nature portfolio

# Peer Review File

## Conserved transcriptional regulation by BRN1 and BRN2 in neocortical progenitors drives mammalian neural specification and neocortical expansion

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**REVIEWER COMMENTS**

Reviewer #1 (Remarks to the Author):

Barão and colleagues report evidence for a general role of transcription factors (TFs) POU3F3 (BRN1) and POU3F2 (BRN2) in cortical development across the mammalian clade. The authors conducted a thorough investigation of Brn1/2 loss-of-function in mouse, concluding that deletion of both TFs leads to severe disruption to cortical architecture which comes about due to progenitors precociously exit the cell cycle, reduction of indirect neurogenesis stemming from aberrant NOTCH signaling, and deregulation of several microcephaly-associated genes. Next, the authors knocked down expression of Brn1/2 in ferrets, which generates a phenotype similar to that seen in mouse. Finally, the authors re-analyze pre-existing scRNAseq data from BRN2 knockout macaques. Using the same pipeline as their mouse data, the authors provide evidence for a reduction of indirect neurogenesis and downregulation of multiple microcephaly-associated genes. Thus, the study suggests that BRN2 has similar function in macaques. Altogether, Barão and colleagues conclude that Brn1/2 function is conserved across lissencephalic and gyrencephalic mammalian species.
This study presents a coherent argument that Brn1/2 functions are conserved across species, and constitutes a novel discovery. The data to support the hypothesis are clear and the methodology is largely appropriate. However, there remain a handful of concerns to be addressed.

Major concerns:

1. The title is quite vague, "Transcriptional Control of Neocortical Size and Microcephaly", and sounds like the authors have solved it all, when in reality this is a very focused study. Please revise the title, tone down overall claims and state explicitly that BRN1/2 transcription factors were studied.
2. Lines 51-53: "Our findings thus establish a mechanistic link between BRN1/2 and genes linked to microcephaly and demonstrate that BRN1/2 are central regulators of gene expression programs in neocortical progenitors critical to determine brain size during evolution." The authors claim that they have discovered the mechanism leading to the downregulation of microcephaly-associated genes. While they have correlative evidence for this, they do not explore the mechanism per se. I suggest reformulating the claim, or presenting evidence that microcephaly-associated genes are direct regulatory targets of Brn1/2.
3. Lines 107-110: "we observed BRN2 expression in murine cortical progenitors already at E11.5 (Extended Data Fig. 1e,f) suggesting that BRN1/2 affects progenitor behavior in mice at earlier time points than previously thought. To test this hypothesis, we inactivated Brn1/2 using timed in utero electroporation (IUE) of pCAG-CRE in Brn1fl/fl;Brn2fl/fl mice at E12.5 and E14.5 (Extended Data Fig. 4a)." The experiments conducted in extended data figure 4 seem inappropriate to test the requirement of Brn1/2 at that time point as they are using progressive interval sampling, which is fine for understanding the cumulative effect of LOF. Instead longitudinal sampling experiments should be performed. One suggestion would be to perform IUE at E11/12/13 and analyze the tissue at E13/14/15, or a similar experimental design. Such an experiment would truly clarify whether Brn1/2 are functionally relevant in that specified window.

Minor concerns/clarifications:

1. Lines 43-45: "Here we show that the transcription factors BRN1 (POU3F3) and BRN2 (POU3F2) act as master regulators of the transcriptional programs in progenitors linked to neuronal specification and neocortex expansion." The claim here is quite strong, and I believe it arises from the data in figure 1 G-I and extended data figure 5. While this argument can be made, the data in figure 1 are not the correct data to show in the main figure as it is rather unclear. Showing deregulation of broad pathways (i.e. grouping deregulated genes into their pathways and showing this) might clarify for readers.

2. Please correct the use of mitosis in lines 58 (mitoses) and line 59 (mitosis).
3. Correct duplicate "the number of glial cells was dramatically increased in in Brn1/2-cKO mice".
4. Which factor plays a more critical role in the lack of ULNs: a depleted progenitor pool or the restriction in fate potential? In other words, if you could keep a larger fraction of cycling progenitors, would a large enough fraction of them progress in their lineage to generate ULNs?
5. This is not a requirement, just would like some clarification. While the RNAscope data for the ferret sufficiently supports the claims and largely mirrors the mouse scRNAseq data, why not perform a sequencing experiment to gather further insights? There could be important ferret-specific transcriptional deregulation you are missing by basing your RNAscope experiment so heavily on the outcome of the mouse scRNAseq data.
6. Figure 3 is the most problematic in terms of information flow. The ferret data might make more sense elsewhere, like figure 4, it feels out of place.
7. Related to the above point, the figures need to be restructured. While having four main figures with ten supplemental figures may be appropriate for certain publication formats, I strongly suggest to show more of the supplemental data (especially data in extended data figures 1-4, but across all data sets in all extended data figures) in main figures, i.e. expanding the number of main figures.
8. The discussion is very succinct and I suggest to expand the discussion to explain and integrate the findings in broader context.

Reviewer #2 (Remarks to the Author):

In the current study, S. Barão and colleagues study the role of Brn1 and 2 during mammalian corticogenesis and the link of these genes with microcephaly. Using single-cell transcriptomics and in utero electroporations, they focus on the role of these two transcription factors in the maintenance of cortical progenitor identity and cycling properties, in relationship with the sequential generation of neuronal types. They show that the function of these genes is maintained across lisencephalic (mouse) and gyrencephalic (ferret and non-human primates) mammalian species, suggesting a strong evolutionary conservation.

This is a well-performed and interesting study; the data are well-presented, and I have no major reservations. Below are some points requiring clarification:

Extended data figure 3 depicts a clear increase in glial population production in Brn1/2 mutants, and from the single-cell transcriptomics analysis, it is not clear if the progenitor pool is depleted, as cell-type proportions appear to be conserved between control and cKO animals (Extended data figure 5D). These findings point towards a possible fate switch of progenitors towards glial production, leading to the absence of superficial layer neurons. To address this possibility, in murine (and if possible, in ferret) in utero electroporations, the authors should quantify the amount of RFP+ cells that express not only superficial or deep layer markers, but also glial ones.

Relating to the increase of deep layer marker expressing neurons (Extended data figure 1e-h), does this reflect a faster cell cycle during early corticogenesis stages or does this relate to an extension of deep layer neuron production in time?

Regarding the cell cycle dynamics of Brn1/2 cKO progenitors, it is striking to see that they proliferate less and exit the cycle faster, but their proportions between E12.5 and E14.5 in relation to all the sampled cells remains constant. Can the authors measure the cell cycle length of both APs and BPs in the two conditions in order to clarify these statements, or at least comment on this?

Brn1/2 cKO animals present a large heterotopia formed by deep-layer neurons; a better characterization of the birthdate (EdU pulse) of these misplaced neurons would be of interest. It would help to understand if only late-born neurons migration is affected or if it is already the case early on.

Regarding the corpus callosum agenesis, it is known that deep layer neurons also project contralaterally. Can the authors speculate on why these deep layer neurons aren't able to project to their targets. Does this relate to a defect in neuronal projections or midline fusion?

The pseudotime analysis, using Monocle3 seems to generate some level of overlap between E12.5 and E14.5 APs (Extended figure 5e). Does PCA1 improve the separation of the two populations? PCA2 seems to relate to something else than exclusively time. Also, relating to transcriptional waves, only wave 4 and 5 seem to be different between conditions. Wave 6, most likely enriched in late progenitors, seems to be conserved. What is the gene composition of each of these waves and what are their function? Could this give hints on the molecular changes that progenitors undergo through time?

In figure 2 C, it is not correct to state that Neurod2+, Tbr2+, Mki67- cells are directly generated neurons. They could be G0/G1 BPs or newborn neurons from cycling BPs. Only the fact that there are cycling less than BPs can be stated from this graphical representation.

In order to better characterize DL-produced neurons in Brn1/2 cKO animals, it would be great to perform a DEG analysis between only DL control and cKO neurons.

