Novel function of Tau in regulating the effects of external stimuli on adult neurogenesis

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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

04 January 2016

Thanks for submitting your manuscript to the EMBO Journal. I am sorry about the delay in getting back to you, but due to the Xmas break things got a bit delayed. Your study has now been seen by two experts and their comments are provided below.

As you can see, the referees find the analysis interesting and are supportive of the work for consideration here. They raise a number of specific and constructive comments that I anticipate you should be able to resolve. Given the positive feedback from the referees, I would like to invite you to submit a suitable revised version of the manuscript, addressing the concerns raised. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the issues raised at this stage.

Regarding referee #2's suggestions for improving the study (at the editor’s discretion) - see below. As the referee indicates point 2 is beyond the scope of this revision, but point 1 is an interesting one and should be doable. Do you have data on hand or would you be able to address this question? We can discuss this point further.

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://emboj.embopress.org/about#Transparent_Process

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

Referee #1:

Review of Pallas-Bazarra et al., 2015, EMBOJ-2015-93518

This interesting article by Pallas-Bazarra et al. addresses important questions related to the role of tau protein on adult hippocampal neurogenesis. In part the paper confirms findings of other groups that have created tau knockout mice (Dawson et al., 2001; Harada et al., 1994) in that loss of tau is related to subtle but potentially important changes in brain development. The authors go beyond this by identifying the cell types and consequences where tau may matter most, such as adult hippocampal neurogenesis and the responses to stress and environmental enrichment.

Comments:

1. The authors should include higher resolution techniques to confirm their findings related to the formation of postsynaptic densities (typically submicron long structures) and mossy fiber terminals in newly born granule cells. It would be appropriate to include electron microscopy to further strengthen the observations.

2. The authors should perform a more rigorous analysis of GABAergic interneurons and their involvement in mediating the pro-neurogenic effects of environmental enrichment in their animal model. Providing an immunofluorescence-based quantification of a series of overexposed images may not be considered sufficient experimentally-based evidence. The authors should include different means of quantification, such as Western blotting and/or qPCR from microdissected fresh tissue.

3. The interpretation of the findings seems to alternate between neurodegeneration and neurodevelopment at several places throughout the manuscript which appears somewhat confusing. The authors intensively discuss the work by Ittner et al., but provide no solid link to it. Neurodegenerative diseases cannot be simply described in the terms of loss of function of a certain protein, there are multilevel events underlying their development. I would recommend concentrating the interpretation in the neurodevelopmental area and including neurodegeneration perhaps briefly in discussion.

4. In this context, the importance of tau is exaggerated in various places by using wording such as crucial, critical, central, necessary, etc. The fact is that the effects of tau are quite subtle, as already described by Hirokawa's group (Harada et al. 1994) and confirmed here. It would also be appropriate to add cautionary notes, for example a correlation does not necessarily mean causality (example on p. 6 bottom).

4. The way how individual figure panels and results are organized could be improved. For example, Figure 6 is divided between two subheadings in results. Furthermore, some paragraphs describing results read more as figure legends. Figure 1 is another example of a strange labelling. The panel 'a' should be labelled the same way as the panel 'b', outside of the image frame rather than on the inside etc. At first glance it may look as if the panel 'a' is missing.

5. Statistical analysis should be described in detail for each condition. In some experiments only three animals were used per experimental group (retroviral injections). The authors should increase the animal number where needed.

Minor points:

- Add figures numbers at top of figures to identify them properly

- Check spelling, there are a number of typos. Entorhinal, RIPA buffer, perforant path, altogether, isoflurane,.....

- Page 14, sentence starting with 'Interestingly' is grammatically incomplete.
Referee #2:

Tau is a protein that is enriched in axons, but also serves important roles in the dendrite. Previously it has been shown that tau depletion delays axonal extension and maturation. Here, the authors focus on hippocampal granule neuron cells in the dentate gyrus, where neurogenesis occurs to show a role for tau in neurogenesis. The papers looks at basal conditions, two forms of stress and environmental enrichment to determine how the absence of tau affects dendritic length, dendritic complexity, and PSD area (as a proxy for spine size). The paper is generally well written, the experiments are technically well done (I have only one comment for one experiment, see below) and the figures are beautifully grafted. The data are very interesting and the system is attractive to dissect the underlying molecular mechanism.

General comments:

I made myself a list of under which conditions (basic, acute stress, Porsolt test, EE) removing tau is good or bad:

(i) Basic conditions: Tau KO: dendritic length and complexity reduced at 4, but not 8 weeks: PSD density/area reduced in 5th branch
(ii) Stress (acute): Tau KO new-born neurons protected form dying
(iii) Porsolt stress test: WT dendritic length goes down branching up; in Tau KO: no reduction of dendritic length.
(iv) EE: WT decrease in number of apoptotic cells; KO: less decrease;
WT: proximal dendrite: PSD density increasing; distal dendrite: PSD area decreasing;
Tau KO: proximal dendrite: PSD density decreasing; PSD area decreasing

These are concrete outcomes (overall a reduction of tau seems to be beneficial) which obviously need some interpretation. In the abstract however the data are summarized by saying 'tau plays a role in maturation' and 'tau mediates the negative consequences’. To me it seems that both the title and the abstract are watering down the interesting findings.

Major concerns:
(1) Page 8: I think the statement that some of the experimental manipulations impact on connectivity is problematic. Connectivity is about functionality and this has not been addressed in this study. Here one could use viruses that spread transsynaptically to make the point or remove the statement. Then, the claim fig. 4o/p cannot be made: The CNP and the P western blots are separate blots so it cannot be claimed that signals in P (Porsolt) differ compared to CNP (controls). Interestingly, the tau signal is reduced in P. The four reactions should be run on one blot and in duplicates.
I am not so convinced of the quantification of the mossy fiber terminals. What defines a terminal? Can this be reliably done as the image will depend on regional difference and the plane of section. Looking at the white arrows pointing at the red structures (MFTs) this look quite variable in size to me. It is also not clear to me how the boundary is determined as the entire neuron is red. The same applies to Fig 6s-x.

(2) Page 9, second paragraph referring to GAD-65 staining and quantification Fig 5l-t: In the methods it states that a circular area has been used for calculation, whereas a rectangular area is shown in the actual figure. Also, it may be me but I am a bit skeptical about quantifying the intensity of these random areas of the layer because looking at the low mag image in fig 5p within this layer there is actually quite some variability in thickness and intensity which also seems to follow a gradient. In other words I am not ultra-convinced of this set of data.

(3) Comments to the Discussion:
page 13, 1st paragraph: The statement on connectivity in my view should be taken out; and page 12, 2nd paragraph: Ittner et al. did not show ‘disappearance of synapse[s]’ in the tau KO.

Minor concerns/comments:
(1) Page 5: Many terms are explained in the methods but not when first mentioned (e.g. PH3, Sox2, BLBP). See also on page 7 (CldU), Page 9: DCX, fractin (also explain for those who don't know these markers)

(2) Page 6: The data have been obtained at 4 and 8 weeks. That PSDs are reduced in the 5th dendritic branching order could simply mean a developmental delay in the tau KO. Do they catch up
at later time-points?
(3) Page 7: CldU administration is labeled as acute stress test. Is the Porsolt test then a chronic test?
(4) Page 7/8: With ref to Fig 4G the authors speak of 'crossings'. What do they mean by that and how is this counted? The sentence ‘it did not cause further alterations in any other point of the dendritic tree’ is not clear.
(5) Page 9: DCX counting: At which age have these data been obtained?
(6) Page 15: Major depression spelt with small letters.

Suggestions for improving the study (at the editor's discretion):

(1) A critical question is whether the difference in dendritic length and arborization and spine density is reflected by functional changes. This could be addressed by electrophysiological recordings.
(2) The authors make the point that hippocampal neurogenesis is associated with 3Rtau expression while the rest of the adult mouse brain expresses 4Rtau. Yet the study looks at presence versus absence of total tau. It would be interesting to generate a mouse model that does not make 3R tau in adulthood yet this is clearly outside the scope of this paper.

We would like to thank the Reviewers and the Editor for having devoted their time to revising our manuscript and for having suggested that we perform several additional experiments, which we consider have contributed to significantly improving the quality of the manuscript.

Referee #1:

This interesting article by Pallas-Bazarra et al. addresses important questions related to the role of tau protein on adult hippocampal neurogenesis. In part the paper confirms findings of other groups that have created tau knockout mice (Dawson et al., 2001; Harada et al., 1994) in that loss of tau is related to subtle but potentially important changes in brain development. The authors go beyond this by identifying the cell types and consequences where tau may matter most, such as adult hippocampal neurogenesis and the responses to stress and environmental enrichment.

We would like to thank the Reviewer for his/her constructive comments. We have made all the changes proposed, which we consider have considerably clarified and improved the content of the manuscript.

Comments:

1. The authors should include higher resolution techniques to confirm their findings related to the formation of postsynaptic densities (typically submicron long structures) and mossy fiber terminals in newly born granule cells. It would be appropriate to include electron microscopy to further strengthen the observations.

Following the Reviewer’s suggestion, we have expanded the aspects of the manuscript related to the formation of postsynaptic densities (PSDs) and mossy fiber terminals (MFTs) in newborn granule neurons. Given that we found the most prominent local alterations in PSD number and size in 8-week-old newborn neurons, we have performed an additional analysis of the morphology of newborn neuron dendritic spines at this cell age under confocal microscopy. In addition, we have analyzed the synapse ultrastructure in both the molecular layer (ML) of the dentate gyrus (afferent synapses, between perforant pathway terminals and granule neurons) and the CA3 hippocampal subfield (efferent synapses, between granule neurons and CA3 pyramidal neurons) by electron microscopy. We consider that these novel data, not reported previously by other groups, strongly support our previous quantifications performed in a select population of newborn granule neurons. The main conclusions of these new determinations are the following:

Regarding afferent synapses (dendritic spines of granule neurons):
As previously mentioned, we performed a complementary analysis of the dendritic spine morphology of 8-week-old mature newborn granule neurons under confocal microscopy. This analysis revealed that Tau deficiency caused a trend towards a reduction in the density of spines in 5th branching order dendrites, in agreement with the decrease in the number of PSDs we had previously found in these dendrites. In addition, we detected an increase in the density of spines in 4th branching order dendrites, which was not reflected by any change in the density of PSDs. It is important to note that PSDs and dendritic spines are not completely equivalent structures, given that, for example, one single dendritic spine can have several (or no) PSDs, as shown in Figure EV 1 A.

Subsequently, we grouped the dendritic spines into three subclasses: stubby, thin and mushroom. Importantly, Tau deficiency reduced the percentage of mushroom spines in 5th branching order dendrites (Figure EV 1 D).

Moreover, a parameter related to synaptic strength and to the PSD size (Arellano et al, 2007), namely the spine head, was reduced in these dendrites due to Tau deficiency (Figure EV 1 C). In general, all these data are in agreement with our previous findings suggesting a marked alteration of dendritic spine and PSD morphology in the distal portions of the dendritic tree.

