nature portfolio

Peer Review File

Retinoic acid drives cell fate specification, maturation and retinal regionality in human retinal organoids

Corresponding Author: Dr Anai Gonzalez-Cordero

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the manuscript by Lim et al., the Authors utilize spatial transcriptomics and single-cell RNA-sequencing of differentiating human retinal organoids to delineate the function of retinoic acid in the control of retinal cell fate specification and regionalization. This work builds on a wealth of data that has determined that retinoic acid signaling plays a crucial role in regional specializations of the human retina, whereby inhibition of retinoic acid signaling results in a rod-free region that differentiates earlier than the rest of the retina. The work provided in this manuscript uses unbiased approaches to examine the consequence of altered retinoic acid pathway activation on retinal development, retinal cell fate specification, and expression of regionally patterned transcripts. The results of the manuscript confirm that low levels of retinoic acid signaling in the human retina bias photoreceptors to differentiate as cones and likely promote a permissive environment for accelerated differentiation of retinal cell types.

Overall, the manuscript provides novel information about how modulating retinoic acid signaling affects cell fate specification, speed of retinal differentiation, and controls spatially restricted transcript expression patterns. However, results are confounded by the use of an automated cell type caller leading to likely mis-annotated cell types, use of markers that are not specific to individual cell types, and limited interpretations of all possible explanations for observed results. These can all be easily addressed within a revised manuscript.

Major Comments:
Cell type annotations and Marker gene analyses:
- Lines 122-124; Line 239-240 - please provide a list of genes that was utilized to attempt spot deconvolution for each cell type and utilized for cell type annotation using ScEiaD. As many transcripts are shared across multiple cell types including RPCs, it would be beneficial to know why so many spots include all cell types. There is also a surprisingly high number of Müller glial cells in the datasets (Figure 1D), especially given the age of the organoids (early). It is unclear how Müller glia were distinguished from RPCs. However, this is recognized as a challenging task as Müller glia and RPCs share a high degree of transcriptional similarities.

The results of the ScEiaD for the automated cell type calling look unusual. NRL should not be highest expressed in cones (RA-10uM; widespread expression in Horizontal cells and Cones in RA- which is seemingly the control). The misannotation of cones is highlighted by the large number of cones clustering with the rods in Figure 3B as dimension reduction is performed on a high number of (ALL?) transcripts instead of singular marker genes. Also see interpretations of results (NRL+/RXRG+ cells) comments below. Not seeing any marker gene that is distinguishing RGCs (POU4F2, RPBMS). Horizontal cells should express LHX1 or ISL1, Sox2/Sox9 are both expressed in RPCs and Müller glia. RLBP1 and SLC1A3 are highly expressed in Müller glia (lower in RPCs, RLBP1 in RPE).

- Lines 252-257 - discrepancy between scRNA-seq experiments and flow cytometry for RGC numbers - 5% of cells are RGCs in scRNA-seq but >18-36% in the flow cytometry. This indicates that either the scRNA-seq cell type calling is incorrect or that gating of the Brn3B.mCherry+ cells is too permissive and is overcounting the mCherry+ cell number. This does not support ‘suppressing premature RGC differentiation in a time independent manner’

- high number of Sox9+ Müller Glial cells. There have been reports of Sox9 antibodies that show ectopic expression in Amacrine cells. Given the high number of Sox9+ cells that are MKI67- (showing a 'Müller glia' nuclear layer that is >4-5

nuclei thick in Figure 3F), it seems like the number of Müller cells is being over estimated. Can the authors provide co-labeling of Sox9 with an Amacrine marker like TFAP2A? Lines 409-412 – 30% Müller glia is too high in a more mature retina, suggesting that Sox9 is not specific.

- Lines 325 – 337; Lines 563-569 – plastic photoreceptors – While RXRG is utilized as a marker of cones, it typically distinguishes cones based on marker co-expression (Thrb, Arr3, etc). Recent evidence (Jin, et al., 2025; FASEB) suggests that NR2E3 directly inhibits RXRG expression. Since NRL is upstream of NR2E3, this suggests that NRL+ cells will co-express RXRG until sufficient levels of NR2E3 accumulates to inhibit RXRG expression and cone fate. This is consistent with NRL and NR2E3 knockout phenotypes where all rods default to an S-cone like fate. Can the authors look at NR2E3 expression within the 'plastic' photoreceptors? Is the retinoic acid treatment causes reduced NR2E3 in NRL+ photoreceptors, thus resulting in the 'plastic' population? Therefore, in the discussion, it is likely better to talk about RXRG expression in early rods (supported by the data and previous publications) instead of a 'surprising' result that NRL is in cones. It is this reviewer's impression that the cones within the rod cluster in the UMAP are mis-annotated based on the automated cell type caller.

- Figures S3F-M. GS staining does not look like typical Muller glia staining in organoids (S3G) or in human retina (S3M). In fact, the GS staining looks like astrocytes in the Human retina, suggesting that the Müller glia are not present within the retina. Prox1 expression is not limited to Horizontal cells, but also stains bipolar cells.

Over-interpretation or not acknowledging alternative explanations:
- Line 199-203 – EdU incorporation assay. The Authors conclude that RA treatment promotes cell cycle progression. This term is ambiguous between two plausible outcomes. 1) Cell cycle progression of RPCs is faster when with RA treatment, resulting in more RPCs incorporating the EdU during the 16-hour treatment window. Alternatively, 2) RA treatment is biasing cell cycle exit. As EdU doesn't indicate if a cell is remaining in the cell cycle, one has to co-label with a proliferative marker like MKI67 to determine if RA is biasing cells to exit the cell cycle (during the EdU treatment period). Determination of cell cycle length can be performed through FACS analysis of DNA content (performed only on cells within the cell cycle through RPC marker co-expression) OR through a dual BrdU EdU pulse chase and co-labeling with an proliferative marker (see Harris et al., 2018; J Mol Histol).

- Line 180-183 – CYP26A1 expression – Is this real staining or an increase in background signal across the ages. The staining seems to be complementary to all nuclear layers. CYP26A1 is localized to the endoplasmic reticulum and is expressed by RPCs and Müller glia. Can the Authors please provide co-labeling with either ER markers in RPCs or definitive Müller glia markers (GLUL)? See Krueger et al., 2024; Differentiation for CYP26A1 staining during primate retina development.

- Line 218-220 – While the presence of Vsx+/Sox9- cells does suggest differentiation of bipolar cells, this does not say anything about cell cycle exit versus proliferation. This suggests that the timing of cell fate specification may be advanced in the BMS-treated retina (bipolar cells being specified during a temporal window when they are not normally generated).

Additional Comments:
Line 108-109 – RGCs and cones are the first-born cell types. While partially accurate, other cell types are also generated very early, including starburst amacrine cells and horizontal cells

Line 116-119 – "Transcriptomic similarity analysis" – Please provide additional detail in the main text, indicating that spatial transcriptomic analysis was performed on 10 week organoids and that Pearson Correlation was performed between the pseudo-bulk of the spatial transcriptomic analysis and previous scRNA-seq on human fetal retina. These details are in the Figure legend, but are unclear when reading the results. The details of spatial transcriptomics are then provided later (Line 120-121).

Line 148-149 – Please also reference Figure S2F, as this is very relevant to the spatial organization of the organoids. It is hard to see from the plots, but to ALDH1A1 and ALDH1A3 display complementary patterns (like what would be expected in vivo) or do many dots contain expression of both transcripts?

Line 194-198 – Further explanation in the figures/legends would be beneficial to distinguish between different experiments For example Figure 2B and Figure S2B both seem to represent Sytox staining but show different results (BMS 1uM is now significant in Figure 2B). One has to carefully read the text to understand that these experiments represent different timing of RA addition and different ages of ROs.

Line 215-216 – 'RA- hROs exhibited the highest percentage of NRL+ rods while no differences were observed in CRX+/NRL- cones across treatments (Figure 2G,H)'. The figure reference should be 2K,L

Line 288 - HOTAIRM1 expression has been previously detected in retina (Lu et al., 2020; Dev Cell) as a human-specific marker of cones.

Lines 395-400 – CellChat analysis cannot suggest 'enhanced functionality' of organoids. While the results are consistent with a more mature organoid with RA- or BMS1uM treatments, it doesn't actually test 1) If synapses are formed, 2) If synapses are functional, 3) if photoreceptors are capable of responding to light and propogating responses to light to the inner retina.

Line 412 – Should Figure 6C,F reference be Figure S6B, F?

Line 437 – Should Figure S6F reference be Figure S6L?

