Hes1 overexpression leads to expansion of embryonic neural stem cell pool and stem cell reservoir in the postnatal brain

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I have now received the reports of three referees on your manuscript 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, all the referees express great interest in your work, but they also have serious criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, they request a more detailed description and better illustrations of the oRGs generated upon Hes1 overexpression. Referee 3 also asks that you exclude the possibility that Hes1 overexpression in fact results in the generation of astrocytes. All referees also request that you quantify more of the data and that you improve the illustration with higher magnification pictures.

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 currently be unable to access the lab to undertake experimental revisions. 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.

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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

Ohtsuka et al explore the relationship between Hes1 overexpression and cell populations within the embryonic and young adult mouse brain, namely: neural stem cells intermediate progenitors, astrocytes, basal radial glia cells, etc. The major findings indicate that forced expression of the Notch effector Hes1 during development maintains the stem cell pool at the expense of intermediate progenitors and neurons.
These observations are interesting but I'm afraid of limited advance relative to what described elsewhere (e.g. Ishibashi et al., 1994; Ohtsuka et al., 1999, 2001, etc).
Mechanistically, the authors show downregulation of Ngn2 and Ccnd1 to explain those phenotypes. Unfortunately, neither these are completely novel as the authors themselves already showed similar results (Shimojo et al., 2008).

Perhaps more novel, this manuscript suggests that basal radial glia (bRG) increase upon Hes1 overexpression. This cell type is evolutionary relevant due to its role in cortical expansion and gyrencephaly. Nevertheless, the functional aspects and/or mechanism behind this observation are poorly explored and even the description of bRG is substandard (i.e. lack of quantitative data) and when identifying the key distinctive feature of these cells (a basal but not apical process) the whole claim turns to be based on one single cell shown, at low resolution, in Supp Fig 6.

More in general, the whole manuscript reads mainly qualitative, with pictures of brains at extremely low magnification and lacking proper quantification or details to support key claims (eg. Fig. 2, 3, 5, 6D, S3, S4B, S5, S6A). As presented, the work does not seem ready for publication at a visible level.

Further aspects for consideration are the following:

I am not convinced about claims on proliferation and Ccnd1 expression: i) the two citations reported about this point (Baek and Shimojo) come to opposite conclusions about the effect of Hes1, ii) quantification of expression of this (and other) markers would require qRT-PCR and specifically after sorting the relevant cell type of the cortex and finally iii) cell cycle markers like Ccnd1 are G1-specific, meaning that a shorter G1 (i.e. faster cell cycle promoting proliferation) may result in a reduction of the proportion of cells expressing them and, hence, a seeming reduction in total expression levels, not necessarily implying reduced proliferation. For making claims on proliferation, the authors should directly measure proliferation, that is cell cycle length.

Under phsyological conditions, Pax6 is not expressed in the ganglionic eminences (Englund et al., 2005), but Hes1 overexpression triggers Pax6 in these areas (Fig. 2A).
Authors could dissect or discuss this.

At P0, there are rosette-like structures at the VZ in the Tg mice (Fig. 2D, and 7D) that were not analyzed or discussed.

Thickness quantification is provided for E15 brain slices (fig. 1E), but is lacking for other stages. Judging from Figs. 2A and 4D (P0 and P14, respectively) the total cortical thickness is comparable between Tg and WT animals, and this aspect is not discussed by the authors.

Panel 4I should be placed in a later figure, after bRG and astrocytes results have been presented.

In Fig. 5B, contrarily to other images and analysis, these images are from the medial cortex (not the dorso-lateral cortex). The rationale for this is not explained.

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Also, in this figure cells are scattered throughout the cortex, contrarily to fig 2B (P7 panel), in which tansgenic cells localize mainly close to the VZ.

In order to truly appreciate author's claims (double positive cells) better resolution in high power images (40x) would be required; for example in: 6B-D, 7A-D.

Fig. 6 should be combined with Fig. 2. Otherwise it is difficult for the reader to go back and forth with the different progenitor types populations (Pax6+, Tbr2+, etc)

Authors claim there is Hes1 attenuation after Dox removal. But is there actually a difference in Hes1 expression 3d after Dox removal? Judging from Fig. 7A it does not seem to be the case. Quantification of this aspect can support their claims.

In Fig 7D there is BrdU signal in midbrain areas of the Tg mice image. I wonder if this represents reliable immunolabeling or perhaps some background artifact.

Fig S2B shows Tbr2 signal lining the ventricular border, even for WT animals (E15.5)
which does not correspond with the typical Tbr2 localization. In our hands, Tbr2 immunolabeling requires antigen retrieval before antibody incubation.

Fig S6C. The DAPI signal suggests this is not E17.5 dorso-lateral cortex (could be medial cortex?). Also, the pointed cell rather than a bRG cell, it looks like an apical RG as an apical extesion can be observed.

Authors point that Hes1 overexpression inhibits both differentiation and proliferation simultaneously. Nevertheless they show more Pax6+ cells at the VZ in the Tg mice compared to WT animals. These inconsistencies are not explained.

The Discussion section seems rather shallow, and repeats some aspects of the Results section. It lacks contrasting evidence nor challenges the current ideas in the field.

Some claims lack examination, for example: "As NSCs underwent symmetric proliferative divisions, the VZ extended tangentially, and the ventricular surface and ventricles were expanded."

Also: "We thus administered Dox from E9.5 and withdrew it at various embryonic stages such as E11.5, E13.5, and E15.5."; which is not shown in the results section.
Additionaly, authors did not test regeneration (damage/insult induced neurogesis), as suggested in the last subtittle; rather they evaluated neurogenesis in young adults.

Sentences like the last one on page 16 are too long and confusing.

