# Presenilin enhancer 2 is crucial for the transition of apical progenitors into neurons but into not basal progenitors in the developing hippocampus

Yingqian Xia, Yizhi Zhang, Min Xu, Xiaochuan Zou, Jun Gao, Mu-Huo Ji and Guiquan Chen
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**Original submission**

First decision letter

MS ID#: DEVELOP/2021/200272

MS TITLE: Presenilin enhancer2 is critical for the transition of apical progenitors to neurons but not basal progenitors in the developing hippocampus

AUTHORS: Yingqian Xia, Yizhi Zhang, Min Xu, Xiaochuan Zou, Jun Gao, Mu-Huo Ji, and Guiquan Chen

Please accept my apologies for the long time it has taken us to receive the reports of referees on your manuscript. I have now received the reports of two referees and I have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, both referees express great interest in your work, but referee 1 also has significant criticisms and recommends a substantial revision of your manuscript before we can consider publication. In particular, they request that you analyse the downstream targets of gamma-secretase (by western blot) and the expression of Hes/Hey genes, Neurod1 and Neurog2 at an earlier stage. They also ask that you analyse Pen-2 deletion in the hippocampus and cerebral cortex at an earlier stage. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost

in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

*Advance summary and potential significance to field*

This is an interesting manuscript that studies the role of Pen-2 in the developing hippocampus, suggesting than Pen-2 regulates the transition of apical progenitors to neurons by controlling Notch cleavage. Using the results from this study and the ones previously obtained in Cheng et al. 2019 the authors also claimed that Pen-2 have different roles during neocortical and hippocampal development.

*Comments for the author*

Comments to the authors,
Major comments,
1) The medial telencephalon phenotype due to Pen-2 deletion by hGFAP-Cre appears quite late (E17.5) after the onset Cre expression (from E13.5). It is possible that the late onset of the phenotype is due to a low Cre efficiency. The samples used to study deletion efficiency should have been taken at earlier stages, ideally before the changes in the number of Pax6+ and Tbr2+ cells by E17.5. Could the authors provide a western blot and/or qRT-PCR from Pen-2 from an earlier stage (E16.5 or E15.5) to address this issue?
2) Pen-2 deletion by hGFAP-Cre does not result in a neocortical phenotype. Could the authors provide a western blot and/or qRT-PCR to show the efficiency of the deletion of Pen-2 in the neocortex, to show that Pen-2 has been removed?
3) Regarding figure 5, the change in NeuroD1 immunoreactivity in the dKO hippocampus is very easy to see in the images, but I can't see it for Tbr1. Please provide better images. It is also very difficult to see clearly the double positive cells in panels 5B and 5D. They authors consider this one of their significant findings therefore new and better images/panels are necessary. Arrows or arrowheads to label double positive cells would help too.
4) The analysis for the downstream targets of y-secretase by western blot should have been done at an earlier stage, preferably before the changes in the number of Pax6+ and Tbr2+ cells by E17.5. By P0 the majority of the cells in the developing hippocampus are neurons; the changes described in the figures may not reflect what it is happening due to the lack of Pen-2 in progenitors.
5) The decrease in Hes1/Hes5/Hey1/Hey2 levels observed by qRT-PCR at E17.5 could be a consequence of the already reduced numbers of progenitor cells at that stage. The qRT-PCRs should have been done at a stage just before the onset of the phenotype, like E16.5. Ngn2 and NeuroD1 levels should have been measured also by E16.5. In addition to qRT-PCRs the authors might want to provide in situ hybridizations for Ngn2 and NeuroD1 at E16.5, like they have done for Hes1 and Hes5 in figure 8C.
6) The authors propose as a novel contribution the different roles of Pen-2 during neocortical and hippocampal development. I do agree that the removal of Pen-2 from E13.5 reveals a role in the developing hippocampus but not in the neocortex. However, I don't think it is proper to compare the results from Emx1-
Cre deletion with those using hGFAP-Cre. Further, looking at the results obtained using Emx1-Cre described in Fig1 in Cheng et al. 2019, it looks like there are no differences in AP/BP phenotype between the dorsal and the medial telencephalon. The description of the distinct roles, at different time points, in the different regions, has to be threaded much more carefully throughout the text and in the discussion. Their model in Figure 9 has to be annotated better to include starting deletion times and/or Cre lines.