Reviewer #2

(Remarks to the Author)
This manuscript examines how retinoic acid affects human retinal development using hPSC-derived retinal organoids (hROs). The authors combine spatial transcriptomics, single-cell RNA-seq, and immunostaining to show that RA levels regulate retinal maturation. They propose that low RA bias organoids toward a cone-rich, macula-like retina, whereas high RA promotes a peripheral-like identity. The work has clear implications for both basic human retinogenesis and optimization of hROs for disease modelling and cell therapy.
Overall, the study is ambitious, methodologically strong, and potentially impactful, but a few conceptual, methodological, and interpretative issues should be addressed to strengthen the paper.

1. Physiological relevance and RA dosing
The study relies heavily on high exogenous RA (up to 10 μM). It would help to better anchor these conditions in a physiological context.
• Can the authors discuss how 0.1–10 μM RA compares to estimated endogenous RA levels during human foetal retinal development? This would help frame if the levels in the study are physiologic developmental ranges.
• The macula/periphery identity claims are intriguing and important, but they rely primarily on transcriptomic signatures and a few marker genes (CALB1, PCP4, etc.).
• It would be helpful to more clearly define what the authors mean operationally by “macula-like” and “peripheral-like” in hROs and remind the reader of the limitations (e.g., no true foveal pit, no RPE, no vasculature).

2. Terminology
Be consistent with terms like “RA–” vs “no exogenous RA” vs “control condition” throughout.

3. Statistics and quantification
• For all quantifications explicitly state n (number of organoids, number of differentiations, number of fields) in figure legends.
• It would help to mention the statistical tests used and whether corrections for multiple comparisons were applied.
• All staining analysis in the paper should have quantification and statistics performed, and not just show a representative image.

4. Line 109 “few RBMPS+ RGCs” should the gene be spelled as RBPMS?

5. Line 1311 RGCs (RBPMS and RXRG). I wasn’t aware that RXRG labels RGCs as I thought of it as a cone marker. Can the authors provide evidence to support that it is an RGC marker or is this an error?

6. Figure 3C should have error bars and statistics.

This is a comprehensive and technically sound study that maps RA’s role in human retinal organoid development across time, dose, and spatial context. The integration of spatial and single-cell transcriptomics with protein-level validation is a major strength, and the translational implications for disease modelling and cell therapy are compelling. Addressing the points above—especially around physiological relevance of RA dosing, more explicit discussion of macula/periphery claims, and providing quantification and statistical analysis will strengthen the manuscript and sharpen its main conceptual contributions and I would be supportive of publication.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The Authors have addressed many of the reviewer comments sufficiently, providing a wealth of data about retinoic acid signaling in the developing human retina. Many limitations of the study (spot size, cell type calling, etc) are addressed within the manuscript and alternate interpretations have been presented.

A few minor comments remain.

Automated segmentation and cell counter: It is unclear how the CellPose counter is dealing with non-nuclear markers for cell type counting and the identity of the denominator in many of the figures that look at cell type proportions. This is extremely evident in Supplemental Figure 8, where the denominator for % cells is unclear, as the '% of cells' far exceeds 100%. Are % S-opsin+ cones (S8J) the % total cells that are s-cones or the % of cones that are also s-opsin+. Just looking at the quantifications in S8J, if total cell number is used, the percentages add up to well over 100% of cells (for example – in the RA1uM condition; %Sox9+ ~30%, % s-opsin+ ~35%, %l/m-opsin+ ~30%, %NRL+ rods ~25%). Is this a result of using non-nuclear markers (ARR3, OPN1SW, OPN1MW)? Looking at the CellPose counting in Figure S2, ARR3 looks to be

annotated in regions where there are no cells (DAPI+) consistent with ARR3 not being a nuclear marker. How does the automated cell counter account for non-overlapping signal?

Line 64 – RA does not generate a rod-free zone. It is the absence of RA (presence of RA degrading enzymes) that coordinates the rod-free zone. “RA and [FGF8] signaling promote the formation of a rod-free zone” suggests that RA promotes this phenotype which is misleading.

Lines 170-172 – ‘more spatially restricted’ – This conclusion hard to interpret from the data given the ‘spatial’ signal seems random (not preferential to region of organoid nor retinal layer). Is the ‘randomness’ more a function of expression levels, and therefore likely a technical artifact due to drop-out?

Line 181-186 – What about CYP26C1? Should be mentioned as it is known to be expressed in retina although maybe not at high levels in early development. Cyp26c1 has been recently shown to express in Müller glia in zebrafish (Lahne, 2026; bioRxiv). If not detected, the authors should state this.

Line 222 – “SOX2+/SOX9+/VSX2+ RPCs were observed in the high RA conditions” no quantitative data shown to support this conclusion

Reviewer #2

(Remarks to the Author)
The authors have satisfactorily addressed the concerns raised in my prior review, and I am now supportive of publication.

Point by point response to reviewers' comments.

We thank the reviewers for their thoughtful and constructive comments on our manuscript. We have carefully reviewed each point raised and made revisions to address concerns. These changes have significantly improved the clarity, structure, and scientific rigor of our work. Below we provide a detailed point-by-point response to each reviewer comment, highlighting the specific data updates and methodological clarifications made. For ease of reference, we have highlighted changes in the text in yellow. Our team anticipates that the data, methods and the overall readability of the manuscript is much improved.

Reviewer #1 (Remarks to the Author)

In the manuscript by Lim et al., the Authors utilize spatial transcriptomics and single-cell RNA-sequencing of differentiating human retinal organoids to delineate the function of retinoic acid in the control of retinal cell fate specification and regionalization. This work builds on a wealth of data that has determined that retinoic acid signaling plays a crucial role in regional specializations of the human retina, whereby inhibition of retinoic acid signaling results in a rod-free region that differentiates earlier than the rest of the retina. The work provided in this manuscript uses unbiased approaches to examine the consequence of altered retinoic acid pathway activation on retinal development, retinal cell fate specification, and expression of regionally patterned transcripts. The results of the manuscript confirm that low levels of retinoic acid signaling in the human retina bias photoreceptors to differentiate as cones and likely promote a permissive environment for accelerated differentiation of retinal cell types.

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Major Comments:

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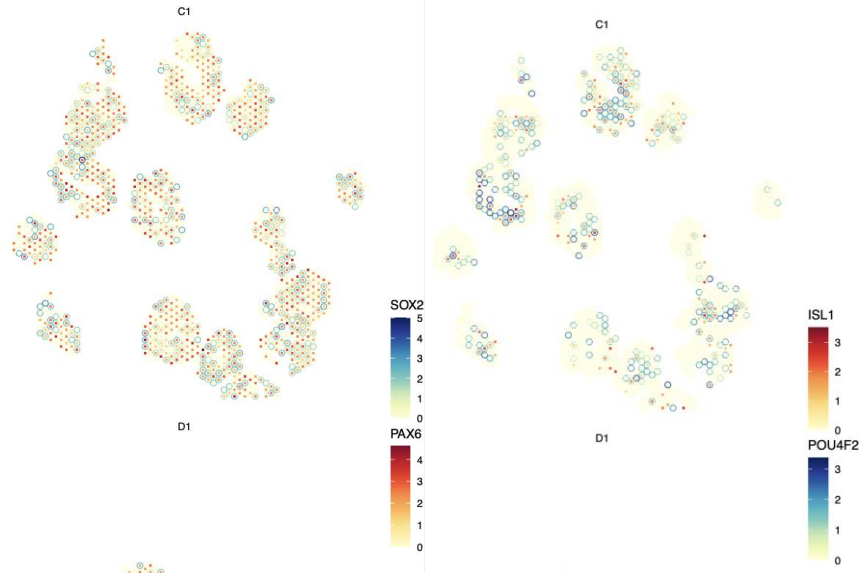
- Lines 122-124; Line 239-240 - please provide a list of genes that was utilized to attempt spot deconvolution for each cell type and utilized for cell type annotation using ScEiaD. As many transcripts are shared across multiple cell types including RPCs, it would be beneficial to know why so many spots include all cell types. There is also a surprisingly high number of Müller glial cells in the datasets (Figure 1D), especially given the age of the organoids (early). It is unclear how Müller glia were

distinguished from RPCs. However, this is recognized as a challenging task as Müller glia and RPCs share a high degree of transcriptional similarities.

