Different Lineage Contexts Direct Common Pro-neural Factors to Specify Distinct Retina Cell Subtypes

Mei Wang, Lei Du, AihCheun Lee, Yan Li, Huiwen Qin, and Jie He

Corresponding Author(s): Jie He, The Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences

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Review Timeline:

Monitoring Editor: Louis Reichardt

Scientific Editor: Tim Spencer

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.)

April 21, 2020

Re: JCB manuscript #202003026

Dr. Jie He
The Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences
Room A0817, New Life Science Building, 320 Yueyang Road
Shanghai 200031
China

Dear Dr. He,

I am attaching to this letter the evaluations by three reviewers of the manuscript that you submitted recently to this journal. While the reviewers do have some scientific concerns that need to be addressed in a possible revision, their overall assessments of your work are quite positive. In the paragraphs below, I will try to indicate the journal's perception of the priority for each of these recommendations.

Concerning reviewer #1, I consider his/her major points #1, #2 and #5 as essential to address convincingly. The suggestions in #3 and #4 seem reasonable, but they are only suggestions. Reviewer #2's two listed concerns appear to be errors in data presentation that somehow slipped past your lab as you proof-read the manuscript and should be straight-forward for you to correct unless this reviewer has made a mistake. Concerning the evaluation of the third reviewer, it does seem to me that you should be able to easily perform the single cell analysis on the early PRCs and present the data. This reviewer's item #2 is just asking for some additional data analysis. IT would clearly help if you could link the single cell ATAC-dsq and RNA-seq link more clearly. The remainder of this reviewer's minor comments appear to be straight-forward for you to address.

In summary, the only major experimental request is the single cell analysis on early RPCs and this seems to require more analysis than data collection by your lab members. I hope that you can address these comments expeditiously as well as convincingly and submit a revision reasonably quickly. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the us once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

I expect to be able to make a final decision editorially without returning the revision to the reviewers provided that you help me by including a copy of the revision in which changes are clearly highlighted and a letter that provides a point-by-point list of the reviewers' comments and your responses.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal

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Sincerely yours,

Louis F. Reichardt, PhD
For the Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript, Wang et al identify fate restricted retinal progenitor cells that generate stereotyped subsets of retinal neurons during their final few cell divisions. Using single-cell RNA-seq and ATAC-seq in zebrafish retina, they identify molecular markers (i.e. Vsx1 and OC1) for these fate-restricted progenitor cell populations. By generating transgenic Vsx1 and OC1 Gal4 lines, they confirm these two genes as molecular markers of the lineage-restricted progenitor populations. Finally, they show that the molecularly identified progenitors not only produce distinct neuronal lineages under normal conditions, but they also do so when forced to overexpress well known fate-determining transcription factors. These findings are significant in two ways. First, the work challenges (or at least complicates) the conventional view that vertebrate neural progenitors do not become fate-committed until their last cell division. While lineage-restricted progenitor cells are well

known in invertebrates, it is typically thought that vertebrate progenitors are multipotent, with their competence to produce particular cell types changing or restricting over time. This paper shows that, in addition to this undoubtedly important mechanism, there also exist certain progenitor cells that are already committed to generating certain cell types but not others. Second, while this paper is one of many that have analyzed neural progenitors by scRNA-seq, the insights gained here - i.e., identification of lineage-restricted progenitors - are unique.

I have several comments on the manuscript that the authors should address:

1. The authors generated Gad1b-GFP and Glyt1-GFP BAC transgenic fish with the goal of labeling GABAergic and glycinergic amacrine cells. It does not appear that the authors confirmed that the GFP+ cells are indeed GABAergic and/or glycinergic. In Fig. S3 there is an analysis of IPL stratification pattern (narrow for GABAergic, diffuse for glycinergic), but even with these images and data it is still not clear how faithfully these two transgenic lines label the targeted cell types. The authors should describe more thoroughly how the lines were validated, and if possible provide additional quantitative information on the fraction of GFP+ cells that were the right cell type (measured by molecular markers of each neurotransmitter phenotype or by stratification pattern).