Reviewer #3 (Remarks to the Author):

This manuscript by Soraia Barão and colleagues showed that BRN1/BRN2 act as master regulators of the transcriptional programs in progenitors that control their proliferative capacity and switch from direct to indirect neurogenesis. Comparative studies in genetically modified mice, ferrets and macaques indicated that BRN1/2 acted in concert with NOTCH and primary microcephaly genes to regulate progenitor behavior. In addition, this research showed BRN1/2 are central regulators of gene expression programs in neocortical progenitors critical to determine brain size. These findings are important to further understanding on the cellular and molecular mechanism of BRN1/2 in cortical progenitors.

The manuscript contains a large amount of in-depth analysis on regulated mechanisms of cortical progenitors by Brn1/2, some interesting finding that BRN1/2 are required for the expression of microcephaly associated genes. While some of the data are intriguing, there is insufficient evidence for many of the claims that are made in this paper. Please see major points and minor points below

My major concerns
1. Line 89, figure 1a-b, the authors concluded that compared to control littermates, the cortex of Brn1/2-cKO mice at postnatal day (P) 13 was reduced in thickness. Considering the knockout samples will be a little smaller, the authors should provide images of same orientation/direction of the cortical layers for ease of comparison and describe in detail the method of slicing. In addition, The authors should show each cortical marker separately to further analyze which layer was reduced in thickness.

2. It is unclear whether Emx1 expression is correlated with BRN2 and BRN1 loss in Brn1/2-cKO mice. Double staining of Emx1 and BRN2/BRN1 in control and mutant mice is required.

3. The number of glial cells was dramatically increased in in Brn1/2-cKO mice at P13. How Brn1/2 to regulate the glial cell specification? How about oligodendrocytes?
4. In Brn1fl/fl;Brn2 fl/fl mice at E12.5 and E14.5 (Extended Data Fig. 4 and 9, Fig 2h), pCAG-CRE and pCAG-RFP were electroporated into brain cells to identify electroporated cells. How to determine that RFP positive cells were CRE electroporated cells? Similar issues were existed in pCS2-NOTCH1, DLL1 and pCAG-Brn-DBD-EnR.
5. Line 228, The authors concluded that BRN1/2 are required for the expression and function of sets of genes linked to microcephaly by using ASPMKO ferrets. Why don't they directly use BRN1/2 knockout ferrets? The comparison would be more convincing by using BRN1/2 knockout ferrets.
6. The evidences about "BRN1/2 are required to maintain the neuronal progenitor pool through the regulation of microcephaly-associated genes" are relatively uncertain. Lack of evidence to distinguish whether ASPM is a direct target of BRN2 or secondary effect. It is important to test whether overexpressing ASPM in Brn1fl/fl;Brn2 fl/fl mice can or partially rescue BRN2/BRN1 mutant phenotypes. It is also important to confirm the relationship between BRN2/BRN1 and ASPM by some specific assays, such as chip-seq experiment and luciferase assay of BRN2/BRN1.

Minor concerns:

1. In extended data fig.5d, the authors should provide a figure legend to explain what different colors represent.
3. Extended data fig.6g, line138, gene ontology (GO) terms that significantly changed in Brn1/2-cKO neurons included axon guidance. The authors did not indicate whether the change gene is up-regulated or down-regulated
4 The images in Extended data, fig.6f and fig.7g are very blurry. The authors should provide high resolution figures, especially for Satb2, Ezh2 and Ngn2.
5 In Fig. 1I, 1-6, what does 1-6 represent, respectively.
6 Lines, 128-129 "observed in wild-type mice similar gene expression changes along the pseudotime axis as reported". Should add the references? Why just present Hmga2 and Cdon
7 In the Fig.s9b, the Hes1 immunostaining is very unclear. Hes1 should be in the nucleus. Please provide the high-resolution images.

**NCOMMS-23-55896-T_Response to reviewer comments**

We thank the reviewers for their insightful comments and the valuable time they invested in their reviews. We are thrilled to report that the resulting changes and additions made to the revised manuscript have further strengthened our main findings and contributed to a better understanding of the role of BRN1/2 in the neocortical progenitor cells. Below we provide a summary of the main added experiments/analysis as well as a summary of the main figure changes. These summaries are followed by a detailed response to the reviewer comments.

**Summary of main added experiments and analysis:**

**EdU cell-fate analysis:** To complement our IUE experiments and better understand the final cortical layer location and cellular identity of the progeny of embryonic progenitors from control and *Brn1/2-cKO* mice, we analyzed the fate of EdU labelled cells at E12.5 and E14.5 in control and *Brn1/2-cKO* P13 cortical sections. These experiments confirmed the significant reduction of ULNs generated from E12.5 and E14.5 *Brn1/2-cKO* progenitors and the migration defects we have previously observed by IUE and analysis of *Brn1/2-cKO* brains at P13. In addition, these experiments allowed us to conclude that there is a shift from neurogenesis to gliogenesis happening at early embryonic ages in *Brn1/2-cKO* brains.

**IUE experiments at earlier embryonic ages:** To further confirm that BRN1/2 affect progenitor behavior at the earliest stages of neurogenesis, we used CRE to acutely inactivate *Brn1/2* by IUE of $Brn1^{fl/fl};Brn2^{fl/fl}$ mice at E12.5 and analyzed neurogenesis at E14.5. We co-expressed pCAG-RFP to identify electroporated cells. Similar to the results we have previously reported for the IUE experiments done at E14.5, the number of mutant $RFP^+$ cells going through indirect neurogenesis was significantly reduced while the number of mutant $RFP^+/NEUROD2^+$ cells was significantly increased consistent with a higher rate of direct neurogenesis when BRN1/2 was inhibited at E12.5.

**BRN1/2 Chromatin Immunoprecipitation (ChIP-qPCR):** To complement our scRNAseq and ISH-RNAscope analysis we did BRN1/2 Chromatin Immunoprecipitation (ChIP-qPCR) to identify the direct targets of BRN1/2 among the different genes characterized in the previously submitted manuscript. These experiments confirmed that BRN1/2 directly binds to the *Ensembl* predicted regulatory regions of *Ngn2*, *Notch1*, *Dll1* and *Hes1*. BRN1/2 did not bind the *Ezh2* region we tested by qPCR. Although we cannot exclude the possibility of BRN1/2 binding to other regulatory regions of *Ezh2* not tested in these experiments, this observation suggests that BRN1/2 effect on *Ezh2* expression is indirect or perhaps results from the precocious neurogenesis induced by BRN1/2 direct regulation of *Ngn2*. In addition, we have confirmed that BRN1/2 directly binds to the *Ensembl* predicted regulatory regions of certain microcephaly-associated genes such as *Aspm*, *Stil* and *Cep135*. BRN1/2 did not bind to the regulatory regions of the other microcephaly-associated genes we tested by qPCR. Once again, although we cannot exclude the possibility of BRN1/2 binding to other regulatory regions of these genes not tested in these experiments, this observation suggests that BRN1/2 effect on the expression of the other microcephaly-associated genes is indirect or perhaps results from the abnormal centrosome function in *Brn1/2-cKO* progenitors.

**ASPM IUE rescue experiments:** To understand the extent to which *Aspm* contributes to the changes of progenitor behavior in *Brn1/2-cKO* mice, we co-expressed pCAG-CRE to inactivate *Brn1/*2 and pBlue-hASPM to overexpress ASPM by IUE of $Brn1^{fl/fl};Brn2^{fl/fl}$ mice at E14.5 and analyzed neurogenesis at E16.5. In these experiments, ASPM overexpression did not rescue the rate of indirect neurogenesis ($RFP^+/TBR2^+$ cells) in the *Brn1/2-cKO* condition. However, it restored the levels of proliferation/cell cycle exit ($RFP^+/Ki67^+$ cells) and neuronal production ($RFP^+/NEUROD2^+$ cells) in the *Brn1/2-cKO* condition to control levels. These results show that BRN1/2-dependent regulation of *Aspm* expression and function primarily controls the proliferation and cell cycle exit of progenitor cells without major contribution to the levels of indirect neurogenesis that is mainly regulated by the tightly regulated NOTCH signaling in these cells.

**Summary of figure changes:**
The first version of the manuscript had four main figures and ten figures of Extended Data. We have now expanded the number of main figures to five to better accommodate the newly added results and improve the flow of the manuscript's narrative and discussion.