Moreover, and given that the identification of individual branching orders cannot be performed under electron microscopy, we analyzed the local differences in the ultrastructure of the afferent synapses of granule neurons in 3 regions of the molecular layer: External (EML), Medial (MML) and Inner (IML). We quantified the number of synapses, the size of the synaptic cleft, and the length, area and depth of the PSDs in Tau -/- and WT granule neurons. In summary, we found that Tau deficiency causes a decrease in these parameters (except the depth of the PSD and the size of the synaptic cleft, which were increased in Tau -/- mice), thus supporting the conclusions of our previous selective analysis of newborn neuron PSDs. These data are shown in Figure EV 2. All together, these data support our previous notion that Tau deficiency alters the morphology of the afferent synapses of granule neurons.

Regarding efferent synapses (mossy fiber terminals (MFTs)):

Following both Reviewers’ suggestion, we have expanded the analysis of the morphology of MFTs, both under confocal and electron microscopy. With this aim, we classified firstly the newborn neuron MFTs by size (Figure EV 3 D) in order to show the natural variability of this parameter occurring equally in mice of both genotypes (this was an observation made by Reviewer 2 that we considered worthy of a separate representation).

In addition, we have analyzed the efferent synapses of granule neurons in the CA3 region at the ultrastructural level under electron microscopy. We quantified the density of presynaptic vesicles, the synaptic cleft size, and the length of the active zone in MFTs. We found a reduction in the density of synaptic vesicles and the length of the active zone, whereas the size of the synaptic cleft was significantly increased in Tau deficiency. Thus, although the analysis of the area of the MFT of newborn neurons did not show any alteration in Tau -/- mice under confocal microscope analyses, the inclusion of high-resolution techniques, following the Reviewer’s suggestion, revealed subtle changes in the MFT ultrastructure that may have important consequences for granule neuron function.

Thus, taken together, all these new data support our previous notion that Tau deficiency causes morphological alterations in the synapses of granule neurons. We would like to thank the Reviewer once more for proposing the inclusion of high-resolution techniques such as electron microscopy, since it has allowed us to provide relevant data related to the synaptic ultrastructure, and we hope these data answer his/her question. In addition, following Reviewer 2’s suggestion, we have performed patch-clamp recordings of mature granule neurons in order to further support the relevance of the morphological alterations we observed in the synapses of Tau -/- mice. Interestingly, we have found electrophysiological alterations caused by Tau deficiency that are in agreement with the alterations in connectivity suggested by the morphological analyses (both at the confocal and electron microscopy levels).
For a detailed description of these alterations, please check our answer to the Reviewer 2’s first suggestion for improving the study (at the editor’s discretion).

We have added the following information to the main text of the manuscript:

- **Appendix Supplementary Methods:**

> “Morphometric analysis of the dendritic spines of newborn granule neurons
>
> Analysis of dendritic spines was performed separately for each branching order of the dendritic tree of 8-week-old retrovirus-labeled newborn neurons (Fig EV 1). Confocal stacks of images were obtained in a LSM710 Zeiss confocal microscope (63x oil immersion objective; XY dimensions: 67.4 µm; Z-axis interval: 0.2 µm). The dendritic length of each segment was measured on merged two-channel Z-projections, and the number of dendritic spines was counted using the NeuronStudio software (CNIC, Mount Sinai School of Medicine, 2007-2009) (Rodriguez et al, 2008a). Prior to their analysis, images were deconvoluted using the Huygens Professional software (Scientific Volume Imaging). A minimum of 30 segments per genotype were examined. Dendritic fragments were automatically constructed by NeuronStudio software, and then individual seed points were rectified manually to more accurately trace the dendrite. Thereafter, the dendritic spines were detected by the software and assigned to one of the following three categories: stubby, thin, and mushroom. We applied the parameters used by NeuronStudio software to classify the spines into the three categories, namely neck ratio 1.100 pixel; thin ratio: 2.500 pixel; mushroom size: 0.350 µm. Each spine was checked manually in order to assure accurate classification. The density of spines (number of spines/µm) and the percentage of each type of spine was calculated in each branching order. Given that the volume of the head of dendritic spines correlates with the size of the PSD, which, in turn, is correlated with synaptic strength (Arellano et al, 2007), the head diameter was also measured.

**Electron microscopy**

After a post-fixation step, 200-µm sagittal sections were obtained on a Leica VT1200S vibratome. Four sections per mouse containing the whole hippocampus were post-fixed in 2% osmium tetroxide (OsO4) for 2 h. They were then rinsed, dehydrated, and embedded in Durcupan (Durcupan, Fluka). Serial semi-thin sections (1 µm) were cut with a diamond knife and stained with 1% Toluidine blue. Subsequently, the area of interest was trimmed, and ultrathin sections (0.06 µm) were obtained with a diamond knife. These sections were then stained with lead citrate and examined under a JEM1010 Jeol electron microscope equipped with a 4Kx4K TemCam-F416 Digital camera. All the images were obtained at 15,000X magnification. To study the ultrastructure of the afferent synapses of granule neurons, 30 images for each subfield of the ML (EML, MML and IML) per animal were obtained. With respect to the ultrastructural organization of the efferent synapses of these neurons, at least 30 images per animal containing the stratum lucidum and the inner boundary of the stratum pyramidale of the CA3 region were obtained.

In the ML, the density of synapses (number of synapses per mm²), size of the synaptic cleft, and the length, depth and total area of the PSDs were measured in each sub-region separately. In the CA3 region, the density of presynaptic vesicles (number of vesicles per µm²), length of the presynaptic active zone, and the size of the synaptic cleft were measured. According to the definitions by Toni et al. (Toni et al, 2008), at the presynaptic level at least 4 presynaptic vesicles fusing with the active zone are required for a structure to be considered a synapse. In order to measure the density of presynaptic vesicles in the CA3 region, given that some MFTs were not fully included in the image due to the high complexity and size of these structures in this zone, a squared region of interest (ROI) was used to count the number of vesicles and to calculate their density. The length of the active zone and also the length, depth and total area of the PSDs were measured manually in the images using ImageJ software. In addition, the size of the postsynaptic cleft was calculated by measuring the distance between the outer borders of both the presynaptic and the postsynaptic membranes. Finally, the density of synapses was calculated by dividing the total number of synapses in each image by the known area of the image. 100-150 synapses per genotype and sub-region were analyzed.”

- **Results:**

> “Tau protein is necessary for the formation of postsynaptic densities (PSDs), dendritic spines, and mossy fiber terminals (MFTs) in granule neurons
>
> In the light of these results, we examined whether the Tau deficiency has additional effects on the morphology of the dendritic spines of 8-week-old newborn neurons, which had shown the most
remarkably alterations in PSDs. It should be noted that each dendritic spine can contain one, none, or more than one PSD (Fig EV 1 A). The absence of Tau increased the density of spines in the 4th branching order dendrites (U= 232; p= 0.022), whereas a trend to decrease this parameter was observed in 5th branching order dendrites (t = 1.815; p= 0.077) (Fig EV 1 B). Moreover, the diameter of the head of the spines located in 5th branching order dendrites was reduced due to Tau deficiency (U= 177552; p < 0.001) (Fig EV 1 C). In addition, we classified the dendritic spines into three categories (stubby, thin and mushroom) and quantified the percentages of each type of spine (Fig EV 1 D). Tau deficiency reduced the percentage of mushroom spines in 3rd ($\chi^2$= 6.109; p = 0.014) and 5th ($\chi^2$= 12.92; p < 0.001) branching order dendrites. Furthermore, it increased the percentage of stubby spines in the 3rd ($\chi^2$= 7.076; p = 0.008) and that of thin spines in the 5th ($\chi^2$= 8.173; p = 0.004), respectively.

In order to further characterize the morphological alterations caused by Tau deficiency on the postsynaptic elements of granule neurons, we next analyzed the ultrastructure of the afferent synapses in their dendritic spines by electron microscopy (Fig EV 2 A). This analysis was performed in three separate regions of the molecular layer (ML) of the DG: external (EML) and inner (IML). We found that Tau deficiency reduced the density of synapses (number of synapses per mm$^2$) in the whole ML ((EML: t= 2.363; p= 0.0019); (MML: t= 9.282; p< 0.001); (IML: t= 2.371; p= 0.019)) (Fig EV 2 B). Moreover, it increased the size of the synaptic cleft ((EML: $t$ = -11.216; p< 0.001); (MML: $t$ = -12.385; p< 0.001); (IML: $t$ = -8.343; p< 0.001)) (Fig EV 2 C) and caused alterations in PSDs, which showed a reduced area ((EML: $t$ = 2.64; p=0.0087); (MML: $t$ = 2.322; p=0.021); (IML: $t$ = 2.641; p=0.0087)) (Fig EV 2 D) and length ((EML: $t$ = 2.668; p=0.0079); (MML: $t$ = 0.818; p=0.413); (IML: $t$ = 4.036; p< 0.001)) (Fig EV 2 E), and an increased depth ((EML: $t$ = -2.669; p=0.0079); (MML: $t$ = -0.488; p=0.625); (IML: $t$ = -3.051; p=0.0024)) (Fig EV 2 F) than WT ones.

In order to evaluate whether Tau deficiency also has an impact on the morphology of newborn neuron MFTs (afferent synapses), we quantified the area of each individual MFT in 8-week-old retrovirally labeled newborn granule neurons. Fig EV 3 A, B show representative images of the whole hippocampus (A), as well as high-power magnifications of MFTs in the CA3 field (B) of WT and Tau-/- mice. No differences were found between WT and Tau-/- animals (U= 17886; p= 0.166) (Fig EV 3 C). In addition, and given the high variability in size of the MFT population, we plotted the percentages of MFT grouped by size in WT and Tau-/- mice (Fig EV 3 D). As shown, no differences in the distribution of MFT sizes were observed to be caused by the absence of Tau (K-S Z= 1.089; p= 0.187).

Finally, we analyzed the effects of Tau deficiency on the ultrastructure of the MFTs under an electron microscope (Fig EV 3 E). In this regard, we studied the stratum lucidum and stratum pyramidale of the CA3 region. The density of presynaptic vesicles (number of vesicles per µm$^2$) (t= 3.548; p < 0.001) (Fig EV 3 F) and the length of the presynaptic active zone (t= 5.589; p < 0.001) (Fig EV 3 G) were reduced, whereas the size of the synaptic cleft (t= -7.409; p < 0.001) (Fig EV 3 H) was increased in Tau-/- animals compared to WT ones.

In summary, these results suggest that Tau participates in the functional maturation of granule neurons, since its absence alters PSD, dendritic spine, and MFT morphology.”

- **Discussion:**

  “Tau knockout specifically reduced not only the number but also the area of most distal PSDs (located on 5th branching order dendrites) in these neurons. In addition, it decreased the percentage of mushroom spines and the spine head diameter in the aforementioned dendrites. These data might be relevant given that the main afferent pathway in the DG is composed by the axons of the pyramidal neurons of the entorhinal cortex (EC) (the perforant pathway). This pathway preferentially ends in the two outer thirds of the ML, where the 5th branching order dendrites of the granule neurons are located. Moreover, Tau deficiency also triggered ultrastructural alterations in the afferent synapses of granule neurons. An enlargement in both the synaptic cleft and the depth of the PSDs was detected. This observation may indicate the destabilization of these elements caused by Tau deficiency. In addition, we showed that absence of this protein also alters the ultrastructure of MFTs (afferent synapses), which may have important consequences for granule neuron functionality.”

