experiment.

>Answer< OA is a general phosphatase inhibitor, with highest affinity for PP2A, followed by PP1 and PP2B. OA also induces rapid missorting of KXGA-Tau, likely due to phosphorylations at many different epitopes, many of which can influence the structure and binding affinity to microtubules of Tau (see e.g. Lichtenberg-Kraag et al. 1992). This is analogous to the multiple pseudo-phosphorylation in the htau40-17EP construct, where the barrier breaks down as well (Fig. 7d, and new Fig. S7).

Referee #2 (Remarks to the Author):

In this manuscript, Li and colleagues of the Mandelkow's laboratory address a long-standing and crucial cell biological/ neurobiology question: spatial restriction of cytoskeletal proteins (tau in this case). This question acquired, in more recent times, relevance to brain pathology, due to the miss-distribution of tau in the neurons of individuals affected by AD. While here authors do not even attempt to investigate to which extent miss-distribution is cause or consequence in this pathology, they do use the AD to justify the notion that mechanisms of retention are affected and therefore ought to exist. Since there is no mention to the disease, it is my recommendation that any reference to it is reduced. The subject of tau restriction to axons is most interesting per se and so there is little need to justify its study based on the miss-localization in a conditions for which there is no clear view of cause-consequence relationship. In addition to the relevance of the question, authors make use of relevant and appropriate and state-of-the-art experimental tools. Thus, Tau diffusion in given axonal segments is not analyzed after bleaching but after activation of photoconvertible GFP. Trhough this, appropriate quantification procedures and the use of pharmacological treatments and tau mutants it is reached the conclusion that tau restriction to axons is due to the existence of a retrograde diffusion barrier. Although most of the results are consistent with this view, some conceptual and experimental clarification and additions are needed before I can recommend acceptance in The EMBO Journal.

1) Authors seem to be concerned about the presence of tau in dendrites after transfections. My recommendation is that they should point out that axonal sorting is tight and precise and therefore that any excess of a cargo, whether a membrane protein or a cytoskeletal, may result in the saturation of the cells' sorting capacity. In fact, authors are expressing high levels of a "cargo" but not the natural complement of molecules involved in the tightness of sorting. This has been observed in many occasions, especially with axonal proteins. The sentences spent to explain this result (missorting into dendrites) reduces the flow of the text in the "right" direction.

>Answer< We agree with the reviewer that the axonal sorting machinery is tight and easily saturated (see reply to referee 1 above). To streamline the text flow we have now changed the wording to put more weight on the axonal distribution and diffusion of Tau. The additional experiments conducted to investigate Tau diffusion and propagation in the distal axon (Fig. S3), in the AIS (Fig. S2), the anterograde and retrograde diffusion of photoconverted TauD2 (quantified in Fig. S7), and the distribution of expressed Tau over time (Fig. S8) also serves this purpose.

2) This is a matter of discussion and I am willing to accept authors explanations but, to me, the term barrier has a "physical" connotation (like that made from the clustering of ankyrins and sodium channels on the plasma membrane of the AIS). Therefore, given that no hints that a post-translational modification are a true physical barrier, capable of preventing the axonal accumulation of other cytoskeletal or other proteins (see below) I would suggest to authors to reconsider the title and final message of the paper, perhaps orienting it towards a more simple still very relevant concept, such as the mechanisms governing the axonal retention of tau.

>Answer< We used the term barrier due to the fact that a similar term has been used before in the field of AIS research (Song & Poo 2009, and others). Apart from that, the term barrier does not need to be a physical structure, it could also be the result of a kinetic process or a change in interactions. For this reason we feel that the term barrier is a good representation of the observations. We now take this point up in the discussion. We also changed the title to emphasize the axonal retention aspect of the barrier.

3) Authors need to define the limit of the mechanism behind tau's impossibility to move retrogradely: is it restricted to the first 50 micometers or is also operative at more distant places? I would like to see data in this regard.

>Answer< To address whether the limitation of Tau to move retrogradely is restricted to the proximal axon, we photoconverted in more distal areas and found that there is no general restriction for tau to move retrogradely. Tau can move in the axon back all the way to the AIS, but not further, emphasizing that the blockage of Tau diffusion is specific for the AIS. To illustrate this we appended a figure with photoactivation of Tau-dendra2 in distal axons (new Fig. S3).

4) The experiment with OA is not very informative because of lack of specificity. The use of mutants make the OA experiment un-necessary. The use of mutants can be easily justified, independently from any OA result (that type of experiment is something one does in the lab but there is no need to show in a final publication).