Main Figures:

**Figure 1. BRN1/2 regulate the competence of neocortical progenitors to generate ULNs:** This figure now includes the neuronal fate changes observed at embryonic stages while the cell cycle results were moved to new Fig. 2. New analysis: gene ontologies for the transcriptomic waves; DL and UL signature scores and expression correlations.

**Figure 2. BRN1/2 regulate cell cycle exit:** New figure including cell cycle results and analysis. New analysis: proliferation after 1h EdU incorporation; cell cycle length.

**Figure 3. BRN1/2 regulate the switch from direct to indirect neurogenesis via NOTCH signaling:** Previous Fig. 2. New analysis: BRN1/2 ChIP-qPCR results for *Notch1*, *Dll1* and *Hes1*.

**Figure 4. BRN1/2 are required for the expression of microcephaly-associated genes and maintenance of the neuronal progenitor pool:** Previous Fig. 3. Upon suggestion of the reviewers, the results from *ASPM*KO ferret were moved to Extended Data Fig. 10. New analysis: luciferase assays for *Aspm* trancriptional activity; BRN1/2 ChIP-qPCR results for some microcephaly-associated genes; IUE ASPM rescue experiments.

**Figure 5. BRN1/2 function is conserved across mammalian species:** Previous Fig. 4. This figure remains the same.

Extended Data Figures:

**Extended Data Figure 1. BRN1 and BRN2 expression:** This figure was adapted to include CRE expression at early embryonic ages.

**Extended Data Figure 2. BRN1/2 are essential for proper neuronal specification and circuit development in the neocortex:** This figure remains the same.

**Extended Data Figure 3. Glial cells are increased in *Brn1/2-cKO*:** This figure was adapted to include the new analysis: EdU cell-fate analysis of glia cells in the control and *Brn1/2-cKO* mice at P13; oligodendrocytes numbers at P13 after IUE of $Brn1^{fl/+};Brn2^{fl/+}$ with pCAG-CRE to inactivate *Brn1/2* at E14.5.

**Extended Data Figure 4. BRN1/2 regulate the competence of progenitor cells to generate ULNs:** This figure was adapted to include the new analysis: EdU cell-fate analysis of DL and UL neurons and their distribution in the cortex of *Brn1/2-cKO* mice.

**Extended Data Figure 5. Altered transcriptional programs in cortical progenitors of *Brn1/2-cKO* mice:** This figure remains the same.

**Extended Data Figure 6. Altered transcriptional programs in *Brn1/2-cKO* cortical neurons:** Part of this figure was moved to main Fig. 1. New analysis: axon guidance defects in *Brn1/2-cKO* mice at P0; DE axon guidance associated-genes in control and *Brn1/2-cKO* neurons at E14.5.

**Extended Data Figure 7. Reduction in proliferation rate and precocious cell cycle exit of cortical progenitors in *Brn1/2-cKO* mice:** Part of this figure was moved to main Fig. 2. New analysis: APs and BPs numbers in control and *Brn1/2-cKO* mice at E12.5 and E14.5.

**Extended Data Figure 8. BRN1/2 regulate the balance between direct and indirect neurogenesis affecting the generation of a specific type of BPs:** This figure was adapted to include the new analysis: Inactivation of *Brn1/2* by IUE at E12.5 and characterization of neurogenesis 48h later.

**Extended Data Figure 9. BRN1/2 are essential to maintain NOTCH signaling balanced during neurodevelopment:** This figure remains the same.

**Extended Data Figure 10. BRN1/2 are required for the expression of microcephaly-associated genes and maintenance of the neuronal progenitor pool:** This figure was adapted to include the *ASPM*KO ferret analysis and the quantification of PAX6$^{+}$ progenitors that were part of main Fig. 3 in the previous version of the manuscript. New analysis: BRN1/2 ChIP-qPCR for the remaining microcephaly-associated genes, *Ngn2* and *Ezh2*, and the positive control *Zic1*.

**Response to reviewer comments**

**Reviewer #1 (Remarks to the Author):**

**Barão and colleagues report evidence for a general role of transcription factors (TFs) POU3F3 (BRN1) and POU3F2 (BRN2) in cortical development across the mammalian clade. The authors conducted a thorough investigation of Brn1/2 loss-of-function in mouse, concluding that deletion of both TFs leads to severe disruption to cortical architecture which comes about due to progenitors precociously exit the cell cycle, reduction of indirect neurogenesis stemming from aberrant NOTCH signaling, and deregulation of several microcephaly-associated genes. Next, the authors knocked down expression of Brn1/2 in ferrets, which generates a phenotype similar to that seen in mouse. Finally, the authors re-analyze pre-existing scRNAseq data from BRN2 knockout macaques. Using the same pipeline as their mouse data, the authors provide evidence for a reduction of indirect neurogenesis and downregulation of multiple microcephaly-associated genes. Thus, the study suggests that BRN2 has similar function in macaques. Altogether, Barão and colleagues conclude that Brn1/2 function is conserved across lissencephalic and gyrencephalic mammalian species. This study presents a coherent argument that Brn1/2 functions are conserved across species, and constitutes a novel discovery. The data to support the hypothesis are clear and the methodology is largely appropriate. However, there remain a handful of concerns to be addressed.**

**Major concerns:**

**1. The title is quite vague, "Transcriptional Control of Neocortical Size and Microcephaly", and sounds like the authors have solved it all, when in reality this is a very focused study. Please revise the title, tone down overall claims and state explicitly that BRN1/2 transcription factors were studied.** We have changed the title to: *BRN1/2 Function in Neocortical Size Determination and Microcephaly.*

**2. Lines 51-53: "Our findings thus establish a mechanistic link between BRN1/2 and genes linked to microcephaly and demonstrate that BRN1/2 are central regulators of gene expression programs in neocortical progenitors critical to determine brain size during evolution." The authors claim that they have discovered the mechanism leading to the downregulation of microcephaly-associated genes. While they have correlative evidence for this, they do not explore the mechanism per se. I suggest reformulating the claim, or presenting evidence that microcephaly-associated genes are direct regulatory targets of Brn1/2.** We took multiple approaches to address this concern in the revised manuscript. First, we did luciferase assays to study the effect of BRN1/2 on *Aspm* promoter/enhancer activity. We cloned the *Ensembl* predicted regulatory region of *Aspm* containing POU-domain binding sites in a luciferase reporter vector and tested its transcriptional activity in absence or presence of pCAG-BRN2 and the dominant negative pCAG-*Brn*-DBD-EnR. The co-expression with BRN2 increased *Aspm*-luciferase activity while the co-expression with *Brn*-DBD-EnR inhibited *Aspm*-luciferase activity (Fig. 4b) suggesting BRN1/2 directly regulates the expression of *Aspm*. To confirm these findings and understand if BRN1/2 directly binds *Aspm* (and the other microcephaly-associated genes) we next did BRN1/2 Chromatin Immunoprecipitation (ChIP) and analyzed the expression of the *Ensembl* predicted regulatory regions for the genes of interest in the immunoprecipitated chromatin by qPCR. We now show that BRN1/2 directly bind to different *Aspm* regulatory regions (*Aspm*, *Aspm-2*) and to at least two more microcephaly-associated genes (*Stil* and *Cep135*; Fig. 4c and Extended Data Fig. 10b). Since we used ChIP-qPCR targeting specific small parts of the regulatory regions for the selected genes and not global ChIP-seq, we cannot exclude that BRN1/2 directly regulates other microcephaly-associated genes by binding to other regulatory regions not analyzed here or guarantee that BRN1/2 only binds to the particular fragments we tested. Furthermore, to understand the extent to which *Aspm* contributes to the changes of progenitor behavior in *Brn1/2-cKO* mice, we expressed pCAG-CRE to inactivate *Brn1/2* and overexpress ASPM by IUE of $Brn1^{fl/fl};Brn2^{fl/fl}$ mice at E14.5 and analyzed neurogenesis at E16.5. We show that ASPM can rescue the proliferative capacity of progenitor cells without affecting the levels of indirect neurogenesis (Fig. 4 d-h) suggesting that BRN1/2-dependent regulation of *Aspm* expression and function primarily controls the proliferation and cell cycle exit of progenitor cells without major contribution to the levels of indirect neurogenesis that is mainly regulated by highly balanced levels of NOTCH signaling in these cells.