2. In the scATAC-seq section of the manuscript, the authors claim that the Vsx1+ progenitor population is represented by the scrt2open and [atoh7open her12closed OC1closed] populations. (This is also noted in Fig. 6D). It wasn't clear to me how the authors determined that this was the case. Did they also have gene expression data within their ATAC-seq datasets? Or was this conclusion based entirely on the data of Fig. 6E? If the latter the evidence seems a bit thin. The authors should explain and more fully rationalize this conclusion.

3. Many readers will be familiar with temporal fate restriction models of cell fate specification. For these readers it would aid understanding if the authors could explain, in the Discussion, how their results might be integrated with such models. E.g. do the authors envision that lineage-restricted progenitors might emerge as part of a temporal fate mechanism? Or is this an additional orthogonal mechanism? Could temporal mechanisms affect the particular neurogenesis patterns observed here (e.g. Fig. 1P)?

4. There has been quite a bit of debate in the cerebral cortex field as to whether lineage-restricted progenitors exist (i.e. are there progenitors that can produce only upper-layer neurons?). The authors may wish to consider adding consideration of this point to the Discussion, as this may broaden the scope of the manuscript.

5. For the Ptf1a Crispr experiments, it was not clear from the Methods section how efficacy of genome editing was validated. Was it simply clear from the phenotype of the Crispr-injected fish? Some molecular assay?

Also one minor point: The authors should consider using page and/or line numbers as a "reviewer-friendly" courtesy.

Reviewer #2 (Comments to the Authors (Required)):

In this manuscript Wang et al. examined how neuronal diversity arises in the zebrafish retina. Using in vivo lineage analysis, the authors identified six major neurogenic lineages, each generating stereotypic retinal cell types and subtypes. They performed scRNA-seq analysis of neurogenic

RPCs and identified several distinct RPC clusters. They showed that the transcript-defined clusters are biased to generating distinct major neurogenic lineages, implicating the existence of lineage-specific late RPCs in the developing retina. They further showed that pro-neurogenic transcription factors, when over-expressed in lineage-specific RPCs, markedly biased the production of different neuronal types or subtypes in a lineage-dependent manner.

In general, this is an exciting study that provides novel insights into the cellular mechanisms for producing neuronal diversity in vertebrate retina. The data quality is high, and supports the authors' conclusion. I only have a few minor concerns.

1. In the first paragraph on page 11, the gene lists for TFs shared between clusters C/D and C/B were reversed.

2. The left panel of Figure 5H should be RGC-2PR.

3. There are many typos and grammatical errors.

Reviewer #3 (Comments to the Authors (Required)):

In this manuscript, Wang et al. reported specific late retina progenitor cell (RPC) lineages in producing distinct and stereotyped neuronal types in the zebrafish retina. The authors developed two lineage tracing methods, mMAZe and atoh7:Switch, and traced ~1000 individual RPCs undergo the final round(s) of neurogenic division. While multiple neuronal types including PRs, HCs, RGCs, ACs and BCs are generated, they arise predominantly from 6 distinct and stereotyped lineages: RGC-2PR, AC-2PR, AC-BC, BC-BC, PR-PR, and HC-HC. More strikingly, distinct lineages give rise to specific neuronal subtypes (i.e., GABAergic vs. glycinergic ACs; off vs. on/on-off BCs) that is controlled by specific transcription factor/TF (i.e., Ptf1a) in the corresponding lineages. These results strongly suggest the existence of RPCs pre-specified/dedicated to distinct neuronal types/subtypes. Indeed, the authors took advantage of single cell RNA-seq and ATAC-seq and identified distinct populations of RPCs and explored the transcription factor program underlying distinct neuronal production.

This manuscript addresses a fundamental question in developing neuroscience and provides important insights into the developmental original of neuronal diversity in the vertebrate nervous system. The authors have performed a comprehensive set of experiments and the manuscript is well written.