- **Legend to Expanded View Supplementary Figures:**
“Figure EV1 – Tau protein deficiency causes morphological alterations in the dendritic spines of newborn granule neurons.

A Representative image of a dendrite of an 8-week-old newborn granule neuron infected by a PSD95-GFP-expressing retrovirus. Arrows indicate examples of the different types of dendritic spines (stubby, thin and mushroom) (shown in red). Note that each dendritic spine can contain several PSDs (shown in green), as indicated by the white asterisk. Scale bar 3 µm.

B-C Quantification of the number of spines/µm (B) and spine head diameter (C) in each dendritic branching order (mean ± SEM; n= 3 mice per genotype; *0.05 > p ≥ 0.01, **0.01 > p ≥ 0.001, ***p < 0.001; Student’s t-test or Mann-Whitney’s U test).

D Quantification of the percentage of each type of spine (stubby, thin and mushroom) in each dendritic branching order (n= 3 mice per genotype; *0.05 > p ≥ 0.01, **0.01 > p ≥ 0.001, ***p < 0.001; Student’s t-test).

Data information: brightness and contrast of representative confocal microscopy image shown in the Figure was minimally adjusted in order to improve visualization.

Figure EV2 – Tau protein deficiency alters the ultrastructure of the afferent synapses of granule neurons in the ML of the DG.

A Representative electron microscopy images of the ML of WT and Tau/-/- mice showing the density (a, b) and the ultrastructural details (c, d) of afferent synapses in granule neurons. Arrows indicate synapses. Brackets indicate PSDs. Asterisks indicate synaptic clefts. White scale bar 1 µm. Red scale bar 250 nm.

B-F Quantification of the number of synapses/mm² (B), the synaptic cleft size (C) and the area (D), length (E) and depth (F) of the PSDs in the IML, MML and EML of WT and Tau/-/- mice (mean ± SEM; n= 3 mice per genotype; *0.05 > p ≥ 0.01, **0.01 > p ≥ 0.001, ***p < 0.001; Student’s t-test). Data information: IML, inner molecular layer; MML, medial molecular layer; EML, external molecular layer. Brightness and contrast of representative images shown in the Figure were minimally adjusted in order to improve visualization.

Figure EV3 – Tau protein deficiency alters the ultrastructure of the efferent synapses of granule neurons in CA3.

A Representative image showing 8-week-old newborn granule neurons in the DG (infected by a PSD95-GFP-expressing retrovirus) projecting axons into the CA3 field (indicated by a white square). Red scale bar 300 µm.

B Representative images of MFTs of 8-week-old newborn granule neurons in the CA3 region of WT and Tau/-/- mice. Green scale bar 20 µm.

C Quantification of the area of MFTs of 8-week-old newborn granule neurons in WT and Tau/-/- mice (mean ± SEM; n= 3 mice per genotype; Mann-Whitney’s U test).

D Representation of the distribution of the size of MFTs in 8-week-old newborn granule neurons of WT and Tau/-/- mice. MFTs were grouped on the basis of size, and the percentage of MFTs in each size group was represented (n= 3 mice per genotype; Kolmogorov-Smirnov’s Z test).

E Representative electron microscopy images of the ultrastructure of efferent synapses of granule neurons in the CA3 region of WT and Tau/-/- mice. Brackets indicate the active zone of the presynaptic terminal. Asterisks indicate synaptic clefts. White scale bar 250 nm.

F-H Quantification of the number of synaptic vesicles/µm² (F), the length of the active zone (G) and the synaptic cleft size (H) of efferent synapses of granule neurons in the CA3 region of WT and Tau/-/- mice (mean ± SEM; n= 3 mice per genotype; **0.01 > p ≥ 0.001, ***p < 0.001; Student’s t-test).

Data information: DG, dentate gyrus; H, hilus; SS, synaptic spine; MFT, mossy fiber terminal. Brightness and contrast of representative images shown in the Figure were minimally adjusted in order to improve visualization.”

2. The authors should perform a more rigorous analysis of GABAergic interneurons and their involvement in mediating the pro-neurogenic effects of environmental enrichment in their animal model. Providing an immunofluorescence-based quantification of a series of overexposed images may not be considered sufficient experimentally-based evidence. The authors should include different means of quantification, such as Western blotting and/or qPCR from microdissected fresh tissue.

Following suggestions made by this Reviewer and Reviewer 2, we have performed more specific quantifications of GABAergic innervation in the revised version of our manuscript.
It is noteworthy that GAD-65 protein has been demonstrated to unambiguously label GABAergic presynaptic terminals (Trejo et al., 2007). However, we understand that the way in which we presented the images in Figure 5 (the old version of the manuscript) led to a methodological misunderstanding of the procedure used to perform the quantifications. The analysis of GABAergic innervation was actually done on high magnification images (Zeiss confocal microscope, oil immersion 63X objective, Image XY dimensions: 24.1 µm). Under this high-power magnification, the 3 layers of the ML can be easily identified on the basis of the different staining pattern of GAD-65 in the three layers. Three high-power magnification images were obtained for each sub-region of the ML (EML, MML and IML) and for each animal. These images were subjected to an invariable threshold, which was previously set up for each sub-region. In order to facilitate interpretation of the results, data corresponding to the number of GABAergic terminals in the ML with respect to the WT under control housing. This analysis has systematically been done by our group and others and has been demonstrated to be a reliable method for measuring GABAergic innervation in the ML (Martinez-Cue et al., 2013; Trejo et al., 2007). However, given that both Reviewers expressed their concerns about this quantification, the previous graph has been removed from the main text in the revised version of the manuscript. Instead of quantifying the total area occupied by GAD-65+ terminals, we have now quantified the density of GAD-65+ GABAergic presynaptic terminals (number of terminals per µm²) and the average area of these terminals. These new quantifications are shown in Figure 7. In addition, we have provided separate quantifications for the EML, MML, and IML in Tau−/− and WT animals, both under control and enriched housing conditions. These data are presented in Figure EV 4. In short, we have found that both Tau deficiency and EE locally alter the three parameters in specific layers of the ML.

Thus, although these subtle changes in GABAergic innervation were not reflected by WB analysis, we consider that we have provided additional experimental evidence that EE modifies GABAergic innervation in the ML layer of the hippocampus—an observation previously made by another group (Llorens-Martin et al., 2010).

In addition to the data presented here, in Figure 7 and Figure EV4, we have included the following information in the main text of the manuscript:

- **Appendix Supplementary methods:**

  **“Analysis of GABAergic innervation**

  GABAergic innervation in the molecular layer (ML) was analyzed by immunohistochemistry of GAD-65+ GABAergic terminals. Images were randomly obtained from the sections comprising the series. Three high magnification confocal images per sub-region of the ML (namely, external (EML), medial (MML) and inner (IML)) were obtained per animal in a LSM710 Zeiss confocal microscope (63X oil immersion objective, XY dimensions: 24.1 µm). In each sub-region, an invariant threshold for fluorescence intensity was established to analyze all images. We measured the number of GAD-65+ GABAergic terminals, as well as the average area of each terminal, using the plugin Particle Analyzor for ImageJ software (ImageJv. 1.33, NIH, Bethesda, MD, USA, http://rsb.info.nih.gov/ij). To simplify the interpretation of the results, data corresponding to the whole ML are shown in Fig 7. These grouped representations required the normalization of individual data from each sub-region with respect to WT control-housed mice. In addition, the regional changes observed in the three sub-regions of the ML are shown in Fig EV 4. 4000-5000 GAD-65+ terminals per genotype, sub-region and experimental condition were studied in order to calculate the average area of the terminals.”

- **Results:**
“It is known that GABA promotes cell survival during AHN (Song et al. 2013). In order to study whether GABAergic innervation is related to the pro-neurogenic effect of EE, we analyzed GAD-65$^-$ GABAergic terminals in the ML of mice in each experimental condition (Fig 7 A). The results showed that EE promoted different effects in WT and Tau/-/- animals in both the density (Interaction $F_{1,208}=12.064; p=0.021$) (Fig 7 B) and the average area (Interaction $F_{1,5822}=62.406; p<0.001$) (Fig 7 C) of GABAergic terminals. While EE promoted an increase in both parameters in WT mice ($p=0.009$ and $p<0.001$, respectively), it caused a reduction in both in Tau/-/- animals ($p=0.023$ and $p<0.001$, respectively). Fig EV 4 shows the regional changes observed in these parameters in the three sub-regions of the ML: EML, MML and IML."

- **Legend to Figures:**

**Figure 7 – Tau protein is involved in the effect of EE on GABAergic innervation.**

A  Representative images of the whole hippocampus showing GABAergic innervation labeled with GAD-65 antibody (red), and high-power magnifications of the DG molecular layer showing GAD-65$^-$ terminals in WT CH, Tau/-/- CH, WT EE and Tau/-/- EE mice. Cell nuclei were labeled with DAPI (blue). White scale bar 300 $\mu$m. Red scale bar 50 $\mu$m. Green scale bar 5 $\mu$m.

B-C  Quantification of the density (B) and area (C) of GAD-65$^-$ terminals in the molecular layer of mice in each experimental condition ($n=10$ mice WT CH, $n=8$ mice Tau/-/- CH, $n=6$ mice WT EE, $n=10$ mice Tau/-/- EE).

Data information: in (B-C) data are presented as mean ± SEM (normalized with respect to WT CH). Colored asterisks indicate differences between the group represented by the same color and the WT CH group: **$0.01 > p \geq 0.001$, ***$p < 0.001$; colored crosses (+) indicate differences between the group represented by the same color and the WT EE group: $+0.05 > p \geq 0.01$; colored pads (#) indicate differences between the group represented by the same color and the Tau/-/- CH group: #0.05 > p $\geq 0.01$, ###$p < 0.001$ (One-way ANOVA + LSD post hoc multiple comparisons). DG, dentate gyrus; ML, molecular layer.”

- **Legend to Expanded View Supplementary Figures:**

**Figure EV4 – Tau protein regulates the effects of EE on GABAergic innervation in a region-dependent manner.**

A  Representative image of the whole hippocampus showing GABAergic innervation labeled with GAD-65 antibody (red) and a magnification of the ML showing the different sub-regions (EML, MML and IML). Cell nuclei were labeled with DAPI (blue). White scale bar 300 $\mu$m. Green scale bar 50 $\mu$m.

B-D  Quantification of the density and area of GAD-65$^-$ terminals in the ML (B), MML (C) and IML (D) ($n=10$ mice WT CH, $n=8$ mice Tau/-/- CH, $n=6$ mice WT EE, $n=10$ mice Tau/-/- EE).

Data information: in (B-D) data are presented as mean ± SEM (normalized with respect to WT CH). Colored asterisks (*) indicate differences between the group represented by the same color and the WT CH group: *$0.05 > p \geq 0.01$, **$0.01 > p \geq 0.001$, ***$p < 0.001$; colored crosses (+) indicate differences between the group represented by the same color and the WT EE group: $+0.05 > p \geq 0.01$; colored pads (#) indicate differences between the group represented by the same color and the Tau/-/- CH group: ##$p < 0.01$, ###$p < 0.001$ (One-way ANOVA + LSD post hoc multiple comparisons). DG, dentate gyrus; ML, molecular layer; IML, inner molecular layer; MML, medial molecular layer; EML, external molecular layer. Brightness and contrast of representative confocal microscopy images shown in the Figure were minimally adjusted in order to improve visualization.”

