Characterization and staging of outer plexiform layer development in human retina and retinal organoids

Sumitha Prameela Bharathan, Angela Ferrario, Kayla Stepanian, G. Esteban Fernandez, Mark W Reid, Justin S Kim, Chloe Hutchens, Narine Harutyunyan, Carolyn Marks, Matthew E Thornton, Brendan H Grubbs, David Cobrinik, Jennifer G Aparicio and Aaron Nagiel
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First decision letter

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MS TITLE: Characterization and staging of outer plexiform layer development in human retina and retinal organoids

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I have now received all the referees reports on the above manuscript, and have reached a decision. 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, two of the referees are happy with your revisions whereas the third still has a couple of issues that he/she considers to be essential to address prior to publication. Please attend to these comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

See prior review

Comments for the author

While the authors have made substantial improvements to the manuscript there are some issues that need to be resolved before I can endorse its publication:

1) The authors state several times that RGCs in retinal organoids undergo apoptosis and are no longer present by day 100. They cite two references neither of which show any data on apoptosis and one of which (2019 Development paper) that clearly demonstrates RGCs (as evidenced using a RGC reporter line) at day 100 and sparse remaining SNCG immune-positive RGCs (that look like the CALB+ cells the authors claim are HCs) at day 160 and day 220. Proof of RGC apoptosis and lack of RGCs in their organoids after day 100 should be provided as requested.

2) The authors need to address our previous comments about cell type markers. Specifically:
a) Their response to 3a needs further clarification: while fetal retina has perfect spatial organization to help confirm cell marker identity, organoids do not. If the authors are to claim that their CALB+ cells in organoids are HCs, they should demonstrate unequivocally that they are not SNCG immune-positive and also that they co-express ONECUT1.

b) Likewise, their response to 3b needs further evidence to support their assertion, since VSX2 immuno-positive may well be proliferative NRPCs. It is straightforward to include a proliferative marker to determine whether VSX2+ cells in fetal retina and in organoids are NRPCs or BPC precursors.
VSX2 immuno-positive post-mitotic cells could also be Müller glia, which share the same spatial location as BPCs, emphasizing the point that one must be careful in assuming that a single marker defines a cell type.

c) Response to 3c also depends on the uncertain assertion that RGCs undergo apoptosis and are not present in their organoids. Once again, definitive evidence of both assertions is necessary.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed my concerns about the structure of the text, the analysis of later timepoints, the staging of subfeatures, quantification consistency across figures, and descriptions of others' work. The publication is ready for publication.

Comments for the author

NA

Reviewer 3

Advance summary and potential significance to field

This study describes and stages synaptogenesis in the outer plexiform layer in the developing human retina and shows that key aspects of human photoreceptor-bipolar cell synaptogenesis are reflected in human pluripotent stem cell derived retinal organoids providing a good framework for the use of retinal organoids to study synaptogenesis in more detail.

Comments for the author

The authors have addressed reviewers' comments very well and the paper is now substantially improved. I have no further suggestions/comments

First revision

Author response to reviewers' comments

	Reviewer 1	Our response
1	The authors state several times that RGCs in retinal organoids undergo apoptosis and are no longer present by day 100. They cite two references, neither of which show any data on apoptosis and one of which (2019 Development paper) that clearly demonstrates RGCs (as evidenced using a RGC reporter line) at day 100 and sparse remaining SNCG immune-positive RGCs (that look like the CALB+ cells the authors claim are HCs) at day 160 and day 220. Proof of RGC apoptosis and lack of RGCs in their organoids after day 100 should be provided as requested.	Thank you for this comment. In response, we have removed any implication of RGC apoptosis or their complete absence in older HROs to agree with the data in the references we cite (Page 15, Line 452-454). To provide evidence for progressive RGC loss in older HROs, we performed additional IF staining on HROs at D70, D100, D130, D160, D220 and D280 (Fig. S6D,F,H,J,L,N). Correlative staining on human retina fetal week 16.6 retina was performed to verify the immunofluorescence pattern (Fig. S6A-B). We first performed IF on human fetal retina with the two markers mentioned in this comment: SNCG (Fig. S6A) and pan-BRN3 (all isoforms; Fig S6B). All cells in the RGC layer showed robust immune-positive signal for BRN3 and SNCG, confirming that these markers can detect a variety of RGC subtypes in the developing retina. BRN3 immunopositivity was specific to RGCs. However, we observed a low level SNCG expression in other retinal cell types, especially in a subset of HCs (Fig. S6A). Our analysis of published single-cell RNA seq in adult human retina (Yan *et al.*, 2020) detected SNCG expression in RGCs and other retinal cell types, including HCs (see RNASeq analysis below). Hence, we opted to use pan-BRN3 to detect RGCs in our HROs. The IF staining on HROs at D70, D100, D130, D160, D220, and D280 (n=5 HROs at each age from two independent experiments), (Fig. S6D,F,H,J,L,N) demonstrated $BRN3^+$ cells in abundance on D70 with a drastic decrease by D100, and only rare cells present in subsequent timepoints. The data presented in Fig. S6D,F,H,J,L,N support the notion that progressive RGC loss is occurring during long-term organoid culture. We address the concern over HC identity of $CALB^+$ cells in the response to Comment 2a below.

We have removed unpublished data given to the reviewers in confidence.

