

Manuscript EMBO-2015-92372

## Relief of hypoxia by angiogenesis promotes neural stem cell differentiation by targeting glycolysis

Christian Lange, Miguel Turrero Garcia, Ilaria Decimo, Francesco Bifari, Guy Eelen, Annelies Quaegebeur, Ruben Boon, Hui Zhao, Bram Boeckx, Junlei Chang, Christine Wu, Ferdinand le Noble, Diether Lambrechts, Mieke Dewerchin, Calvin Kuo, Wieland Huttner and Peter Carmeliet

*Corresponding author: Peter Carmeliet, VIB and KULeuven*

---

### Review timeline:

Submission date:	23 June 2015
Editorial Decision:	03 August 2015
Revision received:	30 October 2015
Accepted:	14 December 2015

---

Editor: Karin Dumstrei

### 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

03 August 2015

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees find the manuscript insightful, interesting and support publication here. They raise a number of specific and constructive points that I do expect you should be able to sort out. Let me know if we need to discuss some of them in more detail.

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

-----  
**REFeree REPORTS**

Referee #1:

This manuscript addresses an important issue, which is the potential effect of irrigation on progenitor cells (radial glia, RGC, and basal progenitors, BP) during fetal cortical development. The authors show that cortical RGCs expand at the expense of BP production in mouse embryos with defective blood vessel formation in the brain (Gpr24 KO). The authors then perform a transcriptomic analysis in a search for genes with altered expression in prominin+ RGCs and find that HIF-1alpha is significantly upregulated and evaluate the role of this transcription factor in cortical progenitor behavior by analyzing mouse mutants (conditional and heterozygous) and embryos electroporated with wild-type and mutant forms of HIF-1alpha. They show how a deficiency in HIF-1alpha favors BP production and that hyperoxia restores the phenotype of the

Gpr124 mutants. Finally, they connect the actions of HIF-1alpha to the hypoxia-inducible activator of glycolysis Pfkfb3.

The functional analyses are well performed and controlled and the results are very interesting. The main criticisms are related to the progenitor analyses.

The authors first show a correlative study in which they analyze markers of progenitors and endothelial cells in the developing brains of mice and ferrets at stages when neurogenesis is starting in the cortex. Taking advantage of the lateral-to-dorsal gradient of blood vessel formation, they find that the number and increment of Tbr2+ basal progenitors (BPs) appears to correlate with the generation of IB4+ capillaries. They further show that the number of cells positive for EdU administered a day before the analysis, but negative for Ki67, as a reflection of newly-generated neurons changes in parallel with vessel growth. The authors should represent this kind of data (Suppl. Figure 1) together with data on Ki67/IB4+ cells in the same sampled regions. Also, panels in Figure S1 should be a bit more contrasted as with the magnification required to illustrate the entire cortex the correlation between the labelings is difficult to perceive.

The authors then go on to analyze whether disturbing blood vessel formation results in alterations in cortical neurogenesis. They analyze embryos from a mouse strain deficient in Gpr124, which has specific alterations in CNS blood vessel formation with the aim of analyzing progenitor proliferation and the neuron generation. Data in Figure 1 is very confusing. The authors show that RGCs, but not BPs are reduced and then they suggest that RGCs do not switch correctly to a neurogenic mode resulting in their overexpansion at the expense of BP generation. This part could be easier to follow if the authors started by showing the general appearance of the developing brain of the mutants (including the staining with Pax6) emphasizing the lateral expansion of the cortex, which is an indication of RGC expansion and then move to the cellular analysis. The change in cerebral architecture is already obvious in panels A and B of Figure 1 and has implications in how the cell counts are performed. In particular, some concerns with this figure include:

- Data shown in the two parts of panel E is redundant as the percentages of Ki67 positive and negative cells are complementary. Instead, the authors should represent one of these counts per area: VZ and SVZ/CP.
- Lateral expansion of the developing cortex needs to be taken into account for the cell scores. RGC expansion is lateral, but BP production is not. The authors should take into account that the area occupied by RGCs is larger to do more accurate comparisons.
- Quantitations shown in panels H and K are difficult to reconcile with data in N.
- The authors need to clarify what "% of all VZ cells" means in each case.

- In Figure S2, panel M, what are the Ngn1+ BPs? Is this how many of the Tbr2+ cells in the VZ are Ngn1? This is confusing. The same for panel N.

- Similar concerns can be raised for Figure 2.

The presence of markers Ngn1 or Tis21 in the Tbr2+ cells cannot be used as a demonstration that the lack of Gpr124 changes BP cell fate. The authors could check if the generation of upper and lower layer neurons is normal.

In situ hybridizations at E10.5 are of lower quality than the rest.

The relationship between absence of assembled blood vessels in Gpr124 KO embryos and a hypoxia/HIF-1alpha/glycolytic pathway in the switch between proliferation and neurogenesis in neural progenitors is very nice. Are avascularized cortical areas in normal mouse embryos hypoxic? The authors could check with the fluorescent probe.

Some other comments:

- The authors hardly use the term NSC in the text and refer to NPCs, RGCs or BPs, still they use it in the title.
- The authors should indicate in their figures what they consider to be the limits between lateral and dorsal cortices.
- Moreover, when explaining the phenotype of the Gpr124 KO mice the authors change from lateral and dorsal to ventral and medial. They should standardize this.
- In Figure S1 switch around panels A-B and C-D.
- The authors could comment on when the Gpr124 KOs die.
- N=3-4 is not appropriate.

- In page 10 "To explore...suppression of the switch of RGCs, BPs or both NPCs to neurogenesis..." add "from proliferation to neurogenesis".
- Indicate the limits between VZ and SVZ also in Figures 1C, D.
- The authors do not mention what is the lifespan of the mutant mice under analysis.
- In page 13, the authors use the term NSC whereas it is not used anywhere else in the text.
- Authors should revise the sentence "other target genes of the Wnt-mediator beta-catenin were even enriched among the genes down-regulated in Gpr124KO NPC (Fig. S3A-D)".

Referee #2:

The manuscript by Lange et al describes the role of angiogenesis and hypoxia in regulating neural stem cell growth and differentiation during CNS development. The manuscript is well written and the conclusions appear to be justified, for the most part, by the data presented. A major strength of the study is the authors focus on obtaining *in vivo* data using genetically engineered models. A few things that would strengthen the study and make it more focused follow:

The data on glycolysis are preliminary and not nearly as strong as the rest of the manuscript concerning the role of angiogenesis, hypoxia and HIF-1. I suggest removing the phrase "by targeting glycolysis" from the title and keeping the focus on angiogenesis and hypoxia. The glycolysis data are mostly correlative, and therefore circumstantial. The Pfkfb3 knockdown data in figure 7 are not sufficient to implicate glycolysis functionally. For example, only a single knockdown vector was employed and off target effects were not excluded. Further, the *in vivo* assay only analyzed GFP expression and not percentages of the various neuronal populations.

The data in Figure 1F to K and Fig 6D to K are confusing. The fact that the neurogenic Ngn1+Tbr2 population, which the authors refer to as radia glia (for example, Fig1H) was reduced by 50% in the Gpr124KO embryos (p10 line 6) seems to be at odds with the author's contention that the RGC NSC population is expanded in these mice. Are the authors are trying to claim that a NSC progenitor population is increased in the KO based on a smaller fraction of Ngn1+, Tbr2- cells (that are presumably somewhat more committed to the neural lineage than Ngn1- NSCs)? If so, this seems very indirect and prone to inaccuracy because the real NSC population may only be a subset of the Ngn1 negative population. Are there no molecular markers that could be used to directly demonstrate the relevant expanded NSC population in the VZ of the KO? Please explain why such staining was not performed. Could the authors subtract all the Ngn1 and Tbr2 positive cells from the total number of DAPI+ cells in the VZ to determine the NSC number more directly?

The authors claim that oxygen rescues the differentiation of NSCs in the Gpr124 KO by showing an increase in Ngn1+, Tbr2- cells in the VZ. However, the role of HIF-1 in this process could be strengthened by crossing the HIF-1 heterozygous mice to Gpr124 KO mice.