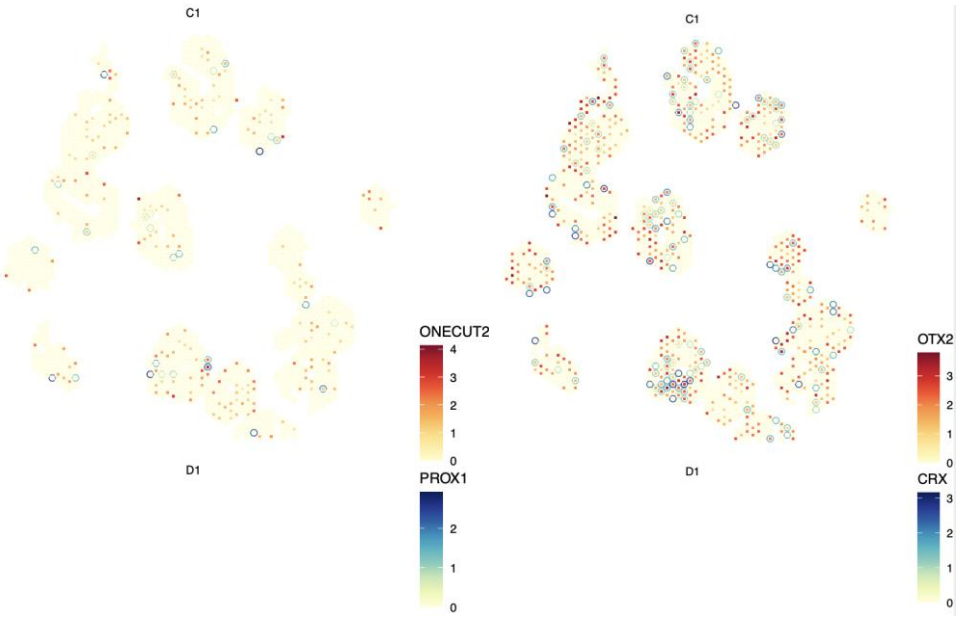
Reply: We thank the reviewer for highlighting the need for greater clarity and caution regarding cell type annotation, particularly for RPCs and Müller Glia, which are known to share substantial transcriptional overlap during early retinal development. To clarify, we reasoned that using the *in vivo* human fetal retina at 11 weeks (Figure 1C) was the most appropriate reference (procedure outlined in *Methods 'Cell deconvolution and cell type annotation of spatial transcriptomics'*). This has now been clarified in the Figure Legend and methods.

CARD[1] algorithm was used to perform cell deconvolution. It performs internal gene filtering and informative gene selection per sample prior to spot deconvolution. As a result, the specific gene set used slightly varies across individual organoids, reflecting organoid-specific signal. While we are unable to provide a single set of genes, we instead provide a list containing the union of genes aggregated across organoids for each slide replicate, as derived from the CARD algorithm, in a new **Table S6.** We have also further clarified the annotation and spot deconvolution procedure in the Methods section (lines 1152-1154) which now states: "Then, the CARD algorithm with default spot and gene filtering parameters was used, keeping spatial locations where at least 100 counts were detected and including genes where at least expressed in 5 spots." Therefore, due to the nature of this approach, individual spots can be assigned proportions owing from multiple cell types as a single spot may encompass more than one cell type, hence the presence of overlapping cell types.

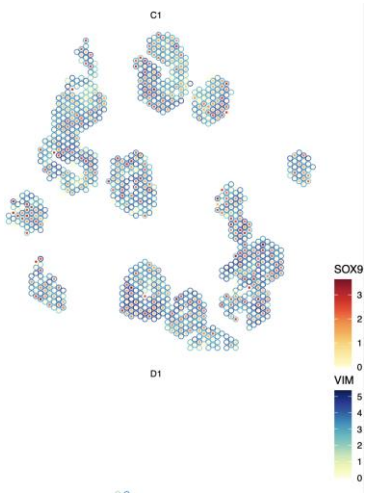
We agree that distinguishing Müller glia from RPCs at immature organoid stages is challenging. To address this, we have (i) added additional marker gene expression plots for the key retinal cell types, including RPCs (SOX2 and PAX6), RGCs (Isl1 and POU4F2), Horizontal (ONECUT2 and PROX1), photoreceptor precursors (OTX2 and CRX) and Müller Glia (SOX9 and Vimentin) (see images below on **Review Response Figure 1**) to **Supplementary Figure 1**, and (ii) revised the Results to interpret Müller Glia proportions cautiously as "Müller glia–like/late progenitor" populations rather than fully mature Müller Glia where mentioned.



(RPCs) (RGCs)



(Horizontal) (Photoreceptor precursors)



(Muller Glia)

Review Response Figure 1. Co-expression of key marker genes for each dominant cell type population present in HROs at 10 weeks profiled by 10x Visium. These panels have been added to **Figure S1**.

The results of the ScEiaD for the automated cell type calling look unusual. NRL should not be highest expressed in cones (RA-10uM; widespread expression in Horizontal cells and Cones in RA- which is seemingly the control). The misannotation of cones is highlighted by the large number of cones clustering with the rods in Figure 3B as dimension reduction is performed on a high number of (ALL?) transcripts instead of singular marker genes. Also see interpretations of results (NRL+/RXRG+ cells) comments below. Not seeing any marker gene that is distinguishing RGCs (POU4F2, RPBMS). Horizontal cells should express LHX1 or ISL1, Sox2/Sox9 are both expressed in RPCs and Müller glia. RLBP1 and SLC1A3 are highly expressed in Müller glia (lower in RPCs, RLBP1 in RPE).

Reply: We appreciate the reviewer's careful evaluation of the photoreceptor annotations, and we agree that the initial presentation may have over-stated the interpretation of NRL expression in Cones.

We clarify that the automated label transfer was performed after PCA and UMAP dimensionality reduction using the top 2000 highly variables genes (Methods, now lines 1130-1132) rather than single marker genes, which can result in partial overlap of early Rod and Cone populations during developmental transitions.

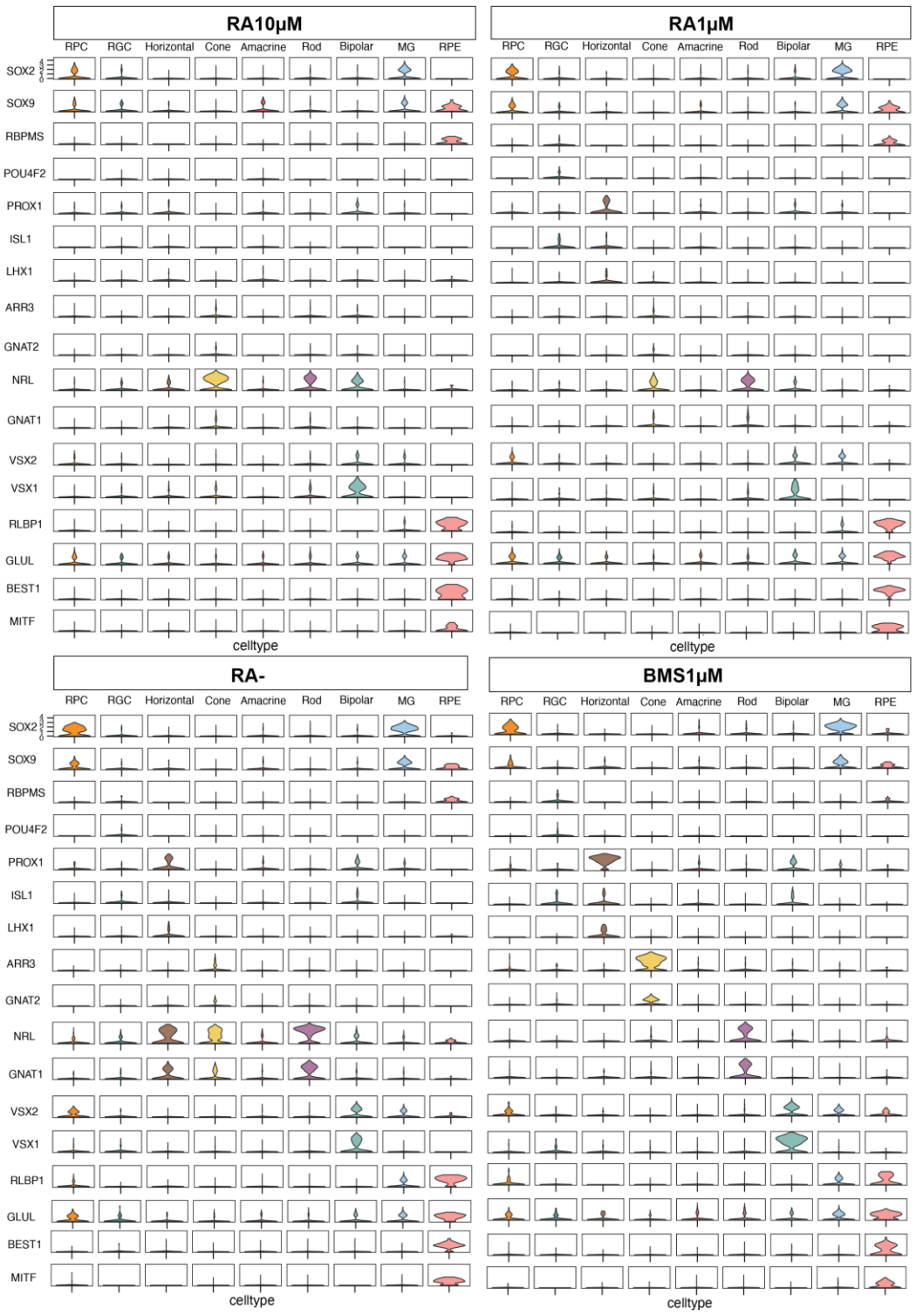
Considering the reviewer's comments and recent literature, we have now revised our interpretation to reflect that NRL+/RXRG+ cells potentially represent immature or early rod photoreceptors prior to NR2E3-mediated rod fate commitment in addition to ectopic expression of NRL within cones (Results lines 348-352, Discussion lines 813-815).

