NF-κB signaling regulates the formation of proliferating Muller glia-derived progenitor cells in the avian retina.

Isabella Palazzo, Kyle Deistler, Thanh V. Hoang, Seth Blackshaw and Andy J. Fischer
DOI: 10.1242/dev.183418

Editor: James Briscoe

Review timeline

Original submission:	2 August 2019
Editorial decision:	16 September 2019
First revision received:	30 January 2020
Editorial decision:	12 February 2020
Second revision received:	4 March 2020
Accepted:	31 March 2020

Original submission

First decision letter

MS ID#: DEVELOP/2019/183418

MS TITLE: NF-κB signaling regulates the formation of proliferating Muller glia-derived progenitor cells in the avian retina.

AUTHORS: Isabella Palazzo, Kyle Deistler, Thanh V. Hoang, Seth Blackshaw, and Andy J. Fischer

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, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. Each of the referees has provided detailed and constructive comments. They ask for several clarifications and more information particularly for the description and interpretation of the single cell transcriptome data, but other experiments should also be introduced and explained more clearly. For example, the difference between the two doses of NMDA used to damage the retina is not clear. In addition, I agree with Referee 2 that measuring directly NFkB signaling in the uninjured and injured retina would greatly strengthen the study.

If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Reviewer 1

Advance summary and potential significance to field

The manuscript titled, “NF-kB signaling regulates the formation of proliferating Müller glia-derived progenitor cells in the avian retina”, by Palazzo et al., identified prolonged NFkB signaling as a potential block to efficient Müller glia reprogramming. Paradoxically, while they also report an instructive role for resident microglia in initiating NF-kB signaling post-damage (which blocks MG reprogramming), elimination of microglia from the retina (the original source NK-kB induction) now promotes MG reprogramming. This finding, while intriguing raises more questions than it answers. This reviewer would not be bothered by this finding, as long as the rest of the study was presented with greater clarity. There are a lot of data in this paper and it’s extremely difficult to digest into a cohesive story. This manuscript would greatly benefit from extensive editing to simplify what I feel are overly complex figures and to provide a clearer narrative. Specific comments are below.

Comments for the author

Figure 1.
• Figure 1 is extremely difficult to decipher. It’s not clear what important information the reader is meant to learn, or what hypotheses were generated by these datasets that lead to subsequent experiments. I understand the significance of Tnfsf15 in the microglia as the potential signaling source for NF-kB induction.
However, I don’t follow the descriptions of the expression patterns for the other NFk-B players involved or their relevance in this context. To my eye, expression between the various MG populations does not seem terribly distinct. Are there any distinctions in the actual NFk-B transcription factors or any known direct target genes?
• The logical flow of the paper would be better if the Muller glia and microglia were more explicitly highlighted on their own in Figure 1. The other retinal cell data can be included as a supplement. This would also allow Figure 1a and 1b to be enlarged and readable. Also, a schematic of NFk-B signaling would help the reader place all of these gene products within that context.
• The authors should define NIRG in the text on page 12.
• In Figure 1a, why are there so few rods and so many MGC? Shouldn’t rods be the majority of the dataset? Were the data filtered? Are the data from populations of purified MGCs? Also, in the text, the authors should state the exact origin of the sc-seq databases along with the GEO accession numbers.
• In Figure 1l, the authors mention the expression of Tnfrsf21 in MG and MGPCs, but not microglia. Is it not important there? Have the authors accounted for all of the possible TNFa orthologs?

Figure 2.
• The pseudotime analysis shows the expected transition states. However, it’s not clear whether the changes or lack of changes in Fig. 2d are statistically or biologically relevant.

Figure 3.
• Did the authors test whether the inhibitors on their own can drive MG proliferation? This could potentially allude to whether the observed changes between NMDA alone and NMDA + NFkB inhibitor is simply additive (both independently boosting proliferation) rather than directly related to a common damage response pathway (possibly downstream of TNFa).
• In Fig. 3e (left panels), the PHH3 staining (in purple) cannot be seen. What is the significance of the neurofilament marker in Fig. 3e?
• I did not see mention of Fig. 3i.
• The increase in Pax6 expression in Sox2+ cells (Fig. i) is not terribly convincing. If these cells were also labeled with EdU, one would have a stronger case that these are reprogrammed MGs.