Minor concerns:

The percent of green radia glia (Ngn1 +, Tbr2 -) in the VZ in Fig 1H (~3 to 6% of total) is smaller than the number of yellow basal progenitors (Ngn1 +, Tbr2 +) (~10 to 12% of total), yet in the figure 1F and G there appear to be more green cells than yellow cells. The same is true in figure 6.

Much of the quantitation is normalized to a particular zone of the developing neopallium, and the overall thickness of the neopallium appears to decrease in the Gpr124 KO. The authors should indicate what criteria were used to mark the borders between the VZ, SVZ and IZ/CP in each of the figures.

Figure 3B. This data is not very informative. Please remove or include the genes and their expression levels in a supplemental figure.

Figure 2E,F. The increase in yellow (Ki67+, Efu+) cells in the ECcKO is not apparent in the figure.

P12, Weigmann reference is missing.

P13, Fig3L should be Fig3K

Figure S5, part F is missing

The methods section is a bit vague. For example, there is no mention of how qPCR was performed.

Referee #3:

The study by Lange et al draws the attention to an intriguing temporal and spatial correlation between the ingrowth of blood vessels and the switch of radial glia from expansion to neuronal differentiation in the cerebral cortex. Using a genetic model in which blood vessel formation is hampered selectively in the cortex, they continue to show that impairment of blood vessel formation results in continued expansion of radial glia at the expense of differentiation via basal progenitors. They further reveal that this effect is due to hypoxia and, on a molecular level, the continued expression of HIF1-alpha. Overexpressing HIF1-alpha mimics the effect of impaired blood vessel formation while reduction of HIF1-alpha by genetic deletion of a single allele can partially rescue the effect of impaired blood vessel formation on neurogenesis. Consistent with this model, elevating O<sub>2</sub> levels results in precocious neurogenesis. Finally, the authors provide evidence that glycolysis plays an important regulatory role in keeping radial glial cells expanding and deleting an enzyme regulating glycolysis favors neuronal differentiation.

This is a very important study adding to growing body of evidence that the metabolite of cells plays a key role in cell fate regulation. The data presented are intriguing and overall convincing. However, there are some points that merit amendment.

Major:

1) Page 10: The authors designate Ngn1+/Tbr2- cells as RGCs. In contrast they designate Ngn1+/Tbr2+ cells as BPs. They state that only the former population is reduced in Gpr124 ko mice, while the latter is only insignificantly reduced (Figure 1H). However in Figure 1N, BPs are significantly reduced. This seems first glance contradictory. It might be due to the different parameters analysed but it would therefore greatly help if the authors provided the reader with a scheme depicting the different marker combinations and how these were assigned to distinct progenitor subtypes. Also it should be explained why there is no contradiction between the lack of reduction in Ngn1+/Tbr2+ cells and the reduced numbers of BPs.

2) The term "neurogenic" needs to be defined. Neurogenic can mean making a neuron as an imminent next step (e.g. referring to cells such as Tis21-GFP+ cells that exit cell cycle and differentiate into neurons) or may refer to the majority of RGCs at 13.5 which are virtually all generating neurons earlier or later. As the term "neurogenic" is often used in the second sense but the reviewer has the impression the authors use in the first sense, this should be clarified.

3) Does deletion of a HIF1-alpha allele result in a smaller cortex as a consequence of a precocious differentiation at the expense of RGC expansion? Does it affect the generation of specific cortical neuron subtypes?

4) What is the evidence that increasing atmospheric O<sub>2</sub> levels to 80% also results in increased O<sub>2</sub> tissue levels? The authors should perform measurements for oxidized thiols and pimonidazole levels; also they should make an estimate how much tissue oxygen will raise when switching from 21 to 80 % oxygen levels.

5) The authors make a point that interfering with glycolysis by knock down of Pfkfb3 increases neuronal differentiation. This means that when a RGC is impaired in glycolysis, this will provide a signal in the RGC to differentiate. Does blocking glycolysis stimulate OX-Phos? What is the effect of knocking down Pfkfb3 in Gpr 124 ko mice?

6) Is there any phenotype regarding astroglialogenesis in the Gpr124 ko mice?

Minor:

1) The quality of the micrographs in Fig S1A-H does not allow to fully assess the temporo-spatial correlation of vessel ingrowth and neurogenesis, at least on a printed version.

- 2) Introduction page 4: referring to "new" neurons during embryonic development is not very meaningful; remove "new"
- 3) The sentence "These RGCs switch from expansion towards differentiation to neurons" sounds blunted and would benefit from further qualification.
- 4) Results: temporo-spatial relationship "between" (instead of to)...
- 5) Figure 1 A and B are apparently taken at different rostro-caudal levels. Same levels should be shown.
- 6) Discussion: The sentence "virtually all cortical NPCs expanded ... and switched..." sounds too strong; it suggests that after vessel growth there is no more expansion going on.

1st Revision - authors' response

30 October 2015

**Referee #1**

This manuscript addresses an important issue, which is the potential effect of irrigation on progenitor cells (radial glia, RGC, and basal progenitors, BP) during fetal cortical development. The authors show that cortical RGCs expand at the expense of BP production in mouse embryos with defective blood vessel formation in the brain (Gpr24 KO). The authors then perform a transcriptomic analysis in a search for genes with altered expression in prominin+ RGCs and find that HIF-1alpha is significantly upregulated and evaluate the role of this transcription factor in cortical progenitor behavior by analyzing mouse mutants (conditional and heterozygous) and embryos electroporated with wild-type and mutant forms of HIF-1alpha. They show how a deficiency in HIF-1alpha favors BP production and that hyperoxia restores the phenotype of the Gpr124 mutants. Finally, they connect the actions of HIF-1alpha to the hypoxia-inducible activator of glycolysis Pfkfb3. The functional analyses are well performed and controlled and the results are very interesting.

**RESPONSE:** We thank the reviewer for his/her positive evaluation of our work.

1) The main criticisms are related to the progenitor analyses. The authors first show a correlative study in which they analyze markers of progenitors and endothelial cells in the developing brains of mice and ferrets at stages when neurogenesis is starting in the cortex. Taking advantage of the lateral-to-dorsal gradient of blood vessel formation, they find that the number and increment of Tbr2+ basal progenitors (BPs) appears to correlate with the generation of IB4+ capillaries. They further show that the number of cells positive for EdU administered a day before the analysis, but negative for Ki67, as a reflection of newly-generated neurons changes in parallel with vessel growth. The authors should represent this kind of data (Suppl. Figure 1) together with data on Ki67/IB4+ cells in the same sampled regions. Also, panels in Figure S1 should be a bit more contrasted as with the magnification required to illustrate the entire cortex the correlation between the labelings is difficult to perceive.

**RESPONSE:** We have added quantifications of intra-parenchymal Ki67/IB4 double-positive cells (proliferating endothelial cells) together with quantifications of the newly generated neurons during cortical development (now presented in the Expanded View Figure EV1L-Q), excluding the endothelial cells in the meningeal vessels. The results show a clear, quantitative correlation between the generation of Ki67-negative neurons from EdU-incorporating dividing precursors on one side and the presence of Ki67-positive, IB4-positive endothelial cells on the other side. These results thus further underscore the congruence between neurogenesis and angiogenesis in the cerebral cortex of the developing brain. Moreover, we increased the size of panels in the former Figure S1 and have split it in two figures EV1 and EV2 to allow a clearer visualization of the congruence between blood vessel ingrowth and radial glia differentiation.

2) The authors then go on to analyze whether disturbing blood vessel formation results in alterations in cortical neurogenesis. They analyze embryos from a mouse strain deficient in Gpr124, which has specific alterations in CNS blood vessel formation with the aim of analyzing progenitor proliferation and the neuron generation. Data in Figure 1 is very confusing. The authors show that RGCs, but not BPs are reduced and then they suggest that RGCs do not switch correctly to a neurogenic mode resulting in their overexpansion at the expense of BP generation. This part could be easier to follow

if the authors started by showing the general appearance of the developing brain of the mutants (including the staining with Pax6) emphasizing the lateral expansion of the cortex, which is an indication of RGC expansion and then move to the cellular analysis.