>Answer< This recommendation of referee 2 is opposite to that of referee 1. We would argue that OA is an established model for Tau hyperphosphorylation and induction of AD-like states since OA treatment induces many different phosphorylations on Tau. There is also a debate in the field that pseudophosphorylations do not mimick phosphorylations properly. For this reason, and since reviewer 1 also proposed more OA experiments, we decided to retain the OA data in the paper.

5) The paper would benefit by showing the relationship between breaking down the barrier for tau retrograde flow and membrane carrier transport specificity (i.e. does it increase dendritic cargoes missorting?).

>Answer< We have shown earlier that elevated Tau bound to microtubules tends to slow down microtubule based transport of vesicles and organelles, both in axons and dendrites (Stamer et al. 2002, Thies and Mandelkow 2007). This can be relieved by phosphorylation of Tau by several kinases (Thies and Mandelkow 2007). In this paper we wanted to focus on the compartmentalization of Tau per se, we do show here however that dendritic sorting of MAP2 is not affected in our paradigm (Fig. 8).

Conclusion: excellent piece of work, of high conceptual impact. Moreover, paper very nicely written, unconventional and logical. A few clarifications and however required before I can recommend final acceptance.

>Answer< We appreciate the positive remarks and the constructive criticism. We addressed and clarified the points brought to our attention by the reviewer.

Referee #3 (Remarks to the Author):

This paper claims to identify a barrier restricting the retrograde trafficking and/or diffusion of axonal Tau. This is an interesting idea, and potentially important as a way to help explain how Tau becomes missorted. The concept that the AIS functions as a regulator of polarity, and that this can be disrupted in disease situations like Alzheimer's, makes this study important and builds on other studies showing AIS dysfunction after disease or injury. It is not completely novel though, as the existence of a diffusion barrier at the AIS has been reported by many different labs. What is new, is that this is claimed to be a retrograde diffusion barrier. Although I'm generally fairly enthusiastic about the concepts and ideas, I find myself unconvinced by the data and have several suggestions for improvement. I list these in no specific order of significance or importance.

1. In figure 2, the authors should show what Dendra staining looks like at t=-1 min. Also, the figures included show dendra staining at 5 min - I see no staining in the region of the AIS, and the noise in panel A4 is very high.

>Answer< There is no signal in the red channel at t=-1 min. A neuron that is not photoconverted can be seen in figure 1. We put an additional mark (asterisk) on that neuron, and added an explanation in the figure legend. Additionally, we included a t=-1min image in Fig. S1, panel A.

2. All the times analyzed should be the same. For example Tau-dendra axonal photoconversion in panel 2B3 is shown at t=30 min, but analysis in Fig. 3 claiming to show the dependence on microtubules is shown for t=75 and 105 minutes. The control Tau-dendra should also be shown at t=75 and 105 minutes. Similarly, the experiments with the mutant Tau proteins (Fig. 6) should be analyzed at 75 and 105 minutes, not 30 minutes.

>Answer< In general, results do not differ depending on the time points of the imaging (regarding the presence or absence of a barrier). Nevertheless, we replaced the figure with an experiment with similar results that was conducted at the time points suggested by the reviewer.

3. Furthermore, the length of axon that is photoconverted is not equivalent in all of these experiments. This should be held constant.

>Answer< For the purpose of comparing the different tau constructs shown in Fig. 7D we held the area of photoconversion constant (also see new Fig. S7).

4. Figure 1E has been modified to 'mask' out another neuron. This should be stated in the text - or more correctly the original rather than the 'doctored' image should be shown.

>Answer< The Figure 1E has not been doctored, and no neuron was masked. The misunderstanding might come from the fact that only one neuron was photoconverted, which therefore appears in the red channel. However, both neurons in the field are apparent in the green channel which shows transfected neurons prior to photoconversion. The neuron that is not photoconverted can be considered as a control. We put an additional mark (asterisk) on that neuron.

5. The experiments with LatA do not address the dependence of this process on F-actin since the authors do not demonstrate that their treatment disrupts the F-actin at the AIS. There are pools of F-actin not susceptible to disruption. Similarly, the authors state in the results and discussion that the assembly/maintenance of all other AIS components is F-actin dependent. This is not true. Indeed, Brachet et al. (2010) demonstrate that the diffusion barrier (actin-dependent) forms after the clustering of AnkG and all other membrane proteins.