3. The interpretation of the findings seems to alternate between neurodegeneration and neurodevelopment at several places throughout the manuscript which appears somewhat confusing. The authors intensively discuss the work by Ittner et al., but provide no solid link to it. Neurodegenerative diseases cannot be simply described in the terms of loss of function of a certain protein, there are multilevel events underlying their development. I would recommend concentrating the interpretation in the neurodevelopmental area and including neurodegeneration perhaps briefly in discussion.
Although one of the most interesting aspects of the roles played by Tau protein is in the point of convergence between neuroplasticity and neurodegeneration, after careful reading of several fragments of the previous version of our manuscript, we agree with Reviewer 1 that the aim of the work is more focused on the developmental roles played by Tau. We have made the appropriate changes in the main text and the following sentences have been deleted in the revised version of our manuscript:

**Abstract:** The sentences “The hippocampus is one of the most affected structures in a group of neurodegenerative diseases known as tauopathies, characterized by impaired Tau metabolism. In particular, alterations in the generation of new neurons in the adult hippocampus appear to be a relevant neuropathological feature of these diseases” have been removed.

**Introduction:** The sentences “Among other neurogenic regions, the hippocampal dentate gyrus (DG) has attracted increasing attention due to the functional relevance of AHN in humans and to its close relation with neurodegenerative diseases (Lepousez et al, 2015)” and “This feature, along with the observation of alterations in AHN in various animal models of tauopathies (Komuro et al, 2015; Llorens-Martin et al, 2011a; Mu & Gage, 2011; Rodriguez et al, 2008b), in which the metabolism of Tau protein is altered (Avila et al, 2004; Hernandez & Avila, 2007), suggest that Tau plays a crucial role in the regulation of AHN” have been removed.

We consider that the link between our work and the study by Ittner et al. is based on the fact that these authors described the molecular mechanism by which the absence of Tau may trigger a functional defect in synapses under pathological conditions. Here we provide experimental evidence supporting the notion that both the alterations described by Ittner et al. and those reported in the present manuscript have an impact on the cellular responses to external stimuli such as acute stress and environmental enrichment.

4. In this context, the importance of tau is exaggerated in various places by using wording such as crucial, critical, central, necessary, etc. The fact is that the effects of tau are quite subtle, as already described by Hirokawa's group (Harada et al. 1994) and confirmed here. It would also be appropriate to add cautionary notes, for example a correlation does not necessarily mean causality (example on p. 6 bottom).

Thank you for pointing this out. We have attempted to be more cautious and have removed all the words over stressing the importance of Tau. The following sentences have been changed:

- **Page 4:** “We also demonstrate, for the first time, that Tau regulates the stimulatory effects of EE on AHN.”
- **Page 6:** “Taken together, these results show that Tau is involved in the morphological maturation of newborn granule neurons, since its absence causes a transient alteration of the dendritic arborization of these cells.”
- **Page 13:** “Tau is involved in the morphological and functional maturation of newborn granule neurons under basal conditions. Tau protein is crucial for the establishment of neuronal polarity during embryonic development, for axonal transport, and for the intracellular trafficking of various molecules (Caceres & Kosik, 1990; Dawson et al, 2001; de Barreda et al, 2010; Hernandez & Avila, 2010; Llorens-Martin et al, 2011b; Sapir et al, 2012). Given the tight control of Tau expression during AHN, it is reasonable to assume that this protein plays a regulatory role in this process.”
- **Page 19:** “Taken together, our data suggest that Tau plays a novel role in the maturation of newborn granule neurons in vivo under basal conditions. Furthermore, we provide evidence that Tau regulates the effects of external stimuli on AHN.”

4. The way how individual figure panels and results are organized could be improved. For example, Figure 6 is divided between two subheadings in results. Furthermore, some paragraphs describing results read more as figure legends. Figure 1 is another example of a strange labelling. The panel ‘a’ should be labelled the same way as the panel ‘b’, outside of the image frame rather than on the inside etc. At first glance it may look as if the panel ‘a’ is missing.

Thank you for these relevant suggestions. We have made several changes to the revised version of the manuscript that we hope will facilitate reading. In brief:
- We considered grouping all the results corresponding to each one of the Figures 2, 4 and 6 (in the old version of the manuscript) under single subheadings, but given the volume of the data included, we believe that this could hinder comprehension of the results. We also considered the possibility of dividing the figures, but this would not match the figure number limit set by *EMBO Journal*. We hope the Reviewer agrees with our decision.

- We have corrected several sentences in the Results section and have added some information that we consider will help guide the reader through a more elaborated train of thoughts.

- Following the Reviewer’s suggestion, we have corrected the erroneous labeling in all the figures in order to comply with *EMBO Journal* requirements.

5. Statistical analysis should be described in detail for every condition. In some experiments only three animals were used per experimental group (retroviral injections). The authors should increase the animal number where needed.

We have made every effort to add the required information in the revised version of the manuscript.

Regarding the animals used for retroviral experiments, we used 3-5 animals per experimental group and time point. However, given that in two of the groups only 3 animals survived the surgery, we considered it more honest to state that 3 animals had been used in general terms. Given the low variability of the morphometric parameters analyzed, this is considered a standard number of mice for this type of experiment. In addition, in these experiments, the n is considered to be each cell or dendrite segment rather than the animal (*Llorens-Martin et al., 2013; Llorens-Martin et al., 2014*). By observing the error bars in the graphs, the Reviewer can verify how morphometric analysis usually gives highly homogenous numbers.

The following information has been included in the revised version of the manuscript (Material and Methods section):

**“Statistical analysis**

Statistical analysis was performed using the SPSS 22 software (SPSS, 1989; Apache Software Foundation, Chicago, IL, USA). The Kolmogorov–Smirnov test was used to test the normality of the sample distribution. Atypical data were detected with box-plots and eliminated when necessary. For comparisons between 2 experimental groups, data from cell counts (PH3, fractin, Sox2, DCX, thymidine analog survival, BLBP and calretinin), morphometric analysis (total dendritic length and Sholl’s analysis), analysis of the PSDs in newborn neurons under a confocal microscope (density and area of PSDs), analysis of dendritic spines under a confocal microscope (density and head diameter of dendritic spines), electron microscopy analysis (number of presynaptic vesicles, length of the active zone, synaptic cleft size, PSD length, depth, and area) and electrophysiological recordings were analyzed by a Student test in the case of normal sample distribution or by a non-parametric test (Mann-Whitney U test) in those cases in which normality could not be assumed. For comparisons between more than 2 experimental groups (Western Blot quantifications, thymidine analog experiments, cell counts, GABAergic innervation, morphometric analysis, and analysis of the PSDs when analyzed in either stressed or enriched mice), data were analyzed by a two-way ANOVA test. In those cases in which the two-way ANOVA interaction was statistically significant, a one-way ANOVA followed by a Fischer LSD post-hoc analysis was used to compare the differences between individual groups. Graphs represent mean values ± SEM. The percentage of the different types of dendritic spines of newborn neurons, as well as the percentage of cells expressing DCX and NeuN, were analyzed by a Chi-squared (χ2) test. The comparison between the cumulative distributions of amplitudes of mEPSCs, as well as the comparison between the distributions of MFT sizes, were performed by a Kolmogorov-Smirnov’s Z test.”

Minor points:

- Add figures numbers at top of figures to identify them properly
  
  Thank you, we have corrected this.

- Check spelling, there are a number of typos. Entorhinal, RIPA buffer, perforant path, altogether, isoflurane...
We would like to apologize for these spelling errors and we have corrected them in the revised version of the manuscript.

- Page 14, sentence starting with 'Interestingly' is grammatically incomplete.
Thank you very much, we have corrected it.

Referee #2:

Tau is a protein that is enriched in axons, but also serves important roles in the dendrite. Previously it has been shown that tau depletion delays axonal extension and maturation. Here, the authors focus on hippocampal granule neuron cells in the dentate gyrus, where neurogenesis occurs to show a role for tau in neurogenesis. The paper looks at basal conditions, two forms of stress and environmental enrichment to determine how the absence of tau affects dendritic length, dendritic complexity, and PSD area (as a proxy for spine size). The paper is generally well written, the experiments are technically well done (I have only one comment for one experiment, see below) and the figures are beautifully grafted. The data are very interesting and the system is attractive to dissect the underlying molecular mechanism.

We would like to thank the Reviewer for his/her constructive comments, which we consider have substantially improved the quality our manuscript.

General comments:

I made myself a list of under which conditions (basic, acute stress, Porsolt test, EE) removing tau is good or bad:
(i) Basic conditions: Tau KO: dendritic length and complexity reduced at 4, but not 8 weeks: PSD density/area reduced in 5th branch.
(ii) Stress (acute): Tau KO new-borne neurons protected from dying.
We would like to apologize for the way in which we presented these Results, which has led to the misunderstanding that thymidine analog administration was an acute-stress paradigm. As we explain later, we have corrected our previous affirmations in the revised version of the manuscript.
(iii) Porsolt stress test: WT dendritic length goes down branching up; in Tau KO: no reduction of dendritic length.
(iv) EE: WT decrease in number of apoptotic cells; KO: less decrease;
WT: proximal dendrite: PSD density increasing; distal dendrite: PSD area decreasing;
Tau KO: proximal dendrite: PSD density decreasing; PSD area decreasing

These are concrete outcomes (overall a reduction of tau seems to be beneficial) which obviously need some interpretation. In the abstract however the data are summarized by saying 'tau plays a role in maturation' and 'tau mediates the negative consequences'. To me it seems that both the title and the abstract are watering down the interesting findings.