Main comments:

1. As the authors stated in the introduction, a fundamental question regarding the progenitor origin of neuronal diversity is predetermined/dedicated progenitors with defined outputs vs. stochastic choices of equipotent progenitors. The authors reported strong evidence of the existence of distinct and dedicated RPCs to produce specific neuronal types/subtypes. Yet, in the discussion the authors suggested that the predetermined/dedicated RPCs emerge from stochastic features (e.g., TF expression) of RPCs and an integrated picture of stochastic and deterministic cell fate regulation. Is there any direct evidence on a stochastic behavior of RPCs in this study?

If the authors perform similar single cell analysis on early RPCs, do they observe any clear

heterogeneity? Given that the authors have collected a substantial number of early lineages (especially in the Atoh7:Switch dataset), they should analyze these lineages thoroughly and report them in the figures.

2. To test the like hood of stochasticity, could the authors estimate/predict the frequencies of the RPC lineages simply based on a random fate choice and the overall abundance of different neuronal types in the retina, and compare with the experimental observation?

3. The link between single cell ATAC-seq and RNA-seq datasets is not obvious. The open accessibility of TF typically refers to the expression regulation of its target genes. It is unclear why scrt2open and atoh7openher12closedOC1closed represented vsx1+ RPCs producing AC-BC and BC-BC lineages, while atoh7openOC1open represented OC1+ RPCs producing RGC-2PR and AC-2PR lineages.

Minor comments:
1. "RPC" first appeared in introduction without the full name being specified.

2. It is unclear what "STAR Methods" refers to.

A point-to-point response to the reviewers

1. The authors generated Gad1b-GFP and Glyt1-GFP BAC transgenic fish with the goal of labeling GABAergic and glycinergic amacrine cells. It does not appear that the authors confirmed that the GFP+ cells are indeed GABAergic and/or glycinergic. In Fig. S3 there is an analysis of IPL stratification pattern (narrow for GABAergic, diffuse for glycinergic), but even with these images and data it is still not clear how faithfully these two transgenic lines label the targeted cell types. The authors should describe more thoroughly how the lines were validated, and if possible provide additional quantitative information on the fraction of GFP+ cells that were the right cell type (measured by molecular markers of each neurotransmitter phenotype or by stratification pattern) (Reviewer 1).

    Thanks for the comment.

    To verify *Tg(gad1b:EGFP)*, we performed the slice immunostaining using the antibody against GAD65/67 and showed that almost all GFP$^+$ ACs in Tg(gad1b:EGFP) were Gad65/67 positive (142/144; Fig. S2B).

    Due to a lack of the available antibody of zebrafish GlyT1, we verified *Tg (glyt1:EGFP)* transgenic fish using *in situ* hybridization of *glyt1* combined with GFP antibody and showed that 98% GFP$^+$ ACs were expressing *glyt1* (130/132; Fig. S2C).

    Together, our analyses indicated two transgenic lines that we generated in this

2. In the scATAC-seq section of the manuscript, the authors claim that the Vsx1+

   progenitor population is represented by the scrt2open and [atoh7open her12closed

   OC1closed] populations. (This is also noted in Fig. 6D). It wasn't clear to me how

   the authors determined that this was the case. Did they also have gene expression

   data within their ATAC-seq datasets? Or was this conclusion based entirely on the

   data of Fig. 6E? If the latter the evidence seems a bit thin. The authors should

   explain and more fully rationalize this conclusion (Reviewer 1).

3. The link between single cell ATAC-seq and RNA-seq datasets is not obvious. The

   open accessibility of TF typically refers to the expression regulation of its target

   genes. It is unclear why scrt2open and atoh7openher12closedOC1closed

   represented vsx1+ RPCs producing AC-BC and BC-BC lineages, while

   atoh7openOC1open represented OC1+ RPCs producing RGC-2PR and AC-2PR

   lineages. (Reviewer 3)