Minor comments,
1) Please clarify in the methods section which cells were quantified in 2C, 2F, 3A, 3C. Every positive cell on the section including the secondary and tertiary matrices? Or just the ones in the ANE and the DNE?. The authors state that there is an "age-related" reduction in the population of APs and BPs in the Pen-2 cKO hippocampus; to conclude that they should have compared the

numbers for the same phenotype at the different stages. Besides according to their quantifications in 2F the number of Tbr2+ cells both for E16.5 and E17.5 looks roughly the same.
2) It would have been better to study the number of pyramidal cells to address whether Pen-2 affects neurogenesis in the hippocampus, as from E16.5 to E17.5 Prox1+ cells constitute only a small fraction of the hippocampal neurons. By E16.5-E17.5 Ctip2/Bcl11b labels CA1/CA1-to-be neurons (for example Abcam ab18465).

Reviewer 2

*Advance summary and potential significance to field*

In the present study, Xia and collegues have analyzed the role of presenilin enhancer2 (Pen-2) in hippocampal morphogenesis. To address this topic, they generated Pen-2 conditional knockout mice with inactivated Pen-2 in neural progenitor cells in the hippocampal primordium. They found hippocampal malformations and a decreased population of neural progenitor cells in the hippocampal neuroepithelium. The authors interprete their findings in the way that that deletion of Pen-2 caused enhanced transition of APs to neurons. Moreover, they found that expression of Notch1 intracellular domain (N1ICD) significantly increased the population of NPCs in the Pen-2 cKO hippocampus. The authors provide evidence for a functional role of Pen-2 in hippocampal development, and that Pen-2 regulates the fate determination of APs in the hippocampus and in the cortex differentially.
In sum, the reviewers impression is that the experiments are well performed, the findings are interesting and significantly contribute to our understanding of molecular mechanisms underlying early hippocampal development.

*Comments for the author*

I have only few criticisms and suggestions to improve the manuscript:
Page 8:
Concerning the dentate gyrus, the authors found that „there was no difference in the 3ry matrix between control and Pen-2 cKO embryos (Fig.3B).“
Given that there was no difference of the 3ry matrix observed, one might expect a normally sized dentate gyrus at an early postnatal stage. This is, however, this not the case, as shown in Fig. 1. Please interpretate of this observation in the discussion.
Page 10:
We observed significantly increased number of Prox1+ cells in the hippocampus in Pen-2 cKO mice compared with littermate controls (Fig.4F), suggesting transiently increased neurogenesis.
Can you exclude that Prox1+ interneurons contribute to the cell counts? Please comment.

Fig.9: A schematic model is important to summarize the findings for the reader and should therefore be clearly arranged. If the represented APs and neurons were simplified as schematic round cells, this would be sufficient and would very much help to cleary distinguish the arrows and abbreviations. Moreover, an additional scheme C) depicting your interpretation of the unaffected dentate 3ry matrix should be included.

Minor:
Page 7:
...please rephrase the wording in the following sentence:
...the thickness of Cux1+, Ctip2+ or Tbr1+ cells was comparable in these two genotypes (Fig.S3A-C). Thus, hGfap-Cre-mediated deletion of Pen-2 did not cause significant effect on cortical development.
For example as follows:
...the thickness of Cux1+, Ctip2+ or Tbr1+ cell layers was comparable in these two genotypes (Fig.S3A-C). Thus, hGfap-Cre-mediated deletion of Pen-2 did not cause significant effect on cortical layering.