Comments for the author

included above in Comments for the authors

Reviewer 2

Advance summary and potential significance to field

This study addresses what are the consequences on cerebral cortex development of sustaining high Hes1 expression levels, here achieved by using an inducible transgenic mouse line. The authors report that the size of the telencephalon is smaller in transgenic postnatal animals, and then investigate the underlying developmental causes. They find that neuron production is reduced and the cortical plate is thinner, while the VZ is thicker and the abundance of Pax6+ RGCs is increased, a phenotype sustained from mid-corticogensis to the neonate.
They find that this correlates with decreased levels of the proneural transcription factor Neurogenin 2. In-depth analysis of this phenotype reveals a reduced proliferation of VZ progenitors linked to reduced levels of the cell cycle protein Ccnd1. Analysis of neuronal fate in postnatal animals reveals that early neurogenesis (at E13.5) is normal, but later neurogenesis is significantly

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delayed, going on well into birth, while gliogenesis is anticipated in transgenic animals, in a cell non-autonomous manner. The authors also report that in Tg embryos Tbr2+ IPCs are decreased while proliferation in the SVZ is increased (contrary to the VZ), essentially due to increased abundance and proliferation of Pax6+ basal Radial Glia Cells in these embryos. Finally, the authors perform BrdU labeling analyses in the adult brain of Tg animals which provide evidence that the pool of adult neural stem cells is expanded in Tg brains, and adult neurogenesis is enhanced by attenuation of Hes1.

Comments for the author

The study is nicely planned and performed, analyses are appropriate for the goals aimed at, and the conclusions are in general well supported by the results, so I am overall supportive of this study. Unfortunately, overexpression experiments are always difficult to interpret in the context of physiological conditions, and the authors neither provide a quantitative estimate of the increase in Hes1 levels across developmental stages, nor frame this overexpression situation in other physiological contexts (i.e., brain development in other species with higher endogenous Hes1 expression). At a more specific level, I believe that addressing the concerns that follow would significantly improve the quality and value of this study.

Major points:
1- The title only refers to postnatal neurogenesis, whereas most of the study is focused on embryonic development and neurogenesis. This must be corrected.

2- The authors find that overexpression of Hes1 in the embryonic cortex increases dramatically the number of Pax6+ cells in VZ (radial glia cells), but the abundance of their (apical) mitoses and expression of Ki67 are greatly diminished (although quantifications are required). The two results seem contradictory, or only make sense if Hes1 OE changes both the proliferation rate of RGCs and their fate decision. Even in this case, the change in cellular lineage needs to be dramatic enough to compensate for the low proliferation rate and still result in increased abundance of Pax6+ cells. Can the authors rule out these possibilities? Is it possible to perform clonal lineage analysis even at just one age of choice, to demonstrate this scenario? This type of experiment would also complement the analyses of Tbr2+ vs Pax6+ cell abundance (Figure 6).

3- Regarding the loss of proliferative activity in Tg embryos, this is very clear and dramatic for apical progenitors, but then the authors also show that this is accompanied by an increase in basal or SVZ proliferation (increased PH3 and pVim in SVZ; Figure 6C). Related to my previous comment, this phenotype seems contrary to increased self-renewal of VZ cells and loss of proliferation (Figure 3). How is this explained?

4- In the analysis of gliogenesis, the abundance of GFAP+ cells is greatly increased in Tg animals compared to WT, but these are not GFP+. Why not? What is the interpretation of this result?

5- Figure S6C – The image needs to be at higher magnification because one cannot really verify the detailed morphology of the cell indicated as bRGC. In fact this one cell indicated as example of bRGCs has the cell body located within the VZ (albeit on its basal side), not in the SVZ. A more appropriate example must be presented, with the cell soma clearly in the SVZ, and a full 3D reconstruction demonstrating the presence of a basal process and absence of apical process. Alternatively, or complementarily, the authors could show an example of a mCherry+ cell with the soma in SVZ and displaying a pVim+ basal process. On this respect, the images shown in Figure 6C and 6D are of insufficient magnification to illustrate co-labeling of pVim and Pax6 or PH3.

Minor concerns:
1- For all quantifications in general, it would be better to show the individual data points, in addition to mean and SEM. This provides a more accurate representation of the datasets and their variance.

2- Figure 1E – This very nice quantification must be accompanied by close-up images from WT and Tg embryos illustrative of these differences in thickness across the different layers of the developing cortex.

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3- Figure 2 – The images show that the thickness and length of VZ is clearly greater in Tg than WT embryos starting at E15.5. Quantifications of this evident overabundance of Pax6+ progenitor cells, and of VZ surface expansion, should be provided.

4- Figure 3B – The results observed on abundance of BrdU+, PH3+ and Ki67+ cells must be quantified, even if they appear obvious in the examples presented in this figure. PH3+ cells are difficult to see in the red channel, the authors should find a better color or color combination to make this much more visible.

5- Figure 6A – It is undefined how the authors identified the borders of SVZ for these quantifications. This should be included in Methods and indicated in the illustrative images. Larger, or higher magnification images, would very much help the reader to appreciate the differences in cell abundance and distribution.

6- Figure 6B – PH3 stains are not really visible in the merged images. For E15.5 this should be presented alone, as is already for E17.5. Co-localization should be demonstrated in higher magnification images, as it is not possible to see in the current images.

7- Figure S2 – it would be much better for the readership if the authors showed high magnifications of the double stains, demonstrating presence or absence of co-localization between BrdU and Pax6 / Tbr2.

8- Figure S3 – the red signal showing Casp3 staining is barely visible. This would much improve by showing the Casp3 stain alone in black-and-white, and next merged with the other channels.

9 Figure S4A – the picture of Tbr1 stain in Tg at E11.5 should be replaced because it appears to have background signal in the VZ, which is not visible in the WT embryo.

Reviewer 3

Advance summary and potential significance to field

This is a very interesting paper describing the effects of Hes1 overexpression (using tet-On) on neocortex development and postnatal neurogenesis in the SVZ. The most important conclusion (although it may not be correct) is that the transgenic animals have increased bRG-like cells late in embryonic development (E17.5). Other points of significance are that Hes1 suppresses mitotic activity, increases the NSC pool in embryonic and postnatal mice, and alters the relation between cell birthday and fate. The data quality is very high, the experiments and methods are well-designed and executed, the paper is well-written, and the figures are clearly presented. Despite all these positive aspects, there are a few areas where the authors have not addressed interesting features of their data, or most importantly, did not consider an alternative hypothesis: that the presumed bRG cells in E17.5 cortex are in fact astrocyte precursors, rather than bRG capable of neurogenesis. As detailed in the concerns below, the bRG cells do not seem to express any neurogenic TFs, nor are they shown to generate neurons, or cells that express neurogenic TFs. The assumption that E17.5 is exclusively devoted to neurogenesis must be questioned in these transgenic mice since upper layer neurogenesis begins prematurely and astrocyte genesis begins early as well. These and other concerns are enumerated below.