4. Many readers will be familiar with temporal fate restriction models of cell fate specification. For these readers it would aid understanding if the authors could explain, in the Discussion, how their results might be integrated with such models. E.g. do the authors envision that lineage-restricted progenitors might emerge as part of a temporal fate mechanism? Or is this an additional orthogonal mechanism? Could temporal mechanisms affect the particular neurogenesis patterns observed here (e.g. Fig. 1P)? (Reviewer 1)

Thanks for this good suggestion. We have extent our discussion on this issue (from line 443 to line 458)

"Temporal generation of different neuron types

In the developing vertebrate retina, distinct retinal types occur in a temporally sequential but overlapping manner (Cepko, 2014). Our analyses revealed the lineage-specific progenitors, providing new insights into this temporal generation of different neurons. Interestingly, our data showed that *OC1*-expressing RPCs produced RGCs, GABAergic ACs, which are early-born. On the contrary, *vsx1*-expressing RPCs gave rise to glycinergic ACs and BCs, which are the late-born neurons. One possibility is the earlier occurrence of *OC1*-expressing RPCs than *vsx1*-expressing RPCs. Alternatively, *onecut1*-expressing RPCs exhibits shorter cell-cycle length than vsx1-expressing RPCs. To distinguish two

possibilities, future analysis is required. Meanwhile, within 3-cell lineages (RGC-2PR and AC2-PR), we observed the sequential generation of RGC/AC and PRs. Thus, within the stereotyped lineages derived from lineage-specific RPCs, the generation of distinct retinal cell types conforms to the conserved temporal order as what we observed at the population level."

5. There has been quite a bit of debate in the cerebral cortex field as to whether lineage-restricted progenitors exist (i.e. are there progenitors that can produce only upper-layer neurons?). The authors may wish to consider adding consideration of this point to the Discussion, as this may broaden the scope of the manuscript. (Reviewer 1)

Thanks for the comments. We have included a new discussion on this topic (from line 426 to line 441).

"Whether similar lineage-specific progenitors exist in developing mammalian cortex is still controversial. Through the lineage tracing using MADM labeling, cortical progenitors marked at the early neurogenic stages were found to produce both deep and superficial layer neurons but be seldom restricted to specific neuron types (Gao et al., 2014). $Cux2^+$ cortical progenitors can be intrinsically specified into only upper-layer neurons (Franco et al., 2012), suggesting the presence of lineage-specific cortical progenitors. On the other hand, the contradicted results were also reported (Eckler et al., 2015;Guo et al., 2013). Recent studies, however,

favor the possibility of the co-existence of progenitors with or without lineage restriction (Garcia-Moreno and Molnar, 2015; Llorca et al., 2019). Using combined approaches, cortical progenitors marked at the onset of the neurogenesis can generate translaminar (~80%), deep layer-restricted (~10%), and superficial layer-restricted (~10%) lineages in the developing mouse neocortex (Llorca et al., 2019). In the future, more systematic single-cell transcriptome and lineage analyses of neural progenitors are needed for a better characterization of lineage-specific progenitors in the developing mammalian cortex."

6. For the Ptf1α Crispr experiments, it was not clear from the Methods section how efficacy of genome editing was validated. Was it simply clear from the phenotype of the Crispr-injected fish? Some molecular assay? (Reviewer 1)

Thanks for the comments. We have included the sequencing result of targeted sequences in ptf1α knockout animals using CRISPR/Cas9, which clearly showed the mutation occurred in 100% (59/59). These mutations include premature stop codon, in frame with deletions, in frame with insertions (Fig.3, B-C).

7. Also one minor point: The authors should consider using page and/or line numbers as a "reviewer-friendly" courtesy. (Reviewer 1)

Thanks for the suggestions. We have included page and/or line numbers in the

8. In the first paragraph on page 11, the gene lists for TFs shared between clusters C/D and C/B were reversed (Reviewer 2).