**First revision**

**Author response to reviewers' comments**

**Point-by-point responses to reviewers' comments**

**Comments from Reviewer 1** and our responses.

**1) The medial telencephalon phenotype due to Pen-2 deletion by hGFAP-Cre appears quite late (E17.5) after the onset Cre expression (from E13.5). It is possible that the late onset of the phenotype is due to a low Cre efficiency. The samples used to study deletion efficiency should have been taken at earlier stages, ideally before the changes in the number of Pax6+ and Tbr2+ cells by E17.5. Could the authors provide a western blot and/or qRT-PCR from Pen-2 from an earlier stage (E16.5 or E15.5) to address this issue?**

Thank you very much for this excellent question. We are sorry that we did not provide data on Pen-2 levels in the cKO group younger than E17.5. As you know, the mouse hippocampus at E15.5 or E16.5 is very tiny. After reading your comments above, we first tried to prepare protein samples from the E16.5 hippocampus for several times, but the resulting protein lysates were really insufficient for us to run Western blotting. Second, since qRT-PCR requires much less amount of samples than Western blotting does, we decided to use qRT-PCR as an alternative way to check whether Pen-2 expression is affected in *Pen-2* cKO mice at E16.5. We dissected the E16.5 hippocampus and prepared RNA samples. From each embryo, we were able to purify a little RNA, but it was sufficient for us to run a few qRT-PCR experiments. Our results revealed robust reduction on *Pen-2* mRNA levels in the hippocampus of *Pen-2* cKO mice at E16.5 compared with littermate controls (Fig.6E). Third, we found that mRNA levels for *Hes1* and *Hes5* were significantly decreased in the hippocampus of *Pen-2* cKO mice at E16.5 compared with controls (Fig.6E). This finding is consistent with our previous *in situ* hybridization results for E16.5 mice (Fig.8C). Fourth, data for mice at E16.5 (Fig.6E) and E17.5 (Figs.1E&6B) revealed equivalent reduction on mRNA levels for *Pen-2*, *Hes1* and *Hes5* in *Pen-2* cKO mice compared with their littermate controls. Together, the above results suggest that *Gfap-Cre*-mediated deletion of *Pen-2* is highly efficient in the hippocampus at E16.5, a stage when *Pen-2* cKO mice have not displayed decreased numbers of Pax6+ cells and Tbr2+ cells in the hippocampal neuroepithelium.

**2) Pen-2 deletion by hGFAP-Cre does not result in a neocortical phenotype. Could the authors provide a western blot and/or qRT-PCR to show the efficiency of the deletion of Pen-2 in the neocortex, to show that Pen-2 has been removed?**

According to your suggestion, we performed qRT-PCR analysis on *Pen-2* using cortical RNA samples at E16.5. Levels of *Pen-2* mRNAs in *Pen-2* cKO cortices were about 50% of those in controls (Fig.S1A). Therefore, the deletion efficiency for *Pen-2* was not high in the cortex in *Pen-2$^{f/f}$;hGfap-Cre* mice as compared to that in the hippocampus (Fig.6E). Overall, Pen-2 is inactivated in the cortex of *Pen-2$^{f/f}$;hGfap-Cre* mice, but its deletion efficiency is lower in the cortex than in the hippocampus.

**3) Regarding figure 5, the change in NeuroD1 immunoreactivity in the cKO hippocampus is very easy to see in the images, but I can't see it for Tbr1. Please provide better images. It is also very difficult to see clearly the double positive cells in panels 5B and 5D. They authors consider this one of their significant findings, therefore new and better images/panels are necessary. Arrows or arrowheads to label double positive cells would help too.**

According to your suggestion, we use a new fluorescence image for the *Pen-2* cKO group (Fig.5E) to replace the previous one for co-staining of Sox2/Tbr1 (in the previous Fig.5C). Second, based on fluorescence signals for Pax6+ cells (Fig.5A) or Sox2+ cells (Fig.5E), we added dashed white lines to outline the hippocampal neuroepithelium in these two panels. Since the boxes used in the previous Fig.5A,5C included non-neuroepithelium regions, new boxes were set within the neuroepithelium in Fig.5A,5E. These new boxed areas were enlarged and then shown in Fig.5B,5F. Third, according to your suggestion, white arrowheads were added to indicate Pax6+/NeuroD1+ cells (Fig.5B) and Sox2+/Tbr1+ cells (Fig.5F), respectively. Fourth, in the RESULTS section, we

provided additional information for statistical analysis results to reveal increased immuno-reactivity for NeuroD1 and Tbr1 in the *Pen-2* cKO hippocampus compared with the control.