Thank you for your suggestion. We have rewritten the abstract and more concrete information has been included in the revised version of the manuscript. We hope you agree with the changes made. The new sentences included in the Abstract are the following:

“Furthermore, we reveal that Tau is involved in the selective cell death of immature granule neurons caused by acute stress. Also, Tau deficiency protects newborn neuron from the stress-induced dendritic atrophy and loss of postsynaptic densities (PSDs). Strikingly, we also demonstrate that Tau regulates the increase in newborn neuron survival triggered by environmental enrichment (EE). Moreover, newborn granule neurons from Tau-/- mice did not show any stimulatory effect of EE on dendritic development or on PSD generation. Thus, our data demonstrate that Tau-/- mice show impairments in the maturation of newborn granule neurons under basal conditions and that they are insensitive to the modulation of adult hippocampal neurogenesis exerted by both stimulatory and detrimental stimuli.”
Major concerns:

(1) Page 8: I think the statement that some of the experimental manipulations impact on connectivity is problematic. Connectivity is about functionality and this has not been addressed in this study. Here one could use viruses that spread transsynaptically to make the point or remove the statement. Following the Reviewer’s first suggestion to improve the manuscript, the revised version now includes patch-clamp recordings of acute slices of granule neurons of WT and Tau−/− mice in order to further experimentally support that Tau deficiency has an impact on granule neuron functionality. Interestingly, we have found electrophysiological alterations caused by the absence of Tau that are in agreement with the morphological alterations in the PSDs previously described. Specifically, we have focused our analyses on the miniature excitatory postsynaptic currents (mEPSCs) in the granule cells of the DG to assess whether synaptic inputs were altered by the absence of Tau. These new data are shown in Figure 3. Although the frequency and amplitude of mEPSC were not affected by Tau deficiency, when we examined the cumulative probability distribution of mEPSC amplitudes, we found two distinct populations of mEPSCs that were altered in opposite directions in Tau−/− mice. Thus, the amplitudes of small mEPSCs in Tau−/− mice were statistically larger than those in WT mice. In contrast, when comparing mEPSCs of larger amplitudes, those in Tau−/− mice were smaller than those in WT animals. This result is indicative of a redistribution of excitatory synaptic weights in Tau−/− mice. Given that other authors did not find basal electrophysiological alterations in CA1 pyramidal neurons (Ittner et al, 2010), we believe that our novel data are relevant and may reveal a specific mechanism by which Tau deficiency causes subtle functional impairments in granule neuron connectivity. We would like to thank the Reviewer for proposing these electrophysiological recordings in order to support the alterations in granule neuron functionality. However, we have made every effort to remove the term “connectivity” and replace it by other equivalent ones throughout the manuscript whenever possible.

On the other hand, we are currently advancing through the initial steps of the legal authorization process to be allowed to work with Rabies viruses at our Institute (it is expected that the authorization process will take around 1 year). While we have made every effort to perform these experiments, we will have to wait a year to be able to address this point in a future study.

The following information has been included in the revised version of the manuscript:

- **Appendix Supplementary methods:**

**“Electrophysiological recordings**

Electrophysiological recordings of granule neurons were made in acute slices using the whole-cell patch clamp technique. The animals were anesthetized by means of an intraperitoneal injection of pentobarbital and perfused intracardially with an ice-cold solution containing (in mM) 92 choline Cl, 2.5 KCl, 1.25 NaH2PO4·2H2O, 0.5 CaCl2, 2 thiourea, 5 Na-Ascorbate, 3 Na-Pyruvate, and 30 NaHCO3, bubbled with 95% O2/5% CO2. The brain was rapidly removed, and 350-µm coronal slices were made using a Leica vibratome. The slices were immersed in recovery solution containing (in mM) 92 NaCl, 2.5 KCl, 2 CaCl2, 2 MgSO4, 1.25 NaH2PO4·2H2O, 30 NaHCO3, 20 Hepes Acid, 2 thiourea, 5 Na-ascorbate, 3 Na-Pyruvate and 25 glucose, bubbled with 95% O2/5% CO2. After <1 h recovery, slices were then transferred to an immersion-recording chamber and superfused with gassed ACSF containing (in mM) 119 NaCl, 2.5 KCl, 1 NaH2PO4·2H2O, 11 glucose, 26 NaHCO3, 1.2 MgCl2, 2.5 CaCl2 and GABA A receptor (picrotoxin 50 µM) blockers and 1 µM TTX to blockade of action potential-dependent synapse activity. Cells were visualized with an Olympus BX50WI microscope (Olympus Optical, Tokyo, Japan) coupled to a 60x water immersion lens. Recordings of excitatory miniature responses (mEPSCs) were obtained with Multiclamp 700A/B amplifiers and pClamp software (Molecular Devices). Patch electrodes (4–7 MΩ) were filled with internal solution that contained (in mM) 115 cesium methanesulfonate, 20 CsCl, 10 Hepes, 2.5 MgCl2, 4 Na2ATP, 0.4 Na3GTP, 10 sodium phosphocreatine, and 0.6 EGTA, pH 7.25. Experiments were performed at room temperature (23–26 °C). Cells were voltage-clamped at −65 mV. mEPSCs were detected using a template with a rise time of 3 ms, decay time of 11 ms, baseline of 10 ms, length of 45 ms. Captured events were manually reviewed. One neuron was recorded per slice and three to seven slices were recorded per mouse.”
Results:

“The absence of Tau modifies the electrophysiological properties of granule neurons

In order to determine whether the morphological alterations caused by Tau deficiency affect the basal electrophysiological properties of granule neurons, we recorded the membrane properties and synaptic activity of these cells under whole-cell configuration. Fig 3 A, B shows representative traces of mEPSCs recorded at a holding potential of \(-65\) mV from WT (black traces) and Tau-/- (red traces) mice. The resting membrane potential of Tau-/- cells was hyperpolarized compared to that of WT cells (t= 2.217; p= 0.038) (Fig 3 C).

We then focused our analyses on the miniature excitatory postsynaptic currents (mEPSCs) to assess whether synaptic inputs were altered by the absence of Tau. Granule cells of the DG of Tau -/- mice did not exhibit alterations in the amplitude (t= -0.256; p = 0.800) (Fig 3 D) neither in the frequency (U= 53.000; p= 0.483) (Fig 3 E) of mEPSCs compared to WT ones. However, examination of the cumulative probability distribution of amplitudes of mEPSCs revealed two distinct populations of mEPSCs altered in opposite directions in Tau-/- mice (Fig 3 F). Thus, the amplitudes of small mEPSCs of Tau-/- mice were statistically larger than those of WT animals (K-S Z= 6.306; p<0.001) (Fig 3 F (i)). In contrast, when comparing the mEPSCs of larger amplitudes, those of Tau-/- mice were smaller than those of WT animals (K-S Z= 9.820; p<0.001) (Fig 3 F (ii)). This result is indicative of a redistribution of excitatory synaptic weights in Tau-/- mice.”

Discussion:

“Indeed, electrophysiological recordings showed that the granule neurons of Tau-/- mice exhibit a hyperpolarized resting membrane potential. Moreover, with respect to synaptic strength, we observed a bidirectional effect in Tau-deficient neurons, in which smaller mEPSCs got larger, and conversely, the larger mEPSCs got smaller. The molecular basis for these alterations is unclear, but they are indicative of a redistribution of synaptic weights, which is consistent with the bidirectional effect of PSD size that we also observed in these neurons. In fact, Tau is present in dendritic spines (Frandemiche et al, 2014; Ittner et al, 2010; Mondragon-Rodriguez et al, 2012). In these structures, it has been shown that activation of NMDA glutamate receptor triggers Tau phosphorylation, thus regulating the interaction of Tau with the actin cytoskeleton, PSD95, and Fyn kinase (Frandemiche et al, 2014; Ittner et al, 2010; Mondragon-Rodriguez et al, 2012). Interestingly, the basal subtle electrophysiological alterations described in this work seem to occur specifically in granule neurons, as it has been demonstrated that Tau deficiency does not affect basic synaptic currents in other regions of the brain (Ittner et al, 2010). Thus, it can be hypothesized that granule neurons are selectively affected by Tau deficiency in terms of their basal synaptic activity. In addition, alterations that may be related to deficient synaptic plasticity (LTP and LTD) have been reported in Tau-/- mice (Ahmed et al, 2014; Kimura et al, 2014; Regan et al, 2015). In fact, hippocampal LTD is considered necessary for clearing old memories, and Tau-/- mice show impairments in learning flexibility and various types of hippocampal-dependent memory (Ahmed et al, 2014; Ikegami et al, 2000; Lei et al, 2014; Ma et al, 2014; Regan et al, 2015). Remarkably, it has been described that granule neurons are important for flexibility of learning strategies (Garthet al, 2009).

Author contributions:

“NPB performed and supervised all the experiments. MLLM performed electron microscopy experiments. JJA performed the retroviral injections. MN performed and analyzed electrophysiological experiments. JAE supervised electrophysiological experiments. MLLM, JA, FH, MN and JAE obtained funding. NPB, MLLM and JA wrote the manuscript.”

Legend to Figures:

“Figure 3 – Tau protein deficiency affects basal synaptic activity in granule neurons.

A  Representative traces of mEPSCs recorded at a holding potential of \(-65\) mV from WT (black traces) and Tau-/- (red traces) mice.
B  Exemplary traces of mEPSCs from one cell from WT (black trace) and Tau-/- (red trace) mice.
C  Resting membrane potential of WT and Tau-/- mice (mean ± SEM; n= 13 cells WT, n= 10 cells Tau-/-; *0.05 > p ≥ 0.01, Student’s t-test).
D-E  mEPSC amplitude (D) and mEPSC frequency (E) of WT and Tau/-/- animals (mean ± SEM; n= 13 cells WT, n= 10 cells Tau-/–; Student’s t test or Mann-Whitney’s U test).

F  Cumulative probability plot of the total mEPSC amplitudes, small mEPSCs (i) and big mEPSCs (ii) from both WT and Tau-/– mice (black and red traces, respectively) (**p < 0.001; Kolmogorov-Smirnov’s Z test)."

Then, the claim fig. 4o/p cannot be made: The CNP and the P western blots are separate blots so it cannot be claimed that signals in P (Porsolt) differ compared to CNP (controls). Interestingly, the tau signal is reduced in P. The four reactions should be run on one blot and in duplicates.

We apologize for the misunderstanding caused by the image shown in Figure 4 (the old one). Six animals per experimental condition were used for this quantification. Due to the number of animals analyzed, samples were processed in parallel in three different blots. Each blot contained the four groups together, and a common loading control was run in the three blots in order to normalize the data. However, due to creative reasons, we wrongly decided to show only one animal per group and remove the animals in between. This is why the blots seem to have been done separately. We have run a new blot including 2 animals per group and have included the new image in the revised version. We must also comment that we quantified Tau signal and did not find statistically significant differences due to the Porsolt test in WT animals.

The following information has been included in the revised version of the manuscript:

- Appendix Supplementary methods:

“Hippocampal extracts for Western blot analysis were prepared in RIPA buffer (radioimmunoprecipitation assay buffer) consisting of 50 mM pH 7.4 Tris-HCl, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, phosphatase inhibitors (1 mM NaF, 1 mM Na3VO4 and 1 µM okadaic acid) and a protease inhibitor cocktail (Roche). Samples were homogenized at 4°C, and protein content was determined by the Pierce® BCA Protein Assay kit (Thermo Scientific). Total protein (25 µg) was electrophoresed on 8% SDS-PAGE gel and transferred to a nitrocellulose membrane (Schleicher and Schuell). After blocking with 5% non-fat dried milk, blots were incubated at 4°C overnight with primary antibodies rabbit anti-GluR1 (Abcam, 1:1000), mouse anti-Tau5 (Calbiochem, 1:1000), and mouse anti-GAPDH (Abcam, 1:2000). Secondary antibodies goat anti-rabbit (Dako, 1:1000) and goat anti-mouse (Dako, 1:1000), followed by ECL detection reagents (Amersham), were used for immunodetection. Six animals per experimental condition were used. Protein levels were determined with the Quantity One software (BioRad). Due to the number of animals analyzed, samples were processed in parallel in three separate blots. Each blot contained all four groups, and a common loading control was run in the three blots in order to normalize the data.”

I am not so convinced of the quantification of the mossy fiber terminals. What defines a terminal? Can this be reliably done as the image will depend on regional difference and the plane of section. Looking at the white arrows pointing at the red structures (MFTs) this look quite variable in size to me. It is also not clear to me how the boundary is determined as the entire neuron is red. The same applies to Fig 6s-x.