**3. Lines 107-110: "we observed BRN2 expression in murine cortical progenitors already at E11.5 (Extended Data Fig. 1e,f) suggesting that BRN1/2 affects progenitor behavior in mice at earlier time points than previously thought. To test this hypothesis, we inactivated Brn1/2 using timed in utero electroporation (IUE) of pCAG-CRE in Brn1fl/fl;Brn2fl/fl mice at E12.5 and E14.5 (Extended Data Fig. 4a)." The experiments conducted in extended data figure 4 seem inappropriate to test the requirement of Brn1/2 at that time point as they are using progressive interval sampling, which is fine for understanding the cumulative effect of LOF. Instead longitudinal sampling experiments should be performed. One suggestion would be to perform IUE at E11/12/13 and analyze the tissue at E13/14/15, or a similar experimental design. Such an experiment would truly clarify whether Brn1/2 are functionally relevant in that specified window.** To address this concern, we acutely inactivated *Brn1/2* by IUE of *Brn1*$^{fl/fl}$;*Brn2*$^{fl/fl}$ mice at E12.5 using CRE and analyzed neurogenesis at E14.5. We co-expressed pCAG-RFP to identify electroporated cells. Similar to the E14.5 IUE results reported in the previous version of the manuscript (and also included in the revised manuscript), at this early embryonic age the number of mutant RFP$^{+}$ cells going through indirect neurogenesis was significantly reduced, while the number of mutant RFP$^{+}$/NEUROD2$^{+}$ cells was significantly increased consistent with a higher rate of direct neurogenesis (Extended Data Fig. 8b,c). These results are consistent with the expression profile of BRN1/2 at the beginning of embryonic neurogenesis and with the additional evidence we have provided for a crucial function of BRN1/2 in progenitor's behavior during early developmental stages.

**Minor concerns/clarifications:**

**1. Lines 43-45: "Here we show that the transcription factors BRN1 (POU3F3) and BRN2 (POU3F2) act as master regulators of the transcriptional programs in progenitors linked to neuronal specification and neocortex expansion." The claim here is quite strong, and I believe it arises from the data in figure 1 G-I and extended data figure 5. While this argument can be made, the data in figure 1 are not the correct data to show in the main figure as it is rather unclear. Showing deregulation of broad pathways (i.e. grouping deregulated genes into their pathways and showing this) might clarify for readers**. Lines 43-45 are part of the summary and refer to our global findings and not particularly to the results presented in Fig. 1 and Extended Data Fig. 5. To clarify it for the readers we have adapted the text and figure legend of Fig. 1g-j and added gene ontology analysis for the transcriptional waves analyzed in Fig. 1i (Fig. 1j). Across the manuscript we have shown that BRN1/2 regulate different transcriptomic programs important for cell cycle, direct and indirect neurogenesis and centrosome function. Specifically, we have shown that BRN1/2 directly regulate the expression of NOTCH signaling components *Notch1*, *Dll1* and *Hes1* that are essential for the balance of direct and indirect neurogenesis and proper neuronal specification of in the neocortex. In addition, we have shown that BRN1/2 directly regulate the expression of *Aspm*, *Stil* and *Cep135*, microcephaly-associated genes that are essential for proper centriole duplication and centrosome function and intrinsically involved in the maintenance of normal numbers of neurogenic progenitors and ultimately appropriately brain size in mammals.

**2. Please correct the use of mitosis in lines 58 (mitoses) and line 59 (mitosis).** It is now correct.

**3. Correct duplicate "the number of glial cells was dramatically increased in in Brn1/2-cKO mic**e". It is now correct.

**4. Which factor plays a more critical role in the lack of ULNs: a depleted progenitor pool or the restriction in fate potential? In other words, if you could keep a larger fraction of cycling progenitors, would a large enough fraction of them progress in their lineage to generate ULNs?** Since 1) the transcriptional profiles of the progenitors are significantly altered resembling, at least in some aspects, a more immature state; 2) the levels of indirect neurogenesis are reduced in the mutant progenitors, and 3) the pool of CUX2$^{+}$ early generated BPs that is fated to generate ULNs is depleted in *Brn1/2-cKO* mice, we predict that even if a larger fraction of cycling progenitors was available, the levels of ULNs would still be reduced. Perhaps there would be more neurogenic progenitors available, a less obvious reduction of cortical size and the cortex would be populated by an even higher number of DLNs.

**5. This is not a requirement, just would like some clarification. While the RNAscope data for the ferret sufficiently supports the claims and largely mirrors the mouse scRNAseq data, why not perform a sequencing experiment to gather further insights? There could be important ferret-specific transcriptional deregulation you are missing by basing your RNAscope experiment so heavily on the outcome of the mouse scRNAseq data.** Although we fully agree that a sequencing experiment in BRN1/2 mutant ferret would help further understand potential BRN1/2 species-specific functions or BRN1/2 role in outer radial glia cells which are very limited in numbers in mice and abundant in ferrets, we do think that the ferret histological and specially the functional results after BRN1/2 inhibition by IUE we present here and their correlation with the mouse and monkey scRNAseq analysis strongly support the findings of the proposed manuscript. In addition, inclusion of yet another data set would further increase the size of an already lengthy manuscript that already consists of 15 Figures.

**6. Figure 3 is the most problematic in terms of information flow. The ferret data might make more sense elsewhere, like figure 4, it feels out of place**. To address this concern and help the narrative of the manuscript, we have changed the text and transferred the ASPMKO ferret results to Extended Data Figure 10.

**7. Related to the above point, the figures need to be restructured. While having four main figures with ten supplemental figures may be appropriate for certain publication formats, I strongly suggest to show more of the supplemental data (especially data in extended data figures 1-4, but across all data sets in all extended data figures) in main figures, i.e. expanding the number of main figures.** To address this concern and help the narrative of the manuscript, we have expanded the number of main figures to five and incorporated several panels from previous Extended Data figures in the new main figures. We were particularly reluctant to include Extended Data Figures 1-4 in the main manuscript because some characterization of the cortex of mice with null mutations in *Brn1/2* had been published previously. Unlike our conditional knock-out mice, the previously published mice die at birth and cannot as fully characterize the neocortex as we did, but presenting the data might dilute the major novel findings of our manuscript that are presented in the main figures. In addition, we tried to stay within the size limits for Nat Commun.

**8. The discussion is very succinct and I suggest to expand the discussion to explain and integrate the findings in broader context.** The discussion is now expanded to better explain and integrate our findings. We also tried to strike a balance with journal requirements. We present a large amount of data that requires substantial explanatory text. The manuscript is thus very lengthy, and we made efforts to focus in the discussion on essential points to stay within page limits.

**Reviewer #2 (Remarks to the Author):**

**In the current study, S. Barão and colleagues study the role of Brn1 and 2 during mammalian corticogenesis and the link of these genes with microcephaly. Using single-cell transcriptomics and in utero electroporations, they focus on the role of these two transcription factors in the maintenance of cortical progenitor identity and cycling properties, in relationship with the sequential generation of neuronal types. They show that the function of these genes is maintained across lisencephalic (mouse) and gyrencephalic (ferret and non-human primates) mammalian species, suggesting a strong evolutionary conservation. This is a well-performed and interesting study; the data are well-presented, and I have no major reservations. Below are some points requiring clarification:**