**4) The analysis for the downstream targets of γ-secretase by western blot should have been done at an earlier stage, preferably before the changes in the number of Pax6+ and Tbr2+ cells by E17.5. By P0 the majority of the cells in the developing hippocampus are neurons; the changes described in the figures may not reflect what it is happening due to the lack of Pen-2 in progenitors.**

First, as we stated in our responses to the # 1 question above, protein lysates prepared from the E16.5 hippocampus were insufficient for us to run Western blotting experiments for Pen-2 and downstream targets of γ-secretase. Therefore, we purified a little RNA to do qRT-PCR analyses. We observed significant reductions on mRNA levels for *Pen-2*, *Hes1* and *Hes5* in *Pen-2* cKO embryos at E16.5 compared with controls. Second, in Fig.8C, we previously presented *in situ* hybridization results, which shows decreased expression of *Hes1* and *Hes5* in *Pen-2* cKO hippocampi at E16.5 compared with littermate controls. Together, the significant reduction on expression levels of *Hes1* and *Hes5* observed in the *Pen-2* cKO hippocampus at E16.5 strongly suggests that molecular changes in Notch downstream targets precede the occurrence of the NPC loss at E17.5.

**5) The decrease in Hes1/Hes5/Hey1/Hey2 levels observed by qRT-PCR at E17.5 could be a consequence of the already reduced numbers of progenitor cells at that stage. The qRT-PCRs should have been done at a stage just before the onset of the phenotype, like E16.5. Ngn2 and NeuroD1 levels should have been measured also by E16.5. In addition to qRT-PCRs the authors might want to provide in situ hybridizations for Ngn2 and NeuroD1 at E16.5, like they have done for Hes1 and Hes5 in figure 8C.**

Thanks very much for the above constructive suggestions! We have conducted additional qRT-PCR analysis using RNA samples from control and *Pen-2* cKO hippocampi at E16.5. In the new Fig.6E, we have shown significant reductions on mRNA levels of *Hes1* and *Hes5* in the E16.5 *Pen-2* cKO hippocampus, indicating efficient deletion of Pen-2.

For *NeuroD1* and *Ngn2*, due to insufficient protein lysates, we were unable to perform Western blotting analysis. First, we conducted qRT-PCR analysis using RNA samples prepared from control and *Pen-2* cKO hippocampi at E16.5. Levels of *NeuroD1* but not *Ngn2* mRNAs were significantly higher in the hippocampus of *Pen-2* cKO mice at E16.5 than those in controls (Fig.6F). Therefore, the increase on the expression of *NeuroD1* (Fig.6C,6F) took place before and after *Pen-2* cKO mice exhibited changes on Pax6+ cells (Fig.2C) and Tbr2+ cells (Fig.2F) in the hippocampal neuroepithelium. Second, fluorescence images for co-staining of Pax6/NeuroD1 allow us to compare relative expression of NeuroD1 in NPCs in the hippocampal neuroepithelium between control and *Pen-2* cKO mice (Fig.5A). Our data revealed that the immuno-reactivity of NeuroD1 was significantly higher in the hippocampal neuroepithelium in *Pen-2* cKO mice than in littermate controls (Fig.5D). Together, these results suggest that NeuroD1 expression is increased in the *Pen-2* cKO hippocampal neuroepithelium prior to the occurrence of significant NPC loss at E17.5.