We regret that the methodological explanation raised the Reviewer’s concerns about these quantifications. In order to measure the area of MFTs, we applied the methodology described by Toni et al. (Toni et al., 2008). As described by these authors, the criteria for MFT quantification are the following (we are citing their original text):

“Mossy fiber boutons that fit the following criteria were selected for quantification:
(i) the diameter of the bouton was more than threefold greater than the diameter of the mossy fiber,
(ii) the bouton was connected to the mossy fiber on at least one end and
(iii) the bouton was relatively isolated from other boutons for accuracy of tracing. These criteria were arbitrary but nevertheless established an unbiased sampling. The contour of the boutons was traced manually and the enclosed area was calculated by the IGL Trace program. A total of 20–21 boutons were analyzed for each time point in CA3 and a total of 20–66 boutons were analyzed for each time point in the hilus”.

Thus, we applied the same criteria to define a MFT and to determine its boundaries. The differences between this paper and ours are, on the one hand, that we measured the traced area with ImageJ instead of with IGL software, and, on the other, that we measured a
minimum of 100 MFTs per experimental condition, whereas 20-60 buttons were analyzed by Toni et al. Following the original description by Toni and collaborators, we restricted the quantifications to equivalent rostrocaudal positions of the DG. However, in addition, we added an additional cautionary measurement that consists of carefully avoiding the CA2 area, given that we have recently demonstrated that the area of MFTs in this region differs from that of the CA3 field (Llorens-Martin et al, 2015). As we made every effort to avoid CA2, it is likely that our measurements were in a region closer to the area considered “Hilus” in Toni’s work. Finally, regarding the comment made by Reviewer regarding the differences attributable to the plane of section, we would like to point out that quantifications were made on Z-projection images and that only those MFTs touching either the upper or lower planes were excluded. We agree with the Reviewer regarding the high variability of MFT size. Importantly, this has been reported by other authors (Toni et al, 2008). However, in order to further explore the effects of Tau deficiency on the variability of the MFT size, we have included an additional representation of MFT grouped by size in Figure EV 3, very similar to that described in the study by Toni et al. As can be observed, the distribution of MFT sizes does not differ between genotypes. It should be noted, however, that our representation is very similar to what Toni et al. considered as Hilus, possibly due to our previously commented cautionary measure of avoiding the part of the CA3 field close to the CA2 region. Moreover, following Reviewer 1’s suggestion, we have analyzed the ultrastructure of MFTs under electron microscopy. Interestingly, Tau deficiency reduced the density of presynaptic vesicles and the length of the active zone and increased the synaptic cleft size in the CA3 region. These quantifications are shown in Figure EV 3. We are pleased to have included this new information in the revised version of our manuscript, since it has allowed us to unravel ultrastructural alterations in the MFTs that we were unable to detect by confocal analysis. We hope that the information related to the methodology used, together with all the new experimental data provided in the revised version of our manuscript, answer Reviewer’s questions regarding these quantifications.

The following data has been included in the main text of the manuscript:

- **Material and Methods:**

  **“Area of MFTs of newborn granule neurons”**

  The area of individual MFTs labeled with GFP was measured in the CA3 region of PSD95-GFP retrovirus-injected animals. A minimum of 20 stacks of images per experimental condition were obtained in a LSM710 Zeiss confocal microscope (63X oil immersion objective; XY dimensions: 100 µm; Z-interval: 0.5 µm). Stacks were randomly obtained among the different sections composing the series. GFP-Z projections were obtained, and the area of each MFT was measured manually in ImageJ software, as previously described (Toni et al, 2008). The following criteria were selected for MFT quantification: a) the diameter of the MFT is more than threefold greater than the diameter of the axon; b) the MFT is connected to the axon on at least one end, and c) the MFT is relatively isolated from other MFTs for accuracy of tracing (Toni et al, 2008). Given that the MFTs of the CA2 region are significantly smaller than those of the CA3 (Llorens-Martin et al, 2015), we avoided the CA2 region when obtaining the images. All the measurements were made in equivalent rostrocaudal positions of the CA3 field which were always closer to the hilus than to the CA2 region. A minimum of 100 MFTs per experimental condition were measured.”

- **Appendix Supplementary methods:**

  **“Electron microscopy”**

  After a post-fixation step, 200-µm sagittal sections were obtained on a Leica VT1200S vibratome. Four sections per mouse containing the whole hippocampus were post-fixed in 2% osmium tetroxide (OsO4) for 2 h. They were then rinsed, dehydrated, and embedded in Durcupan (Durcupan, Fluka). Serial semi-thin sections (1 µm) were cut with a diamond knife and stained with 1% Toluidine blue. Subsequently, the area of interest was trimmed, and ultrathin sections (0.06 µm) were obtained with a diamond knife. These sections were then stained with lead citrate and examined under a JEM1010 Jeol electron microscope equipped with a 4Kx4K TemCam-F416 Digital camera. All the images were obtained at 15,000X magnification. To study the ultrastructure of the afferent synapses of granule neurons, 30 images for each subfield of the ML (EML, MML and IML) and per animal were obtained. With respect to the ultrastructural organization of the efferent synapses of these neurons,
at least 30 images per animal containing the stratum lucidum and the inner boundary of the stratum pyramidale of the CA3 region were obtained. In the ML, the density of synapses (number of synapses per mm²), size of the synaptic cleft, and the length, depth and total area of the PSDs were measured in each sub-region separately. In the CA3 region, the density of presynaptic vesicles (number of vesicles per µm²), length of the presynaptic active zone, and the size of the synaptic cleft were measured. According to the definitions by Toni et al. (Toni et al., 2008), at the presynaptic level at least 4 presynaptic vesicles fusing with the active zone are required for a structure to be considered a synapse. In order to measure the density of presynaptic vesicles in the CA3 region, given that some MFTs were not fully included in the image due to the high complexity of these structures in this zone, a squared region of interest (ROI) was used to count the number of vesicles and to calculate their density. The length of the active zone and also the length, depth and total area of the PSDs were measured manually in the images using ImageJ software. In addition, the size of the postsynaptic cleft was calculated by measuring the distance between the outer borders of both the presynaptic and the postsynaptic membrane. Finally, the density of synapses was calculated by dividing the total number of synapses in each image by the known area of the image. 100-150 synapses per genotype and sub-region were analyzed.”

- **Results:**

“In order to evaluate whether Tau deficiency also has an impact on the morphology of newborn neuron MFTs (efferent synapses), we quantified the area of each individual MFT in 8-week-old retrovirally labeled newborn granule neurons. **Fig EV 3 A, B** show representative images of the whole hippocampus (A), as well as high-power magnifications of MFTs in the CA3 field (B) of WT and Tau−/− mice. No differences were found between WT and Tau−/− animals. Differences were found between WT and Tau−/− animals (U= 17886; p= 0.166) (**Fig EV 3 C**). In addition, and given the high variability in size of the MFT population, we plotted the percentages of MFT grouped by size in WT and Tau−/− mice (**Fig EV 3 D**). As shown, no differences in the distribution of MFT sizes were observed to be caused by the absence of Tau (K-S Z= 1.089; p= 0.187).

Finally, we analyzed the effects of Tau deficiency on the ultrastructure of the MFTs under an electron microscope (**Fig EV 3 E**). In this regard, we studied the stratum lucidum and stratum pyramidale of the CA3 region. The density of presynaptic vesicles (number of vesicles per µm²) (t= 3.548; p < 0.001) (**Fig EV 3 F**) and the length of the presynaptic active zone (t= 5.589; p < 0.001) (**Fig EV 3 G**) were reduced, whereas the size of the synaptic cleft (t= -7.409; p < 0.001) (**Fig EV 3 H**) was increased in Tau−/− animals compared to WT ones.

In summary, these results suggest that Tau participates in the functional maturation of granule neurons, since its absence alters PSD, dendritic spine, and MFT morphology.”

- **Discussion:**

“In addition, we showed that absence of this protein also alters the ultrastructure of MFTs (efferent synapses), which may have important consequences for granule neuron functionality.”

- **Legend to Figures:**

“Figure 8 – Tau protein mediates the stimulatory effects of EE on the dendritic morphology, PSDs and MFTs of newborn neurons.

A  Schematic diagram of the experimental design.

B  Representative images of 8-week-old newborn granule neurons of WT CH, WT EE, Tau−/− CH, and Tau−/− EE mice infected with a PSD95-GFP-expressing retrovirus. In the WT CH image a schematic representation of Sholl’s analysis is shown.

C-D  Quantification of total dendritic length (C) and Sholl’s analysis (D) of 8-week-old newborn granule neurons (n= 3 mice per experimental condition).

E-F  Representative images of an 8-week-old newborn granule neuron in which proximal and distal dendrites are highlighted (E) and of PSDs (green) in distal and proximal dendrites of WT and Tau−/− mice housed under either CH or EE conditions (F).

G-H  Quantification of the number of PSDs/µm (G) and PSD area (H) in each dendritic branching order of 8-week-old newborn granule neurons (n= 3 mice per experimental condition).

I  Representative image showing 8-week-old newborn granule neurons in the DG projecting axons through the mossy fiber tract into the CA3 field (indicated by a white square).
Representative images (J) and quantification of the area (K) of MFTs of 8-week-old newborn neurons in the CA3 region of WT CH, WT EE, Tau–/– CH, and Tau–/– EE mice (n= 3 mice per experimental condition). Arrows indicate MFTs. 

Data information: Data are presented as mean ± SEM. Black asterisks (*) indicate differences between CH and EE groups: **p < 0.001 (Two-way ANOVA). Colored asterisks indicate differences between the group represented by the same color and the WT CH group: *0.05 > p ≥ 0.001, *0.01 > p ≥ 0.001, ***p < 0.001; colored crosses (+) indicate differences between the group represented by the same color and the WT EE group: +++p < 0.001; colored pads (¹) indicate differences between the group represented by the same color and the Tau–/– CH group: ##0.01 > p ≥ 0.001, ###p < 0.001 (One-way ANOVA + LSD post hoc multiple comparisons). In representative images cell nuclei were labeled with DAPI (blue). White scale bar 50 µm. Red scale bar 5 µm. Orange scale bar 300 µm. Green scale bar 20 µm. Brightness and contrast of representative confocal microscopy images shown in the Figure were minimally adjusted in order to improve visualization.”

- **Legend to Expanded View Supplementary Figures:**

“Figure EV3 – Tau protein deficiency alters the ultrastructure of the efferent synapses of granule neurons in CA3.

A Representative image showing 8-week-old newborn granule neurons in the DG (infected by a PSD95-GFP-expressing retrovirus) projecting axons into the CA3 field (indicated by a white square). Red scale bar 300 µm.

B Representative images of MFTs of 8-week-old newborn granule neurons in the CA3 region of WT and Tau–/– mice. Green scale bar 20 µm.

C Quantification of the area of MFTs of 8-week-old newborn granule neurons in WT and Tau–/– mice (mean ± SEM; n= 3 mice per genotype; Mann-Whitney’s U test).