**Extended data figure 3 depicts a clear increase in glial population production in Brn1/2 mutants, and from the single-cell transcriptomics analysis, it is not clear if the progenitor pool is depleted, as cell-type proportions appear to be conserved between control and cKO animals (Extended data figure 5D). These findings point towards a possible fate switch of progenitors towards glial production, leading to the absence of superficial layer neurons. To address this possibility, in murine (and if possible, in ferret) in utero electroporations, the authors should quantify the amount of RFP+ cells that express not only superficial or deep layer markers, but also glial ones.** We took multiple approaches to address this concern in the revised

manuscript. First, to understand if there is a precocious shift from neurogenesis to gliogenesis in *Brn1/2-cKO* mice, we injected EdU into pregnant mice at E12.5 and E14.5 and followed the cell-fate of the labeled progenitors by analyzing the co-labeling of $EdU^+$ cells with the glia markers SOX9 and OLIG2 at P13 (Extended Data Fig. 3b). There is a significant increase of $EdU^+SOX9^+$ and $EdU^+OLIG2^+$ cells in *Brn1/2-cKO* mice at P13 (Extended Data Fig. 3c-f) indicating that precocious gliogenesis is happening in *Brn1/2-cKO* mice during early embryonic development. Second, we have confirmed these results by IUE where we expressed pCAG-CRE to inactivate *Brn1/2* in $Brn1^{fl/+};Brn2^{fl/+}$ mice at E14.5 and analyzed $GFP^+OLIG2^+$ cells at P13 (Extended Data Fig 3g-i). Due to the transient DNA expression of pCAG-RFP in our classical IUE setup, the $RFP^+$ cells labeled primarily neurons at P13 while $GFP^+$ cells in control and mutant littermates from heterozygous crossings where GFP expression is induced by CRE IUE allowed us to label neurons and glia cells. The shift from neurogenesis to gliogenesis was already described for the ferret in the previous version of the manuscript (previous Fig. 4h now Fig. 5h) where we have shown an increase of $RFP^+/OLIG2^+$ cells and total $OLIG2^+$ cells in the cortex of ferrets expressing *Brn*-DBD-EnR. We have now added extra text to highlight these observations more clearly.

**Relating to the increase of deep layer marker expressing neurons (Extended data figure 1e-h), does this reflect a faster cell cycle during early corticogenesis stages or does this relate to an extension of deep layer neuron production in time?** Our results suggest that it results from a combination of three interconnected aspects: 1) a change in the identity of the progenitors (the progenitors that are available at E14.5 are transcriptionally more similar to an E12.5 progenitor and the pool of $CUX2^+$ early generated BPs that is fated to generate ULNs is depleted in *Brn1/2-cKO* mice); 2) a faster cell cycle exit since early development which doesn't allow the progenitors to acquire a late-identity and therefore the neurogenic progenitors available generate DLNs; 3) the reduction of indirect neurogenesis levels which favors the production of DLNs. We have revised the text to clearly describe this.

**Regarding the cell cycle dynamics of Brn1/2 cKO progenitors, it is striking to see that they proliferate less and exit the cycle faster, but their proportions between E12.5 and E14.5 in relation to all the sampled cells remains constant. Can the authors measure the cell cycle length of both APs and BPs in the two conditions in order to clarify these statements, or at least comment on this?** Although due to sample variability we could not observe a significant change in the numbers of progenitors from a simple t-test analysis of the scRNAseq results, we observed a significant reduction of $PAX6^+$ cells overtime when we analyzed PAX6 immunolabeling in *Brn1/2-cKO* brains (Extended Data Fig. 10k). We have also observed a reduction in the numbers of $TBR2^+$ cells starting at E14.5 (Extended Data Fig. 7a). To complement the new cell cycle figure (Fig. 2) and address this concern from Reviewer #2 we added these immunolabeling and quantification results for $PAX6^+$ and $TBR2^+$ cells in control and *Brn1/2-cKO* brains at E12.5 and E14.5 to Extended Data Fig. 7a. In addition, to understand how BRN/2 affect proliferation and cell cycle length, we injected EdU into pregnant mice at E12.5 and E14.5 and analyzed EdU incorporation into DNA 1h later (Fig. 2c). By quantifying the percentage of $Ki67^+$ cells that have incorporated EdU we showed that cell cycle length was unaltered in *Brn1/2-cKO* mice at E12.5 and E14.5 (Fig. 2d,e). This observation further supports the model where the *Brn1/2-cKO* progenitors are precociously being consumed instead of re-entering cell cycle leading to the observed progressive reduction in the number of cells actively dividing and the overall number of progenitors.

**Brn1/2 cKO animals present a large heterotopia formed by deep-layer neurons; a better characterization of the birthdate (EdU pulse) of these misplaced neurons would be of interest. It would help to understand if only late-born neurons migration is affected or if it is already the case early on.** To address this point, we have now included EdU cell-fate analysis in the revised manuscript Extended Data Fig. 4h-n. We injected EdU into pregnant mice at E12.5 and E14.5 and followed the cell-fate of the labeled progenitors by analyzing the co-labeling of $EdU^+$ cells with DL and UL markers at P13 (Extended Data Fig. 4h). Similar to the IUE results presented in Extended Data Fig. 4a-g, there was a reduction in the number of $EdU^+$ cells expressing UL markers with a concomitant increase in the number of $EdU^+$ cells expressing DL markers in *Brn1/2-cKO* brains (Extended Data Fig. 4i,j,l and n). Interestingly, the $EdU^+$ cells labeled at E12.5 and E14.5 lose their preference for DLs or ULs, respectively and present a broader distribution in the cortex of *Brn1/2-cKO* mice at P13 (Extended Data Fig. 4i,j,k

and m). Together with the IUE analysis, these results suggest that the migration of *Brn1/2-cKO* neurons is affected at all stages and that neurons born at E12.5 are also part of the heterotopias present in *Brn1/2-cKO* mice.

**Regarding the corpus callosum agenesis, it is known that deep layer neurons also project contralaterally. Can the authors speculate on why these deep layer neurons aren't able to project to their targets. Does this relate to a defect in neuronal projections or midline fusion?** To address this comment, the text was adapted to include "The increase in DL neurons (DLNs) should have preserved at least some of the DL callosal projections but instead we observed an abnormal ventral misrouting of $L1^+$ projections in *Brn1/2-cKO* mice (Extended Data Fig. 2b - open arrowhead), suggesting that axon guidance is affected in these neurons." and the legend of Extended Data Fig. 2 was adapted to include "Open arrowhead shows the abnormal ventral misrouting of $L1^+$ projections in *Brn1/2-cKO* mice.". Additionally, we have included midline analysis of control and *Brn1/2-cKO* mice at P0 (Extended Data Fig. 6d). Pioneer neurons and glia guidepost cells were present in *Brn1/2-cKO* mice at P0 suggesting that the axonal projection defects observed in the mutants are primarily related to the abnormal expression of axon guidance-associated genes in these neurons at E14.5 (Extended Data Fig. 6b,e; SI_2). To further support this hypothesis, we have included a more comprehensive list of DE axon guidance-associated genes as part of Extended Data Fig. 6 (panel e).

**The pseudotime analysis, using Monocle3 seems to generate some level of overlap between E12.5 and E14.5 APs (Extended figure 5e). Does PCA1 improve the separation of the two populations? PCA2 seems to relate to something else than exclusively time. Also, relating to transcriptional waves, only wave 4 and 5 seem to be different between conditions. Wave 6, most likely enriched in late progenitors, seems to be conserved. What is the gene composition of each of these waves and what are their function? Could this give hints on the molecular changes that progenitors undergo through time?** For the pseudotime analysis we used our python script as described in the GitHub link provided. PCA1 shows a slight correlation with the developmental time but doesn't help with the separation of the two time points. We hypothesized that PCA2 mostly resembles cellular maturity and PCA2 should contain information about both the developmental time point and cellular state. However, this hypothesis is largely based on the correlation between the principal components and the metadata we have. Therefore, it's also possible that PCA2 relates to other biological information, which we couldn't tell from the current metadata, contributing to the partial overlap between E12.5 and E14.5 APs.