Moreover, we include a bubble plot visualisation of the key Cone and Rod markers (ARR3, CRX, RXRG, NR2E3) to emphasize that this transitory population is distinct to mature Cones and Rods (now added as a **Supplementary Figure S5D**, see below).

To confirm our annotation we also include additional marker genes to individual cell populations previously shown in **Supplementary Figure S3C** (now **Figure S4**). POU4F2, RPBMS, LHX1, ISL1, SOX2, SOX9 and RLPBP1 were added to delineate our clustered populations. Unfortunately, SLC1A3 did not pass our filtering thresholds during pre-processing so we are unable to include it as most likely the expression was not meaningfully detected.

Nevertheless, we show that POU4F2 and RBPMS are relatively highly expressed in RGCs. We also observed that LHX1 and ISL1 are distinguishing markers for Horizontal cells and RLBP1 is more highly expressed in Muller Glia compared to RPCs, and it is also consistently highly expressed in RPE. Lastly, SOX2 and SOX9 are co-expressed in both RPCs and Muller Glia as expected. These markers together with other that were already depicted in **Supplementary Figure S3C** are now a new **Supplementary Figure 4** (see below).

Most importantly, we emphasize the central conclusion that retinoic acid signalling influences the timing and regional bias of photoreceptor differentiation remains unchanged.



New Figure S4.

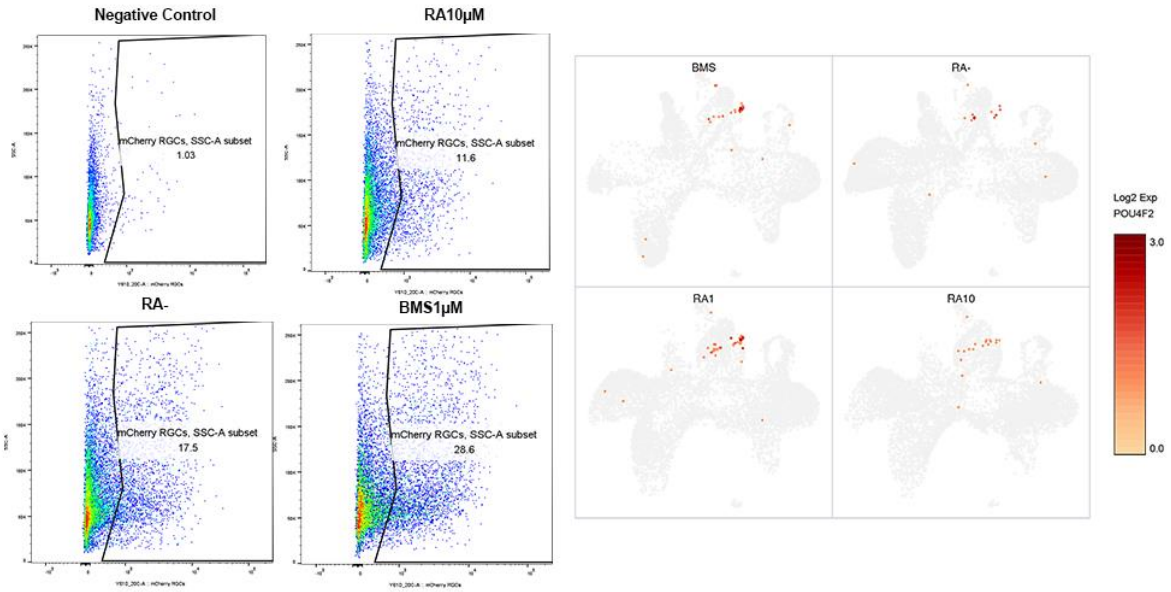
- Lines 252-257 - discrepancy between scRNA-seq experiments and flow cytometry for RGC numbers - 5% of cells are RGCs in scRNA-seq but >18-36% in the flow cytometry. This indicates that either the scRNA-seq cell type calling is incorrect or that gating of the Brn3B.mCherry+ cells is too permissive and is overcounting the mCherry+ cell number. This does not support ‘suppressing premature RGC differentiation in a time independent manner’.

Reply: Discrepancies between scRNA-seq and validation experiments at protein level (proteomics/flow/IHC) have been widely discussed in the literature, with possible biological and technical reasons.

Technical biases can be explained by scRNA-seq requires enzymatic dissociation**,** which disproportionately affects certain cell types for example fragile or large cells are more prone to loss (e.g., RGCs are large cells). However, in this case samples were went through similar dissociation procedure for both techniques. However, a striking difference between both techniques is the filtering. Removal of low-quality cells with low RNA content or high mitochondrial reads can add filtering biases. Leading to scRNA-seq cell proportions that no longer reflect true tissue composition. On a biological context protein level may not correlate with transcripts due to post-transcriptional regulation, translation efficiency, protein half-life, or trafficking.

To confirm correct RGC identity in the transcriptomic data, we have confirmed the flow cytometry analysis is adequate (see below **Review Response Figure 2** for representative scatterplots of BRN3B mCherry+ cells). We also checked heatmaps for POU4F2 (BRN3B)expression within scRNA-seq data and observed a very small number of RGCs expressing this marker (see below).