D Representation of the distribution of the size of MFTs in 8-week-old newborn granule neurons of WT and Tau–/– mice. MFTs were grouped on the basis of size, and the percentage of MFTs in each size group was represented (n= 3 mice per genotype; Kolmogorov-Smirnov’s Z test).

E Representative electron microscopy images of the ultrastructure of efferent synapses of granule neurons in the CA3 region of WT and Tau–/– mice. Brackets indicate the active zone of the presynaptic terminal. Asterisks indicate synaptic clefts. White scale bar 250 nm.

F-H Quantification of the number of synaptic vesicles/µm² (F), the length of the active zone (G) and the synaptic cleft size (H) of efferent synapses of granule neurons in the CA3 region of WT and Tau–/– mice (mean ± SEM; n= 3 mice per genotype; **0.01 > p ≥ 0.001, ***p < 0.001; Student’s t-test).

Data information: DG, dentate gyrus; H, hilus; SS, synaptic spine; MFT, mossy fiber terminal. Brightness and contrast of representative images shown in the Figure were minimally adjusted in order to improve visualization.”

(2) Page 9, second paragraph referring to GAD-65 staining and quantification Fig 5l-t: In the methods it states that a circular area has been used for calculation, whereas a rectangular area is shown in the actual figure. Also, it may be me but I am a bit skeptical about quantifying the intensity of these random areas of the layer because looking at the low mag image in fig 5p within this layer there is actually quite some variability in thickness and intensity which also seems to follow a gradient. In other words I am not ultra-convinced of this set of data.

We apologize for the misunderstanding caused by the images in Figure 5. Following your suggestion and that of Reviewer 1, we have clarified the information relative to GAD-65 quantifications. In the revised version of the manuscript, we have performed more specific quantifications instead of quantifying the total area occupied by GAD-65+ GABAergic terminals, a parameter that raised concerns by Reviewers. Here we have copied a fragment of our response to Reviewer 1 regarding this matter:

“It is noteworthy that GAD-65 protein has been demonstrated to unambiguously label GABAergic presynaptic terminals (Trejo et al, 2007). However, we understand that the way in which we presented the images in Figure 5 (the old version of the manuscript) led to a methodological misunderstanding of the procedure used to perform the quantifications. The analysis of GABAergic innervation was actually done on high magnification images (Zeiss confocal microscope, oil immersion 63X objective, Image XY dimensions: 24.1 µm). Under this high-power magnification, the 3 layers of the ML can be easily identified on the basis of the different staining pattern of GAD-65 in the three layers. Three high-power magnification
images were obtained for each sub-region of the ML (EML, MML and IML) and for each animal. These images were subjected to an invariant threshold, which was previously set up for each sub-region. In order to facilitate interpretation of the results, data from the three sub-regions were normalized and presented as % of area occupied by GAD-65+ terminals in the ML with respect to the WT under control housing. This analysis has systematically been done by our group and others and has been demonstrated to be a reliable method for measuring GABAergic innervation in the ML (Martínez-Cue et al., 2013; Trejo et al., 2007). However, given that both Reviewers expressed their concerns about this quantification, the previous graph has been removed from the main text in the revised version of the manuscript. Instead of quantifying the total area occupied by GAD-65+ terminals, we have now quantified the density of GAD-65+ GABAergic presynaptic terminals (number of terminals per µm²) and the average area of these terminals. These new quantifications are shown in Figure 7. In addition, we have provided separate quantifications for the EML, MML, and IML in Tau−/− and WT animals, both under control and enriched housing conditions. These data are presented in Figure EV 4. In short, we have found that both Tau deficiency and EE locally alter the three parameters in specific layers of the ML.”

In addition to the data presented here, in Figure 7 and Figure EV4, we have included the following information in the main text of the manuscript:

Appendix Supplementary methods:

“Analysis of GABAergic innervation

GABAergic innervation in the molecular layer (ML) was analyzed by immunohistochemistry of GAD-65+ GABAergic terminals. Images were randomly obtained from the sections comprising the series. Three high magnification confocal images per sub-region of the ML (namely, external (EML), medial (MML) and inner (IML)) were obtained per animal in a LSM710 Zeiss confocal microscope (63X oil immersion objective, XY dimensions: 24.1 µm). In each sub-region, an invariant threshold for fluorescence intensity was established to analyze all images. We measured the number of GAD-65+ GABAergic terminals, as well as the average area of each terminal, using the plugin Particle Analizer for ImageJ software (ImageJ, v. 1.33, NIH, Bethesda, MD, USA, http://rsb.info.nih.gov/ij). To simplify the interpretation of the results, data corresponding to the whole ML are shown in Fig 7. These grouped representations required the normalization of individual data from each sub-region with respect to WT control-housed mice. In addition, the regional changes observed in the three sub-regions of the ML are shown in Fig EV 4. 4000-5000 GAD-65+ terminals per genotype, sub-region and experimental condition were studied in order to calculate the average area of the terminals.”

Results:

“It is known that GABA promotes cell survival during AHN (Song et al., 2013). In order to study whether GABAergic innervation is related to the pro-neurogenic effect of EE, we analyzed GAD-65+ GABAergic terminals in the ML of mice in each experimental condition (Fig 7 A). The results showed that EE promoted different effects in WT and Tau−/− animals in both the density (Interaction F1,308= 12.064; p= 0.021) (Fig 7 B) and the average area (Interaction F1,58237= 62.406; p< 0.001) (Fig 7 C) of GABAergic terminals. While EE promoted an increase in both parameters in WT mice (p=0.009 and p< 0.001, respectively), it caused a reduction in both in Tau−/− animals (p= 0.023 and p< 0.001, respectively). Fig EV 4 shows the regional changes observed in these parameters in the three sub-regions of the ML: EML, MML and IML.”

Legend to Figures:

“Figure 7 – Tau protein is involved in the effect of EE on GABAergic innervation.

A Representative images of the whole hippocampus showing GABAergic innervation labeled with GAD-65 antibody (red), and high-power magnifications of the DG molecular layer showing GAD-65+ terminals in WT CH, Tau−/− CH, WT EE and Tau−/− EE mice. Cell nuclei were labeled with DAPI (blue). White scale bar 300 µm. Red scale bar 50 µm. Green scale bar 5 µm.

B-C Quantification of the density (B) and area (C) of GAD-65+ terminals in the molecular layer of mice in each experimental condition (n= 10 mice WT CH, n= 8 mice Tau−/− CH, n= 6 mice WT EE, n= 10 mice Tau−/− EE).
Data information: in (B-C) data are presented as mean ± SEM (normalized with respect to WT CH). Colored asterisks indicate differences between the group represented by the same color and the WT CH group: **0.01 > p ≥ 0.001, ***p < 0.001; colored crosses (+) indicate differences between the group represented by the same color and the WT EE group: +0.05 > p ≥ 0.01; colored pads (#) indicate differences between the group represented by the same color and the Tau-/- CH group: #0.05 > p ≥ 0.01, ###p < 0.001 (One-way ANOVA + LSD post hoc multiple comparisons). DG, dentate gyrus; ML, molecular layer.

Legend to Expanded View Supplementary Figures:

"Figure EV4 – Tau protein regulates the effects of EE on GABAergic innervation in a region-dependent manner.

A Representative image of the whole hippocampus showing GABAergic innervation labeled with GAD-65 antibody (red) and a magnification of the ML showing the different sub-regions (EML, MML and IML). Cell nuclei were labeled with DAPI (blue). White scale bar 300 µm. Green scale bar 50 µm.

B-D Quantification of the density and area of GAD-65⁺ terminals in the EML (B), MML (C) and IML (D) (n= 10 mice WT CH, n= 8 mice Tau-/- CH, n= 6 mice WT EE, n= 10 mice Tau-/- EE). Data information: in (B-D) data are presented as mean ± SEM (normalized with respect to WT CH). Colored asterisks (*) indicate differences between the group represented by the same color and the WT CH group: *0.05 > p ≥ 0.01, **0.01 > p ≥ 0.001, ***p < 0.001; colored crosses (+) indicate differences between the group represented by the same color and the WT EE group: +++p < 0.001; colored pads (#) indicate differences between the group represented by the same color and the Tau-/- CH group: ###p < 0.001 (One-way ANOVA + LSD post hoc multiple comparisons). DG, dentate gyrus; ML, molecular layer; IML, inner molecular layer; MML, medial molecular layer; EML, external molecular layer. Brightness and contrast of representative confocal microscopy images shown in the Figure were minimally adjusted in order to improve visualization."

3) Comments to the Discussion:
page 13, 1st paragraph: The statement on connectivity in my view should be taken out; and page 12, 2nd paragraph: Ittner et al. did not show ‘disappearance of synapse[s]’ in the tau KO. Thank you very much. We have clarified the sentence relative to Ittner’s work. Furthermore, following the Reviewer’s suggestion, we have performed patch-clamp electrophysiological recordings of granule neurons. As mentioned previously, the results obtained support the notion that Tau deficiency alters newborn neuron functionality. However, we have made every effort to remove the term “connectivity” and replace it by other equivalent ones throughout the manuscript whenever possible.

Minor concerns/comments:

(1) Page 5: Many terms are explained in the methods but not when first mentioned (e.g. PH3, Sox2, BLBP). See also on page 7 (CldU), Page 9: DCX, fractin (also explain for those who don’t know these markers)

Thank you. We have included these definitions and a brief explanation of each marker.

(2) Page 6: The data have been obtained at 4 and 8 weeks. That PSDs are reduced in the 5th dendritic branching order could simply mean a developmental delay in the tau KO. Do they catch up at later time-points?

We would like to thank the Reviewer for drawing out attention to this point. Following his/her suggestion, we addressed whether Tau-/- newborn neurons would follow maturation dynamics similar to that of WT neurons. In order to tackle this question, we quantified the percentage of 1-, 4- and 8-week-old cells that expressed either DCX or NeuN. We did not find statistically significant differences between Tau-/- and WT mice at any of the cell ages studied. These quantifications are shown in Figure 1 H. In the light of the results obtained, and given that the dendritic alterations are no longer observed at 8 weeks of cell age, it is considered unlikely that newborn neurons in Tau-/- mice show maturational retardation at times longer than this period, but rather that they experience a selective synaptic impairment due to Tau deficiency.
Moreover, we aimed to address whether the general population of granule neurons also exhibit alterations in synapse morphology. As we further detailed in our responses to Reviewer 1, we examined the ML (afferent synapses) and the CA3 (efferent synapses) regions of the hippocampus of Tau −/− and WT mice under electron microscopy. Interestingly, we found that Tau deficiency altered numerous morphological parameters in the afferent and efferent synapses of granule neurons. These results are summarized in Figures EV 2 and EV 3. Thus, we believe that the PSD alterations observed in newborn granule neurons are not due to a maturational delay but to the specific effects of Tau on synapse development and stabilization.

The following information has been added to the revised version of the manuscript:

- **Appendix Supplementary methods:**

  “In order to study the maturation of newborn neurons along time, the percentage of 1-, 4-, and 8-week-old newborn neurons (labeled with thymidine analogs) that expressed DCX and NeuN was analyzed in WT and Tau −/− mice. A minimum of 100 cells per experimental condition and time point were examined.”