Comments for the author

Major:
1. Fig. 1E demonstrates markedly decreased thickness of SVZ/IZ/CP of E15.5 Tg mice. Cortical surface area is equally interesting and relevant, and should also be measured at E15.5 (at least), and possibly additional ages (e.g. using data from Fig. 2A).
2. Fig. 2C (and Fig. S3, E13.5) show "salt-and-pepper" pattern of Hes1 expression in Tg mice; this does not match Nestin expression (which is not divided into radial columns) as seen in AIBS (E13.5) or Genepaint (E14.5) databases. Is Hes1 shut off in some cells by lateral inhibition, despite transgene expression?

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3. Results state: "the onset of generation of early-born neurons (Tbr1+ layer VI neurons and Ctip2+ layer V neurons) was roughly comparable in Tg and WT mice (Fig. S4A)." But also, importantly, Fig. S4A shows ectopic expression of Tbr1 in VZ of Tg mice. This points to disorganization of the VZ, an important effect that may correlate with salt-and-pepper Hes1; does Tbr1/GFP double IHC on E11.5 show complementary expression of these two markers in VZ?
4. Results p. 9 has the heading: "Generation of superficial layer neurons was decelerated and prolonged" but this is opposite to the previous sentence that "switching from deep to superficial layer neurogenesis occurred earlier in the Tg cortex compared to that in the WT cortex (Fig. 4C)." This suggests an accelerated switch and should not be described as decelerated.
5. Concerning the prolongation of UL neurogenesis in E17.5 Tg: the authors assume that it is "(mostly neurons generated at these stages)" but do not test the (very possible) alternative that there is a premature shift at E17.5 to astrocyte genesis, similar to the premature shift to UL neurogenesis, and also exactly as suggested by the very next section. This very important point should be tested by double labeling for BrdU-E17.5/GFAP colocalization on P14 or later. A quantitative study of BrdU+/Cux+ and BrdU+/GFAP+ cells should be done comparing WT and Tg.
6. Results p. 11: The properties of bRG cells are summarized: "bRGCs manifest similar characteristics to those of aRGCs; they are positive for Hes1, Pax6, and Sox2 but negative for Tbr2." In addition, it is very important that what sets bRG cells apart from classic astrocyte precursors (which also contact the basal surface) is that bRGs generate neurons, generally via IPCs (Tbr2+). The authors do not show that the putative bRG cells in Tg mice generate neurons or IPCs. Indeed, the decrease of Neurog2+ cells and Tbr2+ IPCs, and their absence from the IZ, indicate that the cells do not generate neurons. Importantly, neuronal differenttiation is driven by Neurog2, which in turn activates Tbr2 expression, and both TFs would be expected in a neurogenic zone. These observations further support the alternative hypothesis, that these supposed bRGs are actually astrocyte precursors.
7. Results p. 11: It is suggested that pVim is "a specific marker of RGCs in the M phase" but pVim is also expressed by IPCs in M-phase (Englund et al., J. Neurosci., 2005). It would be better to say pVim is a marker of M-phase.
8. Was the presence of increased NSCs confirmed in the postnatal and adult hippocampus? Perhaps the authors wish to report hippocampal abnormalities in a separate paper, but since it is an obvious question, at least some mention would be appropriate here, if only to indicate confirmation and that details will be reported separately.
9. Discussion: The authors refer to "the smooth brain surface of primitive mammals, such as rodents (lissencephalic)." This is an oversimplification. Some rodents, such as capybara, have a gyrencephalic brain; some primates, such as marmoset, are essentially lissencephalic. Even some non-placental mammals are gyrencephalic. There is no evidence of "evolution from lissencephalic to gyrencephalic mammals" and indeed early mammals may have been gyrencephalic. Also, this is not a dichotomy, but there is a continuous variation of gyrencephaly. "Primitive" and "rodents" are not useful here.
10. Is ventricle size still increased on P31 in Tg mice, as suggested by Fig. 7?
11. Discussion should address the question: How do we understand how both Hes1 KO and Hes1 overexpression cause premature genesis of neurons, especially upper layers? Would the authors care to speculate on what is downstream of Hes1 in the relation between cell birthday and DL, UL, and astrocyte fates?

Minor:
1. Introduction: neural tube is not a 'monolayer" but is pseudostratified 2. Introduction: states that "generation of later-born neurons (superficial layer neurons) was decelerated and prolonged" but results show accelerated shift to genesis of UL neurons (Fig. 4C); the statement should be changed to be clearer about this effect.
3. Results p. 7: phrase " Tbr2, a marker of IPCs in the SVZ" should be changed; Tbr2+ IPCs are located in both VZ and SVZ, as shown in many papers (e.g., Englund et al., J. Neurosci., 2005; Kowalczyk et al.,
Cerebral Cortex, 2009).
4. Fig. 4I: spelling error in figure "astorocytes."

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First revision

Author response to reviewers' comments

MS ID#: DEVELOP/2020/189191
MS TITLE: Hes1 overexpression leads to expansion of neural stem cell reservoir and enhanced neurogenesis in the postnatal brain
AUTHORS: Toshiyuki Ohtsuka and Ryoichiro Kageyama

We would like to thank the reviewers for their scrupulous review and the constructive comments. According to the reviewer's critique and suggestions, we have performed some additional experiments and added modifications in the manuscript.

Here, we tried to address all the issues and eliminate the uncertainties raised by the reviewers one by one on separate pages. We hope you will find that we have answered all the comments and incorporated the modifications into the revised manuscript.

To Reviewer 1:
More in general, the whole manuscript reads mainly qualitative, with pictures of brains at extremely low magnification and lacking proper quantification or details to support key claims (eg. Fig. 2, 3, 5, 6D, S3, S4B, S5, S6A). As presented, the work does not seem ready for publication at a visible level.)

We provided higher magnification images and added quantification results in Fig. 2, 3, 5, 6, and Fig. S6.