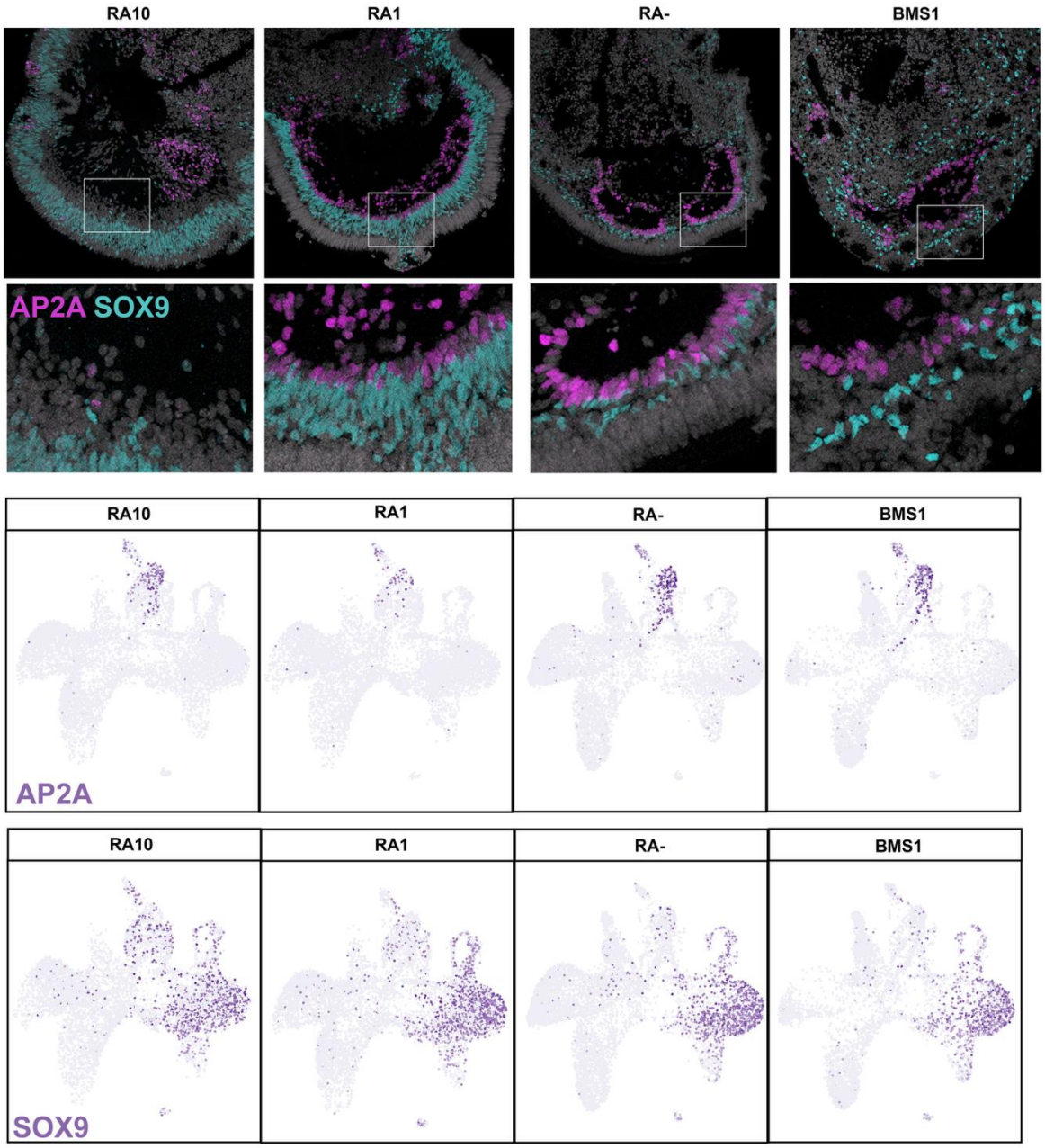
Importantly, we acknowledge in the text that that RGCs are underrepresented within our scRNA-seq dataset. To our knowledge a comparatively quantification of the proportions of RGCs in hROS with both scRNA-seq and protein datasets has not yet been performed. Other studies such as Sridhar et al. 2020[2] also detected less than 10% RGCs on their scRNA-seq dataset at a similar timepoint in development, however validation at protein level was not performed. Further studies, need to be performed to confirm if RGCs are biased to discrepancies.



Review Response Figure 2. Representative BRN3B.mCherry flow plots and POU4F2 scRNA-seq UMAPs.

- high number of Sox9+ Müller Glial cells. There have been reports of Sox9 antibodies that show ectopic expression in Amacrine cells. Given the high number of Sox9+ cells that are MKI67- (showing a 'Müller glia' nuclear layer that is >4-5 nuclei thick in Figure 3F), it seems like the number of Müller cells is being over estimated. Can the authors provide co-labeling of Sox9 with an Amacrine marker like TFAP2A? Lines 409-412 – 30% Müller glia is too high in a more mature retina, suggesting that Sox9 is not specific.

Reply: We have now checked our scRNAseq dataset as well as performed extra IHC experiments for TFAP2A /SOX9 and in both techniques we observed very little co-localisation of these markers. Perhaps not all amacrine subtypes are present in hROs. Therefore, we are convinced that our current quantification for Muller glia cells is accurate. It is interesting that hROs contain high number of Muller Glia cells and this is a characteristic that should be explored in further studies.



Review Response Figure 3. IHC of SOX9 and AP2A colocalisation in 16 week old hROs as well as integrated UMAP representation of RA treatment samples, coloured by TFAP2A expression (top) or SOX9 expression (bottom).

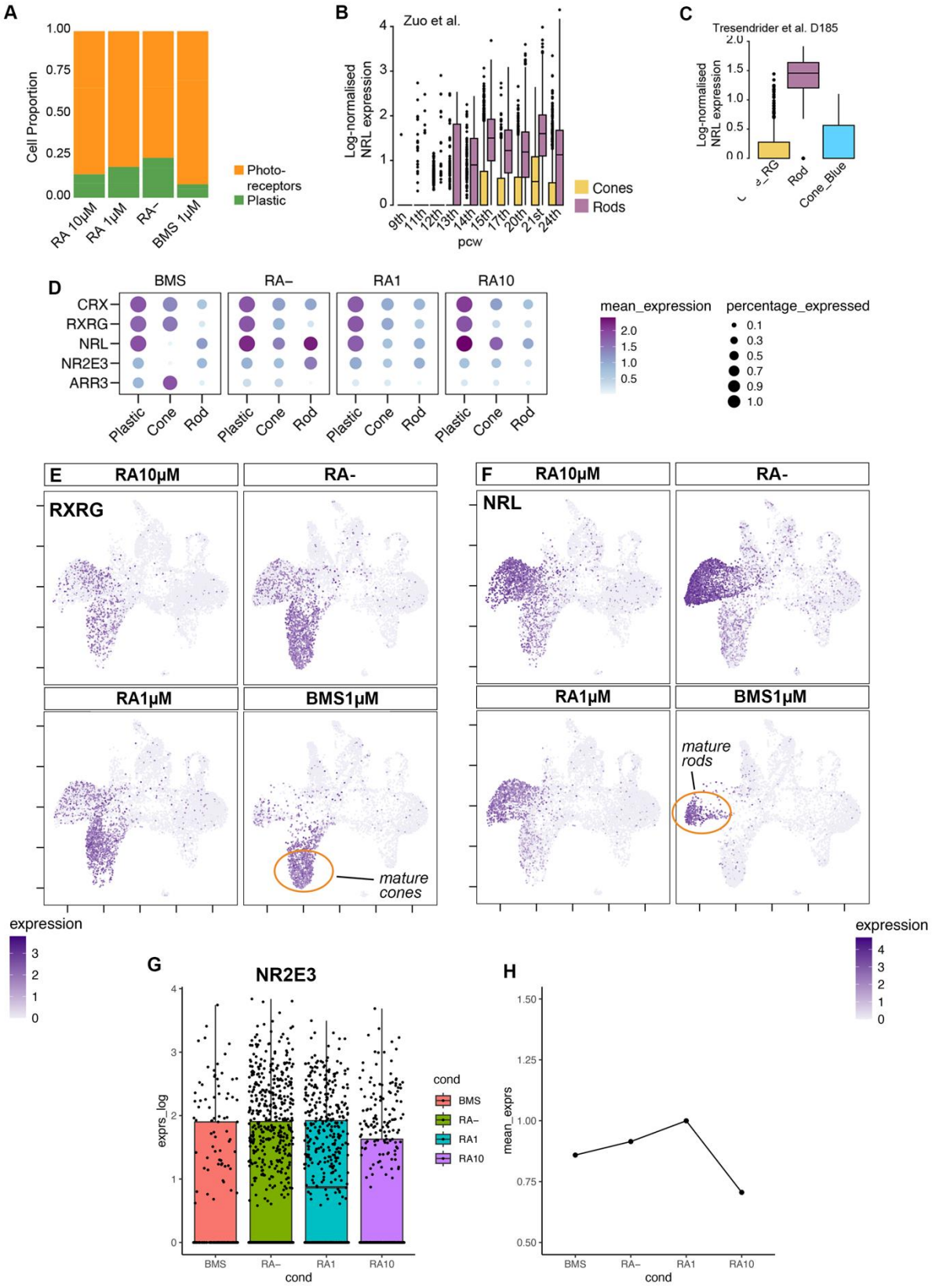
- Lines 325 – 337; Lines 563-569 – plastic photoreceptors – While RXRG is utilized as a marker of cones, it typically distinguishes cones based on marker co-expression (Thrb, Arr3, etc). Recent evidence (Jin, et al., 2025; FASEB) suggests that NR2E3 directly inhibits RXRG expression. Since NRL is upstream of NR2E3, this suggests that NRL+ cells will co-express RXRG until sufficient levels of NR2E3 accumulates to inhibit RXRG expression and cone fate. This is consistent with NRL and NR2E3 knockout phenotypes where all rods default to an S-cone like fate. Can the authors look at NR2E3 expression within the ‘plastic’ photoreceptors? Is the retinoic acid treatment causes reduced NR2E3 in NRL+ photoreceptors, thus resulting in the

‘plastic’ population? Therefore, in the discussion, it is likely better to talk about RXRG expression in early rods (supported by the data and previous publications) instead of a ‘surprising’ result that NRL is in cones. It is this reviewer’s impression that the cones within the rod cluster in the UMAP are mis-annotated based on the automated cell type caller.