Analysis of candidate gene expression in retinal cell types in human adult retina (data from study by Yan *et al.*, 2020). Bubble plots showing relative gene expression in horizontal cells (HCs), retinal ganglion cells (RGCs), photoreceptors (PRs), bipolar cells (BCs) and amacrine cells (ACs) in human adult retina. The genes with known cell-type specific gene expression are shown in green boxes for each retinal cell type (CALB1 and ONECUT1 for HCs; SNCG, POU4F1, POU4F2 and RXR□ for RGCs; RXR□, ARR3 and RHO for PRs; VSX2, PCP2 and GNG13 for BCs; and CALB2 and TFAP2A for ACs). Of note, SNCG expression is detected in RGCs but also in a subset of HCs (H1), PRs (rods), BCs (all cone bipolar cells), and ACs. Bubble plots were generated from publicly available scRNAseq data on human adult retina by Yan et al., 2020 using the Broad Institute Single Cell portal (https://singlecell.broadinstitute.org/). The bubble size indicates the percentage of cells exhibiting the specific expression profile. The bubble color indicates scaled mean expression, and the scaling is relative to each gene's expression across all cells of a given retinal cell type.

2	**The authors need to address our previous comments about cell type markers.**	
a	Their response to 3a needs further clarification: while fetal retina has perfect spatial organization to help confirm cell marker identity, organoids do not. If the authors are to claim that their CALB+ cells in organoids are HCs, they should demonstrate unequivocally that they are not SNCG immune-positive and also that they co- express ONECUT1. Comment 3a: Figure 1G, H and I use CALB as a marker of horizontal cells, but it also is expressed by ganglion cells and amacrine cells (which are quite a bit more abundant than HCs in retinal organoids). Since the spatial organization that supports the authors' argument that these CALB+ cells are indeed HCs is only reliably in fetal tissue, a more specific HC marker (or series of markers) is necessary for the organoid evaluation.	Thank you for this comment. To evaluate the cellular identity of CALB$^+$ cells, we performed additional immunofluorescence studies on human retina fetal week 16.6 (Fig S6A) and HROs at ages D70, D100, D130, D160, D220 and D280 (Fig S6C,E,G,I,K,M). We used CALB, SNCG, and ONECUT1 antibodies, as suggested by the reviewer. In human fetal retina, many cells along the apical portion of the INL were ONECUT1$^+$, and a subset of these were also CALB$^+$ (Fig S6A). A subset of HCs was actually triple positive for ONECUT1, CALB and SNCG (weakly). In HROs at age from D100 to D280 (n=5 HROs at each age from two independent experiments), we found that all CALB$^+$ cells present in the apical aspect of the INL co- expressed ONECUT1 (Fig. S6E,G,I,K,M) and lacked expression of BRN3 (Fig. S6,H,J,L,N). This confirms that CALB$^+$ cells adjacent to the OPL of HROs at ages D130 to D280 in Fig.5, Fig.6 and Fig. 8 represent HCs.

b	Likewise, their response to 3b needs further evidence to support their assertion, since VSX2 immuno-positive may well be proliferative NRPCs. It is straightforward to include a proliferative marker to determine whether VSX2+ cells in fetal retina and in organoids are NRPCs or BPC precursors. VSX2 immuno-positive post-mitotic cells could also be Müller glia, which share the same spatial location as BPCs, emphasizing the point that one must be careful in assuming that a single marker defines a cell type. Comment 3b: Figure 3B identifies VSX2+ INL progenitors, but these are much more likely to be proliferating retinal progenitors.	Thank you for this comment. We agree that "VSX2+ INL progenitors" could mislead one to think of VSX2 as a marker for cells destined to become bipolar cells. As the reviewer points out, the cells inhabiting the presumptive INL could include maturing bipolar cells, bipolar precursors, Müller glia and even proliferating retinal progenitors. As illustrated in Fig. 1A-B, our intention for using VSX2 in combination with Recoverin (RCVRN) on fetal retina is to show that photoreceptor precursors ($RCVRN^{+}VSX2^{-}$) are already segregated from the rest of the cell types in the outer neuroblastic layer. We believe that confirming the exact identity of these $VSX2^{+}$ cells lies outside the scope of this manuscript. We have therefore modified the text to remove any implications regarding the exact identity of $VSX2^{+}$ cells (Page 5, Line 127-133).

c	Response to 3c also depends on the uncertain assertion that RGCs undergo apoptosis and are not present in their organoids. Once again, definitive evidence of both assertions is necessary. Comment 3c: RXRγ clearly has significant non-cone immunofluorescence in the fetal retina (e.g., figure 3S), so it is not a good choice for identifying cones in retinal organoids.	Thank you for this comment. We agree with the reviewer and have responded to the issue of RGC loss in long-term HRO culture in Comment 1. To confirm the cellular identity of $RXR\gamma^{+}$ cells in the HROs, and to distinguish RXRγ expressing cones from RGCs, we have performed additional immunofluorescence experiments. IF staining on human fetal retina with a pan-BRN3 antibody showed lack of expression of BRN3 in the photoreceptor layer, thereby confirming that the combination of RXRγ and BRN3 can be used to distinguish $RXR\gamma^{+}$ cones from $RXR\gamma^{+}$ RGCs in this setting. In HROs at ages D100, D130, D160, D220 and D280 (n=5 HROs at each age from two independent experiments), we found that all $RXR\gamma^{+}$ cells in the apical layer were negative for BRN3. Thus, we can confirm that $RXR\gamma^{+}$ cells in the apical layer of HROs at D100 and D130 in Fig. 7 are cone precursors.

Second decision letter

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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. The referee report on this version is appended below.

Reviewer 1

Advance summary and potential significance to field

Please see original review

Comments for the author

All concerns were addressed.