1) I am not convinced about claims on proliferation and Ccnd1 expression: i) the two citations reported about this point (Baek and Shimojo) come to opposite conclusions about the effect of Hes1, ii) quantification of expression of this (and other) markers would require qRT-PCR and specifically after sorting the relevant cell type of the cortex and finally iii) cell cycle markers like Ccnd1 are G1-specific, meaning that a shorter G1 (i.e. faster cell cycle promoting proliferation) may result in a reduction of the proportion of cells expressing them and, hence, a seeming reduction in total expression levels, not necessarily implying reduced proliferation. For making claims on proliferation, the authors should directly measure proliferation, that is cell cycle length.

i) It was revealed from other studies (Baek et al. and Shimojo et al. etc.) that high levels of Hes1 expression suppress cell proliferation, but low levels of Hes1 expression (due to oscillating expression) promote/support cell proliferation.
ii) Although we have the pHes1-d2EGFP Tg mice in which embryonic NSCs are visualized and can be isolated by FACS, but GFP is also expressed under the Nestin promoter in this Hes1-overexpressing Tg mice, and in addition, Hes1 overexpression could affect the Hes1 promoter activity; that makes it difficult to isolate the relevant cell type from WT and Hes1-OE brain. Instead, we counted the number of $Ccnd1^+$ cells in the radial column of constant width (200um) and provided the data in Fig. 3F, and further we performed RT-PCR for Ccnd1 by using RNAs prepared from the neocortical regions of WT vs Tg mice, and provided the data in Fig. 3G.
iii) We think that the data of BrdU incorporation experiments and immunostaining of pH3 and Ki67 (Fig. 3A-C) provide a sufficient evidence that indicates low proliferation rate of the VZ cells. In addition, we measured cell cycle length in $Pax6^+$ NSCs and provided the data in Fig. 3D.

2) Under physiological conditions, Pax6 is not expressed in the ganglionic eminences (Englund et al., 2005), but Hes1 overexpression triggers Pax6 in these areas (Fig. 2A). Authors could dissect or discuss this.

We added these sentences in the "Result and Discussion" section (page 7); "It seemed that Pax6 expression was enhanced in some of ventricular cells in the ganglionic eminences. It is possible that overexpression of Hes1 ectopically induced Pax6 expression in neural progenitors in the ventral telencephalon."

3) At P0, there are rosette-like structures at the VZ in the Tg mice (Fig. 2D, and 7D) that were not

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analyzed or discussed.

Rosette-like structures were sometimes observed when the phenotype was considerably severe, but this phenotype was not typical nor reproducible.
We added discussion about this phenotype (page 14); "Pax6$^+$ clusters sometimes exhibited rosette-like structures in the Tg brain as shown in Fig. 7D, probably due to the heterogeneous organization within the VZ/SVZ, although this phenotype was not typical nor reproducible."

4) Thickness quantification is provided for E15 brain slices (fig. 1E), but is lacking for other stages. Judging from Figs. 2A and 4D (P0 and P14, respectively) the total cortical thickness is comparable between Tg and WT animals, and this aspect is not discussed by the authors.

We provided the data of layer thickness at various embryonic stages in Fig. 2B.
We added description that "the cortical thickness is generally thinner during embryonic stages but the difference became less after postnatal stages, probably because mice with severe phenotype could not survive and ones with mild phenotype only survived after postnatal stages" (page 10).

5) Panel 4I should be placed in a later figure, after bRG and astrocytes results have been presented.

We moved Fig. 4I to Fig. 6G.

6) In Fig. 5B, contrarily to other images and analysis, these images are from the medial cortex (not the dorso-lateral cortex). The rationale for this is not explained. Also, in this figure cells are scattered throughout the cortex, contrarily to fig 2B (P7 panel), in which transgenic cells localize mainly close to the VZ.

We replaced the panels showing the medial cortex to those showing the dorso-lateral cortex.
We provided a typical image in Fig.5B, although phenotypes of Tg mice were varied due to the difference in the amount of Doxycycline that was taken from drinking water.

7) In order to truly appreciate author's claims (double positive cells) better resolution in high power images (40x) would be required; for example in: 6B-D, 7A-D.

We provided figures of higher magnification in Fig. 6 A-D and higher magnification of merged images in Fig. 7 C,D.

8) Fig. 6 should be combined with Fig. 2. Otherwise it is difficult for the reader to go back and forth with the different progenitor types populations (Pax6+, Tbr2+, etc)

We think it is better to integrate all the data in one figure (in Fig. 6) to highlight the increase of bRG-like cells, because there would be too many panels if we combine Fig. 2 and Fig. 6.

9) Authors claim there is Hes1 attenuation after Dox removal. But is there actually a difference in Hes1 expression 3d after Dox removal? Judging from Fig. 7A it does not seem to be the case. Quantification of this aspect can support their claims.

We quantified Hes1 expression levels (in 500 um x 100 um rectangle strips including the SVZ lining the lateral ventricular surface) and compared them between 4w and 4w+3d after removal of Dox.
We added the data in Fig. 7A.

10) In Fig 7D there is BrdU signal in midbrain areas of the Tg mice image. I wonder if this represents reliable immunolabeling or perhaps some background artifact.

BrdU signals were observed in the deep layers of neocortex, a part of medial telencephalon, and a part of dorsal diencephalon. High levels of BrdU signals mark all the cells that exited cell cycle (and differentiated mainly into neurons such as deep layer neurons) just after BrdU incorporation at E11.5.

11) Fig S2B shows Tbr2 signal lining the ventricular border, even for WT animals (E15.5) which

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does not correspond with the typical Tbr2 localization. In our hands, Tbr2 immunolabeling requires antigen retrieval before antibody incubation.

We always perform antigen retrieval before Tbr2 staining.
We retried immunostaining and provided better figures in Fig. S2B.

12) Fig S6C. The DAPI signal suggests this is not E17.5 dorso-lateral cortex (could be medial cortex?). Also, the pointed cell rather than a bRG cell, it looks like an apical RG as an apical extension can be observed.

The image we provided in Fig. S6C is a coronal section of E17.5 dorso-lateral cortex. However, it was relatively caudal telencephalon, because it was easy to obtain better figures depicting full-length of basal radial fibers in coronal sections.
Therefore, the cortical structure looked different (e.g. cortical wall and cortical layers were much thinner) compared to other figures, most of which were images of more rostral part of telencephalon.
It is very difficult to acquire full-length images of basal radial fibers, because they run long distance in the rostral telencephalon; further they run obliquely and are frequently winding and bending. Therefore, we could obtain clear images of full-length basal radial fibers only in the caudal telencephalon.
We provided additional images of typical bRG-like cells in Fig. 6 and Fig. S6, and a higher resolution image of mCherry+ cells with 3D reconstruction in Supplementary Movie 1 (Movie S1).

13) Authors point that Hes1 overexpression inhibits both differentiation and proliferation simultaneously. Nevertheless, they show more Pax6+ cells at the VZ in the Tg mice compared to WT animals. These inconsistencies are not explained.

Hes1 overexpression increased the number of Pax6+ NSCs by elongating the period of symmetric proliferative divisions due to inhibition of neuronal differentiation. It is likely that this increase of Pax6+ NSCs prevailed over the low proliferation rate.
We provided the data of symmetric vs asymmetric division mode of NSCs in Fig. 3E and added this discussion in the Discussion section (page 15).

14) The Discussion section seems rather shallow, and repeats some aspects of the Results section. It lacks contrasting evidence nor challenges the current ideas in the field.

We reduced the overlapping parts in the Discussion section, and added discussion regarding the challenges to the current ideas.
As contrasting evidence or challenges to the current ideas in the field,
we added discussion regarding the generation mechanism of RGCs (page 16-17).
we discussed more about the new challenges to maintain adult neurogenesis by expanding neurogenic NSCs; that is a contrasting strategy to the conventional methods to compensate the declined neurogenesis by activating pre-existing quiescent NSCs (page 17-18).
we already discussed the mechanism regulating the origin of adult NSCs (slowly-dividing NSCs) with contrasting to previous reports (page 18).

15) Some claims lack examination, for example: "As NSCs underwent symmetric proliferative divisions, the VZ extended tangentially, and the ventricular surface and ventricles were expanded."

We performed BrdU incorporation experiments and estimated the proportion of symmetric proliferative divisions vs asymmetric neurogenic divisions. We confirmed that NSCs continued symmetric proliferative divisions in the Tg brain and provided the data in Fig. 3E.

16) Also: "We thus administered Dox from E9.5 and withdrew it at various embryonic stages such as E11.5, E13.5, and E15.5."; which is not shown in the results section. Additionally, authors did not test regeneration (damage/insult induced neurogenesis), as suggested in the last subtitle; rather they evaluated neurogenesis in young adults.

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We added a description; “However, we failed to observe larger brains in Tg mice, although the cortical wall became slightly thicker compared to that in Tg mice that received continuous Dox administration (data not shown).”
We modified the last subtitle in the Discussion section (page17); “Hes1 overexpression leads to expansion of the NSC reservoir and enhanced neurogenesis in the adult brain”.
We actually tested the regeneration capacity by using a stab wound injury model, but we could not observe significant increase in neurogenesis in the injured adult cortex. And unfortunately, we could not investigate the adult neurogenesis in the dentate gyrus (hippocampus), because rtTA was not expressed in the pNestin-rtTA Tg mice. (We added this discussion in the Discussion section (page 18).) We would like to estimate the regeneration capacity with other measures as our future challenges.

17) Sentences like the last one on page 16 are too long and confusing.

We split the long sentence into two.

To Reviewer 2:

the authors neither provide a quantitative estimate of the increase in Hes1 levels across developmental stages, nor frame this overexpression situation in other physiological contexts (i.e., brain development in other species with higher endogenous Hes1 expression.

We performed RT-PCR for Hes1 by using RNAs prepared from the neocortical regions of WT vs Tg mice at E15.5, and found that expression levels of Hes1 was markedly higher in the Tg brain than in the WT brain. The data was provided in Fig. 2C.
It is quite difficult to compare Hes1 expression levels in developing NSCs across species, because it is difficult to match the developmental stages across species and purely isolate NSCs from developing brains.

Major points:
1- The title only refers to postnatal neurogenesis, whereas most of the study is focused on embryonic development and neurogenesis. This must be corrected.

We changed the title to underscore the effect on embryonic neural development.

2- The authors find that overexpression of Hes1 in the embryonic cortex increases dramatically the number of Pax6+ cells in VZ (radial glia cells), but the abundance of their (apical) mitoses and expression of Ki67 are greatly diminished (although quantifications are required). The two results seem contradictory, or only make sense if Hes1 OE changes both the proliferation rate of RGCs and their fate decision. Even in this case, the change in cellular lineage needs to be dramatic enough to compensate for the low proliferation rate and still result in increased abundance of Pax6+ cells. Can the authors rule out these possibilities? Is it possible to perform clonal lineage analysis even at just one age of choice, to demonstrate this scenario? This type of experiment would also complement the analyses of Tbr2+ vs Pax6+ cell abundance (Figure 6).

We quantified the number of BrdU+, pH3+, and Ki67+ cells in Fig. 3A-C.
Hes1 overexpression increased the number of Pax6+ NSCs by elongating the period of symmetric proliferative divisions due to inhibition of neuronal differentiation. It is likely that this increase of Pax6+ NSCs prevailed over the low proliferation rate.
We added this discussion in the Discussion section (page 15).
We performed BrdU incorporation experiments and estimated the proportion of symmetric proliferative divisions vs asymmetric neurogenic divisions. We confirmed that NSCs continued symmetric proliferative divisions in the Tg brain and provided the data in Fig. 3E.

3- Regarding the loss of proliferative activity in Tg embryos, this is very clear and dramatic for apical progenitors, but then the authors also show that this is accompanied by an increase in basal or SVZ proliferation (increased PH3 and pVim in SVZ; Figure 6C). Related to my previous comment, this phenotype seems contrary to increased self-renewal of VZ cells and loss of

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proliferation (Figure 3). How is this explained?

Increase in basal or SVZ proliferation (increased pH3+ and pVim+ cells in SVZ) was caused by increase in generation of bRG-like cells in the SVZ only at later embryonic stages. We did not estimate the proliferation rate of these bRG-like cells (Pax6+ cells) in the SVZ, because it is difficult to compare with that in the WT brain (there are only few Pax6+ cells in the SVZ), but instead we estimated the proliferation rate of Tbr2+ cells in the SVZ (already shown in Fig. S2D), indicating comparable proliferation rates in WT and Tg brains.

4- In the analysis of gliogenesis, the abundance of GFAP+ cells is greatly increased in Tg animals compared to WT, but these are not GFP+. Why not? What is the interpretation of this result?

We added an explanation in the Results section (page 11); "These results suggest that downregulation of Hes1 is necessary for terminal differentiation into mature ($GFAP^+$) astrocytes, and that those $GFP^+;Pax6^+$ cells were immature astrocytes in which terminal differentiation/maturation was prevented. The ectopic distribution of immature astrocytes ($Pax6^+$) might have enhanced the production of $GFAP^+$ astrocytes throughout the cortex."

5- Figure S6C – The image needs to be at higher magnification because one cannot really verify the detailed morphology of the cell indicated as bRGC. In fact, this one cell indicated as example of bRGCs has the cell body located within the VZ (albeit on its basal side), not in the SVZ. A more appropriate example must be presented, with the cell soma clearly in the SVZ, and a full 3D reconstruction demonstrating the presence of a basal process and absence of apical process. Alternatively, or complementarily, the authors could show an example of a mCherry+ cell with the soma in SVZ and displaying a pVim+ basal process. On this respect, the images shown in Figure 6C and 6D are of insufficient magnification to illustrate co-labeling of pVim and Pax6 or PH3.

We provided higher magnification images of mCherry+ cells in Fig. 6 and Fig. S6, and a 3D reconstruction image in Supplementary Movie 1 (Movie S1).
pVim is a nuclear marker and is absent in radial fibers.

Minor concerns:
1- For all quantifications in general, it would be better to show the individual data points, in addition to mean and SEM. This provides a more accurate representation of the datasets and their variance.

We tried to make graphs with individual data points, but we think that it is better to show most of our data in conventional bar graphs, because the number of each samples (mice) were not so many in most of our analyses.

2- Figure 1E – This very nice quantification must be accompanied by close-up images from WT and Tg embryos illustrative of these differences in thickness across the different layers of the developing cortex.

We provided higher magnification images in Fig. 1E.

3- Figure 2 – The images show that the thickness and length of VZ is clearly greater in Tg than WT embryos starting at E15.5. Quantifications of this evident overabundance of Pax6+ progenitor cells, and of VZ surface expansion, should be provided.

We quantified the thickness of layers, number of Pax6+ cells, length of ventricular surface, and length of cortical surface on coronal sections, and provided the data in Fig. 2B.

4- Figure 3B – The results observed on abundance of BrdU+, PH3+ and Ki67+ cells must be quantified, even if they appear obvious in the examples presented in this figure. PH3+ cells are difficult to see in the red channel, the authors should find a better color or color combination to make this much more visible.

We quantified the number of BrdU+, pH3+ and Ki67+ cells and provided the data in Fig. 3A-C.

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We provided better figures of pH3 staining in grey scale images in Fig. 3B.

5- Figure 6A – It is undefined how the authors identified the borders of SVZ for these quantifications. This should be included in Methods and indicated in the illustrative images. Larger, or higher magnification images, would very much help the reader to appreciate the differences in cell abundance and distribution.

We added the explanation of "borders of the VZ and SVZ" in the Figure legend (Fig. 6); "Borders of the VZ and SVZ were estimated based on the continuity of $Pax6^+$ cells and were shown by white dashed lines in green channels", and indicated the borders with white dashed lines in images showing Pax6 staining (green channels).
We provided higher magnification views of merged images.

6- Figure 6B – PH3 stains are not really visible in the merged images. For E15.5 this should be presented alone, as is already for E17.5. Co-localization should be demonstrated in higher magnification images, as it is not possible to see in the current images.

We provided separated figures of pH3 staining in grey scale images and provided higher magnification images in Fig. 6B.

7- Figure S2 – it would be much better for the readership if the authors showed high magnifications of the double stains, demonstrating presence or absence of co-localization between BrdU and Pax6 / Tbr2.

We provided better figures of higher magnification in Fig. S2A,B.

8- Figure S3 – the red signal showing Casp3 staining is barely visible. This would much improve by showing the Casp3 stain alone in black-and-white, and next merged with the other channels.

We provided better figures to show Casp3 staining more clearly in grey scale images.

9 Figure S4A – the picture of Tbr1 stain in Tg at E11.5 should be replaced because it appears to have background signal in the VZ, which is not visible in the WT embryo.

We provided a better figure to show Tbr1 expression in the Tg brain.

To Reviewer 3:
Major:
1. Fig. 1E demonstrates markedly decreased thickness of SVZ/IZ/CP of E15.5 Tg mice. Cortical surface area is equally interesting and relevant, and should also be measured at E15.5 (at least), and possibly additional ages (e.g. using data from Fig. 2A).

We quantified the thickness of SVZ/IZ/CP and measured the length of cortical surface and ventricular surface (from dorsal to lateral border on coronal sections) of WT and Tg mice at different developmental stages, and provided the data in Fig. 2B.

2. Fig. 2C (and Fig. S3, E13.5) show "salt-and-pepper" pattern of Hes1 expression in Tg mice; this does not match Nestin expression (which is not divided into radial columns) as seen in AIBS (E13.5) or Genepaint (E14.5) databases. Is Hes1 shut off in some cells by lateral inhibition, despite transgene expression?

Expression of Hes1 and d2EGFP was homogeneous throughout the VZ in most cases, if Dox intake was sufficient. But the transgene expression was sometimes observed in the mosaic pattern when ingestion of Dox water by pregnant mice was insufficient or inconstant.

3. Results state: "the onset of generation of early-born neurons (Tbr1+ layer VI neurons and Ctip2+ layer V neurons) was roughly comparable in Tg and WT mice (Fig. S4A)." But also, importantly, Fig. S4A shows ectopic expression of Tbr1 in VZ of Tg mice. This points to disorganization of the VZ, an important effect that may correlate with salt-and-pepper Hes1; does Tbr1/GFP double IHC

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on E11.5 show complementary expression of these two markers in VZ?

Expression patterns of Tbr1 were comparable between the WT and Tg embryos at E11.5. We provided a better figure to show the Tbr1 expression in the Tg brain in Fig. S4A.

