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Regulation of Cerebral Cortex Size and Folding by Expansion of Basal Progenitors

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 02 April 2013

Thank you for submitting your manuscript to The EMBO Journal. Two referees have now reviewed your paper and their comments are provided below. I am still waiting for a third report, but as I have no heard back from this referee yet I am taking a decision on the paper based on the two reports on hand.

As you can see below, both referees find the analysis very interesting, well done and suitable for publication in the EMBO Journal. They raise minor concerns that shouldn't involve too much additional work to resolve. Given these comments, I would therefore like to invite you to submit a suitably revised manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you very much for submitting your interesting work to The EMBO Journal.

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In their manuscript "Regulation of Cerebral Cortex Size and Folding by Expansion of Basal Progenitors" Nonaka-Kinoshita et al use a '4D' mouse with controlled overexpression of Cdk4 and cyclinD1 which leads to basal progenitor (BP) expansion. This transient expansion of BPs increases cortical thickness of the embryonic brain and cortical surface area of the mature brain. The postnatal brain still has correct layering and organization, though they are hydrocephalic. The increase in cortical surface area however does not result in an increase in cortical folds in the brain of a naturally lissencephalic mice. In contrast, overexpression of Cdk4 and cyclinD1 in a naturally gyrencephalic ferret however results in increased cortical folds.
Based on these transgenesis- and virus-based data the authors conclude that basal progenitors exert an influence on surface area size in both lissencephalic and gyrencephalic species. However increasing basal progenitors does not induce gyrification of normally lissencephalic brains.
It has been previously suggested that one of the key differences between lissencephalic and gyrencephalic brains is the diffference in the relative proportions of basal radial glia to progenitor cells. The authors suggest that their current findings supports the notion that variation in surface area versus folding is caused by differential contribution of basal radial glia and basal progenitor cells. We believe that they present convincing evidence that this is true.
However, a few points should be addressed to strengthen the manuscript.

1. Figure 1: 1E- RFP expression is mosaic in the VZ.
In the text the authors mention that RFP is detected in Tbr2- RGCs and Tbr2+ BPs. It would be beneficial to further characterize which cells are being targeted.

2. Staining for RGs (Pax6, Sox2) and focusing on the IZ might suggest whether bRGs are expanded in the 4D mouse in addition to the basal progenitors.

3. Figure 2: Authors suggest there is an increase in bRGs in the IZ (Figure 2D), bRG specific marker staining - Pax6, Sox2 - may verify this claim.

4. In figure 5B: authors should include images of controls.
5. Overall: the authors should tone down certain statements in their manuscript. Even though we agree that the authors present convincing evidence that challenges previous concepts we suggest to use a wording such as "challenging" and not "refuting" (throughout the manuscript).

Referee #3 (Remarks to the Author):

A previous study in mouse showed that over-expression of Cdk4 and cyclin D1 (4D) in a small proportion of cortical progenitor cells promoted basal progenitor (BP) expansion without altering cortical lamination and shape (Lange et al., 2009).

Here the authors generated a transgenic mouse line that uses the tetracycline regulatable system to turn or, and off, expression of both of Cdk4 and cyclin D1 (4D) in mouse cortical progenitors. This led to increases in BP cell proliferation and led to larger cortical surface area in the mouse, without the induction of cortical folding.

On the other hand, increased 4D expression in cortical progenitors of the gyrencephalic ferret also increased in BP cell proliferation and larger cortical surface area, but did increase cortical folding. Thus, they present evidence that factors other than increased BP proliferation underlie the formation/evolution of cortical gyri.

Suggestions/Comments organized according to figure number.

Figure 1

The authors used fibroblasts to study the induction of 4D by doxycycline; it is likely that fibroblasts are very different then neurons and therefore the authors need to acknowledge this weakness in the experimental design.

In panel F they show that the expression of the RFP mRNA is not homogenous. The mosaicism in the ventricular zone could reflect that the transgene was introduced into the X chromosome and that they are analyzing a female embryo. Has this possibility been excluded?

Figure 4 Have the authors investigated whether or not there is a change in the numbers of astrocytes that are generated as a function of the doxycycline induction of 4D; these could account for some of the surface area changes.

Panel D shows that the size of the ventricles of the 4DG specimen are enlarged; can the authors account for this based solely on the increased surface area of the cortex or is there some evidence for hydrocephalus. Another possibility is that there defects in the axons connecting the cortex to the striatum, which then lead to weakening of the tissue at the pallial-subpallial boundary. Likewise there maybe problems in the formation of the commissures; what is the effect on the corpus callosum?

The nestin regulatory elements will lead to 4D expression throughout central nervous system; what is the effect on size of the basal ganglia and other regions of the CNS? Could this contribute to the ventriculomegally?

Figure 5

The authors need to clarify why viral transduction transduction of 4D gives a stronger phenotype than electroporation of 4D.

1st Revision - authors' response 04 April 2013

POINT-BY-POINT RESPONSE TO THE REVIEWERS

We thank the reviewers for their enthusiasm and interest in our work, constructive points of criticism and insightful comments. We believe that our revised manuscript (changes in RED) addresses all the reviewers' comments as summarized in the point-by-point response below.