Figure 4.
• What is the consequence of prostratin treatment without NMDA? As the author’s point out, it’s curious that TNFSF15 +NMDA did not have an effect. Yes, it’s possible that levels are saturated. This is testable by injecting TNFSF15 without NMDA.

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Figure 5.
• Figure 5f and 5g are mislabeled as 4f and 4g in the text. Also, these data for prostratin would fit better in Figure 4 to illustrate that treatment does not cause cell death which might secondarily result in fewer proliferative MGPCs?
• The authors claim that TNFSF15 caused a significant increase in dying cells, but earlier in the manuscript they suggest that levels within the damaged retina are possibly saturating.

Figure 8.
• I don't entirely understand the inclusion of the FGF and Insulin data. It seems to further muddy the waters as many gene regulatory networks would be expected to be downstream of these pathways. Maybe I'm missing something, but it's not clear how this specifically relates to NFkB signaling at a molecular level.
• Just show MG and astrocyte data for sc-seq and put the rest in a supplement.

Finally, this manuscript would greatly benefit from genetic loss-of-function experiments to corroborate some of the findings with the various inhibitors. AAV-mediated siRNA may be an option?

Reviewer 2

Advance summary and potential significance to field

Injury to the mammalian retina can lead to blindness. In fish, Muller glia respond to retinal injury by dividing and producing a multipotent progenitor population that regenerates all major retinal neuron types. In postnatal chicks, Muller glia also divide in response to retinal injury, but, in comparison to fish, they exhibit a very limited ability to regenerate new neurons. The Fischer lab has been using postnatal chicks to study the signaling pathways underlying Muller glial cell reprogramming, cell division, and neurogenesis. The paper by Palazzo et al., investigates a role for NF-kB signaling in regulating Muller glial cell proliferation in the postnatal chick retina. They report on single cell RNA sequencing data indicating expression of NF-kB signaling components in Muller glia and Muller glia-derived progenitor cells in the chick retina. They also take advantage of a variety of putative NF-kB signaling inhibitors and activators to manipulate NF-kB signaling in the chick retina and conclude NF-kB signaling inhibits formation of MGPCs. Interestingly, it is reported that Muller cells respond differently to activators and inhibitors of NF-kB signaling when microglia are ablated, but the underlying mechanism for these different responses remains unexplored. Overall this paper suggests that NF-kB signaling is important for Muller cell proliferation and that this signaling is regulated by microglia-derived ligands. However, concerns center around the indirect nature of the experiments and the lack of detailed analysis.

Comments for the author

Major concerns:
1. A major concern is that the main conclusion of the paper - that NF-kB signaling is regulated in the injured retina to control Muller cell proliferation - was never directly tested. There is not a single experiment designed to measure NF-kB signaling in the uninjured and injured chick retina, so how do we know it changes with injury or with the drugs that are used in this study? Gene expression changes do not necessarily reflect signaling pathway activity, and most of the pharmacological inhibitors used like sulfasalazine, PGJ2, and prostratin, act on multiple targets and also are metabolized to pharmacologically active compounds.
2. NMDA is used at low (63 nM) and high (1 microMolar) concentrations for different experiments, yet Muller cells responded similarly to both concentrations. It seems to this reviewer that the lower concentration that stimulates Muller cell proliferation is preferable to minimize potential off target effects and for consistency.
3. The distinction between different labels all seemingly marking proliferating Müller glia is not clear. Why are Sox2+/EdU+ cells and Sox2+/NF+/pHisH3+ cells mentioned separately? The authors seem to want to make the data more complete, but clarification would help.
4. Fig. 3h: Please provide the % of Sox2+ cells that are also Pax6+ in NMDA-treated retina +/- sulfasalazine.
5. There is no TUNEL labeling with Colchicine, why? This should be included.