**6) The authors propose as a novel contribution the different roles of Pen-2 during neocortical and hippocampal development. I do agree that the removal of Pen-2 from E13.5 reveals a role in the developing hippocampus but not in the neocortex. However, I don't think it is proper to compare the results from Emx1-Cre deletion with those using hGFAP-Cre. Further, looking at the results obtained using Emx1-Cre described in Fig1 in Cheng et al. 2019, it looks like there are no differences in AP/BP phenotype between the dorsal and the medial telencephalon. The description of the distinct roles, at different time points, in the different regions, has to be threaded much more carefully throughout the text and in the discussion. Their model in Figure 9 has to be annotated better to include starting deletion times and/or Cre lines.**

Thank you very much for your comments on the cellular model we proposed in Fig.9. We agree with you on that different Cre lines and different timings for Pen-2 deletion need to be

taken into account for different phenotypes observed in *Pen-2$^{f/f}$;Emx1-Cre* and *Pen-2$^{f/f}$;hGfap-Cre* mice.

More specifically, *Emx1-Cre*-mediated deletion of *Pen-2* causes increased transition of APs to BPs in the ventricular zone of the telencephalon at E12.5 and E13.5 (Cheng et al. 2019). Whereas *hGfap-Cre*-mediated deletion of *Pen-2* results in enhanced transition of APs to neurons in the hippocampal neuroepithelium at E17, it does not affect populations of APs and BPs in the cortex (Figs.S1&S2). The above discrepancy may be due to the following reasons. First, the starting time for Cre expression driven by the human *Gfap* promoter is later than that by the *Emx1* promoter (E13.5 vs E10.5). Second, *hGfap-Cre*-mediated deletion efficiency of Pen-2 is lower in the cortex than in the hippocampus. Third, deletion efficiency of Pen-2 in the cortex is lower in *Pen-2$^{f/f}$;hGfap-Cre* mice than in *Pen-2$^{f/f}$;Emx1-Cre* mice. The above discussions were added in the third paragraph in the DISCUSSION section.

Overall, our aim was to investigate mechanisms by which Pen-2 regulates hippocampal development. Therefore, the hippocampus but not the cortex is the focus in this study. After reading your comments above, we made the following changes. First, we deleted "Our findings further suggest that Pen-2 regulates the fate determination of APs in the hippocampus and in the cortex differentially", the last sentence in the previous ABSTRACT section. Second, we deleted "These results suggest that Pen-2 may differentially regulate the fate determination of APs during corticogenesis and hippocampal morphogenesis", the last sentence in the third paragraph in the previous DISCUSSION section. Third, we re-wrote the last paragraph in the DISCUSSION section. Fourth, we removed the graph for the cortex from the previous Fig.9. The new Fig.9 focuses on the hippocampus, and it may better reflect main findings in this study than the previous one did.

**Minor comments**

**1) Please clarify in the methods section which cells were quantified in 2C, 2F, 3A, 3C. Every positive cell on the section including the secondary and tertiary matrices? Or just the ones in the ANE and the DNE? The authors state that there is an "age-related" reduction in the population of APs and BPs in the Pen-2 cKO hippocampus; to conclude that they should have compared the numbers for the same phenotype at the different stages. Besides according to their quantifications in 2F the number of Tbr2+ cells both for E16.5 and E17.5 looks roughly the same.**

In Fig.2, we counted cells positive for Pax6 or Tbr2 in hippocampal sub-regions including the ANE, the DNE, the 2ry matrix and the 3ry matrix in each brain section. The number of Pax6+ cells or Tbr2+ cells in the hippocampus was the sum of positive cells from four sub-regions. In Fig.3, the number of Pax6+ cells or Tbr2+ cells was presented separately for each sub-region. As shown in Fig.3A, four different hippocampal sub-regions were outlined by dashed lines. Additional information for cell counting was added in the MATERIALS AND METHODS section.