Thank you to the reviewer for your insight and for pointing us to this literature. As mentioned above we have amended to include NR2E3 expression analysis which demonstrated a decreased in RA10uM (albeit minimally). The number of plastic cells expressing NR2E3 is also diminished in RA10um conditions (new data now shown in Figure S5).

Additionally, when examining UMAPs of RXRG and NRL expression both expression of NRL within the cone population as well as RXRG expression within the rod population, indicating that the plastic population of photoreceptors observed has the propensity to commit to either fates (new data shown in Figure **S5E,F**).

The discussion now states the following: ‘Our scRNA-seq data revealed unexpected NRL transcript expression in cone cells, despite NRL’s established role in rod specification[3,4]. We also observed as well as RXRG expression within the rod population. This led to the identification of a population of ‘plastic’ photoreceptors co-expressing CRX, NRL, and RXRG, with their abundance being RA concentration-dependent. While transcript contamination during dissociation may contribute to this phenomenon[5-7], RA may also directly induce NRL expression ectopically in cones[8]. These plastic cells could also represent early rod photoreceptor precursors which express RXRG prior to NR2E3-mediated inhibition of RXRG and thus cone-fate commitment[9].’



New Figure S5.

- Figures S3F-M. GS staining does not look like typical Muller glia staining in organoids (S3G) or in human retina (S3M). In fact, the GS staining looks like astrocytes in the Human retina, suggesting that the Müller glia are not present within the retina. Prox1 expression is not limited to Horizontal cells, but also stains bipolar cells.

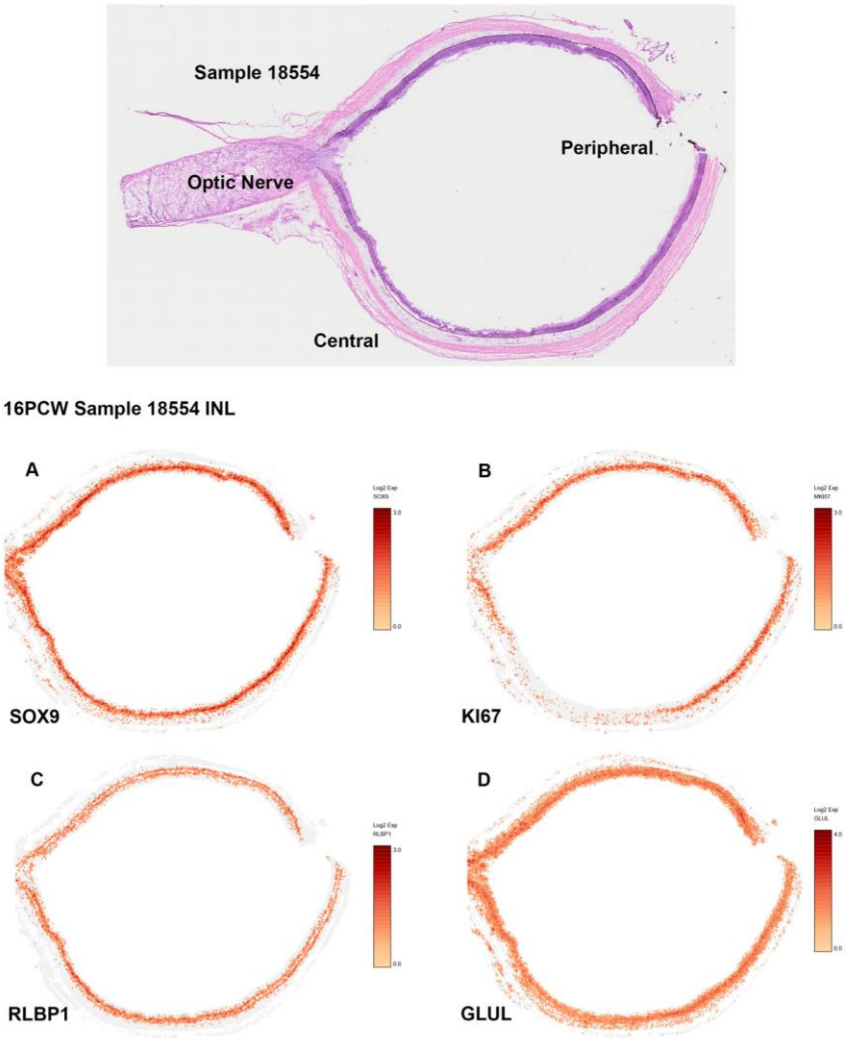
Reply: Müller glia phenotyping, as mentioned by the reviewer, can be challenging at early developmental stages due to overlapping gene expression with RPCs, as highlighted by the reviewers. Typical Müller glia markers such as GS and CRALBP are generally used to delineate Müller glia in the postnatal and adult retina, and expression at these early stages is only beginning. To emphasize this, we explain in the text with the following sentence:
"Albeit weak, possibly due to the onset of expression at this stage, Glutamine Synthetase (GS) validated the identity of Müller glia cells in 16-week-old hROs."

Additionally, for the reviewer's consideration, we now have access to spatial data from the 16 PCW samples, and preliminary analyses indicate that MGs are present at this stage (see figure **Reviewer Response Figure 4** below). Inclusion of this data is beyond the scope of the current manuscript, but we would like to provide it as reassurance.

Müller glia phenotyping, as noted by the reviewer, can indeed be challenging at early developmental stages due to overlapping gene expression with RPCs. Common Müller glia markers such as GS and CRALBP are typically used to delineate these cells in the postnatal and adult retina, and their expression at earlier stages is only beginning to emerge.

To clarify this point, we now explicitly stated this in the manuscript:
Line 288: "Albeit weak, possibly due to the onset of expression at this stage, Glutamine Synthetase (GS) validated the identity of Müller glia cells in 16-week-old hROs."

Figure S3J and K and figure legends have been amended to include the label Bipolar cells.



Reviewer Response Figure 4. H&E brightfield images paired with spatial heatmap visualisation of transcripts of the 16PCW foetal eye highlighting prevalence of mg markers

Over-interpretation or not acknowledging alternative explanations:
- Line 199-203 – EdU incorporation assay. The Authors conclude that RA treatment promotes cell cycle progression. This term is ambiguous between two plausible outcomes. 1) Cell cycle progression of RPCs is faster when with RA treatment, resulting in more RPCs incorporating the EdU during the 16-hour treatment window. Alternatively, 2) RA treatment is biasing cell cycle exit. As EdU doesn't indicate if a cell is remaining in the cell cycle, one has to co-label with a proliferative marker like MKI67 to determine if RA is biasing cells to exit the cell cycle (during the EdU treatment period). Determination of cell cycle length can be performed through FACS analysis of DNA content (performed only on cells within the cell cycle through RPC marker co-expression) OR through a dual BrdU EdU pulse chase and co-labeling with an proliferative marker (see Harris et al., 2018; J Mol Histol).

Reply: We thank the reviewers for pointing this out and we agreed with the alternative explanations we have now changed the text to highlight the results that the experimental data presented is showing. The text now says: Line 214 "This suggests that in elevated RA levels there are more cells entering or progressing

through S-phase pointing out to a larger pool of proliferative progenitors in this condition."

- Line 180-183 – CYP26A1 expression – Is this real staining or an increase in background signal across the ages. The staining seems to be complementary to all nuclear layers. CYP26A1 is localized to the endoplasmic reticulum and is expressed by RPCs and Müller glia. Can the Authors please provide co-labeling with either ER markers in RPCs or definitive Müller glia markers (GLUL)? See Krueger et al., 2024; Differentiation for CYP26A1 staining during primate retina development.

Reply: We thank the reviewer for raising this important point. We observe an increase in signal with CYP26A1 becoming specific to Müller glia as organoids age. We have now also performed additional IHC in 26 weeks old hROs with CYP26A1 and CRALBP showing not only the increase in expression but the specificity to these cells. This has been added to **Figure S2** as panel **N.**

Line 214 now states: 'This suggests that in elevated RA levels there are more cells entering or progressing through S-phase pointing out to a larger pool of proliferative progenitors in this condition.'

Thank you for pointing us out to the Krueger et al. study. We believe this data matches their findings in which "At later stages of development, *CYP2*6A1 expression is restricted to the Müller glia."

- Line 218-220 – While the presence of Vsx+/Sox9- cells does suggest differentiation of bipolar cells, this does not say anything about cell cycle exit versus proliferation. This suggests that the timing of cell fate specification may be advanced in the BMS-treated retina (bipolar cells being specified during a temporal window when they are not normally generated).