9. The left panel of Figure 5H should be RGC-2PR (Reviewer 2).

10. There are many typos and grammatical errors (Reviewer 2).

11. As the authors stated in the introduction, a fundamental question regarding the progenitor origin of neuronal diversity is predetermined/dedicated progenitors with defined outputs vs. stochastic choices of equipotent progenitors. The authors reported strong evidence of the existence of distinct and dedicated RPCs to produce specific neuronal types/subtypes. Yet, in the discussion the authors suggested that the predetermined/dedicated RPCs emerge from stochastic features

(e.g., TF expression) of RPCs and an integrated picture of stochastic and deterministic cell fate regulation. Is there any direct evidence on a stochastic behavior of RPCs in this study? (Reviewer 3)

Thanks for the question. Actually, we are extremely interested in whether lineage-specific neurogenic RPCs are specified in a random manner or in earlier RPCs. We raise this outstanding question in the Discussion for the future investigation. We do not have any direct evidence to support either hypothesis yet.

12. If the authors perform similar single cell analysis on early RPCs, do they observe any clear heterogeneity? Given that the authors have collected a substantial number of early lineages (especially in the Atoh7:Switch dataset), they should analyze these lineages thoroughly and report them in the figures (Reviewer 3).

Thanks for this great suggestion. Here we defined her4-expressing progenitors as early RPCs (Cluster A; Fig. 4 C). We further performed the new analysis on early RPC heterogeneity in terms of single-cell transcriptomes. Interestingly, we found that there were two subpopulations of early RPCs that may contribute to the generation of *onecut1-* and *vsx1*-expressing RPCs, respectively (Fig. S3 E), and made the following text (from line 226 to line 234):

"In addition, we performed the further analysis on early RPCs (Cluster A; Fig. 4 C). They could be divided into three clusters (Fig. S3 E). Clusters 1 and 2 highly

expressed early markers (e.g. her6, her12). Interestingly, they also weakly expressed different neurogenesis-related TFs, suggesting that different early RPCs might generate distinct neurogenic RPCs. On the other hand, we also observed that some large-size lineages (>3 cells per lineages) occurred frequently in the lineages traced by atoh7:Switch and mMAZe, such as 4BCs, RGC-AC-BC-2PR, RGC-2AC-BC-2PR (Table S1 and Table S2), suggesting that lineage-specific progenitors might exist in earlier RPCs. The link between early RPCs and neurogenic lineages needs the future investigation."

13. To test the like hood of stochasticity, could the authors estimate/predict the frequencies of the RPC lineages simply based on a random fate choice and the overall abundance of different neuronal types in the retina, and compare with the experimental observation? (Reviewer 3)

Thanks for the suggestion. We are very interested in the question as to whether lineage-specific RPCs are specified stochastically. Since we observed heterogeneity in early RPCs which may contribute to different lineage-specific RPCs (Fig. S3 E), the generation of these lineages is unlikely to be simply based on a random fate choice. However, it is possible that stochastic determination might occur at earlier stage before neurogenic phase. To clarify this stochasticity, we are currently analyzing the generation of stereotyped neurogenic lineages derived from sister progenitors at earlier stages.

14. "RPC" first appeared in introduction without the full name being specified (Reviewer 3).

Sorry for this ignorance. We have corrected it.

15. It is unclear what "STAR Methods" refers to (Reviewer 3).

Thanks for the comment. We have corrected it in the revised manuscript.

May 21, 2020

RE: JCB Manuscript #202003026R

Dr. Jie He
The Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of
Sciences
Room A0817, New Life Science Building, 320 Yueyang Road
Shanghai 200031
China

Dear Dr. He,

I want to thank you for returning such a complete description of your responses and modifications
made in response to the evaluations provided by the reviewers of the initial version of your
manuscript. I just finished reading these as well as your revised manuscript and think that you and
your lab members did a thorough, even exemplary job in addressing the reviewers'
recommendations. All of us have thought from receipt of your initial submission that your
manuscript had the potential to be published in this journal and I am glad to let you know that I am
satisfied with your revisions, am recommending editorial acceptance, and additionally look forward
to your future work.
Please congratulate your lab members on my behalf for an excellent set of important and
informative experiments.

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8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

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10) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely yours,

Louis F. Reichardt
For the JCB

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology