Single-cell transcriptomics of the early developing mouse cerebral cortex disentangles the spatial and temporal components of neuronal fate acquisition

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MS TITLE: Single-cell transcriptomics of the early developing mouse cerebral cortex disentangles the spatial and temporal components of neuronal fate acquisition

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I have now received all the referees' reports on the above manuscript, and have reached a decision. I apologize for the delays with your manuscript. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

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

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing

how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Moreau et al., present a thorough transcriptional exploration to deeper understand the emergence of cortical neuronal diversity during development. Nowadays, the dorsal pallium neurogenesis has been intensively investigated and it is well established that temporal progression of apical progenitors is one of the main sources for driving neuronal diversity emergence. However, temporal regulations are not sufficient to account for all the cortical neuronal diversity and this study investigated the contribution of spatial diversity. The data collected and analysed in this article are of high interest in the field of cortical development as it focuses on a region often marginalized in cortical development studies despite its great importance for its complexity.
Indeed, most of the studies using single-cell transcriptomics focused only on dorsal pallium and the contribution of both lateral and ventral pallial apical progenitors to glutamatergic cortical cells is not sufficiently investigated. Overall, the relevance of this new findings is important and represents a fundamental resource and aiming at improving our knowledge on cortical neurogenesis. In conclusion I'm willing to support the publication of this article but I have important concerns detailed below which need to be addressed before publication. I would like also to note the important bioinformatic work done by the authors. The code which has been used to generate figures is really clear and well written making the publication all the more appreciated. This is definitely a plus to convince me about the accuracy of the provided figures. In addition, this represents a great tool/pipeline for people interested in the topic.

Comments for the author

Major concerns:
- The spatial diversity investigated by the authors relies on the microdissection of tissue around the PSB region. However, it is unclear to me which tissue has been taken regarding the rostro-caudal axis. The spatial patterning along rostro-caudal axis is well described and I am really surprised that the authors observed it only partially (Figure S5C). I would really appreciate if the authors could complement this analysis and, at least, discuss why they did not observe this important well known spatial contribution.

- In page 13, the authors performed a regression model on the temporal score from Telley et al., 2019 and used this to score their data. From these predictions, with the subsequent data the authors claim that dorso-ventral patterning in the cortex is correlated with temporal progression. This is really interesting and represents for me the main finding that needs to be more investigated. The main issue, here, resides in the fact that the data are collected at one time point only, E12.5. How is this still appliable later for example at E15? The temporal modules as called by the authors represent few overlapping genes (n=109). What about the other temporal genes? Do other temporal modules (overlapping spatial and temporal patterning) exist at E15? I think that the addition of another time point at E15 would definitely improve the global understanding of the interaction between temporal and spatial patterning.

- In Figure 7, the authors identified two trajectories giving rise to Bhlhe22 and Nr4a2 neurons corresponding to dorsal and ventral cells respectively. The dorso-ventral patterning identified in APs supports these two neuronal populations emergence. However, we can observe that Nr4a2 population used the direct neurogenesis trajectory (without cell division) whereas Bhlhe22 one goes through the indirect neurogenesis following proper BPs enrichment. If true, the authors should experimentally demonstrate that at E12.5 dorsal pallium has more indirect neurogenesis than ventral pallium. It is known that indirect neurogenesis increases with time so this will contribute to strongly support the correlation observed between temporal progression and dorso-ventral patterning. In the same line, regarding my previous comment, would it be also true at E15?

Reviewer 2

Advance summary and potential significance to field

This is an interesting manuscript reporting on scRNAseq analysis of one specific timepoint (E12.5) during development of the pallial-subpallial boundary, including some neighboring tissue from nearby developing cortex and developing subpallium. The authors microdissected this region from E12.5 mouse embryos and sequenced 4225 cells, followed by clustering of cells, cell classification and comparison. The authors also use in their analysis some of the scRNAseq data from a prior study of developing cortex that collected single cells at E12.5 and E15.5.

Comments for the author

The paper is beautifully written and very clear in its descriptions and figures. There are some interesting discoveries that emerge from the work. In particular the identification of distinct clusters of Cajal Retzius cells present in ventral tissue versus dorsal one. Or the fact that Apical progenitors appear to spread across a continuum mostly defined by dorso-lateral differences rather than anterior-posterior ones.