4. Results p. 9 has the heading: "Generation of superficial layer neurons was decelerated and prolonged" but this is opposite to the previous sentence that "switching from deep to superficial layer neurogenesis occurred earlier in the Tg cortex compared to that in the WT cortex (Fig. 4C)." This suggests an accelerated switch and should not be described as decelerated.

We use the word “decelerated” to mean that “the speed of UL neurogenesis was reduced”. Switching from DL to UL neurogenesis was actually “accelerated”, but the speed of production of UL neurons was “decelerated” after the switching.
We modified some misleading descriptions in Abstract, Introduction (page 5), and Results (page 9) sections.

5. Concerning the prolongation of UL neurogenesis in E17.5 Tg: the authors assume that it is "(mostly neurons generated at these stages)" but do not test the (very possible) alternative that there is a premature shift at E17.5 to astrocyte genesis, similar to the premature shift to UL neurogenesis, and also exactly as suggested by the very next section. This very important point should be tested by double labeling for BrdU-E17.5/GFAP colocalization on P14 or later. A quantitative study of BrdU+/Cux+ and BrdU+/GFAP+ cells should be done comparing WT and Tg.

We performed BrdU labeling at E17.5 and quantified the proportion of BrdU+;NeuN+/BrdU+ and BrdU+;GFAP+/BrdU cells in the cortex and SVZ. We observed that a majority of cells generated at this stage (E17.5) differentiated into neurons in the Tg cortex and provided the data in Fig. 4H.

6. Results p. 11: The properties of bRG cells are summarized: "bRGCs manifest similar characteristics to those of aRGCs; they are positive for Hes1, Pax6, and Sox2 but negative for Tbr2." In addition, it is very important that what sets bRG cells apart from classic astrocyte precursors (which also contact the basal surface) is that bRGs generate neurons, generally via IPCs (Tbr2+). The authors do not show that the putative bRG cells in Tg mice generate neurons or IPCs. Indeed, the decrease of Neurog2+ cells and Tbr2+ IPCs, and their absence from the IZ, indicate that the cells do not generate neurons. Importantly, neuronal differentiation is driven by Neurog2, which in turn activates Tbr2 expression, and both TFs would be expected in a neurogenic zone. These observations further support the alternative hypothesis, that these supposed bRGs are actually astrocyte precursors.

We considered the lineage tracing of bRG-like cells, but we thought that it is too difficult to label only bRG-like cells and track the fates of their progeny to examine whether neurons or Tbr2+ IPCs are produced from bRG-like cells.
Instead, we performed double-labeling of Pax6 and Neurog2 at E17.5 and found that many of bRG-like cells (Pax6+ cells in the SVZ) were Neurog2+, suggesting that those cells are neurogenic, and provided the data in Fig. 6F.
We speculate that those bRG-like cells directly produce neurons not via Tbr2+ IPCs because Hes1 overexpression constantly suppresses generation of Tbr2+ cells, although the mechanism is unclear.

7. Results p. 11: It is suggested that pVim is "a specific marker of RGCs in the M phase" but pVim is also expressed by IPCs in M-phase (Englund et al., J. Neurosci., 2005). It would be better to say pVim is a marker of M-phase.

Englund et al. showed a selected image of double-positive (Tbr2+;pVim+) cells, but did not describe that all (or a majority of) Tbr2+ IPCs in M-phase express pVim. Some of apical progenitors in the VZ also express both Pax6 and Tbr2, and some of newly-generated Tbr2+ cells (immature IPCs) still retain Pax6 expression.
As you can see in Fig. 6D, pVim is observed in a subset of pH3 cells in the SVZ, whereas most of apically dividing cells (mostly apical RGCs) were double-positive for pVim and pH3. And pVim is mostly overlapped with Pax6 and segregated from Tbr2 in our samples.

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We estimated the proportion of pVim+;Pax6+/pVim+ cells and found that over 86% of pVim+ cells in the SVZ were Pax6+.
Therefore, we think that pVim does not similarly label RGCs and Tbr2+ IPCs and that it is more specific to RGCs (aRGCs and bRGCs), although it is expressed in a limited subset of Tbr2+ cells in M-phase.

8. Was the presence of increased NSCs confirmed in the postnatal and adult hippocampus? Perhaps the authors wish to report hippocampal abnormalities in a separate paper, but since it is an obvious question, at least some mention would be appropriate here, if only to indicate confirmation and that details will be reported separately.

We added in the Discussion section (p18) that “Unfortunately, rtTA expression in the hippocampus was very weak and we could not detect GFP expression in the hippocampus in the postnatal Tg brain. Therefore, it was difficult to analyze the effect of Hes1 overexpression on NSCs in the hippocampus including the dentate gyrus”.

9. Discussion: The authors refer to "the smooth brain surface of primitive mammals, such as rodents (lissencephalic)." This is an oversimplification. Some rodents, such as capybara, have a gyrencephalic brain; some primates, such as marmoset, are essentially lissencephalic. Even some non-placental mammals are gyrencephalic. There is no evidence of "evolution from lissencephalic to gyrencephalic mammals" and indeed early mammals may have been gyrencephalic. Also, this is not a dichotomy, but there is a continuous variation of gyrencephaly. "Primitive" and "rodents" are not useful here.

We removed those words (“primitive” and “rodents”) and changed the sentences; “In certain mammalian species such as dogs, sheep, and primates, the brain surface is convoluted (gyrencephalic), in contrast to the smooth brain surface of lissencephalic mammals such as mice, and it has been thought that mammals have developed gyrencephalic brain during mammalian evolution.”

10. Is ventricle size still increased on P31 in Tg mice, as suggested by Fig. 7?

Yes, ventricle size is always larger in the postnatal and adult Tg brain compared to the WT brain.

11. Discussion should address the question: How do we understand how both Hes1 KO and Hes1 overexpression cause premature genesis of neurons, especially upper layers? Would the authors care to speculate on what is downstream of Hes1 in the relation between cell birthday and DL, UL, and astrocyte fates?