**RESPONSE:** We have changed the representation of the figure panels in Figure 1 and the flow of the text according to the reviewer's suggestions. We now first mention the lateral expansion of the Gpr124<sup>KO</sup> cortex (new Figure 1C-E) directly after describing the vascular phenotype of these mutants (new Figure 1A,B) and then go on to investigate the underlying cellular mechanisms: i.e. neurogenesis (new Figure 1F-H), BP generation from RGCs (new Figure 1 I-K) and RGC differentiation using Ngn1 (new Figure 1L-O) and *Tis21*-GFP (new Figure 1P-S) as differentiation markers.

3) The change in cerebral architecture is already obvious in panels A and B of Figure 1 and has implications in how the cell counts are performed. In particular, some concerns with this figure include:

3a) Data shown in the two parts of panel E is redundant as the percentages of Ki67 positive and negative cells are complementary. Instead, the authors should represent one of these counts per area: VZ and SVZ/CP.

**RESPONSE:** We have changed the representation of this panel (now Figure 1H of the current manuscript) and now show the proportion of EdU+/Ki67+ cells in the VZ and SVZ/CP, respectively. For reasons of completeness, we also show now the Ki67- cells. The results now show significantly elevated abundance of EdU+/Ki67+ cells in the VZ, while the proportion of Ki67+ cells in the SVZ and CP is not significantly changed. These results further support our conclusion that radial glia expand more and differentiate less in the Gpr124<sup>KO</sup> cortex, as more EdU+/Ki67+ cells are retained in the VZ. The corresponding panels in Figure 2 (Figure 2J of the current manuscript) and Figure 5 (Figure 5D of the current manuscript) were adapted in the same way.

3b) Lateral expansion of the developing cortex needs to be taken into account for the cell scores. RGC expansion is lateral, but BP production is not. The authors should take into account that the area occupied by RGCs is larger to do more accurate comparisons.

**RESPONSE:** The referee rightly points out that neurogenesis and BP production occur in radial units, while RGC expand laterally. Hence, to analyze RGC differentiation in any given part of the cortex, one has to take into account the differentiated progeny (i.e. BPs and to a minor extent neurons) that is delaminating from the neuroepithelium and migrates basally within the same radial unit. Even though RGCs occupy a larger area, as a result of their increased expansion, their differentiation at any level along the ventricular zone will be radially and if they expand more, they will generate less differentiated progeny that will migrate in the radial direction. Thus, we quantified the abundance of expanding RGCs, neurogenic RGCs and BPs within a particular field as a percentage of all VZ cells in that field to determine RGC differentiation. Even if we would measure a larger area occupied by RGCs in the GPR124<sup>KO</sup> brains (for instance, proportional to the fold-enlargement of the lateral extension of the cortex), we would still obtain the same results, as the counts of the differentiated progeny are always expressed as a percentage of all VZ cells in the field analyzed.

We have also added now a detailed description of our methodology of cell quantification in the VZ to the additional materials and methods on page 10-11 of the Appendix.

3c) Quantitations shown in panels H and K are difficult to reconcile with data in N.

**RESPONSE:** We assume that the referee refers to the fact that the proportion of Ngn1+ BPs is only mildly (insignificantly) reduced in Gpr124<sup>KO</sup> mice (Fig. 1H,K of the original manuscript; now Fig. 1N,R), while the total number of BPs is significantly reduced in Gpr124<sup>KO</sup> mice (original Fig. 1N; now Fig. 1K).

For the new panel 1N,K, this apparent discrepancy can be explained as follows: panel K shows the fraction of all BPs (i.e. the entire population; expressed as a percentage of all VZ cells), while panel N shows the fraction of Ngn1+ BPs (percentage of all VZ cells). However, in panel K, the proportion of Ngn1+ BPs is larger in the Gpr124<sup>KO</sup> (38%) than WT (31%) brains, even though this increase does not reach statistical significance; this is shown separately in Fig. S2M. As a result, when showing the fraction of Ngn1+ cells separately in panel N, the difference in this fraction

between both genotypes becomes visually smaller and no longer statistically significant. Though the precise reason hereof remains unknown, these data may suggest various possible explanations: (i) the regulation of Ngn1 protein stability in the different cell cycle phases is altered in Gpr124<sup>KO</sup> brains; and/or (ii) the Gpr124<sup>KO</sup> BPs have longer G1 phase, in which Ngn1 is expressed ([Britz et al., 2006](#)), thereby reducing the fraction of Ngn1<sup>-</sup> BPs in Gpr124<sup>KO</sup> brains.

In the original panel 1K, the difference in the fraction of Tis21+ cells was not statistically significant between the WT and Gpr124<sup>KO</sup> brains (note that this is a separate group of mice as those analyzed for the new panel 1N,K). However, this lack of statistical difference was due to the presence of a high proportion of BPs in one of the Gpr124<sup>KO</sup> brains analyzed. We now remedied this situation by counting more sections from the same brains to have a broader statistical basis. These new results now reveal a significant reduction of the fraction of Tis21+ BPs in the VZ of Gpr124<sup>KO</sup> cortices as compared to WT, in agreement with the data shown for the total BP population in the new Fig. 1K.

3d) The authors need to clarify what "% of all VZ cells" means in each case.

**RESPONSE:** We counted all Hoechst-positive nuclei in the VZ, defined using nuclear morphology and Tbr2-immunostaining to separate the VZ from the overlying SVZ. We have now added a section in the supplementary materials and methods (page ... of the Appendix) to fully describe our approach of cell quantification in the VZ.

This total cell number in the VZ was then used as a denominator for determining the percentages of marker-positive cells (Ngn1+; Tbr2+; Tis21+; etc.) in order to correct for small differences in location and tissue architecture between the controls and the different mutants.

4) In Figure S2, panel M, what are the Ngn1+ BPs? Is this how many of the Tbr2+ cells in the VZ are Ngn1? This is confusing. The same for panel N.

**RESPONSE:** Indeed, we calculated the percentage of BPs in the VZ that are positive for Ngn1 (Ngn1+/Tbr2+) or Tis21 (Tis21-GFP+/Tbr2+) in the figure panels S2M or S2N (of the original submission), respectively, in order to investigate whether the proportion of neurogenic marker-positive cells within the newborn BPs (still located in the VZ) changed upon Gpr124 deficiency, as a proxy for cell fate change. However, the results show no significant change of cell fate in the BP population under these conditions. For clarity, we have changed the labeling of the graphs from "Ngn1+ BPs" to "Ngn1+/Tbr2+ BPs". The same applies for Tis21-GFP+. See Appendix Figure S2M and S2N.

5) Similar concerns can be raised for Figure 2.

**RESPONSE:** We assume that this comment relates to two comments of the reviewer on Figure 1:

1. We now also changed the order of the data representation: first reporting the vascular phenotype (new Figure 2A,B), then the lateral expansion of the cortex (new Figure 2E-G), followed by the changes in neurogenesis (new Figure 2H-J) and NSC differentiation (new Figure 2L-O).
2. We now also show the proportion of EdU+/Ki67+ cells in the VZ and SVZ+CP separately (new Figure 2J).

6) The presence of markers Ngn1 or Tis21 in the Tbr2+ cells cannot be used as a demonstration that the lack of Gpr124 changes BP cell fate. The authors could check if the generation of upper and lower layer neurons is normal.