- **Results:**

  “Tau protein is not involved in the regulation of the basal rate of adult hippocampal neurogenesis (AHN)

We first examined whether Tau protein is involved in the regulation of AHN under basal conditions. **Fig 1 A-D** shows that the absence of Tau did not affect the number of proliferative phospho-histone 3 (PH3)+ cells (t= 0.757; p= 0.471) or the number of apoptotic caspase-cleaved actin (fractin)+ cells (t= -0.771; p= 0.452) in the DG. However, as proliferation and death rates are general parameters that may not reflect changes in specific cell subpopulations, we also analyzed the survival of 1-, 2-, 4-, 6- and 8-week-old cells labeled with the thymidine analogs 5-Iodo-2′-deoxyuridine (IdU) or 5-Chloro-2′-deoxyuridine (CldU). **Fig 1 E** shows the experimental design. Tau depletion did not alter cell survival at any of the ages studied (**Fig 1 F**). Hence, we conclude that Tau protein is not involved in the regulation of newborn neuron survival rate. **Fig 1 G** summarizes the main cell markers expressed during the sequential differentiation stages that newborn neurons go through before they become fully mature. We addressed whether Tau deficiency affects the differentiation of newborn granule neurons and their commitment to neural lineage. To explore this notion, the percentage of cells that expressed doublecortin (DCX) and Neuronal nuclei (NeuN) markers was evaluated in 1-, 4-, and 8- week-old CldU− newborn neurons. As expected, DCX expression decreased with time, whereas the opposite effect was observed for NeuN (**Fig 1 H**). No differences in the expression of either of these markers were found in response to Tau deficiency. Thus, it can be concluded that Tau is not involved in newborn neuron differentiation under basal conditions. Moreover, the absence of Tau did not alter the number of Sex determining region Y-box 2 (Sox2)+ (t= -1.180; p= 0.261) or Brain lipid binding protein (BLBP) (t= -1.015; p= 0.330) neural progenitor cells. In addition, no differences in the number of DCX− neuroblasts (t= -1.201; p=0.253) or in the number of calretinin− immature neurons (t= -1.427; p= 0.179) were found in Tau− mice compared to WT (**Fig 1 I-N**). All together, these data suggest that neither is Tau involved in the regulation of AHN rate under basal conditions.”

- **Legend to Figures:**

  “Figure 1 – Tau protein is not involved in regulating the rate of adult hippocampal neurogenesis under basal conditions.

  **H** Quantification of the percentage of 1-, 4- and 8-week-old newborn neurons that express the DCX neuroblast marker and the NeuN mature neuron marker in WT and Tau−/− mice (χ² test).”

(3) Page 7: CldU administration is labeled as acute stress test. Is the Porsolt test then a chronic test? **We are sorry for having caused a misunderstanding of this term. We did not intend the reader to understand that the administration of thymidine analogs was an acute stress model. The experiments including CldU and IdU administration were performed under basal, stress and enriched conditions and are merely a methodological tool by which to study generation and survival rate of newborn neurons. In contrast, the Porsolt test is a model of...
acute stress. We have clarified the pertinent sentences in the text. The following text appears in the revised version of our manuscript.

- **Results:**

“**Tau protein is required for stress-induced death of newborn granule neurons**

Given that AHN is regulated by external stimuli, we next examined whether Tau protein is involved in the negative regulation of AHN exerted by acute stress. We administered CldU thymidine analog to WT and Tau−/− animals in order to label dividing newborn neurons and then split these animals into two experimental conditions: mice subjected to the Porsolt test (P), which is considered an acute stress model; and control mice not subjected to this test (CNP). Animals were sacrificed 1 week after CldU administration. Mice were subjected to the Porsolt test on the 2 days prior to sacrifice (Fig 4 A, B)”

(4) Page 7/8: With ref to Fig 4G the authors speak of 'crossings'. What do they mean by that and how is this counted? The sentence ‘it did not cause further alterations in any other point of the dendritic tree’ is not clear.

We are sorry for not including this definition in the previous version of our manuscript. In order to analyze the morphology and branching of the dendritic trees of newborn granule neurons, Sholl’s analysis was performed using a Sholl’s analysis plugin in ImageJ. This analysis consists of placing a central point on the cell soma and tracing concentric circles (separated by a fixed interval, which was, in our case, 10 µm). Then, the number of times that the dendritic tree intersects each circle is represented in the graphs. Thus, the word “crossings” actually refers to the number of intersections that the dendritic tree makes with each circumference. We have clarified this information and also corrected the sentence suggested by Reviewer.

The following information has been included in the revised version of the manuscript:

- **Material and Methods:**

“This analysis consists of placing a central point on the cell soma and tracing concentric circles (separated by a 10 µm distance interval). The number of times that the dendritic tree intersects each circle is represented in the graphs (number of crossings).”

(5) Page 9: DCX counting: At which age have these data been obtained?

All cell counts were done at 4 months of age. We have added this information to the revised version of the manuscript:

- **Material and Methods:**

“Animals were sacrificed at 4 months of age.”

(6) Page 15: Major depression spelt with small letters.

Thank you, we have corrected it.

Suggestions for improving the study (at the editor's discretion):

(1) A critical question is whether the difference in dendritic length and arborization and spine density is reflected by functional changes. This could be addressed by electrophysiological recordings.

Thank you for this interesting suggestion. We would like to thank the Reviewer for having suggested that we perform electrophysiological recordings in order to further strengthen our previous observations. As previously mentioned, we have performed patch-clamp recordings of granule neuron in acute slices. As shown in Figure 3, Tau−/− neurons showed basal electrophysiological alterations that are in agreement with the morphological alterations we had previously described (both by confocal and electron microscopy analyses). Thus, we consider that these data, taken together, further support the notion that Tau deficiency has important consequences for the maturation of granule neurons both at the morphological and the functional levels.

(2) The authors make the point that hippocampal neurogenesis is associated with 3Rtau expression while the rest of the adult mouse brain expresses 4Rtau. Yet the study looks at presence versus
absence of total tau. It would be interesting to generate a mouse model that does not make 3R tau in adulthood yet this is clearly outside the scope of this paper.

Thank you for your suggestion. We also believe that it would be interesting to have a genetic system that could allow the flexible switch of the expression of either 3R- or 4R- Tau, in order to analyze the putative changes in newborn maturation triggered by the expression of both isoforms. It can be hypothesized that 4RTau expression might accelerate the maturation of these cells, whereas 3RTau may retain them in a more undifferentiated (or plastic) stage. However, more importantly, this system would provide crucial information regarding Tau functions that no current murine models can offer. In fact, it is known that compensatory roles played by other MAPs take place in Tau knock-out murine models (Dawson et al, 2001; Fujio et al, 2007; Harada et al, 1994). However, by using the murine model suggested by Reviewer, the effects of having a “wrong isoform” of Tau at the “wrong maturational time point” could be examined. Thank you again for outlining the design of this original murine model. We hope we will be able to use such a valuable tool in our future experiments.

REFERENCES RELATIVE TO THIS DOCUMENT


Thank you for submitting your manuscript to The EMBO Journal. Your revision has now been seen by the two referees and as you can see below they appreciate the introduced changes and support publication here. I am therefore very pleased to accept the manuscript for publication here.

Before we can transfer the manuscript to our publisher there are just a few minor things to sort out.

I don't think that there are any callouts to the expanded view figures in the text. Could you take a look at this and send us a modified word document.

We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Could you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

It would also be good if you could provide me with a summary figure that I can place in the synopsis. The size should be 550 wide by 400 high (pixels). If it becomes too much work then just ignore it and I can choose an image from the paper.

I think that should be it. You can send me the above info by email. Just contact me if you have any further questions.

REFeree REPORTS

Referee #1:

The authors have amended previous weak points and added extensive new data. The paper could be published as is.
Referee #2:

The paper has been extensively and thoroughly revised (providing a very detailed rebuttal). In particular, the electrophysiological recordings as part of the revision strengthen the manuscript significantly, supporting the main conclusions of the study.
### A- Figures

1. **Data**
   - The data shown in figures should satisfy the following conditions:
     - The data were obtained and processed according to the field’s best practice and are presented to reflect the results of the experiment in an accurate and unbiased manner.
     - Figure panel(s) should include all data points, measurements, or observations that can be compared to each other in a scientifically meaningful way.
     - Graphs should include error bars only for independent experiments and sample sizes where the application of statistical tests is warranted; error bars should not be shown for technical replicates.
   - When n is small (< 5), the individual data points from each experiment should be plotted alongside an error bar.
   - Source data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. **Captions**
   - Each figure caption should contain the following information, for each panel where they are included:
     - A specification of the experimental system investigated (e.g., cell line, species name).
     - An explicit mention of the biological and chemical entity(ies) that are altered/variable/subjected in a controlled manner.
     - The exact sample size [n] for each experimental group/condition, given as a number, not a range.
     - A description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
     - A statement of how many times the experiment was independently replicated in the laboratory.
     - Definitions of statistical methods and measures:
       - Common tests, such as t-tests (please specify whether paired vs. unpaired), simple χ² tests, Wilcoxon and Mann–Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section.
       - Are tests one-sided or two-sided?
       - Are there adjustments for multiple comparisons?
       - Are statistical test results, e.g., if P-values > 0.05, then P-values < 0.05.
       - Definition of “center values” as median or average.
       - Definition of error bars as s.d. or s.e.m.

### B- Statistics and general methods

- **A.** How was the sample size chosen to ensure adequate power to detect a prespecified effect size?
- **B.** Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g., blinding of the investigator)? If yes, please describe.
- **C.** Describe in detail/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?

### C- Reagents

- **A.** To show that antibodies were profiled for use in the system under study (assay and species), provide: a station, catalog number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., Antibodypedia (search name), 10mg/100g.
- **B.** Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

### D- Animal Models

- **A.** Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.
- **B.** For experiments involving live animals, include a statement of compliance with ethical guidelines and identify the committee(s) approving the experiments.
- **C.** Follow the recommended animal research guidelines (Post-Bat Act) x100542, 2010 to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines under ‘Reporting Guidelines’ for details on reporting guidelines. See also: NIH, see link at top right.

### E- Human Subjects

- **A.** Please confirm compliance.
11. Identify the committee(s) approving the study protocol.

12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

13. For publication of patient photos, include a statement confirming that consent to publish was obtained.

14. Report any restrictions on the availability (and/or on the use) of human data or samples.

15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'.

17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'.

F. Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition' (see link list at top right).

Data deposition in a public repository is mandatory for:

a. Protein, DNA and RNA sequences
b. Macromolecular structures
c. Crystallographic data for small molecules
d. Functional genomics data
e. Proteomics and molecular interactions

19. Deposite is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) in Figures.

20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA.

21. As far as possible, primary and referenced data should be formally cited in a Data Availability section.

Examples:

- Primary Data
  - Huang L, Braun AF, Leit M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4C05
  - AP-MS analysis of human histone-deacetylase interactions in CEM-T cells (2013). PDBX PDB002038

- Referenced Data
  - Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4C05

22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (i.e., CellML) should be used instead of scripts (e.g., MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as BioModels (see link list at top right) or BioModels Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.

G. Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosafety documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosafety guidelines, provide a statement only if it could.