Reply: We have now amended the sentence to remove cell cycle exit /proliferation text and to restrict our comment to indicate the early specification of bipolar cells. The text now states: Line 231 'The presence of bipolar cells at this early 10-week timepoint in BMS hROs denoted a premature differentiation of these cells.'

Additional Comments:
Line 108-109 – RGCs and cones are the first-born cell types. While partially accurate, other cell types are also generated very early, including starburst amacrine cells and horizontal cells

Reply: Indeed, we believe this to be more accurate and therefore we have amended the text accordingly. The text now says: Lanes 108-109 'Here, we analysed RGCs and cone photoreceptors specifically, which, together with starburst amacrine cells and horizontal cells, are the first-born cell types of the retina.'

Line 116-119 – "Transcriptomic similarity analysis" – Please provide additional detail in the main text, indicating that spatial transcriptomic analysis was performed on 10 week organoids and that Pearson Correlation was performed between the pseudo-bulk of the spatial transcriptomic analysis and previous scRNA-seq on human fetal

retina. These details are in the Figure legend, but are unclear when reading the results. The details of spatial transcriptomics are then provided later (Line 120-121).

Reply: We understand how this might have caused confusion as the text on the transcriptomic similarity analysis was before we described the spatial transcriptome dataset. We have now moved the text and amalgamated the text in lines 120-121.

The whole passage now reads: 'To capture the relationship of these nascent cells and RPCs we performed 10x Visium spatial transcriptomics on 10-week-old hROs. Recently, Zuo et al., performed scRNA-seq on the foetal retina at various stages of development (9 to 24 PCW)[10]. Due to the large spot diameter of the Visium spatial technology (~5 cells per spot), we performed spot deconvolution of hROs using the Zuo et al., 11-PCW foetal dataset for annotation. Pearson Correlation was performed between the pseudo-bulk transcriptomes of the hROs at 10 weeks and Zuo et al. human foetal retina timepoints which showed that hRO development at 10 weeks most closely resembled the foetal retina at 11-13 PCW (**Figure 1C** and **Table 1,** PSC line 1 in two z-positions A and B, PSC line 2 in two z-positions A and B) validating hROs as a good representation of the native retinal tissue.'

Line 148-149 – Please also reference Figure S2F, as this is very relevant to the spatial organization of the organoids. It is hard to see from the plots, but to ALDH1A1 and ALDH1A3 display complementary patterns (like what would be expected in vivo) or do many dots contain expression of both transcripts?

Reply: We have now referenced Figure S2F. To maintain the order of figure mentions in the text, we have reassigned Panel F as Panel A. This modification also allowed us to enlarge the ALDH1A1 and ALDH1A3 panels, improving visualization and clarity of their expression patterns. Figure legends panels have also been corrected.

Line 194-198 – Further explanation in the figures/legends would be beneficial to distinguish between different experiments. For example Figure 2B and Figure S2B both seem to represent Sytox staining but show different results (BMS 1uM is now significant in Figure 2B). One has to carefully read the text to understand that these experiments represent different timing of RA addition and different ages of ROs.

Reply: We appreciate the highlighting of this potential for confusion. We confirm that Figure 2B shows % of Sytox+ cells in 10-week-old hROs while Figure S2 shows % of Sytox+ cells in 16-week-old hROs and have amended both the figure legend as well as labelled the figure for clarity.

Line 215-216 – 'RA- hROs exhibited the highest percentage of NRL+ rods while no differences were observed in CRX+/NRL- cones across treatments (Figure 2G,H)'. The figure reference should be 2K,L

Reply: This has been amended.

Line 288 - HOTAIRM1 expression has been previously detected in retina (Lu et al., 2020; Dev Cell) as a human-specific marker of cones.

Reply: The text has been amended to state that both HOXB2 and HOTAIRM1 have been seldom described in the context of retinal development.

Lines 395-400 – CellChat analysis cannot suggest 'enhanced functionality' of organoids. While the results are consistent with a more mature organoid with RA- or BMS1uM treatments, it doesn't actually test 1) If synapses are formed, 2) If synapses are functional, 3) if photoreceptors are capable of responding to light and propagating responses to light to the inner retina.
Reply: We have amended the text to remove 'suggesting enhanced functionality of these cells.'

Line 412 – Should Figure 6C,F reference be Figure S6B, F?
Reply: Yes, this has been amended.

Line 437 – Should Figure S6F reference be Figure S6L?
Reply: Yes, this has been amended.

Reviewer #2 (Remarks to the Author):

This manuscript examines how retinoic acid affects human retinal development using hPSC-derived retinal organoids (hROs). The authors combine spatial transcriptomics, single-cell RNA-seq, and immunostaining to show that RA levels regulate retinal maturation. They propose that low RA bias organoids toward a cone-rich, macula-like retina, whereas high RA promotes a peripheral-like identity. The work has clear implications for both basic human retinogenesis and optimization of hROs for disease modelling and cell therapy.
Overall, the study is ambitious, methodologically strong, and potentially impactful, but a few conceptual, methodological, and interpretative issues should be addressed to strengthen the paper.

1. Physiological relevance and RA dosing
The study relies heavily on high exogenous RA (up to 10 μM). It would help to better anchor these conditions in a physiological context.

• Can the authors discuss how 0.1–10 μM RA compares to estimated endogenous RA levels during human foetal retinal development? This would help frame if the levels in the study are physiologic developmental ranges.

Reply: We appreciate the reviewer's request to contextualize our RA dosing. Direct quantitative measurements of endogenous all-trans retinoic acid (atRA) in the *human foetal retina* are scarce; most human studies infer RA activity from enzyme expression (e.g., CYP26A1) rather than absolute concentrations. Nevertheless, available embryonic and neonatal data across vertebrates consistently place physiological RA in the low nanomolar range:

- Live imaging with genetically encoded RA probes in zebrafish embryos estimated peak endogenous RA concentrations at ~6 nM within the hindbrain field, demonstrating a linear source–sink gradient during early development (Shimozono S. et al., 2013, Nature).

- The predicted scleral atRA in mouse and human concentrations in the approximately 3–40 nM range (Dovoriashyna et al., 2025, J. R. Soc. Interface).

On this basis, our applied doses **(**0.1–10 µM**,** i.e., 100–10,000 nM**)** are supraphysiologic relative to endogenous developmental concentrations. This is consistent with widely noted practice in the field that exogenous RA used in experimental systems is often ~1,000-fold higher than endogenous to overcome rapid catabolism, protein binding, and diffusion constraints *in vitro* (Horton and Maden, 1995 and Ghyselinck & Duester, 2019).

Importantly, to anchor our interpretation to developmental physiology, we (i) use RA- conditions as our baseline (no exogenous RA) and (ii) limit RA exposure to defined windows.

We have added sentence to the Methods stating: 'Endogenous RA concentrations during vertebrate embryogenesis are in the low-nanomolar range (~1–10 nM), whereas the 0.1–10 µM doses applied here are supraphysiologic and commonly used to probe RA-dependent pathways in vitro; interpretations are therefore made relative to RA- (no exogenous RA) conditions and constrained exposure windows.'

• The macula/periphery identity claims are intriguing and important, but they rely primarily on transcriptomic signatures and a few marker genes (CALB1, PCP4, etc.).

Reply: We appreciate the reviewer's observation regarding the reliance on transcriptomic signatures and a limited set of marker genes.
While the development of the macula has been well described in terms of morphological features, studies investigating the signalling pathways and molecular cues underlying macular development have been relatively scarce, gaining momentum only in recent years with the advent of scRNA-seq and the isolation of specific retinal regions during development. To date, transcriptional comparisons between macular and peripheral regions have yet to fully elucidate the signalling pathways involved in establishing these domains. Constraints related to loss of spatial integrity during scRNA-seq are now beginning to be addressed through spatial transcriptomics and bespoke computational tools. These advances highlight the need for continued investigation and underscore the novelty and relevance of our findings. To strengthen our claims, we have incorporated additional validation approaches, including immunohistochemistry for CALB1 and PCP4, along with other region-specific markers, to confirm spatial expression patterns at the protein level. Furthermore, we have performed comparative analyses using a high quality publicly available dataset and integrated functional annotations to support macular versus peripheral identity.

• It would be helpful to more clearly define what the authors mean operationally by "macula-like" and "peripheral-like" in hROs and remind the reader of the limitations (e.g., no true foveal pit, no RPE, no vasculature).