The MS however also makes substantial claims about cell relationships (i.e. progenitor diversity to neuronal progeny or relationship over the space-time continuum), that are interesting but cannot, in my opinion, be answered with this data. There simply is not enough cells sequenced nor enough timepoints sequenced to claim this specific spatio-temporal relationships. And the claims are almost uniquely based on computational analysis (again on limited number of cells and one collection timepoint). Any lineage relationship claim, for example, would require some sort of a lineage tree, even if built in pseudotime. The warping of time and space model in Figure 8 remains very speculative and it is unclear how much it can be trusted. This model could very much be reinforced if some additional timepoints and more cells were sequenced as input for the analysis.

The claim that the data “provide a high resolution atlas of gene expression along the DV axis of the early developing telencephalic VZ” (and other statements of this broad nature) are very stretched given the limited dataset used here and they detract from the work. Similarly, the title is grandiose (Single-cell transcriptomics of the early developing mouse cerebral cortex disentangles the spatial and temporal components of neuronal fate acquisition) given the limited dataset.

Overall, a nice piece of work but with several over-reaching conclusions. My suggestion would be to increase the number of cells/time points, build lineage trees for ventral and dorsal lineages, and validate the spatio-temporal model. Also valuable would be to provide the readers with clear definitions of molecular divergence among similar classes of cells in ventral vs dorsal telencephalon.

First revision

Author response to reviewers' comments

Reviewer 1
We are very happy that the referee considers our paper of “high interest” and that it represents a “fundamental resource”. We are also delighted that she/he acknowledges the efforts we made to share our bioinformatics codes as a resource for the community. We thank her/him for comments that we found very constructive and that helped us improve the manuscript. Since some of the comments made by both reviewers are similar, Reviewer #1 may also find it useful to refer to our response to Reviewer #2.
“it is unclear to me which tissue has been taken regarding the rostro-caudal axis”
We have sampled the entire rostro-caudal axis. This is confirmed by the spatial mapping of neuronal populations that is shown in Fig. S4, where the reader can visualize the rostral position of Etv1+ neurons and compare with that more caudal of Lhx5+ neurons. In order to make it more

comprehensible to the reader, we have modified Fig. 1A to clearly show the dissection we performed. We have also modified the main text (lines 91 and 253-255).

“The spatial patterning along rostro-caudal axis is well described and I am really surprised that the authors observed it only partially (Figure S5C). I would really appreciate if the authors could complement this analysis and, at least, discuss why they did not observe this important well known spatial contribution.”

In order to respond to the Reviewer’s comment, we have regressed the variations observed among apical progenitors along the DV axis to reveal other sources of variation that might not have been captured by our initial analysis. Indeed, we identified a rostro-caudal (RC) signature. Our approach was validated by the observation that the expression of well-known cortical patterning genes such as Pax6, Nr2f1 (COUP-TFI) or Lhx2 along the pseudo-DV and pseudo-RC axes perfectly match the patterns described in vivo (Liu et al., 2000; Nakagawa et al., 1999; Stoykova and Gruss, 1994). When examining RC variable genes, we found three times less than DV variable genes and most of them (~80%) also changed in the DV dimension. In other words, there is a stronger contribution of the DV axis than the RC axis to spatial diversity and we confirm that our pseudo-DV score captures most of the spatial variations. The new data are presented in the revised version of Fig. S5C. We also show in situ hybridization along the RC axis for some genes (Fig. S5D) and provide the complete list of RC variable genes in Table S7. The main text has been amended accordingly (lines 407-418).

“the authors claim that dorso-ventral patterning in the cortex is correlated with temporal progression. This is really interesting and represents for me the main finding that needs to be more investigated. The main issue, here, resides in the fact that the data are collected at one time point only, E12.5. How is this still appliable later for example at E15? […] Do other temporal modules (overlapping spatial and temporal patterning) exist at E15? I think that the addition of another time point at E15 would definitely improve the global understanding of the interaction between temporal and spatial patterning.”

We agree with the Referee’s suggestion that another time point would significantly improve our study. We took advantage of a recently published dataset (La Manno et al., 2020) from which we could extract 12k APs obtained from E11 to E15 forebrains. We used this dataset to validate our findings at E12.5: we found that genes belonging to the “temporal module” indeed display temporal variation from E12 to E15 whereas those from the “spatial module” remain constant over time. We also provide comparative in situ hybridisation experiments between E12 and E15 to complement our bioinformatics findings. The data are presented at the bottom of Fig. 6 (panels I and J) as well as in an entirely new Fig. S6. The main text has been amended accordingly (lines 455-464). We thank the Reviewer for the suggestion as we believe this is a very important addition to the manuscript.

“The temporal modules as called by the authors represent few overlapping genes (n=109). What about the other temporal genes?”
It is true that only a small fraction of the 729 Telley et al. (2019) temporal variable genes overlaps with our DV axis variable genes. We can see several reasons for this. First, genes subjected to strictly temporal variation certainly exist. Actually, the sharp distinction between E12/E13 and E14/E15 APs shown in Fig. 6I argues in favor of “major changes due to temporal maturation” as we now mention (lines 458-460). Second, genes which are completely absent at E12 were obviously not captured as DV variable in our dataset whereas they could be classified as time-variable in the Telley et al. study. Third, Telley et al. used a different technical approach (Fluidigm vs 10X) that allowed them to sequence more genes per cell (although the difference is more in UMI/cell than genes/cell). Fourth, we applied a relatively stringent statistical cut-off (qval < 1e-3) to ensure that the genes we consider variable along the DV axis display a strong spatial variation at E12. We therefore chose to favour specificity over sensitivity.

“the authors should experimentally demonstrate that at E12.5 dorsal pallium has more indirect neurogenesis than ventral pallium. […] would it be also true at E15?”
We performed immunostaining using antibodies against Tbr2 to identify BPs and Ki67 to identify those that are cycling. Since Tbr2 immunoreactivity persist to some extent in neurons, we also used a Tbr1 antibody to exclude Tbr1+/Tbr2+ young neurons from our analysis. We confirmed our bioinformatics analysis by showing that the fraction of cycling BPs is higher in the DP than in the

VP. This will further support our statement that direct neurogenesis is more abundant in the VP than the DP at E12.5. The new data are presented in Fig. 7C and Fig. S7C. The main text has been amended accordingly (lines 489-493). We thank the Reviewer for encouraging us to perform such a quantification as it very nicely supports our initial finding.
We did not extend the analysis to E15 as proposed by the Reviewer, as the point of this experiment was to validate our lineage reconstructions performed at E12 that showed a BP bias for direct neurogenesis in the VP lineage. Although the question raised by Reviewer makes perfect sense and quantitative studies on the spatio-temporal changes in direct/indirect neurogenesis remain fragmentary, we thought this would go beyond the scope of our paper.
We thank again the Reviewer for the constructive comments and hope she/he will find our revisions convincing.

Reviewer 2
We are pleased that the referee found our paper of "beautifully written and very clear", we thank her/him for comments that helped us improve our manuscript. In addition to the point by point response provided below, Reviewer #2 may also refer to our answers to similar comments made by Reviewer #1 (especially regarding time points).

"My suggestion would be to increase the number of cells/time points […] and validate the spatio-temporal model"
The Reviewer will be pleased to see that the revised version of our manuscript now includes the analysis of apical progenitors extracted from the dataset of (La Manno et al., 2020). The dataset contains 12k cells that were collected at stages ranging from E11 to E15. Almost 10k are pallial progenitors, the remainder are subpallial. Within pallial APs, we found a clear separation between young (E11-E12) and older (E13-E15) progenitors. Importantly, we found that genes belonging to the "spatial module" remain constant over time whereas those from the "temporal module" change with the stage according to our initial findings. In addition, we validated our spatio-temporal model by performing comparative in situ hybridization at E12 and E15 for genes spatially variable and subjected, or not, to temporal changes. These data are shown in Fig. 6I, J as well as in a completely new Supplementary Fig. S6. The main text has been amended accordingly (lines 455-464). We thank the Reviewer for encouraging us to perform this additional work that, we believe, definitely improves our manuscript.

"Any lineage relationship claim, for example, would require some sort of a lineage tree, even if built in pseudotime."
"[My suggestion would be to…] build lineage trees for ventral and dorsal lineages"
Since we show that APs differ between the VP and DP, we are not sure about how to interpret the Reviewer's comment. Our understanding of lineage trees is that they mostly make sense when identical progenitors generate distinct cell types and that one wants to identify the molecular events occurring around branch points. Clearly, our reconstructions of Nr4a2 and Bhlhe22 lineages argue for a model where APs already display an initial bias in their fate potential, making lineage trees irrelevant in this context. This, however, does not rule out the existence of decision points at boundaries between spatio-temporal domains, where precursors commit to either one of two adjacent lineages. Perhaps the Reviewer's suggestion refers to such situations (e.g. the commitment to Bhlhe22 vs Ppp1r14c fate). Although they are not difficult to implement, we are reluctant to conduct such analyses as we have little indication on the identity of the last common progenitor we should consider as a starting point, and we would end up in producing lineage trees tricky to validate in the absence of lineage tracing tools. We chose to reconstruct only lineages for which we have a high confidence and that are supported by histology as well as lineage tracing. Other studies will be required to address the specific point of lineage bifurcations and we concur with the Reviewer that they will definitely require much larger cellular resolution (cell numbers and timepoints), in addition to some sort of clonal analysis. A fair part of our discussion is dedicated to this point (lines 587-612)