Thank you very much for reminding us of no significant age-related reduction on BPs in *Pen-2* cKO mice. When we stated age-related reduction, we actually meant significant reduction in the number of BPs in *Pen-2* cKO mice at E17.5 but not E16.5 compared with littermate controls. According to your suggestion, we deleted "Overall, the above results suggest that there is age-related reduction on the population of APs in the *Pen-2* cKO hippocampus" and "there is an age-related loss of BPs in *Pen-2* cKO mice" in the RESULTS section.

**2) It would have been better to study the number of pyramidal cells to address whether Pen-2 affects neurogenesis in the hippocampus, as from E16.5 to E17.5 Prox1+ cells constitute only a small fraction of the hippocampal neurons. By E16.5-E17.5 Ctip2/Bcl11b labels CA1/CA1-to-be neurons (for example Abcam ab18465).**

Thanks very much for this great suggestion. In this study, we found that the CA1, the CA3 and the DG were all significantly affected in *Pen-2$^{f/f}$;hGfap-Cre* mice (Fig.1F). Therefore, it would be ideal to use a marker which can label both pyramidal neurons and granule neurons. We agree with you on that Ctip2 is a well-known marker for pyramidal neurons in the CA1. However, we chose Tbr1 but not Ctip2 for the following reason. It has recently been shown that Tbr1 not only labels pyramidal neurons in the CA1 but also neurons in the CA3 and the DG in the developing hippocampus at E14, E16 and E18 (Fig. 8 in Barry et al., 2008). In agreement with the above study,

our results showed that Tbr1 labels neurons in the CA1, the CA3 as well as the DG in the hippocampus at E17 (Fig.5E).

**Comments from Reviewer 2** and our responses.

**Page 8:**
**Concerning the dentate gyrus, the authors found that „there was no difference in the 3ry matrix between control and Pen-2 cKO embryos (Fig.3B). Given that there was no difference of the 3ry matrix observed, one might expect a normally sized dentate gyrus at an early postnatal stage. This is, however, this not the case, as shown in Fig. 1. Please interpretate of this observation in the discussion.**

This is a very interesting question. As you know, neurons in the DG are generated from NPCs localized in the 3ry matrix as well as the DNE in the hippocampus. Whereas a proportion of DG neurons may be generated from NPCs in the 3ry matrix and are called DG-born neurons, the rest are generated from NPCs in the DNE and migrate to the 3ry matrix to form the DG (Nakahira and Yuasa, 2005). First, equal amount of NPCs in the 3ry matrix in control and *Pen-2* cKO mice at E17.5 (Fig.3A-C) suggest that, the number of DG-born neurons may be comparable between two genotypes. Second, the robust reduction on NPC populations in the DNE in *Pen-2* cKO mice at E17.5 (Fig.3A-C) suggests that the number of DNE-born neurons may be significantly decreased in *Pen-2* cKO mice at and after E17.5. The decrease on DNE-born neurons in *Pen-2* cKO mice at and after E17.5 may explain why the DG is very small in the *Pen-2* cKO hippocampus in Fig.1F.

**Page 10:**
**We observed significantly increased number of Prox1+ cells in the hippocampus in Pen-2 cKO mice compared with littermate controls (Fig.4F), suggesting transiently increased neurogenesis. Can you exclude that Prox1+ interneurons contribute to the cell counts? Please comment.**

Thanks very much for this question. Since the DG is significantly affected in *Pen-2* cKO mice and Prox1 is a well-known marker for granule neurons in the DG, we examined Prox1+ cells (Fig.8A). To answer your question, we used GAD67 to label interneurons. We performed double-staining of Prox1/GAD67. However, no Prox1+/GAD67+ cells were observed in the DG in *Pen-2* cKO mice at E17.5, suggesting that Prox1+ granule neurons in the DG are negative for GAD67. The image on the right is a representative one for double-staining of Prox1/GAD67. Therefore, there may be no interneurons to be excluded from Prox1+ cells in Fig.8A.