**RESPONSE:** We fully agree with the reviewer. Indeed, we already stated in our original manuscript that the proportion of Ngn1-positive and Tis21-positive BPs does not change between control and Gpr124<sup>KO</sup> embryos, and that this finding argues against a change of BP cell fate upon impaired brain angiogenesis. Hence, we do not wish to say that lack of Gpr124 changes BP cell fate. We agree that it is interesting to examine whether impaired brain angiogenesis alters the generation of upper versus lower layer neurons. Unfortunately however, it is experimentally not feasible to provide a reliable trustworthy response to this question in the Gpr124<sup>KO</sup> model. Indeed, upper layer neurons start to be generated from proliferating precursors around E14.5 ([Molyneaux et al., 2007](#)). However, homozygous Gpr124<sup>KO</sup> embryos die already around E15.5 ([Kuhnert et al., 2010](#)) and the organization of the brain cortex is already disturbed from E14.5 onwards in part through widespread hemorrhages, causing severe hypoxia and inflammation ([Cullen et al., 2011](#)) (unpublished findings).

Thus, the Gpr124<sup>KO</sup> model enables us to study early to mid-neurogenesis, but the embryonic lethality precludes us from investigating upper layer neuron formation later in cortical development.

7) In situ hybridizations at E10.5 are of lower quality than the rest.

**RESPONSE:** We have replaced 2 of these panels with better images (see new Appendix Figure S4).

8) The relationship between absence of assembled blood vessels in Gpr124 KO embryos and a hypoxia/HIF-1 $\alpha$ /glycolytic pathway in the switch between proliferation and neurogenesis in neural progenitors is very nice. Are avascularized cortical areas in normal mouse embryos hypoxic? The authors could check with the fluorescent probe.

**RESPONSE:** Using labeling of oxidized thiols as a proxy for oxygen levels, we had already detected lower tissue oxygenation in the avascular areas of the early developing cortex (see Figure 4A-F of the current submission). We have now also performed immunostaining for HIF-1 $\alpha$  in the early developing cortex, as another readout of cellular hypoxia. These data show abundant VZ cells with HIF-1 $\alpha$  protein localized in the avascular areas of the cortex. Upon vascularization, HIF-1 $\alpha$  becomes excluded from the nucleus, similar to the situation in the control cortex in Figure 3I-I' in the current submission. Together, these data show lower oxygen levels and HIF-1 $\alpha$  stabilization and nuclear localization in avascular areas of the early developing cortex and suggest that vessel derived oxygen controls the stability and/or nuclear localization of HIF-1  $\alpha$  in the developing cortex. The HIF-1 $\alpha$  immunostaining data have now been incorporated into Figure 4G-I' of the current submission and the in-situ hybridization panels have been moved to the supplement (new Appendix Figure S4).

Some other comments:

a) The authors hardly use the term NSC in the text and refer to NPCs, RGCs or BPs, still they use it in the title.

**RESPONSE:** We have now adapted the text and use more the term neural stem cell, where appropriate see for example pages 3-4 and 18-22 of the current submission). We also explain on page 3 of the manuscript that radial glia are the NSCs of the embryonic cortex).

b) The authors should indicate in their figures what they consider to be the limits between lateral and dorsal cortices.

**RESPONSE:** We have now inserted arrowheads to indicate the border between lateral and dorsal cortex in the Figure 4G,H,I, Figure EV1A-H and Appendix Figure S4A-I of the current submission.

c) Moreover, when explaining the phenotype of the Gpr124 KO mice the authors change from lateral and dorsal to ventral and medial. They should standardize this.

**RESPONSE:** We have now adapted the phrasing according to the referee's suggestions (see page 7 of the manuscript text).

d) In Figure S1 switch around panels A-B and C-D.

**RESPONSE:** We have now adapted the figure panels according to the referee's suggestions as well as panels S1F and S1G which are now Figure EV1A,B, EV1C,D or EV1F,G, respectively We have also included the IB4 channel in each of these panels.

e) The authors could comment on when the Gpr124 KOs die.

**RESPONSE:** No living Gpr124<sup>KO</sup> embryos are collected from E 15.5 on, as described ([Kuhnert et al., 2010](#)). We have now added this information to the main text (see page 7 of the manuscript text).

f) N=3-4 is not appropriate.

**RESPONSE:** We have changed the legend of Figure 1, now explaining that N=4 refers to controls and N=3 refers to Gpr124<sup>KO</sup> brains (see page 33 of the manuscript text).



g) In page 10 "To explore...suppression of the switch of RGCs, BPs or both NPCs to neurogenesis..." add "from proliferation to neurogenesis".

**RESPONSE:** We have changed the text accordingly (see page 8 of the manuscript text).

h) Indicate the limits between VZ and SVZ also in Figures 1C, D.

**RESPONSE:** We added approximate boundaries of VZ and SVZ (in the new panels Figure 1F,G) and also in the new panels Figure 2H,I and the new Figure 5B,C.

i) The authors do not mention what is the lifespan of the mutant mice under analysis.

**RESPONSE:** We added information concerning the embryonic lethality of Gpr124<sup>KO</sup> mice (see page 7 of the manuscript text) and normal survival and fertility of HIF-1 $\alpha$ <sup>CC-/-</sup> mice (see page 13 of the manuscript text), in the text. HIF-1 $\alpha$ <sup>CC+/-</sup> mice are viable and fertile as well. Gpr124<sup>ECCKO</sup> mice used in this study were generated by injecting pregnant dams carrying PdgfB-iCre x Gpr124<sup>-lox</sup> embryos with tamoxifen at E10.5 and all Gpr124<sup>ECCKO</sup> embryos generated in this way were collected at E13.5 for analysis, so their survival at later fetal and postnatal stages is actually not known.

j) In page 13, the authors use the term NSC whereas it is not used anywhere else in the text.

**RESPONSE:** As explained above, we now use more the term neural stem cell, where appropriate (see for example pages 3-4 and 18-22 of the current submission).

k) Authors should revise the sentence "other target genes of the Wnt-mediator beta-catenin were even enriched among the genes down-regulated in Gpr124KO NPC (Fig. S3A-D)".

**RESPONSE:** We have revised this sentence (see page 11 of the manuscript text).

## Referee #2

The manuscript by Lange et al describes the role of angiogenesis and hypoxia in regulating neural stem cell growth and differentiation during CNS development. The manuscript is well written and the conclusions appear to be justified, for the most part, by the data presented. A major strength of the study is the authors focus on obtaining *in vivo* data using genetically engineered models.

**RESPONSE:** We thank the referee for the positive evaluation of our work.

1) A few things that would strengthen the study and make it more focused follow: The data on glycolysis are preliminary and not nearly as strong as the rest of the manuscript concerning the role of angiogenesis, hypoxia and HIF-1. I suggest removing the phrase "by targeting glycolysis" from the title and keeping the focus on angiogenesis and hypoxia. The glycolysis data are mostly correlative, and therefore circumstantial. The Pfkfb3 knockdown data in figure 7 are not sufficient to implicate glycolysis functionally. For example, only a single knockdown vector was employed and off target effects were not excluded. Further, the *in vivo* assay only analyzed GFP expression and not percentages of the various neuronal populations.

**RESPONSE:** We respectfully disagree with the referee. In fact, we believe that the functional implication of glycolysis in the increased expansion of neural stem cells by hypoxia is an important part and novelty of the manuscript, not documented in previous publications. To strengthen the data, we have now performed Pfkfb3 knockdown *in vivo* using a second shRNA sequence, yielding similar results. Moreover, we now show that Pfkfb3 knockdown reduces glycolysis, but does not affect mitochondrial respiration (oxygen consumption) in NSCs *in vitro*, thus showing that Pfkfb3 knockdown does not indiscriminately (via off target effects) affects any type of metabolic pathway. These data are now incorporated in Fig. 7 and Figure EV5I. Together, these data provide strong evidence that Pfkfb3-driven glycolysis is important for normal NSC expansion and is particularly required for increased NSC maintenance upon HIF stabilization. Further, we would like to clarify that we have analyzed the differentiation of RGCs *in vivo* by determining the proportion of electroporated, GFP+ RGC progeny in the germinal zones (VZ/SVZ)

and in the the neuronal layers. There, we distinguished between (i) GFP+ cells that had already migrated into the cortical plate (CP) and thus were designated as early born neurons, and (ii) cells that were still migrating in the intermediate zone (IZ) and had not yet reached the CP, identifying them as recently born. This approach has been validated ([Lange et al, 2009](#)) and is commonly used in the field ([Dai et al, 2013](#); [Fasano et al, 2009](#); [Hasan et al, 2013](#); [Sun et al, 2011](#); [Yabut et al, 2010](#)). Importantly, the fact that we find more GFP+ cells in the CP after Pfkfb3 knockdown is in line with the increased RGC differentiation and omits the concern that cell migration might be impaired in these conditions.