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

6. Is pax6 the only reprogramming factor induced in MGPCs? It seems reasonable to conclude that Muller cell reprogramming precedes proliferation and thus measuring the induction of reprogramming factors prior to proliferation would be a better indicator of reprogramming. Does sulfasalazine have an effect on expression of other reprogramming factors?
Assaying one factor after Muller cells enter the cell cycle is not sufficient to conclude that Nf-kB inhibition promotes Muller glia reprogramming. Also, how does PCG2, SC757, and prostratin affect these genes?
7. Investigating how NF-kB signaling fits into the network of cell-signaling pathways that regulate the formation of MGPCs was limited to Notch signaling, but this group has also shown involvement of MAPK glucocorticoid, Jak/Stat, and retinoic acid. Is NF-kB signaling acting downstream of all these pathways? Also, in the experiments with DAPT and sulfasalazine, a DAPT control should be included. Finally, it is not clear if the pharmacological reagents used in this study also affect Notch signaling; this should be tested.
8. Experiments describing microglia ablation and treatment with pharmacological agents meant to regulate NF-kB signaling suggest these agents act on microglia who are responsible for mediating their effects on Muller cells. How can the authors be sure chlodrinate liposomes don't act on both microglia and Muller cells? Just because Muller cells survive does not mean they do not phagocytose the liposomes and are unaffected by the drug.
In fact, the Hyde lab has shown Muller cells have phagocytic activity.
Finally, the authors have sited an article for >99% microglia ablation within two days. But the following experiments are performed within 24 hours. In this context, the percentage of microglia ablated should be shown for confirmation.
9. p25, top paragraph, last sentence: States "…. signals provided by microglia are required to initiate Muller glial reactivity as a first step toward reprogramming….." This sentence does not reflect the finding that NF-kB acts downstream of Notch signaling in the injured chick retina as suggested in Fig. 3i (it does not appear to be an initiator or a first step).
10. Overall the error bars in most figures are very large with significant overlap in control and experimental conditions, which reduces confidence in data interpretation.

Minor comments.
1. Names of genes are sometimes listed without much explanation and some of them are listed twice with different names and roles. A table detailing everything would have been great.
2. Please expand on the value of the pseudotime analysis. It seems to not add much and we already know Muller glia change over time with injury.
3. p19, top paragraph: change "P112" to P12.
4. p20, top paragraph, penultimate sentence should end with …..retinas missing reactive microglia (Fig. 7c). "microglia" was missing from original.
5. p26, top paragraph, lines 7-9: Please add Ramachandran et al., PNAS 2011 reference to sentence referring to "Wnt signaling promotes formation of proliferating MGPCs in….. which is consistent with findings in mouse and fish retinas…."
6. Text states: "Tnfsf15 was detected only in microglia (Fig. 8c)". But the fig does not show that.
7. Fig 6d, co-localization of GS+EDU does not seem to be increasing 2-fold in prostratin treated retinae as claimed in Fig 6f.

Reviewer 3

Advance summary and potential significance to field

This work advances our knowledge about the molecular signalling pathways that regulate the potential for
regeneration of neural retina. The work is performed in chicken that has a retina with a moderate capability to regenerate and the results may contribute to advance our knowledge how to increase the potential for human retina to regenerate.

Comments for the author

MS ID#: DEVELOP/2019/183418 NF-κB signaling regulates the formation of proliferating Müller glia-derived progenitor cells in the avian retina

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

In this impressive and important work by Palazzo and co-workers, they have profiled and studied Müller glia-derived progenitor cells (MGPCs) in the chicken retina with respect to NF-κB signaling and regulation of Müller cells in neuronal regeneration. They use single cell transcriptome profiling and histological analyses and address the involvement and importance of microglia in the process. They conclude that activation of NF-κB suppress the reprogramming of Müller glia into proliferating MGPCs and that the effect depends on the presence of reactive microglia. Taken together, their findings suggest that NF-κB-signaling is a key pathway in regulating reprogramming of Müller glia into MGPC.

Comments.

1. The authors use 10X single cell profiling to describe the cellular events in a damaged chicken retina. They have used NMDA-induced excitotoxic damage that trigger the response of Müller cells. Single cell transcriptomes were analyzed using tSNE, scatterplots and pseudotime trajectories.
a. The appearance of tSNE clustering plots is known to be highly dependent on both the algorithm and the chosen parameters but no information is provided how the process was performed or on what parameters were used. Sufficient info should be provided so that the analysis may be repeated using the deposited data.
b. It should be described what known cell-type specific markers were employed to identity the generated tSNE clusters (Fig 1).