Reply: We thank the reviewer for this suggestion. In the revised manuscript, we now provide an explicit operational definition of 'macula-like' and 'peripheral-like' regions in hROs, clarifying that these designations are based solely on cell-type composition and molecular readouts. Importantly, we emphasize that key anatomical features of the human macula and fovea, such as the foveal pit, presence of retinal pigment epithelium (RPE), and vasculature are absent in current organoid models. These limitations are now clearly stated in the Discussion to ensure readers interpret our findings within the appropriate context. The text now states: 'However, we emphasise that these regional 'macula-like' and 'peripheral-like' phenotypes refer to transcriptomic profiles enriched for gene signatures and cell type analysis only as key features of the retina, such as typical morphological features of the macula and fovea, such as foveal pit is not present in current organoids. Moreover, the absence of RPE in hRO models may limit macula-like region formation. Future work should explore combinatorial signalling modulation and incorporation of additional cell types such as RPE, microglia, and endothelial cells to enhance hRO structural and functional fidelity.'

2. Terminology
Be consistent with terms like "RA–" vs "no exogenous RA" vs "control condition" throughout.
Reply: We have amended the manuscript to consistently use 'RA-' as the standard notation. To avoid confusion and ensure clarity, we specify that 'RA-' refers to the absence of exogenous RA only when introducing a new set of results, and this is reiterated in the figure legends and methods.

3. Statistics and quantification
• For all quantifications explicitly state n (number of organoids, number of differentiations, number of fields) in figure legends.
• It would help to mention the statistical tests used and whether corrections for multiple comparisons were applied.
• All staining analysis in the paper should have quantification and statistics performed, and not just show a representative image.

Reply: We appreciate the reviewer's comment and have carefully revised the manuscript to ensure that all missing *n* numbers are included and that appropriate statistical tests are referenced. The only stainings not quantified were GNAT1 and PDE6A/B in Figure 5. These markers are strongly expressed in the outer segment regions, which are inherently difficult to quantify due to their elongated and overlapping morphology, making accurate segmentation and cell-based counting unreliable. Furthermore, our analysis was focused not on cell counts but on the enhanced presence of these markers within the outer segments, which corroborates the findings from the transcriptomic data.

4. Line 109 "few RBMPS+ RGCs" should the gene be spelled as RBPMS?
Reply: This has been added: RNA-binding protein with multiple splicing (RBPMS)

5. Line 1311 RGCs (RBPMS and RXRG). I wasn't aware that RXRG labels RGCs as I thought of it as a cone marker. Can the authors provide evidence to support that it is an RGC marker or is this an error?

Reply: We appreciate the reviewer's comment. The RXRγ antibody used in our study was obtained from Santa Cruz (catalog number sc-365252). A good example of RXRG expression in RGCs in the human retina is shown in Bharathan et al., 2021[11].

Our data from human fetal retina at 10 PCW **(Figure S1H,I)** show that this antibody only weakly labels RGCs and strongly labels photoreceptors. Similarly, in human retinal organoids (hROs), co-localization of RXRγ with RBPMS was rarely observed. This likely reflects the inherent cellular composition of hROs, where ganglion cells represent a small fraction of the population, while cones constitute a larger proportion. Nevertheless, RGCs were accurately quantified using Brn3b.mCherry flow cytometry, ensuring robust identification and analysis of this cell population.

6. Figure 3C should have error bars and statistics.

We agree with the reviewer that inclusion of error bars and statistical analysis is important where biological replicates are available. To generate the single-cell RNA-sequencing data, organoids were pooled prior to sequencing for each condition, resulting in a single biological replicate per condition. Thus, error bars and statistics cannot be meaningfully applied.

To acknowledge this limitation, we have now clarified in the corresponding Methods to explicitly state that the single-cell RNA-sequencing data generated are derived from one differentiation batch with pooled samples of five to six organoids.

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Point by point response to reviewers' comments.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The Authors have addressed many of the reviewer comments sufficiently, providing a wealth of data about retinoic acid signaling in the developing human retina. Many limitations of the study (spot size, cell type calling, etc) are addressed within the manuscript and alternate interpretations have been presented.

A few minor comments remain.

Automated segmentation and cell counter: It is unclear how the CellPose counter is dealing with **non-nuclear markers for cell type counting** and **the identity of the denominator** in many of the figures that look at cell type proportions. This is extremely evident in Supplemental Figure 8, where the denominator for % cells is unclear, as the '% of cells' far exceeds 100%.

Q1. For non-nuclear markers such as ARR3 and opsins, the model was trained using two channels, DAPI and the non-nuclear marker. By analysing both channels simultaneously, the model was able to identify individual cells based on the presence of a single nucleus and accurately distinguish cell boundaries, enabling determination of where one cell ended and another began. Furthermore, to ensure counting fidelity, all images were manually screened, and any images that were not properly segmented were excluded from downstream analysis.
This has now been clarified on Methods.

Q2. The identity of the denominator and cell counting exceeding 100%.

We thank the reviewer for bringing this up. We did not expect that these proportions would sum to 100%, as the different measurements were performed using stains across separate organoids, cell lines, and tissue sections. We did not quantify the relative proportion of a marker-positive cells within the same organoid or section. Instead, we aggregated data from a broad range of samples across multiple organoids and cell lines to construct an estimate of the average proportion of cells expressing each marker.

Q3. Are % S-opsin+ cones (S8J) the % total cells that are s-cones or the % of cones that are also s-opsin+. Just looking at the quantifications in S8J, if total cell number is used, the percentages add up to well over 100% of cells (for example – in the RA1uM condition; %Sox9+ ~30%, % s-opsin+ ~35%, %l/m-opsin+ ~30%, %NRL+ rods ~25%). Is this a result of using non-nuclear markers (ARR3, OPN1SW, OPN1MW)?

Reply: We appreciate the source of confusion regarding these proportions, which likely arises because Supplementary Figure S8 presents two different types of quantitative analyses. Specifically, in Figure S8J we show the proportion of the cone photoreceptor population (ARR3-positive cells) that expresses S-OPSIN, rather than the proportion of S-OPSIN positive cells relative to the total cell population (DAPI). In contrast, Figures S8E-H show the proportion of each cell type normalised to DAPI, representing percentages relative to all cells.

To address this ambiguity, we have relabelled the y-axes of all relevant graphs to explicitly indicate the denominator used for normalisation. Measurements expressed as a proportion of the total cell population are now labelled as “normalised to DAPI,” while measurements expressed as a proportion of the cone photoreceptor population (for L/M-OPSIN and S-OPSIN) are labelled as “normalised to ARR3.”

Q4. Looking at the CellPose counting in Figure S2, ARR3 looks to be annotated in regions where there are no cells (DAPI+) consistent with ARR3 not being a nuclear marker. How does the automated cell counter account for non-overlapping signal?

Reply: See question 1 reply.

Q5. Line 64 – RA does not generate a rod-free zone. It is the absence of RA (presence of RA degrading enzymes) that coordinates the rod-free zone. “RA and [FGF8] signaling promote the formation of a rod-free zone” suggests that RA promotes this phenotype which is misleading.

Reply: We thank the reviewer for bringing this to our attention. This has been amended on the text. The text now states: ‘In the chick retina, fibroblast growth factor 8 (FGF8) signalling and a lack of RA signalling promoted the formation of a rod-free zone composed exclusively of cones, analogous to the fovea at the centre of the primate macula.’

Q6. Lines 170-172 – ‘more spatially restricted’ – This conclusion hard to interpret from the data given the ‘spatial’ signal seems random (not preferential to region of organoid nor retinal layer). Is the ‘randomness’ more a function of expression levels, and therefore likely a technical artifact due to drop-out?

Reply: To avoid confusion we now re-phrased the sentence to state the fo.llowing: “RDH10 and RDH11 were the most abundant, although less abundant than RBP1 (**Figure S2E,F**)…’

Q7. Line 181-186 – What about CYP26C1? Should be mentioned as it is known to be expressed in retina although maybe not at high levels in early development. Cyp26c1 has been recently shown to express in Müller glia in zebrafish (Lahne, 2026; bioRxiv). If not detected, the authors should state this.

Reply: There was little to no expression of CYP26C1. This has been added to the text and referenced.

Line 222 – “SOX2+/SOX9+/VSX2+ RPCs were observed in the high RA conditions” no quantitative data shown to support this conclusion

Reply: In this sentence specifically we were referring to the appearance of bipolar cells which expressed VSX2 only and we highlighted the presence of RPCs (SOX2+/SOX9+/VSX2+) just as a comparison as RPCs have already been quantified via Edu in Figure 2D.

Reviewer #2 (Remarks to the Author):

The authors have satisfactorily addressed the concerns raised in my prior review, and I am now supportive of publication.