>Answer< We concur that it is important to show that the LatA treatment disrupted the F-actin. We therefore repeated the LatA treatments and stained afterwards with phalloidin to visualize F-actin, as well as AnkyrinG. The data show that treatment with LatA efficiently and globally disrupted F-actin (Fig. S5).

Concerning our previous statement that the AIS is F-actin dependent, we find that comparison of the different papers published on the composition of the AIS shows that different components are subject to different assembly kinetics and developmental stages. Thus the paper by Brachet et al. (2010) focuses on membrane-associated components, whereas others (e.g. Song et al., 2009, and ourselves) focus on cytosolic components. F-actin may not be necessary for the assembly of all components, but it is nevertheless recognized as one of the key features of the AIS, especially with regard to the cytosolic assemblies. In order to resolve the issue we have now changed the wording to emphasize that we focus on cytosolic components rather than membrane-bound components of the AIS.

6. Experiments with the mutant Tau constructs should have half-times measured both proximal and distal to the region of photoconversion (and the proximal region should be outside of where the AIS would be expected to be found).

>Answer< We added an analysis of the proximal regions outside the AIS (Fig. S7) and found that there is diffusion of tau retrogradely, and to similar extents as anterogradely, in agreement with our

previous study using a FRAP-based approach (Konzack et al. 2007). However, we also found that constructs that have a high affinity for microtubules have a somewhat shorter half time (i.e. faster spreading) in the anterograde direction. We believe that this is due to the fact that at this stage the axon is still growing, requiring constant anterograde growth and transport of microtubules (Baas and Buster 2004; Baas et al. 2006), and Tau tugs along (Konzack et al. 2007). This effect is superimposed on that of diffusion.

7. What are the times waited after photoconversion in Figs. 6E and 6F.

>Answer< The time points are the same as in the other experiments, we now included them in the figures.

Overall, the concept is interesting, but the experiments to show it is F-actin independent are not convincing, and the images demonstrating exclusion from the AIS are not convincing since they are all done at different time points.

>Answer< Although we found that the different timepoints do not change the essential outcome of the experiment, we replaced the figure which showed different incubation lengths (Fig. 3, nocodazole experiment). We added experiments with Latrunculin A treatment and F-actin staining and found that, in addition to maintaining the Tau diffusion barrier, F-actin staining was dramatically decreased, including in the AIS region (Fig. S5).

2nd Editorial Decision 21 September 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I have asked the original three referees to review the manuscript and have now received their comments. As you can see below, the referees appreciate the introduced changes and support publication in the EMBO Journal. I am therefore very pleased to proceed with the acceptance of your paper for publication in the EMBO journal.

You will receive the formal acceptance letter shortly. Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

In this revision the authors have performed additional experiments along with the line of previous reviewers' suggestions, which considerably improved the manuscript. Now this reviewer recommends acceptance in the EMBO Journal.

Referee #2

The authors have satisfactorily addressed my concerns, conceptual and experimental, and therefore find this work highly suitable for The EMBO Journal. The new data showing diffusion of tau in distal axons convincingly shows that the phenomenon of restriction is spatially defined. Moreover, the new data further showing missorting of tau even under conditions where the exogenous protein is not in great excess (roughly) ought to be taken as a simple consequence of saturation of the sorting machinery. I understand that these new experiments obey to the request of reviewer No. 1 yet, from my experience in the field of axonal sorting I have to agree with the authors in that axonal sorting is a rather difficult to study process and that spill-over to other domains (dendrites) will likely happen. In any event, the important point of the work is that axonal tau is retained in the axon

by a mechanical/dynamic barrier at the AIS. To me, this is the main contribution of this work. It will be a venue for future research to determine the mechanism responsible for tau entry into axons, specifically. Finally, I thought the OA acid experiments were not informative. On the other hand, Reviewer No. 1 thought this aspect had to be further clarified. To me, the work with the phpspho mutants is far more precise to address the issue of phosphorylation. Not only more precise but also because the use of OA today somewhat takes away part of the technological "grandeur" of this work. In any event, I leave this decision to the authors.

Referee #3

This is a very interesting and timely paper that identifies a novel aspect of the AIS diffusion barrier, namely that it can also function to prevent retrograde transport of certain proteins. This has not been reported previously and should be of interest to any neuroscientists and cell biologists since the function, assembly, and role of the AIS has been the focus of much attention in recent years.

The authors have been responsive to my concerns and have addressed my concerns in this revised version.