2. The transcriptome databases were probed for different genes in the studied pathways such Nfkabia and the results are displayed as tSNE clusters and violin-cluster plots. The presentation of the data is not clear.
a. It was concluded in the result part that:
"Nfkbia was prominently expressed in microglia, bipolar cells and NIRG cells, and had scattered expression, at relatively high levels, in Müller glia, MGPCs, amacrine cells and photoreceptors (Fig. 1d,h)".
It is not clear from the text or figs how this conclusion was reached and what the quantitative thresholds were used for the statement. Just by watching fig 1d it is not obvious that the NIRG cell cluster have more prominent expression than several of the other clusters. The quantitative assessment should be explained and objectively supported.
On the same line: It is stated that:
"By comparison, Nfkbib was predominantly expressed by Müller glia in normal and damaged retinas, and by MGPCs, but was less abundant in Müller glia at 48 and 72 hrs after NMDA-treatment (Fig. 1e,h). Similarly Nfkbiz was predominantly expressed by Müller glia in control and damaged retinas, and was less abundant in MGPCs (Fig. 1f,h). "
In Fig. 1e and 1f, the expression pattern is obviously different, although similar. From Fig. 1e, Nfkbib does not seem to be expressed in most Müller glia but only part of the Müller glia. In Fig. 1e, the expression of Nfkbib is higher at the edge of the cluster, while in Fig. 1f the expression of Nfkbiz is more evenly distributed. It is not clear how the result and conclusion was reached based on the patterns presented in the clusters.
b. Part of the methods as well as results is presented in the figure legends and only in the figure legends. This organization makes it very difficult to follow and to find information. This reviewer prefer that relevant methods should be in the methods section or as a supplementary info and results should be in the results part/figures.
c. Page 12. It is stated that:
Among Müller glia, the number of Nfkbia-expressing cells were most abundant at 24 hrs after NMDA-treatment (Fig. 1h). This statement should be supported by a statistical analysis between the groups. The violin/cluster plots may look different but it is not clear what makes the 24 hs time point different from the MGPCs. This comment is valid for several of the claimed differences.
d. The combined violin/cluster plots may be explained to facilitate for the reader.
e. Fig. 1h shows relative expression level in Müller cells /MGPCs. It should be clarified what the relative expression level is.

3. Two different doses of NMDA was used to inflict damage (1 µmole and 63 nmol) without explanation.
Should be motivated. Does the two quite different doses inflict a similar damage.

Statements in the result are not congruent with the presented figures. Examplified:

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

4. Page 15 2d para. "Treatment of NMDA-damaged retinas with PGJ2 or SC757, but not sulfasalazine, resulted in decreased proliferation of microglia (Fig S1). Data for sulfasalazine is not presented so it is not clear how the comparison/conclusion can be made. PGJ2 has both immune and cell count. SC757 has only cells counted.
5a. Page 16 mid para. "There was no significant difference in the number of Sox2+/Edu+ cells in damaged retinas treated with sulfasalazine and DAPT compared to damaged retinas treated with sulfasalazine alone (Fig. 3k)." Figure 3k shows number of Sox9+ cells, not Sox2+ cells.
b. Figure 4a+b shows number of Sox9+ cells, not Sox2+ cells as claimed in results.
6. Page 18, 1st para: It is stated. "Consistent with these findings, numbers of TUNEL-positive cells were significantly reduced in NMDA-damaged retinas treated with PGJ2 compared to controls (Fig 5.e)
TUNEL data is shown for 72h but for sulfasalazine its 4h +24h + 72h. The conclusion may be overstated. Same applies to Fig 4f+g.
7. Page 22, mid para. "Tnfsf15 was detected only in microglia (Fig. 8c)". Figure 8c only show Nfkbia, Nfkbib Nfkbiz, Tnfsf10, Tnfrsf1a, Tnfrsf21
8. Page 23 mid para. "Four consecutive daily treatments with NF-kB inhibitors (sulfasalazine, PGJ2, or SC757) in combination with FGF2 significantly increased the numbers of Edu+/Sox9+ proliferating MGPCs compared to treatment with FGF2 alone (Fig 9a-c)" Data for SC757 is not shown.

9. Minor comments:
a. Introduction, row 12, reference gallina et all., 2014a, 201 ? Truncated reference?
b. Page 22/23. Sentence is truncated. "Similar to trends seen in damaged retinas, levels of Nfkbib and Mfkbiz were relatively high in resting MG, decreased in transitional glia, and Nfkbib increased while Nfkbiz decreased toward (Fig. 8e) -
c. Fig 3. The dose NMDA is indicated in a) (63 nmol) but not in i). Should be clarified. See also comment 3.
d. The use of figure abbreviation should be congruent. Fig vs Figs (Ex. Figs 7, Fig 6-f)
e. The use of reagent nomenclature should be congruent. pHisH3 (Fig 4) vs pHH3 (fig 3)

First revision

Author response to reviewers' comments

Please find attached the revised research article titled "*NF-kB signaling regulates the formation of proliferating Muller glia-derived progenitor cells in the avian retina*" authored by Isabella Palazzo, Kyle Deistler, Thanh V. Hoang, Seth Blackshaw and Andy Fischer to be considered for publication in *Development*.