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REVIEWER 1 COMMENTS FOR THE AUTHOR:

In their manuscript "Regulation of Cerebral Cortex Size and Folding by Expansion of Basal Progenitors" Nonaka-Kinoshita et al use a '4D' mouse with controlled overexpression of Cdk4 and cyclinD1 which leads to basal progenitor (BP) expansion. This transient expansion of BPs increases cortical thickness of the embryonic brain and cortical surface area of the mature brain. The postnatal brain still has correct layering and organization, though they are hydrocephalic. The increase in cortical surface area however does not result in an increase in cortical folds in the brain of a naturally lissencephalic mice. In contrast, overexpression of Cdk4 and cyclinD1 in a naturally gyrencephalic ferret however results in increased cortical folds. Based on these transgenesis- and virus-based data the authors conclude that basal progenitors exert an influence on surface area size in both lissencephalic and gyrencephalic species. However increasing basal progenitors does not induce gyrification of normally lissencephalic brains. It has been previously suggested that one of the key differences between lissencephalic and gyrencephalic brains is the diffference in the relative

proportions of basal radial glia to progenitor cells. The authors suggest that their current findings support the notion that variation in surface area versus folding is caused by differential contribution of basal radial glia and basal progenitor cells. We believe that they present convincing evidence that this is true. However, a few points should be addressed to strengthen the manuscript.

AUTHORS REPLY
We are glad that the reviewer has found our data convincing and important.

REVIEWER 1 – POINT 1
Figure 1: 1E- RFP expression is mosaic in the VZ. In the text the authors mention that RFP is detected in Tbr2- RGCs and Tbr2+ BPs. It would be beneficial to further characterize which cells are being targeted.

AUTHORS REPLY
We found that within the two subpopulations of RFP+ and RFP– cells the proportion of Tbr2– RGCs and Tbr2+ BPs was essentially identical (page 7, line 8). Since RGCs and BPs represent essentially the totality of cells in the VZ, this implies that mosaicism in the 4D line does not introduce bias with regard to targeting a specific cell type (see also reply to reviewer 3, point 2 on this issue). This is now clarified in the revised manuscript.

REVIEWER 1 – POINT 2
Staining for RGs (Pax6, Sox2) and focusing on the IZ might suggest whether bRGs are expanded in the 4D mouse in addition to the basal progenitors.

AUTHORS REPLY
We agree with this reviewer on the need to unequivocally identify bRGs based on specific markers. In mouse, bRGs have been defined as cells in the IZ expressing Pax6 but not Tbr2 (Pax6+/Tbr2-) (Wang et al., 2011; Shitamukai et al., 2011), so we went back to our original Pax6/Tbr2 stains (the same originally used for Fig S1B) and counted in the IZ. We have found that the density of Pax6+/Tbr2- cells in IZ is similar in control and 4D embryos, indicating that the population of bRGs is actually not affected in 4D embryos. Moreover, our new counts reveal that the increased PH3+ mitotic cells in the IZ of 4D embryos are Tbr2+, further confirming that the effect of 4D is restricted to BP. The result and interpretation of this analysis is now included in the relevant Results section (page 9), and we thank the reviewer for suggesting us to better dissect this important aspect.

REVIEWER 1 – POINT 3
Figure 2: Authors suggest there is an increase in bRGs in the IZ (Figure 2D), bRG specific marker staining - Pax6, Sox2 - may verify this claim.
AUTHORS REPLY
See reply to point 2 above.

REVIEWER 1 – POINT 4
In figure 5B: authors should include images of controls.

AUTHORS REPLY
This has been done as suggested.

REVIEWER 1 – POINT 5
Overall: the authors should tone down certain statements in their manuscript. Even though we agree that the authors present convincing evidence that challenges previous concepts we suggest to use a wording such as "challenging" and not "refuting" (throughout the manuscript).

AUTHORS REPLY
This has been changed as suggested.

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REVIEWER 3 COMMENTS FOR THE AUTHOR:

A previous study in mouse showed that over-expression of Cdk4 and cyclin D1 (4D) in a small proportion of cortical progenitor cells promoted basal progenitor (BP) expansion without altering cortical lamination and shape (Lange et al., 2009). Here the authors generated a transgenic mouse line that uses the tetracycline regulatable system to turn or, and off, expression of both of Cdk4 and cyclin D1 (4D) in mouse cortical progenitors. This led to increases in BP cell proliferation and led to larger cortical surface area in the mouse, without the induction of cortical folding. On the other hand, increased 4D expression in cortical progenitors of the gyrencephalic ferret also increased in BP cell proliferation and larger cortical surface area, but did increase cortical folding. Thus, they present evidence that factors other than increased BP proliferation underlie the formation/evolution of cortical gyri.

AUTHORS REPLY

We are glad that also this reviewer has found our data convincing and important.

REVIEWER 3 – POINT 1

Figure 1. The authors used fibroblasts to study the induction of 4D by doxycycline; it is likely that fibroblasts are very different then neurons and therefore the authors need to acknowledge this weakness in the experimental design.

AUTHORS REPLY

We realize that this figure was misleading in that no functional relevance should be drawn between fibroblasts and neurons. The only purpose of this figure was to show that overexpressed Cdk4 and cyclin D1 are catalytically active because both were fused to 17 aminoacids of a 2A peptide and it was therefore possible that this addition would have impaired their catalytic activity. This was not the case as shown by hyperphosphorylation of retinoblastoma (Fig. 1B). Similarly, fibroblasts in 1C were used as a proxy to screen the several lines obtained after pronuclear injection. This is now explained in the text.

REVIEWER 3 – POINT 2

In panel F they show that the expression of the RFP mRNA is not homogenous. The mosaicism in the ventricular zone could reflect that the transgene was introduced into the X chromosome and that they are analyzing a female embryo. Has this possibility been excluded?

AUTHORS REPLY

It is correct that mice are mosaics, at least as judged by RFP detection (see also reply to reviewer 1, point 1, on this issue). Even if this was a good suggestion, mosaicism was not due to X inactivation because both genders showed a similar degree of mosaicisms. We frankly do not know the reason for this since rtTA in situ hybridization showed homogeneous expression of the transactivator and our 4D line has a homogeneous genetic background. One possibility is that this reflects limitations with regard to RFP+ detection but also this seems unlikely because endogenous fluorescence and antibody enhancement led to similar outcomes. Apparently, mosaicism is not unusual in doxycycline inducible lines and, whatever the reason, mosaicism makes our data even more significant because effects on brain size are triggered by a relatively small proportion of cells.

REVIEWER 3 – POINT 3

Figure 4 Have the authors investigated whether or not there is a change in the numbers of astrocytes that are generated as a function of the doxycycline induction of 4D; these could account for some of the surface area changes.

AUTHORS REPLY

We found that in the mature brain the proportion of neurons (Satb2+ or Citip2+), astrocytes (S100b+) and oligodendrocytes (Olig2+) was unchanged (Fig. 4E). This was surprising because it is thought that BPs are exclusively neurogenic and not gliogenic. Therefore, our data suggest that

enhanced gliogenesis occurs as a consequence of an increased and prolonged neurogenesis, which we are investigating in the frame of another project. Moreover, while increased gliogenesis will certainly increase brain size, it is likely that such contribution is homogeneous in the 3D space because, differently to neurons, glia distributes homogeneously in the cortex. Thereofore, despite potential secondary effects of gliogenesis on tissue morphology, we believe that our data strongly support the notion that the primary effect of BP expansion is to increase cortical surface (or gyrification in ferret) but not cortical thickness, which is the main message of our paper.

REVIEWER 3 – POINT 4

Panel D shows that the size of the ventricles of the 4DG specimen is enlarged; can the authors account for this based solely on the increased surface area of the cortex or is there some evidence for hydrocephalus. Another possibility is that there defects in the axons connecting the cortex to the striatum, which then lead to weakening of the tissue at the pallial-subpallial boundary. Likewise there maybe problems in the formation of the commissures; what is the effect on the corpus callosum?

AUTHORS REPLY

In hydrocephalus a bigger brain is caused by an increase in cerebrospinal fluid, which does not necessarily imply an increase in tissue mass. In contrast, 4D mice display both enlarged ventricles and brain tissue, which is typically referred to as megalencephaly. Our data show that increased brain tissue arises well before any detectable increase in ventricular volume e.g. at E18 the cortex is visibly thicker but an increase in the ventricular volume is only detected by P21 (Fig. 3 vs. 4). Therefore, we conclude that the increase in ventricular volume is a topological consequence of a brain in which cortical surface, but not thickness, was increased resulting in bigger ventricles. We entirely agree that the most important next question to address is about synaptic connectivity and neuronal circuitry of 4D mice! We are currently performing these experiments in particular to address the potential effects of the extranumerary neurons in the occurrence of seizures, locomotor function, information processing, cognitive performance, and several other behaviors. Yet, we hope that the reviewer will agree that these experiments fall beyond the purpose of the current study that solely addresses the role of BPs in brain topology in lissencephalic and gyrencephalic species.

REVIEWER 3 – POINT 5

The nestin regulatory elements will lead to 4D expression throughout central nervous system; what is the effect on size of the basal ganglia and other regions of the CNS? Could this contribute to the ventriculomegally?

AUTHORS REPLY

Our previous work (Lange et al., *Cell Stem Cell*, 2009) has shown, and our current study (Nonaka et al.) confirms, that 4D triggers the expansion solely of BPs and not neuroepithelial/RGCs. The fact that BPs are specifically restricted to the forebrain explains why we could never detect any obvious effect in any other region of the CNS except for the forebrain.

REVIEWER 3 – POINT 6

Figure 5. The authors need to clarify why viral transduction transduction of 4D gives a stronger phenotype than electroporation of 4D.

AUTHORS REPLY

Viral transduction showed a stronger phenotype because of stable integration. In contrast, electroporated plasmids are diluted over time in cycling cells as it is now explained in page 13-14.