"Also valuable would be to provide the readers with clear definitions of molecular divergence among similar classes of cells in ventral vs dorsal telencephalon."
We assumed that the Reviewer is referring to the differences between pallial and subpallial neurogenic programs. In the revised version of our manuscript, we included pseudotime reconstruction of the two lineages to extract specific or common signatures. We identified genes which are common to AP, BP, EN or LN stages regardless of their pallial or subpallial identity, as

well as genes distinguishing the two branches. They are now presented in the revised version of Fig. S1A. Lists of genes common to pallial and subpallial differentiation, or distinguishing them, are now provided in Table S2-S4.

“There simply is not enough cells sequenced […] to claim this specific spatio-temporal relationships.”
We respectfully disagree with the Reviewer statement. Although very large datasets obviously allow to better resolve spatio-temporal relationships, we firmly believe that the dataset we provide, despite its relatively modest size, allows us to draw the conclusions we present in our manuscript. Actually, the additional analysis of the much larger (La Manno et al., 2020) dataset presented in the revised version of the paper clearly confirmed our initial findings. Furthermore, decent sequencing depth, rigorous use of the existing analysis pipelines, parsimonious data interpretation and histological validations are equally important to ensure successful scRNAseq approaches and we honestly think that we met such standards.

“the claims are almost uniquely based on computational analysis”
We have confirmed our computational analyses with histological work, including lineage tracing experiments using four distinct transgenic mouse lines, that represent two full main figures as well as several panels in other main and supplementary Figures. We therefore respectfully disagree that our study is “almost uniquely” based on computational analysis. We hope that adding the quantification of cycling BPs along the DV axis as suggested by Reviewer #1 (shown in Fig. 7C and S7C) as well as additional in situ hybridisation panels at E12 and E15 in Fig. S6C, D will further convince the Reviewer.

“The warping of time and space model in Figure 8 remains very speculative”
This is intentional. In the final stage of preparing the manuscript, we attempted to summarise our findings graphically and faced the issue that the Waddington landscape metaphor is not ideally suited to represent both temporal and spatial dimensions (unless one manages to represent a landscape changing with time). We took inspiration from the classical genotype/phenotype map to represent a progenitor/neuron map, and thought it could be useful for the reader to extrapolate our findings to any other kind of dimension than DV and time. We have amended the sentence referring to this model that now reads “In an attempt to graphically represent our findings we propose to adapt the classical Waddington metaphor to integrate the influence of both space and time on the same landscape” (lines 613-614).

“The claim that the data “provide a high resolution atlas of gene expression along the DV axis of the early developing telencephalic VZ” (and other statements of this broad nature) are very stretched given the limited dataset used here and they detract from the work.”
We have rephrased the sentence that now reads “Our data therefore provide an atlas of gene expression along the DV axis of the early developing telencephalic VZ…” (lines 416-418).

We hope that the Reviewer will find our answers to her/his comments as well as the modification made to the manuscript appropriate. We appreciate the constructive inputs that brought us to submit this revised version.

La Manno, G., Siletti, K., Furlan, A., Gyllborg, D., Vinsland, E., Langseth, C. M., Khven, I., Johnsson, A., Nilsson, M., Lönnerberg, P., et al. (2020). Molecular architecture of the developing mouse brain. bioRxiv 2020.07.02.184051.
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Stoykova, A. and Gruss, P. (1994). Roles of Pax-genes in developing and adult brain as suggested by expression patterns. J. Neurosci. 14, 1395–1412.

Second decision letter

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MS TITLE: Single-cell transcriptomics of the early developing mouse cerebral cortex disentangles the spatial and temporal components of neuronal fate acquisition

AUTHORS: Matthieu X Moreau, Yoann Saillour, Andrzej W Cwetsch, Alessandra Pierani, and Frederic Causeret
ARTICLE TYPE: Research Article

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

Reviewer 1

Advance summary and potential significance to field

Moreau et al., present a thorough transcriptional exploration to deeper understand the emergence of cortical neuronal diversity during development. The relevance of this study is important and represents a fundamental resource aiming at improving our knowledge on how spatial and temporal patterning shape cortical neurogenesis.

Comments for the author

The new data provided by the authors answer all my concerns.