**Fig.9: A schematic model is important to summarize the findings for the reader and should therefore be clearly arranged. If the represented APs and neurons were simplified as schematic round cells, this would be sufficient and would very much help to cleary distinguish the arrows and abbreviations. Moreover, an additional scheme C) depicting your interpretation of the unaffected dentate 3ry matrix should be included.**

According to your suggestions above, first, we changed the shape for AP in Fig.9. Second, we added the following sentences in the DISCUSSION section.

It is worth mentioning that the pool of NPCs in the 3ry matrix was unchanged in the *Pen-2* cKO hippocampus at E17.5, suggesting that the number of DG-born neurons may be comparable between two genotypes. Since neurons in the DG are generated from NPCs localized in the 3ry matrix as well as the DNE in the hippocampus (Nakahira and Yuasa, 2005), the reduction on NPC populations in the DNE in *Pen-2* cKO mice at E17.5 suggests that the number of DNE-born neurons may be significantly decreased in *Pen-2* cKO mice at and after E17.5. Overall, depletion of NPCs in the *Pen-2* cKO hippocampal neuroepithelium may result in deficient neurogenesis in hippocampal sub-regions and consequently lead to hippocampal malformation.

**Page 7:**

**...please rephrase the wording in the following sentence:**

**...the thickness of Cux1+, Ctip2+ or Tbr1+ cells was comparable in these two genotypes**

**(Fig.S3A-C). Thus, hGfap-Cre-mediated deletion of Pen-2 did not cause significant effect on cortical development. For example as follows: ...the thickness of Cux1+, Ctip2+ or Tbr1+ cell layers was comparable in these two genotypes (Fig.S3A-C). Thus, hGfap-Cre-mediated deletion of Pen-2 did not cause significant effect on cortical layering.**

According to your advice, we re-wrote the above sentence as follows: the thickness of Cux1+, Ctip2+ or Tbr1+ cell layers was comparable in these two genotypes (Fig.S3A-C). Thus, *hGfap-Cre*-mediated deletion of Pen-2 did not significantly affect cortical lamination.

Thanks very much!

**References:**

**Barry, G., Piper, M., Lindwall, C., Moldrich, R., Mason, S., Little, E., Sarkar, A., Tole, S., Gronostajski, R. M. and Richards, L. J.** (2008). Specific glial populations regulate hippocampal morphogenesis. *J Neurosci* **28**, 12328-12340.
**Nakahira, E. and Yuasa, S.** (2005). Neuronal generation, migration, and differentiation in the mouse hippocampal primoridium as revealed by enhanced green fluorescent protein gene transfer by means of in utero electroporation. *J Comp Neurol* **483**, 329-340.

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Second decision letter

MS ID#: DEVELOP/2021/200272

MS TITLE: Presenilin enhancer2 is critical for the transition of apical progenitors to neurons but not basal progenitors in the developing hippocampus

AUTHORS: Yingqian Xia, Yizhi Zhang, Min Xu, Xiaochuan Zou, Jun Gao, Mu-Huo Ji, and Guiquan Chen
ARTICLE TYPE: Research Article

I am delighted to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

*Advance summary and potential significance to field*

This is an interesting manuscript from Xia et al. that analyzes the role of Pen-2 in the developing hippocampus. Using a Pen-2 cKO by GFAP-CRE they have found evidence for Pen-2 regulating the transition of apical progenitors to neurons. Mechanistically, they have found evidence supporting deficient Notch signaling. In my opinion these findings contribute to our knowledge of hippocampal development.

*Comments for the author*

The authors have addressed all the comments that I have raised during the first revision of the manuscript.
They have produced additional evidence to further support their findings. Moreover, they have provided a revised version of the manuscript with changes in the result and discussion sections that describe more accurately their findings. I do not have any other major concerns and I can support its publication in Development in its current form.
Minor comment: The authors might want to re-visit their figures and make their graph y-axis legends uniform (for example sometimes they write "No. of", then "Number of" and later "Average No. of", sometimes within the same figure)