To address the second part of this question and better represent the gene composition of each of the transcriptomic waves represented in Fig. 1i, we have adapted the text and the figure legend for Fig. 1g-h and have included the gene ontology analysis for the waves (Fig. 1j). Additionally, Supplementary Information_1 (SI_1) includes the gene lists of each wave and highlights the genes that are more significantly altered in *Brn1/2-cKO* mice per wave. Although visually wave 4 and 5 are the ones where the changes are more obvious, several genes are dramatically changed in all the waves. The most obvious change we can highlight from this temporal transcriptomic analysis is that there are major transcriptional changes across all the waves in *Brn1/2-cKO* progenitors and therefore the temporal pattern is lost. We cannot bluntly say that these progenitors are more or less mature but rather that they are transcriptionally abnormal. One of the most obvious changes from wave 1-3 is that a lot of the genes that are expressed at high levels in control at E12.5 and should rapidly go down as embryonic ages progress are expressed at even higher levels in *Brn1/2-cKO* progenitors or remain at a constant level instead of going up or down (e.g. *Hmag2*, *Cdon*, *Top2a*, *Wnt5a*, *Foxo1*, *Insm1*, *Zbtb20*, *Kif11*…). Although there are several other molecular pathways altered in *Brn1/2-cKO* progenitors and we try to tackle some of them and their cellular a functional relevance in the subsequent sections of the manuscript, this observation suggests that *Brn1/2-cKO* progenitors are, at least in some aspect, transcriptionally more "intrinsic"[27] or closely related to a younger progenitor and therefore more prone to generate DLNs at E14.5 than ULNs.

**In figure 2 C, it is not correct to state that Neurod2+, Tbr2+, Mki67- cells are directly generated neurons. They could be G0/G1 BPs or newborn neurons from cycling BPs. Only the fact that there are cycling less than BPs can be stated from this graphical representation.** This classification is based on classical pseudotime analysis where the TRIcycle scores combined with different marker genes are used to infer trajectories as described by Moreau et al.[28]. However, we do agree with the reviewer's comment that a less stringent statement should be

made, and to address this concern we have adapted the text to include a more detailed description of these group of cells. In line with this, when we refer to the groups represented on Fig. 3c (previous Fig. 2c) throughout the manuscript we always highlight the decrease in indirect neurogenesis instead of stating any strong conclusions on direct neurogenesis from this representation. Nonetheless, we still consider that, in similarity to what is classically used to analyze indirect neurogenesis in brain sections by immunostaining, using the proportion of diving BPs (*Neurod2*$^{+}$;*Tbr2*$^{+}$;*MKi67*$^{-}$) and non-dividing BPs (*Neurod2*$^{+}$;*Tbr2*$^{+}$;*MKi67*$^{-}$) allows us to predict the levels of indirect neurogenesis versus direct neurogenesis among the tested groups. Therefore, we kept the classification presented in Fig. 2c and revised its description in the main text accordingly with these concerns and considerations. The altered proportions we got from this initial scRNAseq analysis and classification were further validated by three independent approaches represented in Fig. 3 and Extended Data Fig. 8.

**In order to better characterize DL-produced neurons in Brn1/2 cKO animals, it would be great to perform a DEG analysis between only DL control and cKO neurons.** To address this point, we did DEG analysis of *Brn1/2-cKO* total neurons compared to control DLNs and have included these tables as part of the supplemental information file (SI_7 and SI_8). As expected from our histological analysis of *Brn1/2-cKO* that reveal an abnormal brain with a large heterotopia and highly defasciculated neuronal projections, the *Brn1/2-cKO* neurons still present transcriptional differences when compared to control DLNs. Nonetheless, when we analyzed the signature score of the different neuronal populations, we observed that the average DL and UL signature score for *Brn1/2-cKO* total neurons was closely related to the control DLNs signature score (Fig. 1o) and their gene expression strongly correlated with the DL marker gene expression of control DLNs at E12.5 and E14.5 (Fig. 1p). These results indicate that, although the *Brn1/2-cKO* neurons are still transcriptionally abnormal, the genes defining their identity are more closely related with the DL signature of control DLNs.

**Reviewer #3 (Remarks to the Author):**

**This manuscript by Soraia Barão and colleagues showed that BRN1/BRN2 act as master regulators of the transcriptional programs in progenitors that control their proliferative capacity and switch from direct to indirect neurogenesis. Comparative studies in genetically modified mice, ferrets and macaques indicated that BRN1/2 acted in concert with NOTCH and primary microcephaly genes to regulate progenitor behavior. In addition, this research showed BRN1/2 are central regulators of gene expression programs in neocortical progenitors critical to determine brain size. These findings are important to further understanding on the cellular and molecular mechanism of BRN1/2 in cortical progenitors.**

**The manuscript contains a large amount of in-depth analysis on regulated mechanisms of cortical progenitors by Brn1/2, some interesting finding that BRN1/2 are required for the expression of microcephaly associated genes. While some of the data are intriguing, there is insufficient evidence for many of the claims that are made in this paper. Please see major points and minor points below.**

**My major concerns:**
**1. Line 89, figure 1a-b, the authors concluded that compared to control littermates, the cortex of Brn1/2-cKO mice at postnatal day (P) 13 was reduced in thickness. Considering the knockout samples will be a little smaller, the authors should provide images of same orientation/direction of the cortical layers for ease of comparison and describe in detail the method of slicing. In addition, The authors should show each cortical marker separately to further analyze which layer was reduced in thickness.** To address this concern, the materials and methods were adapted to include a more detailed description of how the cortical thickness was determined. Briefly, P13 postnatal brains were embedded in 3% low melting point agarose in PBS and sectioned coronally at 60 µm with a vibrating microtome. The sections were sequentially collected over 10 wells and therefore each well had a full representation of the brain. For the quantifications, three equivalent midbrain sections were used per mice per genotype. The midbrain sections were classified in reference to their proximity to the ventricle, hippocampus, and corpus callosum/midline (1$^{st}$ section ~Interaural 3.46mm; Bregma -0.34mm; 2$^{nd}$ section ~Interaural 2.58mm; Bregma -1.22mm; 3$^{rd}$ section ~Interaural 1.74mm; Bregma -2.06mm). The images provided

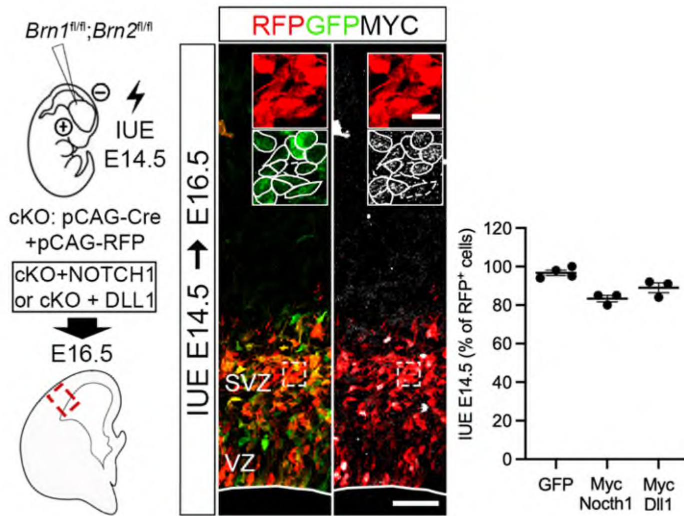
in Fig1a-b are representations of the 1st section level in control and *Brn1/2-cKO* mice. The *Brn1/2-cKO* brains present different anatomical defects in the cortex, midline, and hippocampus that are broadly documented across Fig. 1a-c and Extended Data Fig.2a-d. The separated layer markers are shown and quantified in Extended Data Fig.2e-g. The representative images shown and considered for quantification correspond to equivalent coronal midbrain sections determined the same way as explained for cortical thickness quantifications (the materials and methods were adapted to make this point clear). Considering the ~20% cortical thickness reduction, the presence of a large cortical heterotopia and the broad expression of DL markers with total absence of UL markers observed in the *Brn1/2-cKO* mice, we do not feel confident dividing the *Brn1/2-cKO* cortex in specific layers for independent quantification. From the immunostaining of the separated layer markers (Extended Data Fig.2e-g), the most evident observation is that *Brn1/2-cKO* mice did not simple lose all ULNs and therefore have a dramatically reduced cortex, but instead *Brn1/2-cKO* mice has a slightly smaller cortex with only DL-like neurons that now populate the whole cortex instead of being confined to their specific DL location.