Premature neurogenesis occurred from the onset of neurogenesis in Hes1 KO mice, but the transition timing from UL to DL neurogenesis and that from neurogenesis to gliogenesis were not elaborately examined. In contrast, premature onset of neurogenesis was not observed and only the sift of transition timing was observed in the Hes1-overexpressing Tg mice.
We speculated the involvement of Hmga1/2 genes that were found to be downregulated in the Hes5-overexpressing Tg mice (Bansod et al., 2017) and examined their expression levels, but we found that only Hmga1 was downregulated, whereas Hmga2 was slightly upregulated in the Tg brain at E15.5. Therefore, we could not conclude that Hmga genes were responsible to the sift of transition timing in the Hes1-overexpressing Tg mice.

Minor:
1. Introduction: neural tube is not a 'monolayer" but is pseudostratified

We understand that “pseudostratified” means a monolayer that looks like “stratified”.
We revised the description from “monolayer cells” to “monolayer (pseudostratified) cells”

2. Introduction: states that "generation of later-born neurons (superficial layer neurons) was decelerated and prolonged" but results show accelerated shift to genesis of UL neurons (Fig. 4C); the statement should be changed to be clearer about this effect.

We use the word “decelerated” to mean that “the speed of UL neurogenesis was reduced”.

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Transition from DL to UL neurogenesis prematurely occurred, but the speed of production of UL neurons was “decelerated” after the transition.
We modified some misleading descriptions in Abstract, Introduction (page 5), and Results (page 9) sections.
We remove “decelerated” from Abstract and revised the description in Introduction; “later-born neurons (superficial layer neurons) were slowly produced and neurogenesis was prolonged”.

3. Results p. 7: phrase " Tbr2, a marker of IPCs in the SVZ" should be changed; Tbr2+ IPCs are located in both VZ and SVZ, as shown in many papers (e.g., Englund et al., J. Neurosci., 2005; Kowalczyk et al., Cerebral Cortex, 2009).

We changed the phrase to "Tbr2, a marker of IPCs most of which are located in the SVZ".

4. Fig. 4I: spelling error in figure "astorocytes."

We revised “astorocytes” to “astrocytes”.

Second decision letter

MS ID#: DEVELOP/2020/189191

MS TITLE: Hes1 overexpression leads to expansion of embryonic neural stem cell pool and stem cell reservoir in the postnatal brain

AUTHORS: Toshiyuki Ohtsuka and Ryoichiro Kageyama

My apologies for the very long time it has taken us to receive the report of one of the referees who reviewed the earlier version of your manuscript. I have now receive the report and I have reached a decision. The report is appended below and you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The reviewer's evaluation is positive and we would like to publish a revised manuscript in Development, provided that you satisfactorily address the remaining suggestions and comments of the referee. Please attend to these two comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 2

Advance summary and potential significance to field

This manuscript studies what are the consequences on cerebral cortex development of sustaining high Hes1 expression levels, here achieved by using an inducible transgenic mouse line. The authors report that the size of the telencephalon is smaller in transgenic postnatal animals, and then investigate the underlying developmental causes. They find that neuron production is reduced and the cortical plate is thinner, while the VZ is thicker and the abundance of Pax6+ RGCs is increased, a phenotype sustained from mid-corticogensis to the neonate. They find that this correlates with decreased levels of the proneural transcription factor Neurogenin 2. In-depth analysis of this

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phenotype reveals a reduced proliferation of VZ progenitors linked to reduced levels of the cell cycle protein Ccnd1.
Analysis of neuronal fate in postnatal animals reveals that early neurogenesis (at E13.5) is normal, but later neurogenesis is significantly delayed, going on well into birth, while gliogenesis is anticipated in transgenic animals, in a cell non-autonomous manner. The authors also report that in Tg embryos Tbr2+ IPCs are decreased while proliferation in the SVZ is increased (contrary to the VZ), essentially due to increased abundance and proliferation of Pax6+ basal Radial Glia Cells in these embryos.
Finally, the authors perform BrdU labeling analyses in the adult brain of Tg animals which provide evidence that the pool of adult neural stem cells is expanded in Tg brains, and adult neurogenesis is enhanced by attenuation of Hes1.

The study is nicely planned and performed, analyses are appropriate for the goals aimed at, and the conclusions are in general well supported by the results, so I am overall supportive of this study.

Comments for the author

The authors have more or less satisfactorily responded to most of my comments (many of the high magnifications requested are still not of sufficient enlargement to confirm co-localization), but two others remain completely unresolved:

Major point 5 – No high magnification is provided for co-labeling of pVim and Pax6 or PH3, which remains necessary to demonstrate this point in Figure 6C and 6D.

Minor concern 1 – Individual data points must be displayed in all quantitative analyses of the study. The authors acknowledge that the number of data points were not so many in their analyses, and argue that this is the reason for not showing them individually. But, precisely, this makes my point even more relevant. Plots should be the combination of bar histograms with individual data points, as is now customary in most serious journals.

Second revision

Author response to reviewers' comments

MS ID#: DEVELOP/2020/189191
MS TITLE: Hes1 overexpression leads to expansion of neural stem cell reservoir and enhanced neurogenesis in the postnatal brain AUTHORS: Toshiyuki Ohtsuka and Ryoichiro Kageyama

We would like to thank the reviewers for their scrupulous review.
According to the reviewer’s suggestions, we added modifications in the figures and manuscript.

To Reviewer 2:
The authors have more or less satisfactorily responded to most of my comments (many of the high magnifications requested are still not of sufficient enlargement to confirm co-localization), but two others remain completely unresolved:

Major point 5 – No high magnification is provided for co-labeling of pVim and Pax6 or PH3, which remains necessary to demonstrate this point in Figure 6C and 6D.

We added higher magnification images of separated channels (green/red/merged) to more clearly demonstrate the co-labeling of pVim and Pax6 or pH3 in Fig. 6C and D.

Minor concern 1 – Individual data points must be displayed in all quantitative analyses of the study. The authors acknowledge that the number of data points were not so many in their analyses, and argue that this is the reason for not showing them individually. But, precisely, this

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makes my point even more relevant. Plots should be the combination of bar histograms with individual data points, as is now customary in most serious journals.

We added individual data points on the bar charts for all quantitative analyses.

Third decision letter

MS ID#: DEVELOP/2020/189191

MS TITLE: Hes1 overexpression leads to expansion of embryonic neural stem cell pool and stem cell reservoir in the postnatal brain

AUTHORS: Toshiyuki Ohtsuka and Ryoichiro Kageyama
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.

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