2) The data in Figure 1F to K and Fig 6D to K are confusing. The fact that the neurogenic Ngn1+Tbr2 population, which the authors refer to as radia glia (for example, Fig1H) was reduced by 50% in the Gpr124KO embryos (p10 line 6) seems to be at odds with the author's contention that the RGC NSC population is expanded in these mice. Are the authors are trying to claim that a NSC progenitor population is increased in the KO based on a smaller fraction of Ngn1+, Tbr2- cells (that are presumably somewhat more committed to the neural lineage than Ngn1- NSCs)?

#### RESPONSE:

**Figure 1F-K:** First, we would like to clarify that Fig. 1H (original manuscript, now Fig. 1N) shows the proportion of Ngn1+ RG, and not the total RG population. Since the proportion of neurogenic RG is decreased and the proportion of BPs (the differentiated progeny of RG) is also decreased, it follows that the population of expanding RGs is increased (now shown in Fig. 1O).

Second, in the original manuscript, it is indeed correct that the proportion of Ngn1+ BPs is only mildly reduced in Gpr124<sup>KO</sup> mice (Fig. 1H,K of the original manuscript; now Fig. 1N,R), while the total number of BPs is significantly reduced in Gpr124<sup>KO</sup> mice (Fig. 1N of the original manuscript; now Fig. 1K).

For the new panel 1N,K, this apparent discrepancy can be explained as follows: panel K shows the fraction of all BPs (i.e. the entire population; expressed as a percentage of all VZ cells, while panel N shows the fraction of Ngn1+ BPs (percentage of all VZ cells). However, in panel K, the proportion of Ngn1+ BPs is larger in the Gpr124<sup>KO</sup> (38%) than WT (31%) brains, even though this increase does not reach statistical significance; this is shown separately in Fig. S2M. As a result, when showing the fraction of Ngn1+ cells separately in panel N, the difference in this fraction between both genotypes becomes visually smaller and no longer statistically significant. Though the precise reason herefore remains unknown to us, these data may suggest various possible explanations: (i) the regulation of Ngn1 protein stability in the different cell cycle phases is altered in Gpr124<sup>KO</sup> brains; and/or (ii) the Gpr124<sup>KO</sup> BPs have longer G1 phase, in which Ngn1 is expressed ([Britz et al, 2006](#)), thereby reducing fraction of Ngn1- BPs in Gpr124<sup>KO</sup> brains.

In the original panel 1K, the difference in the fraction of Tis21+ cells was not statistically significant between the WT and Gpr124<sup>KO</sup> brains (note that this is a separate group of mice as those analyzed for the new panel 1N,K). However, this lack of statistical difference was due to the presence of a high proportion of BPs in one of the Gpr124<sup>KO</sup> brains analyzed. We now remedied this situation by counting more sections from the same brains to have a broader statistical basis. These new results now reveal a significant reduction of the fraction of Tis21+ BPs in the VZ of Gpr124<sup>KO</sup> cortices as compared to WT, in agreement with the data shown for the total BP population in the new Fig. 1K.

**Fig 6D-K:** These figures basically show the opposite phenotype, induced by the hyperoxia, and the same arguments as used above for Fig. 1 explain these results.

**Analysis of NSC expansion:** Our conclusion of increased NSC expansion upon inhibition of angiogenesis in Gpr124<sup>KO</sup> brains relies on: (i) a reduced proportion of RGs that switched from expansion towards neurogenesis, indicated by the expression of neurogenic markers, such as Ngn1 and *Tis21*-GFP, specifically in the Tbr2-negative RGC population; (ii) reduced generation of BPs from RGs; and (iii) a correlation of the latter result with the increased lateral expansion of the cortices.

3) If so, this seems very indirect and prone to inaccuracy because the real NSC population may only be a subset of the Ngn1 negative population. Are there no molecular markers that could be used to directly demonstrate the relevant expanded NSC population in the VZ of the KO? Please explain why such staining was not performed.

#### RESPONSE:

**Analysis of expanding NSCs:** Indeed, this approach is rather indirect, as we defined expanding NSCs by the absence of differentiation markers (Tbr2, Ngn1, Tis21). This approach leads to an

over-estimation of the number of expanding NSCs when Ngn1 is used as marker, because the Ngn1 protein is only present during the G1 phase of the cell cycle. As a result, differentiating but Ngn1<sup>-</sup> RGCs in S, G2 or M-phase are counted as expanding NSCs. Importantly, even though Ngn1 only labels a subset of the neurogenic precursors, it is noteworthy that *Tis21*-GFP is a pan-neurogenic marker, and that the observed changes in the proportion of neurogenic RG in Gpr124<sup>KO</sup> mice are congruent when using both markers (compare Figure 1N and 1R), suggesting that both markers can be reliably used to detect changes of neurogenic RGs.

**Absence of reliable molecular markers to identify expanding NSCs:** Pax6, Prominin1 (CD133) and Sox2 mark NSCs, but the protein of these markers is retained in newborn BPs. Therefore, stainings for these markers in the VZ of the developing cortex, where NSCs and committed progenitors co-exist, show a significant overlap of NSC markers with the expression of committed progenitors (Arai et al, 2011; Englund et al, 2005; Glickstein et al, 2009; Hutton & Pevny, 2011; Wilsch-Brauninger et al, 2012). For example, about 30% of Tbr2-positive newborn BPs in the VZ is still labeled for Pax6 at E14.5 and, conversely, more than 15% of Pax6-positive cells are also positive for Tbr2 (Englund et al, 2005; Glickstein et al, 2009). These data preclude the use of classical NSC markers alone to selectively identify expanding NSCs directly.

In fact, differentiation markers provide a better resolution to identify the expanding NSC population by exclusion, as their expression by definition is absent from expanding NSCs, allowing their identification with minimal ambiguity. Thus, we used the combination of location in the cortical VZ together with the absence of differentiation markers to define expanding NSCs. This is a common and widely used approach in the field (Aprea et al, 2013; Arai et al, 2011).

4) Could the authors subtract all the Ngn1 and Tbr2 positive cells from the total number of DAPI+ cells in the VZ to determine the NSC number more directly?

**RESPONSE:** We have included the results of this calculation in Figures 1O,S, 2O, 5I and 6L,Q as additional quantifications of expanding NSCs. In all cases, NSC numbers are increased when differentiation markers are decreased (in Gpr124<sup>KO</sup> and Gpr124<sup>ECcKO</sup> cortices) and they are decreased when the differentiation markers are increased (in HIF-1 $\alpha$ <sup>CC+/-</sup> mice and upon hyperoxia treatment).

5) The authors claim that oxygen rescues the differentiation of NSCs in the Gpr124 KO by showing an increase in Ngn1+, Tbr2- cells in the VZ. However, the role of HIF-1 in this process could be strengthened by crossing the HIF-1 heterozygous mice to Gpr124 KO mice.

**RESPONSE:** Our experiments show that increasing tissue oxygenation rescued the differentiation of NSCs in Gpr124<sup>KO</sup> mice. We admit that this does not directly demonstrate an involvement of HIF-1 $\alpha$  in this process, but preferred to perform this high oxygenation rescue experiment rather than to interfere with HIF-1 $\alpha$  activity genetically, as homozygous loss of HIF-1 $\alpha$  alleles in the developing cortex compromises the survival of cortical cells.