**2. It is unclear whether Emx1 expression is correlated with BRN2 and BRN1 loss in Brn1/2-cKO mice. Double staining of Emx1 and BRN2/BRN1 in control and mutant mice is required.** Available Emx1 antibodies suitable for immunohistochemistry were raised in rabbits and so were the BRN1/2 antibodies. Therefore, we have decided to proceed with the mouse CRE antibody that allowed us to co-stain CRE and BRN1 or BRN2 and efficiently address this point from Reviewer #1. We have adapted Extended Data Fig. 1 to include CRE expression at early embryonic ages E11.5 and E12.5 (Extended Fig. 1b,h) in control heterozygous and *Brn1/2-cKO* mice. We confirmed that BRN1/2 expression overlaps with the CRE expression in heterozygous and that CRE expression induces an efficient depletion of the *Brn1/2* genes in *Brn1$^{fl/fl}$;Brn2$^{fl/fl}$* mice.

**3. The number of glial cells was dramatically increased in in Brn1/2-cKO mice at P13. How Brn1/2 to regulate the glial cell specification? How about oligodendrocytes?** In Extended Data Fig. 3a we showed that all SOX9$^{+}$, OLIG2$^{+}$ and GFAP$^{+}$ glial cells are increased in *Brn1/2-cKO* mice compared to the control at P13. In Fig. 4k, we showed that OLIG2$^{+}$ cells are also increased in *Brn1/2-cKO* mice compared to the control at P0. In addition, we have shown an increase of RFP$^{+}$/OLIG2$^{+}$ cells and total OLIG2$^{+}$ cells in the cortex of ferrets expressing *Brn*-DBD-EnR (New Fig 5h (previous Fig. 4h)). Based on the evidence we provided showing an abnormal centrosome function and fast consumption of progenitor cells in *Brn1/2-cKO* mice, we have hypothesized that the neuronal progenitors are going through terminal differentiation faster in *Brn1/2-cKO* mice which results in a depletion of the neurogenic pool and a quicker transition to gliogenesis in these mutants. In the revised manuscript, to further understand if there is a precocious shift from neurogenesis to gliogenesis in *Brn1/2-cKO* mice, we injected EdU into pregnant mice at E12.5 and E14.5 and followed the cell-fate of the labeled progenitors by analyzing the co-labeling of EdU$^{+}$ cells with the glia markers SOX9 and OLIG2 at P13 (Extended Data Fig. 3b). There is a significant increase of EdU$^{+}$SOX9$^{+}$ and EdU$^{+}$OLIG2$^{+}$ cells in *Brn1/2-cKO* mice at P13 (Extended Data Fig. 3c-f) indicating that precocious gliogenesis is happening in *Brn1/2-cKO* mice during early embryonic development. In addition, we confirmed these results by IUE where we expressed pCAG-CRE to inactivate *Brn1/2* in *Brn1$^{fl/+}$;Brn2$^{fl/+}$* mice at E14.5 and analyzed GFP$^{+}$OLIG2$^{+}$ cells at P13 (Extended Data Fig 3g-i). Due to the transient DNA expression of pCAG-RFP in our classical IUE setup, the RFP$^{+}$ cells labeled primarily neurons at P13 while GFP$^{+}$ cells in control and mutant littermates from heterozygous crossings where GFP expression is induced by CRE IUE allowed us to label neurons and glia cells. The new results combined with the evidence included in the previous submitted manuscript further support our hypothesis that the neurogenic pool of progenitor cells is depleted faster resulting in a quicker transition to gliogenesis in the *Brn1/2-cKO* mice.

**4. In Brn1fl/fl;Brn2 fl/fl mice at E12.5 and E14.5 (Extended Data Fig. 4 and 9, Fig 2h), pCAG-CRE and pCAG-RFP were electroporated into brain cells to identify electroporated cells. How to determine that RFP positive cells were CRE electroporated cells? Similar issues were existed in pCS2-NOTCH1, DLL1 and pCAG-Brn-DBD-EnR**. We and others have previously shown that co-expression of plasmids is highly efficient by IUE. To address this concern, we provide below some examples that that was also the case in our experiments. When we electroporate pCAG-CRE in *Brn1$^{fl/fl}$;Brn2$^{fl/fl}$* mice we induce GFP expression under the BRN2 promoter

(see Extended Data Fig. 1a).We show below the percentage of RFP⁺GFP⁺ cells (~95%). In addition, we show an example of RFP co-expression with MYC after IUE of pCS2-NOTCH1-MYC (>80%).




**5. Line 228, The authors concluded that BRN1/2 are required for the expression and function of sets of genes linked to microcephaly by using ASPMKO ferrets. Why don't they directly use BRN1/2 knockout ferrets? The comparison would be more convincing by using BRN1/2 knockout ferrets.** We fully agree that generating a BRN1/2 knockout ferret would help further understand potential BRN1/2 species-specific functions or BRN1/2 role in outer radial glia cells which are very limited in numbers in mice and abundant in ferrets. In addition, this goes in our opinion beyond the goal of the current manuscript. Just the generation and characterization of these animals would constitute a massive body of work that would lead to a multi-figure paper. Furthermore, the targeted genetic manipulations by IUE that we use here substantially support our conclusions. The ferret histological and functional results we present here after BRN1/2 inactivation by IUE and the correlation of these results with the *ASPM*KO ferret phenotypes and the mouse and monkey scRNAseq analysis strongly support the findings of the proposed manuscript. To help with the narrative of the manuscript and the contextualization of the *ASPM*KO phenotypes in our study, we have moved the *ASPM*KO ferret characterization to Extended Data Fig. 10. In line 228, we hypothesized that, since *Brn1/2-cKO* mice have a more obviously microcephalic brain than several mouse models of microcephaly, BRN1/2 could be required for the expression and function of sets of genes linked to this disease. Using our mouse scRNAseq analysis we have confirmed that several microcephaly-associated genes (including *Aspm*) are indeed reduced in *Brn1/2-cKO* progenitors at E14.5. The results from the *Brn1/2-cKO* mice were then compared to the *ASPM*KO ferret phenotypes aiming to gain insights on the molecular and cellular mechanisms that could overlap between the two models and perhaps be conserved across species. We have confirmed that several of the phenotypes overlap between the two models (Fig. 4 and Extended Data Fig. 10) and used IUE to inactivate BRN1/2 in ferrets and confirm that some of these cellular mechanisms are dependent of BRN1/2 activity in the ferret and that likely BRN1/2 acts upstream of ASPM in ferret to regulate these functions (Fig. 5g,h). We have now showed that *Aspm* is a direct transcriptional target of BRN1/2 (see answer to point 6) and have provided additional evidence to support that BRN1/2-dependent regulation of *Aspm* expression and function primarily controls the proliferation and cell cycle exit of progenitor cells.

**6. The evidences about "BRN1/2 are required to maintain the neuronal progenitor pool through the regulation of microcephaly-associated genes" are relatively uncertain. Lack of evidence to distinguish whether ASPM is a direct target of BRN2 or secondary effect. It is important to test whether overexpressing ASPM in Brn1fl/fl;Brn2 fl/fl mice can or partially rescue BRN2/BRN1 mutant phenotypes. It is also important to confirm the relationship between BRN2/BRN1 and ASPM by some specific assays, such as chip-seq experiment and luciferase assay of BRN2/BRN1.** We took different approaches to address this concern in the revised manuscript. First, we did luciferase assays to study the effect of BRN1/2 on *Aspm* promoter/enhancer

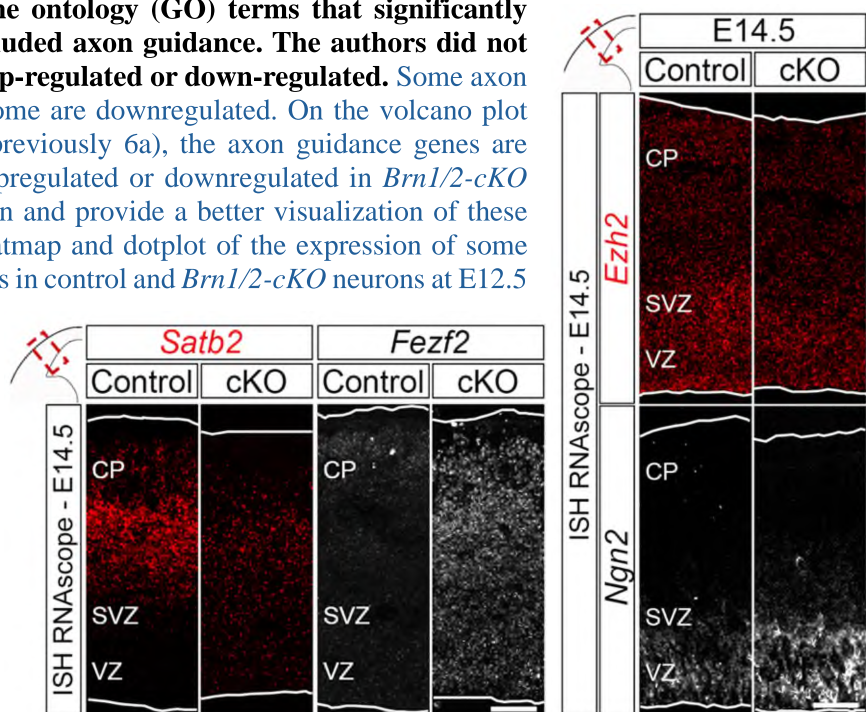
activity. We cloned the *Ensembl* predicted regulatory region of *Aspm* containing POU-domain binding sites in a luciferase reporter vector and tested its transcriptional activity in absence or presence of pCAG-BRN2 and the dominant negative pCAG-*Brn*-DBD-EnR. The co-expression with BRN2 increased *Aspm*-luciferase activity while the co-expression with *Brn*-DBD-EnR inhibited *Aspm*-luciferase activity (Fig. 4b) suggesting BRN1/2 directly regulates the expression of *Aspm*. To confirm these findings and understand if BRN1/2 directly binds *Aspm* (and the other microcephaly-associated genes) we next did BRN1/2 Chromatin Immunoprecipitation (ChIP) and analyzed the expression of the *Ensembl* predicted regulatory regions for the genes of interest in the immunoprecipitated chromatin by qPCR. We now show that BRN1/2 directly bind to different *Aspm* regulatory regions (*Aspm*, *Aspm-2*) and to at least two more microcephaly-associated genes (*Stil* and *Cep135*; Fig. 4c and Extended Data Fig. 10b). Since we used ChIP-qPCR targeting specific small parts of the regulatory regions for the selected genes and not global ChIP-seq, we cannot exclude that BRN1/2 directly regulates other microcephaly-associated genes by binding to other regulatory regions not analyzed here or guarantee that BRN1/2 only binds to the particular fragments we tested. Furthermore, to understand the extent to which *Aspm* contributes to the changes of progenitor behavior in *Brn1/2-cKO* mice, we expressed pCAG-CRE to inactivate *Brn1/2* and overexpress ASPM by IUE of *Brn1*$^{fl/fl}$;*Brn2*$^{fl/fl}$ mice at E14.5 and analyzed neurogenesis at E16.5. We show that ASPM can rescue the proliferative capacity of progenitor cells without affecting the levels of indirect neurogenesis (Fig. 4 d-h) suggesting that BRN1/2-dependent regulation of *Aspm* expression and function primarily controls the proliferation and cell cycle exit of progenitor cells without major contribution to the levels of indirect neurogenesis that is mainly regulated by tightly balanced levels of NOTCH signaling in these cells.

**Minor concerns:**

**1. In extended data fig.5d, the authors should provide a figure legend to explain what different colors represent**. The figure legend was adapted.

**3. Extended data fig.6g, line138, gene ontology (GO) terms that significantly changed in Brn1/2-cKO neurons included axon guidance. The authors did not indicate whether the change gene is up-regulated or down-regulated.** Some axon guidance genes are upregulated, and some are downregulated. On the volcano plot presented in Extended Data Fig. 6b (previously 6a), the axon guidance genes are highlighted in blue and classified as upregulated or downregulated in *Brn1/2-cKO* neurons. To further address this concern and provide a better visualization of these changes, we have now included an heatmap and dotplot of the expression of some classical axon guidance-associated genes in control and *Brn1/2-cKO* neurons at E12.5 and E14.5 (Extended Data Fig. 6e).

**4. The images in Extended data, fig.6f and fig.7g are very blurry. The authors should provide high resolution figures, especially for Satb2, Ezh2 and Ngn2.** The compression of the data for the first submission affected the quality of the figures and definitely the visualization of the ISH RNAscope results for *Satb2*, *Ezh2* and *Ngn2*. Here we provide a .tif high resolution crop for the reviewer and the high-resolution images would be included in the last version of the manuscript.

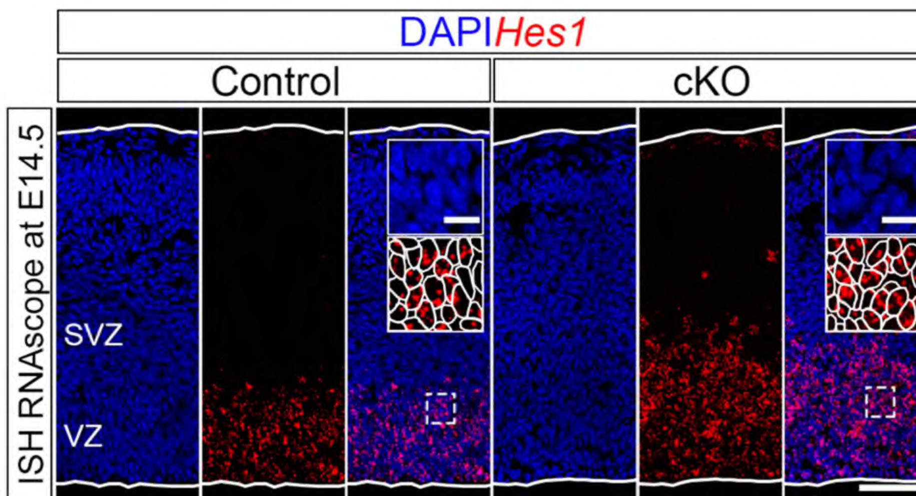



**5. In Fig. 1I, 1-6, what does 1-6 represent, respectively.** In Fig. 1i, 1-6 represent the different transcriptomic waves along the pseudotime axis previously described by Telley et al.[27] to reflect the temporal progression of APs. The gene list for each of these transcriptomic waves and the genes more significantly changed in *Brn1/2-cKO* apical

progenitors along the waves are included in Supplementary Information_1 (SI_1). To make this point clear for the readers, we have adapted the text and the figure legend of Fig. 1g-h and included a gene-ontology description of these waves in Fig. 1 (Fig. 1j).

**6. Lines, 128-129 "observed in wild-type mice similar gene expression changes along the pseudotime axis as reported". Should add the references? Why just present Hmga2 and Cdon?** We have included the reference and adapted the text and the figure legend to make this point clear for the readers. *Hmga2* and *Cdon* are part of the genes used as an example in the original publication[27] to characterize the temporal progression of the waves. We have included these two in the main figure as a proof of principle that we could reproduce the results from Telley et al.[27] in the control APs. A more extensive gene list is included in Extended Data Fig.5 and the complete gene list is included in Supplementary Information_1 (SI_1).

**7. In the Fig.s9b, the Hes1 immunostaining is very unclear. Hes1 should be in the nucleus. Please provide the high-resolution images.** The results presented in Extended Data Fig. 9b are from ISH RNAscope analysis which sometimes can be less clear than the immunolabeling analysis where a sharp nuclear staining for HES1 is more obvious (Fig.3g and Extended Data Fig. 9c). To address this concern, we provide below the co-immunostaining of *Hes1* ISH RNAscope with DAPI where we confirm that the *Hes1* ISH staining mostly overlaps with the DAPI nuclear staining.



**REVIEWERS' COMMENTS**

Reviewer #1 (Remarks to the Author):

The authors have done a thorough job for their revision and addressed most points that were raised in the initial review well. The present version has improved significantly and we have no more concerns.

Reviewer #2 (Remarks to the Author):

I am satisfied with the new experiments and analyses performed by the authors and congratulate them on this excellent work.

Reviewer #3 (Remarks to the Author):

The author have addressed my main concerns and I have no any question.