We have carefully revised the manuscript according to the comments of the editor and reviewers as follows:

The Editor asks that we explain the difference between the two doses of NMDA used throughout the paper, as well as asking that we "*directly measure NFkB in the uninjured and injured retina*". We have performed several dozen Western Blots, and all blots for phospho- specific epitopes of different components of the NF-κB-signaling pathway have failed. Many of the phosphorylated epitopes are not conserved in chick orthologues. Further, none of the antibodies produced plausible patterns of labeling in fixed retinal tissues. However, we obtained antibodies to IκBα which, in Western blot analyses, produced a band at the expect molecular weight. Levels of IκBα are expected to decrease with increased NF-κB signaling activity.
Indeed, we found that the intensity of the IκBα band was significantly decreased in NMDA treated samples compared to saline control samples and was significantly increased by NF-κB antagonists. These data have been added to a new figure (Fig. 3). Additionally, we have elaborated on the rational for using the two different doses in response to reviewer comments below and expanded explanation in the manuscript at relevant points.

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Reviewer 1 Comments.

1. The reviewer states "*Figure 1 is extremely difficult to decipher. It's not clear what important information the reader is meant to learn, or what hypotheses were generated by these datasets that lead to subsequent experiments. I understand the significance of Tnfsf15 in the microglia as the potential signaling source for NF-kB induction. However, I don't follow the descriptions of the expression patterns for the other NFk-B players involved or their relevance in this context.*"
-To provide a cellular context for NF-κB signaling we queried single cell RNA- sequencing (scRNA-seq) databases generated from chick retinas at different times after NMDA- treatment. Among cell-signaling pathways it is common that expression of components of the pathway are dynamically regulated when the pathway is active (reviewed by Purvis and Lahav, 2013 Cell 152(2): 945-956). We have added text to the Results to clarify this issue.

2. The reviewer states "*The logical flow of the paper would be better if the Muller glia and microglia were more explicitly highlighted on their own in Figure 1. The other retinal cell data can be included as a supplement. This would also allow Figure 1a and 1b to be enlarged and readable. Also, a schematic of NFk-B signaling would help the reader place all of these gene products within that context.*"
-The violin plots include scRNA-seq data for only Muller glia and MGPCs, whereas the tSNE plots provide data for retina-wide patterns of expression. We have streamlined the figure to focus on the data relevant to Muller glia. We have generated a new Figure 1 providing a schematic of NF-κB signaling, names of different proteins and corresponding genes, and cites of action of pharmacological agents used in the study.

3. "*The authors should define NIRG in the text on page 12.*"
- We have defined NIRG cells as non-astrocytic inner retinal glia on page 12 and cited the original papers that first characterized these cells.

4. The reviewer asks "*In Figure 1a, why are there so few rods and so many MGC? Shouldn't rods be the majority of the dataset? Were the data filtered? Are the data from populations of purified MGCs?*"
-Unlike the rodent retina, the populations of cell-types in chick retina are not dominated by rod photoreceptors. Nonetheless, the Muller glia have an over-abundant representation in the scRNA-seq databases. This likely results from fortuitous capture-bias and/or tolerance of the Muller glia to the dissociation process. We have added this information to the Methods and Materials.

5. "*In Figure 1l, the authors mention the expression of Tnfrsf21 in MG and MGPCs, but not microglia. Is it not important there? Have the authors accounted for all of the possible TNFa orthologs?*"
- TNFα has not been identified in the chick genome, but it has been suggested that chicken Tumor Necrosis Factor Super Family 15 (TNFSF15)/TL1A may function in its place (Migone et al., 2002; Takimoto et al., 2005). We added and edited text in the Results to describe the different TNF orthologues in the chick.