Furthermore, in consultation with the editor, we did not embark on such genetic intercross because of the following additional reasons:

First, we already provide substantial evidence for an important role of HIF-1 $\alpha$  in RGC expansion because: (i) HIF-1 $\alpha$  is a major activator of gene expression in NPCs of Gpr124<sup>KO</sup> embryos (see Fig 3D of the current submission); (ii) only HIF-1 $\alpha$ , but not HIF-2 $\alpha$  is expressed in the embryonic cortex and is stabilized in the hypoxic Gpr124<sup>KO</sup> cortex (Fig 3F-I' of the current submission); (iii) stabilization of HIF-1 $\alpha$  via transgenic overexpression is sufficient to delay neurogenesis and increase RGC maintenance/expansion (Fig 5O-R of the current submission); while (iv) partial conditional knockout of HIF-1 $\alpha$  decreases RGC expansion and induces differentiation in the cortex (Fig 5E-I of the current submission); and (v) finally, we show that exposing Gpr124<sup>KO</sup> embryos to hyperoxia rescues RGC differentiation (Fig 6H-L of the current submission) and at the same time reduces the expression of HIF-1 $\alpha$  target genes (Fig 6B,C of the original submission). Hence, we used 5 different experimental strategies to imply a role for HIF-1 $\alpha$  in RGC expansion in WT and Gpr124<sup>KO</sup> embryos. (vi) In addition, we now confirmed HIF-1 $\alpha$  downregulation in the Gpr124<sup>KO</sup> cortex under conditions of hyperoxia compared to mutants at normoxia (Fig 6F,G of the current submission), thus directly demonstrating that hyperoxia treatment reduced HIF-1 $\alpha$  stability and abundance.

Second, we acknowledge that crossing HIF-1 heterozygous mice to Gpr124<sup>KO</sup> mice is an appealing experiment, but generating the triple-transgenic embryos needed for this analysis (Gpr124<sup>-/-</sup>;

Emx1-Cre Tg/+; HIF-1 $\alpha$  lox/+) from the existing strains will require at least 50 weeks. Moreover, since the homozygous Gpr124<sup>KO</sup> mice are embryonically lethal, only heterozygous Gpr124<sup>KO</sup> mice can be used for the mating. Such a breeding scheme leads to a frequency of only 25% of homozygous Gpr124<sup>KO</sup>, and a frequency of homozygous Gpr124<sup>KO</sup> with additional heterozygous HIF-1 $\alpha$  deficiency of 1/8 or 1/16, depending on the available genotype of the parents. Since such breeding strategy would jeopardize timely revision, the editor advised us that it was not necessary to perform this genetic intercross.

Together, these experiments provide strong evidence for an involvement of HIF-1 $\alpha$  in regulation of RGC differentiation by oxygen levels.

Minor concerns:

a) The percent of green radial glia (Ngn1 +, Tbr2 -) in the VZ in Fig 1H (~3 to 6% of total) is smaller than the number of yellow basal progenitors (Ngn1 +, Tbr2 +) (~10 to 12% of total), yet in the figure 1F and G there appear to be more green cells than yellow cells. The same is true in figure 6.

**RESPONSE:** Indeed, the proportion of Ngn1-positive BPs is notably larger than that of Ngn1-positive RGs under all experimental conditions tested. However, the signal for Tbr2 immunostaining is much stronger than that for Ngn1 under our conditions, so that the red color of the Tbr2 masks the green Ngn1 staining and many Ngn1+/Tbr2+ cells appear only red or orange at best. If necessary or appropriate, we can include a separate panel in the Appendix, showing only the green Ngn1 signal.

b) Much of the quantitation is normalized to a particular zone of the developing neocortex, and the overall thickness of the neocortex appears to decrease in the Gpr124 KO. The authors should indicate what criteria were used to mark the borders between the VZ, SVZ and IZ/CP in each of the figures.

**RESPONSE:** We have added a section in the supplementary materials and methods (see page 10-11 of the Appendix), explaining our definition criteria for the different cortical zones and methodology of cell quantification.

c) Figure 3B. This data is not very informative. Please remove or include the genes and their expression levels in a supplemental figure.

**RESPONSE:** We have moved this panel to the supplement as new Appendix Figure S3A and supply the list of regulated genes and their expression levels as source data.

d) Figure 2E,F. The increase in yellow (Ki67+, Efu+) cells in the ECcKO is not apparent in the figure.

**RESPONSE:** We have replaced these panels (new Figure 2 H,I) with more representative images.

e) P12, Weigmann reference is missing.

**RESPONSE:** We added this reference to the manuscript.

f) P13, Fig3L should be Fig3K

**RESPONSE:** We have changed this mistake and panel 3K is now 3J (see page 11 of the manuscript text).

g) Figure S5, part F is missing

**RESPONSE:** This figure legend has been moved to Figure EV6J, where it refers to.

h) The methods section is a bit vague. For example, there is no mention of how qPCR was performed.

**RESPONSE:** We have moved the description for qRT-PCR to the Main methods. Details and the codes for the used pre-made TaqMan assays and in-situ probe sequences can be now found in the Appendix on pages 20 and 18, respectively .

**Referee #3**

The study by Lange et al draws the attention to an intriguing temporal and spatial correlation between the ingrowth of blood vessels and the switch of radial glia from expansion to neuronal differentiation in the cerebral cortex. Using a genetic model in which blood vessel formation is hampered selectively in the cortex, they continue to show that impairment of blood vessel formation results in continued expansion of radial glia at the expense of differentiation via basal progenitors. They further reveal that this effect is due to hypoxia and, on a molecular level, the continued expression of HIF1-alpha. Overexpressing HIF1-alpha mimics the effect of impaired blood vessel formation while reduction of HIF1-alpha by genetic deletion of a single allele can partially rescue the effect of impaired blood vessel formation on neurogenesis. Consistent with this model, elevating O<sub>2</sub> levels results in precocious neurogenesis. Finally, the authors provide evidence that glycolysis plays an important regulatory role in keeping radial glial cells expanding and deleting an enzyme regulating glycolysis favors neuronal differentiation. This is a very important study adding to growing body of evidence that the metabolite of cells plays a key role in cell fate regulation. The data presented are intriguing and overall convincing.

**RESPONSE:** Thank you.

However, there are some points that merit amendment.

Major:

1) Page 10: The authors designate Ngn1+/Tbr2- cells as RGCs. In contrast they designate Ngn1+/Tbr2+ cells as BPs. They state that only the former population is reduced in Gpr124 ko mice, while the latter is only insignificantly reduced (Figure 1H). However in Figure 1N, BPs are significantly reduced. This seems first glance contradictory. It might be due to the different parameters analysed but it would therefore greatly help if the authors provided the reader with a scheme depicting the different marker combinations and how these were assigned to distinct progenitor subtypes. Also it should be explained why there is no contradiction between the lack of reduction in Ngn1+/Tbr2+ cells and the reduced numbers of BPs.

**RESPONSE:** First, we have added such a scheme to the manuscript as Appendix Figure S1. Second, we would like to clarify that Fig. 1H (original manuscript, now Fig. 1N) shows the proportion of Ngn1+ RG, and not the total RG population. Since the proportion of neurogenic RG is decreased and the proportion of BPs (the differentiated progeny of RG) is also decreased, it follows that the population of expanding RGs is increased (now shown in Fig. 1O). Third, in the original manuscript, it is indeed correct that the proportion of Ngn1+ BPs is only mildly reduced in Gpr124<sup>KO</sup> mice (Fig. 1H,K of the original manuscript; now Fig. 1N,R), while the total number of BPs is significantly reduced in Gpr124<sup>KO</sup> mice (Fig. 1N of the original manuscript; now Fig. 1K).

For the new panel 1N,K, this apparent discrepancy can be explained as follows: panel K shows the fraction of all BPs (i.e. the entire population; expressed as a percentage of all VZ cells, while panel N shows the fraction of Ngn1+ BPs (percentage of all VZ cells). However, in panel K, the proportion of Ngn1+ BPs is larger in the Gpr124<sup>KO</sup> (38%) than WT (31%) brains, even though this increase does not reach statistical significance; this is shown separately in Fig. S2M. As a result, when showing the fraction of Ngn1+ cells separately in panel N, the difference in this fraction between both genotypes becomes visually smaller and no longer statistically significant. Though the precise reason herefore remains unknown to us, these data may suggest various possible explanations: (i) the regulation of Ngn1 protein stability in the different cell cycle phases is altered in Gpr124<sup>KO</sup> brains; and/or (ii) the Gpr124<sup>KO</sup> BPs have longer G1 phase, in which Ngn1 is expressed (Britz et al, 2006), thereby reducing fraction of Ngn1- BPs in Gpr124<sup>KO</sup> brains.

In the original panel 1K, the difference in the fraction of Tis21+ cells was not statistically significant between the WT and Gpr124<sup>KO</sup> brains (note that this is a separate group of mice as those analyzed for the new panel 1N,K). However, this lack of statistical difference was due to the presence of a high proportion of BPs in one of the Gpr124<sup>KO</sup> brains analyzed. We now remedied this situation by counting more sections from the same brains to have a broader statistical basis. These new results now reveal a significant reduction of the fraction of Tis21+ BPs in the VZ of Gpr124<sup>KO</sup> cortices as compared to WT, in agreement with the data shown for the total BP population in the new Fig. 1K.

2) The term "neurogenic" needs to be defined. Neurogenic can mean making a neuron as an imminent next step (e.g. referring to cells such as Tis21-GFP+ cells that exit cell cycle and differentiate into neurons) or may refer to the majority of RGCs at 13.5 which are virtually all generating neurons earlier or later. As the term "neurogenic" is often used in the second sense but the reviewer has the impression the authors use in the first sense, this should be clarified.

**RESPONSE:** We now clarify in the introduction, on page 3 of the manuscript, our "working definition" of neurogenic, which is commitment towards the neurogenic lineage (generation of a neuron or neuronally-determined BP) in the next division.

3) Does deletion of a HIF1-alpha allele result in a smaller cortex as a consequence of a precocious differentiation at the expense of RGC expansion? Does it affect the generation of specific cortical neuron subtypes?

**RESPONSE:** We have now measured the size of the cortex (see the new Figure 5J-N) and the relative proportion of (Tbr1+ and Ctip2+) early-born deep layer neurons and (Brn2+) late-born upper layer neurons in HIF-1 $\alpha$ <sup>CC+/-</sup> mice at postnatal day 5 (reported in the new Figure EV4H-N). The results indicate small, but consistent changes in cortical size and thickness, but no change in deep layer vs. upper layer neurons in the cortex in the cortex of HIF-1 $\alpha$ <sup>CC+/-</sup> mice.

These data show that precocious differentiation of RGCs reduces cortical size, but that the proportion of deep vs. upper layer neurons is not changed. While we do not know the precise reason of why there is no change in the deep vs. upper layer neurons, we can only speculate about possible hypothetical mechanisms. For instance, if the HIF-1 $\alpha$ <sup>CC+/-</sup> BPs would self-generate themselves more intensely, then they could perhaps still produce sufficient upper layer neurons, which are generated the last and are most prone to be underrepresented upon exhaustion of the precursors. Perhaps, the HIF-1 $\alpha$ <sup>CC+/-</sup> BPs may do so, because BPs have only low levels of hypoxia signaling (Fig. 5U) and therefore might be less affected by the heterozygous loss of HIF-1 $\alpha$ . However, ultimately, the cortex becomes thinner, because the HIF-1 $\alpha$ <sup>CC+/-</sup> RGCs become exhausted.

A common reason why a particular cortical layer is underrepresented in mutant mouse models of microcephaly is apoptosis ([Lizarraga et al, 2010](#); [Pulvers & Huttner, 2009](#)). However, apoptosis was not changed in the cortex of HIF-1 $\alpha$ <sup>CC+/-</sup> mice, at least not at E13.5, providing an additional / alternative explanation of why precocious RGC differentiation in HIF-1 $\alpha$ <sup>CC+/-</sup> mice reduces cortex size, without changing the deep layer versus upper layer neurons. Because of the speculative nature of this hypothetical mechanism, we have not included any such discussion in the revised manuscript.

4) What is the evidence that increasing atmospheric O2 levels to 80% also results in increased O2 tissue levels? The authors should perform measurements for oxidized thiols and pimonidazole levels; also they should make an estimate how much tissue oxygen will raise when switching from 21 to 80 % oxygen levels.

**RESPONSE:** We performed the hyperoxia treatment of prenatal fetuses according to a published protocol ([Ream et al, 2008](#)). In this report, the authors show reduced PIMO staining in the forebrain of hyperoxia-treated embryos at E 12.5.

We have now also measured tissue oxygenation of Gpr124<sup>KO</sup> and control brains upon hyperoxia treatment using oxidized thiols and pimonidazole. The results suggest normalization of tissue oxygenation in Gpr124<sup>KO</sup> brains by the hyperoxia treatment, comparable to that of control embryos (Fig 6B-E; Appendix Figure S5 of the current submission). Oxygenation of control brains did further increase slightly upon hyperoxia treatment, consistent with previous results ([Ream et al, 2008](#)).

In addition, we found that HIF-1 $\alpha$  is destabilized in the Gpr124<sup>KO</sup> cortex upon hyperoxia treatment (Fig 6F,G of the current submission), confirming that raising ambient oxygen concentrations can influence tissue oxygenation in the developing mammalian embryo and rescue the aberrant stabilization of HIF-1 $\alpha$  upon defective blood vessel formation or function.

**Estimation of oxygen levels:** Estimations of the actual level of tissue oxygen changes upon hyperoxia treatments are technically challenging, if not impossible, because of several reasons. First, pimonidazole labeling is known to be not linear over different oxygen tensions, so that only the presence of pimonidazole staining can indicate the presence of hypoxia qualitatively, but not quantitatively. Second, it is also unknown whether the measurement of tissue oxygen levels by oxidized thiols is linear and, if so, over what range. We therefore choose to display the results from the tissue oxygenation stainings for the different genotypes and treatments relative to each other

from stainings in parallel. Measurement of absolute tissue oxygenation is theoretically possible using  $^{19}\text{F}$ -MRI oxymetry (Takeda et al, 2011), but we did not consider this technique, because tissue oxygenation is expected to normalize to room air conditions when mice are taken out of the hyperoxia chamber and brought to the MRI instrument.

5) The authors make a point that interfering with glycolysis by knock down of Pfkfb3 increases neuronal differentiation. This means that when a RGC is impaired in glycolysis, this will provide a signal in the RGC to differentiate. Does blocking glycolysis stimulate OX-Phos?

**RESPONSE:** As requested, we have analyzed mitochondrial oxygen consumption of NSCs in vitro after infection with scr or Pfkfb3 shRNA lentiviruses. Besides OCR coupled to ATP synthesis ( $\text{OCR}_{\text{ATP}}$ ; sensitive to oligomycin), we also measured mitochondrial OCR ( $\text{OCR}_{\text{MITO}}$ ; sensitive to antimycin A). None of these parameters significantly changed upon Pfkfb3 knockdown. We report this findings in Figure 7E and Figure EV5I of the current submission.

6) What is the effect of knocking down Pfkfb3 in Gpr 124 ko mice?

**RESPONSE:** In addition to silencing Pfkfb3 via in-utero electroporation of a shRNA together with HIF-1 $\alpha$  overexpression in wild type brains, it would be interesting to silence Pfkfb3 in Gpr124<sup>KO</sup> brains. However, this experiment is not feasible, since we can electroporate embryos with a high rate of targeting only at E12.5, while Gpr124<sup>KO</sup> brains can only be analyzed until E14.5 due to embryonic lethality. Hence, less than two days are available to express sufficient amounts of the shRNA, to lower Pfkfb3 mRNA levels, to degrade preformed Pfkfb3 protein in order to achieve Pfkfb3 loss-of-function, which then still needs time to induce the change in cell fate. It is very unlikely that 48 hours are a sufficiently long period, therefore precluding us from utilizing this experimental strategy. Another substantial practical problem is the low fraction of homozygous Gpr124<sup>KO</sup> embryos (1 out of 4) per litter, which will necessitate us to perform a huge amount of experiments in order to obtain a sufficient amount of well electroporated Gpr124<sup>KO</sup> embryos – both for scr and (multiple) Pfkfb3 shRNAs likely requiring multiple months, if not more than half a year. However, our experiments of Pfkfb3 knockdown in the presence of HIF-1 $\alpha$  overexpression already provide strong evidence that Pfkfb3 upregulation is required for increased RGC maintenance under these conditions.