6. With respect to Figure 2, the reviewer states "*The pseudotime analysis shows the expected transition states. However, it's not clear whether the changes or lack of changes in Fig. 2d are statistically or biologically relevant.*"
-The pseudotime analysis segregated the cells into resting Muller glia, reactive Muller glia and MGPCs across pseudotime. Mapping expression levels of NF-kB components to Muller glia/MGPCs that are scattered across pseudotime, independent of their library of origin, implies discrete functions of NF-kB in Muller glia across the different states. We have performed branched pseudotime analyses and re-mapped the cells from different pseudotime states back into Seurat to perform statistical analyses of expression levels between the different pseudotime states. The text in the Results has been revised to reflect these revisions.

7. The reviewer asks "*Did the authors test whether the inhibitors on their own can drive MG proliferation? This could potentially allude to whether the observed changes between NMDA alone and NMDA + NFkB inhibitor is simply additive (both independently boosting proliferation) rather than directly related to a common damage response pathway (possibly downstream of TNFa)*".
-We applied NF-κB inhibitors to undamaged, normal retinas. We failed to observe any changes in glial phenotype or proliferation and added this data to supplemental Fig. 2. There is little to no

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

active NF-κB signaling in resting Muller glia, as indicated by Western Blots for IκBα (added to Fig. 3), thus the NF-κB-inhibitors had nothing to inhibit and resulted in no changes in Muller glia, including no reprogramming to proliferating MGPCs. We have added text to the Results to describe these outcomes and Discussion of interpretations.

8. The reviewer states "*In Fig. 3e (left panels), the PHH3 staining (in purple) cannot be seen. What is the significance of the neurofilament marker in Fig. 3e?*"
-Expression of neurofilament and phospho-histone H3 (pHH3) are known to be transiently expressed by proliferating MGPCs (Fischer and Reh, 2001). pHH3 is expressed by proliferating cells during late G2 and M phase of the cell cycle. Labeling for pHH3 and neurofilament serves to corroborate that the EdU-labeled Sox9$^+$ cells are, indeed, proliferation MGPCs. We have added text to the Results to improve clarity. We have edited Figure 3E to change the color of the pHH3 from dark red to blue to better permit visualization.

9. The reviewer states "*I did not see mention of Fig. 3i.*"
-On page 15 we added a reference to Fig. 3i in the following sentence: "In addition, application of sulfasalazine following NMDA resulted in increased expression of stem cell associated transcription factor Pax6 in Sox2-positive cells (Fig. 3h-j)…"

10. The reviewer states "*The increase in Pax6 expression in Sox2+ cells (Fig. 3i) is not terribly convincing. If these cells were also labeled with EdU, one would have a stronger case that these are reprogrammed MGs.*"
-We have added text to clarify to use of this technique and cited previous papers wherein levels of a nuclear label are measured within ROI's defined by Sox2 or Sox9 in the nuclei of Muller glia. The Pax6 is increased in every Sox2+ nuclei. Text has been added to the Results and Methods to better describe and support the implementation of this technique.

11. With respect to Figure 4, the reviewer asks "*What is the consequence of prostratin treatment without NMDA? As the author's point out, it's curious that TNFSF15 +NMDA did not have an effect. Yes, it's possible that levels are saturated. This is testable by injecting TNFSF15 without NMDA.*"
-Consecutive daily injections of prostratin or TNFSF15 to undamaged, normal retinas failed to stimulate reprogramming of Muller glia. We propose that damage or treatment with FGF2 is required to "gate" or recruit NF-κB signaling, which is likely mediated by signals derived from activated microglia, into a network of signaling pathways that regulate the formation of MGPCs. This "gating" effect is similar to that seen with the effects of EGF on mouse Müller glia, wherein damage was required for Muller glia to up-regulate receptor and render these glia responsive to EGF (Close et al., 2006 Glia). Similarly, activation of BMP/Smad-, Jak/Stat, Wnt/ß-catenin, and retinoic acid-signaling must be combined with retinal damage or FGF2- treatment to drive the proliferation of MGPCs in the chick retina (Todd et al., 2017 Glia; Todd et al., 2016 Sci Rep; Gallina et al., 2016 Dev Neurobiol; Todd et al., 2018 Stem Cells). Text has been added to the Results and Discussion to address this issue. Prostratin alone and TNFSF15 alone showed no induction of proliferation. These data have been added to supplemental Figure 2.

12. The reviewer indicates that "*Figure 5f and 5g are mislabeled as 4f and 4g in the text. Also, these data for prostratin would fit better in Figure 4 to illustrate that treatment does not cause cell death which might secondarily result in fewer proliferative MGPCs?*"
-We have correctly referred to the figure panels in the manuscript.