7) Is there any phenotype regarding astrogliogenesis in the Gpr124 ko mice?

**RESPONSE:** As mentioned above, Gpr124<sup>KO</sup> mice die around E15.5, thus precluding analysis of gliogenesis in this model.

Minor:

a) The quality of the micrographs in Fig S1A-H does not allow to fully assess the temporo-spatial correlation of vessel ingrowth and neurogenesis, at least on a printed version.

**RESPONSE:** We have resized these figure panels in order to increase visibility and clarity (see Figure EV1A-H).

b) Introduction page 4: referring to "new" neurons during embryonic development is not very meaningful; remove "new"

**RESPONSE:** We have adapted this sentence (see page 4 of the manuscript).

c) The sentence "These RGCs switch from expansion towards differentiation to neurons" sounds blunted and would benefit from further qualification.

**RESPONSE:** We have changed this sentence on page 3 of the manuscript.

d) Results: temporo-spatial relationship "between" (instead of to)...

**RESPONSE:** We have changed this sentence (see page 5 of the manuscript text).

e) Figure 1 A and B are apparently taken at different rostro-caudal levels. Same levels should be shown.

**RESPONSE:** These sections are taken at the same rostro-caudal levels, indicated by the appearance of the hippocampal anlage and the choroid plexus. However, the lateral expansion of the cortex and the degeneration of the ventral telencephalon may cause the impression that the sections are from different rostro-caudal levels.

f) Discussion: The sentence "virtually all cortical NPCs expanded ... and switched..." sounds too strong; it suggests that after vessel growth there is no more expansion going on.

**RESPONSE:** We have adapted this sentence, emphasizing that vessel ingrowth correlates with the initiation of differentiation, rather than a complete switch of all NSCs.

## References

- Apra J, Prenninger S, Dori M, Ghosh T, Monasor LS, Wessendorf E, Zocher S, Massalini S, Alexopoulou D, Lesche M, Dahl A, Groszer M, Hiller M, Calegari F (2013) Transcriptome sequencing during mouse brain development identifies long non-coding RNAs functionally involved in neurogenic commitment. *The EMBO journal* **32**: 3145-3160
- Arai Y, Pulvers JN, Haffner C, Schilling B, Nusslein I, Calegari F, Huttner WB (2011) Neural stem and progenitor cells shorten S-phase on commitment to neuron production. *Nature communications* **2**: 154
- Britz O, Mattar P, Nguyen L, Langevin LM, Zimmer C, Alam S, Guillemot F, Schuurmans C (2006) A role for proneural genes in the maturation of cortical progenitor cells. *Cerebral cortex* **16 Suppl 1**: i138-151
- Cullen M, Elzarrad MK, Seaman S, Zudaire E, Stevens J, Yang MY, Li X, Chaudhary A, Xu L, Hilton MB, Logsdon D, Hsiao E, Stein EV, Cuttitta F, Haines DC, Nagashima K, Tessarollo L, St Croix B (2011) GPR124, an orphan G protein-coupled receptor, is required for CNS-specific vascularization and establishment of the blood-brain barrier. *Proc Natl Acad Sci U S A* **108**: 5759-5764
- Dai Q, Andreu-Agullo C, Insolera R, Wong LC, Shi SH, Lai EC (2013) BEND6 is a nuclear antagonist of Notch signaling during self-renewal of neural stem cells. *Development* **140**: 1892-1902
- Englund C, Fink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T, Hevner RF (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **25**: 247-251
- Fasano CA, Phoenix TN, Kokovay E, Lowry N, Elkabetz Y, Dimos JT, Lemischka IR, Studer L, Temple S (2009) Bmi-1 cooperates with Foxg1 to maintain neural stem cell self-renewal in the forebrain. *Genes & development* **23**: 561-574
- Glickstein SB, Monaghan JA, Koeller HB, Jones TK, Ross ME (2009) Cyclin D2 is critical for intermediate progenitor cell proliferation in the embryonic cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**: 9614-9624
- Hasan SM, Sheen AD, Power AM, Langevin LM, Xiong J, Furlong M, Day K, Schuurmans C, Opferman JT, Vanderluit JL (2013) Mcl1 regulates the terminal mitosis of neural precursor cells in the mammalian brain through p27Kip1. *Development* **140**: 3118-3127
- Hutton SR, Pevny LH (2011) SOX2 expression levels distinguish between neural progenitor populations of the developing dorsal telencephalon. *Developmental biology* **352**: 40-47



- Kuhnert F, Mancuso MR, Shamloo A, Wang HT, Choksi V, Florek M, Su H, Fruttiger M, Young WL, Heilshorn SC, Kuo CJ (2010) Essential regulation of CNS angiogenesis by the orphan G protein-coupled receptor GPR124. *Science* **330**: 985-989
- Lange C, Huttner WB, Calegari F (2009) Cdk4/cyclinD1 overexpression in neural stem cells shortens G1, delays neurogenesis, and promotes the generation and expansion of basal progenitors. *Cell stem cell* **5**: 320-331
- Lizarraga SB, Margossian SP, Harris MH, Campagna DR, Han AP, Blevins S, Mudbhary R, Barker JE, Walsh CA, Fleming MD (2010) Cdk5rap2 regulates centrosome function and chromosome segregation in neuronal progenitors. *Development* **137**: 1907-1917
- Molyneaux BJ, Arlotta P, Menezes JR, Macklis JD (2007) Neuronal subtype specification in the cerebral cortex. *Nature reviews Neuroscience* **8**: 427-437
- Pulvers JN, Huttner WB (2009) Brca1 is required for embryonic development of the mouse cerebral cortex to normal size by preventing apoptosis of early neural progenitors. *Development* **136**: 1859-1868
- Ream M, Ray AM, Chandra R, Chikaraishi DM (2008) Early fetal hypoxia leads to growth restriction and myocardial thinning. *American journal of physiology Regulatory, integrative and comparative physiology* **295**: R583-595
- Sun G, Ye P, Murai K, Lang MF, Li S, Zhang H, Li W, Fu C, Yin J, Wang A, Ma X, Shi Y (2011) miR-137 forms a regulatory loop with nuclear receptor TLX and LSD1 in neural stem cells. *Nature communications* **2**: 529
- Takeda Y, Costa S, Delamarre E, Roncal C, Leite de Oliveira R, Squadrito ML, Finisguerra V, Deschoemaeker S, Bruyere F, Wenes M, Hamm A, Serneels J, Magat J, Bhattacharyya T, Anisimov A, Jordan BF, Alitalo K, Maxwell P, Gallez B, Zhuang ZW, Saito Y, Simons M, De Palma M, Mazzone M (2011) Macrophage skewing by Phd2 haplodeficiency prevents ischaemia by inducing arteriogenesis. *Nature* **479**: 122-126
- Wilsch-Brauninger M, Peters J, Paridaen JT, Huttner WB (2012) Basolateral rather than apical primary cilia on neuroepithelial cells committed to delamination. *Development* **139**: 95-105
- Yabut O, Domogauer J, D'Arcangelo G (2010) Dyrk1A overexpression inhibits proliferation and induces premature neuronal differentiation of neural progenitor cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30**: 4004-4014

2nd Editorial Decision

14 December 2015

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

-----  
REFeree REPORTS

Referee #1:

The authors have addressed all the concerns and nicely revised the manuscript. The results are well presented and sustain interesting conclusions in a relevant topic.

Referee #2:

The manuscript is much improved over the original version and in this reviewers opinion is now in a form suitable for publication in EMBO.

Referee #3:

The authors addressed my remaining concerns and questions satisfactorily.