13. The reviewer states "*The authors claim that TNFSF15 caused a significant increase in dying cells, but earlier in the manuscript they suggest that levels within the damaged retina are possibly saturating*."
- We have adjusted our interpretation as follows "It is possible that TNF-receptors on Müller glia were saturated in damaged retinas and, thus, addition of exogenous TNFSF15 had no significant effect upon the formation of MGPCs."

14. With respect to Figure 8, the reviewer states "*I don't entirely understand the inclusion of the FGF and Insulin data. It seems to further muddy the waters as many gene regulatory networks would be expected to be downstream of these pathways. Maybe I'm missing something, but it's not clear how this specifically relates to NFkB signaling at a molecular level.*"
-We use FGF2+insulin treatment to stimulate the formation of MGPCs in the absence of damage.

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Treatment with FGF2+insulin is known to selectively activate MAPK-signaling and a network of additional cell-signaling pathways thereafter, while activating microglia, but not incurring neuronal damage (Fischer et al., 2009a,b). The purpose of these studies was to investigate whether NFkB suppresses the formation of MGPCs without damaging the retina, or whether NFkB activity is dependent upon neuronal damage. Our findings suggest NFkB is part of the network of pathways that are activated during the formation of MGPCs in retinas treated with FGF2 in the absence of retinal damage.

15. The reviewer states "*Just show MG and astrocyte data for sc-seq and put the rest in a supplement.*"
-All of the violin plots, pseudotime analyses, and tSNE plots in Figure 7 represent scRNA-seq data from only the Muller glia. There are no astrocytes in the chick retina (Fischer et al 2010 *Glia*; Rompani and Cepko 2010, *J Neurosci*). The tSNE plots in Figures 1 and 8 show all retinal cell types that express NFkB components and TNF-related ligands and receptors to demonstrate predominant expression within the Muller glia.

16. The reviewer states "*Finally, this manuscript would greatly benefit from genetic loss-of-function experiments to corroborate some of the findings with the various inhibitors. AAV-mediated siRNA may be an option?*"
-This is a great idea. However, the post-hatch chick retina is refractory to gene transfer. Over the past 20 years we have tried electroporation, sonoporation, nanoparticles, liposomes, AV5, AAV2, AAV9, scAAV6, A3C, Shh10, RCAS, etc and failed miserably. The trophism of viruses is drastically different in chick retina compared to that of rodent retina, and the chick microglia are hypersensitive and destroy any AAV-transfected cells within one week of transfection. We have preliminary data indicating that delivery or RCAS to embryonic retina or electroporation of Tol2 transposase, and hatching the chicks to generate transgenic retinas is possible. However, this technique is far from wide-spread implementation.

Reviewer 2

1. The reviewer states "*A major concern is that the main conclusion of the paper - that NF-kB signaling is regulated in the injured retina to control Muller cell proliferation - was never directly tested. There is not a single experiment designed to measure NF-kB signaling in the uninjured and injured chick retina, so how do we know it changes with injury or with the drugs that are used in this study? Gene expression changes do not necessarily reflect signaling pathway activity, and most of the pharmacological inhibitors used, like sulfasalazine, PGJ2, and prostratin, act on multiple targets and also are metabolized to pharmacologically active compounds.*"
-We agree that this is a necessary piece of data to validate this study. Given the failure of antibodies to provide convincing labeling in retinal tissue, we relied upon gene expression patterns to assess what cell types that are involved and changes in express of the necessary signaling components of NFkB. Given that this pathway is post-translationally regulated, we agree that gene expression changes alone are not sufficient to indicate signaling activity. Thus we probed western blots for expression of total P65 and phospho-P65 to asses active NFkB. Unfortunately the chick primary amino acid sequence is not conserved at phosphorylation sites and we failed to identify antibodies that recognize chicken phospho-P65 in immunohisto- chemistry or western blot analyses. We next probed western blots for total IkBa levels. When NFkB-signaling is activated, IkBa is degraded, allowing NFkB transcription factors to be freed in the cytoplasm allowing for nuclear translocation. Thus, when NFkB is inactive IkBa levels should be high, and when NFkB-signaling is active IkBa levels should decrease. We probed western blots from protein extracts from saline control, NMDA, Prostratin, TNFSF15, and NMDA+Sulf treated retinas. The findings from these western blot analyses are consistent with notion that NFkB-signaling is low in normal retinas, high in damaged retinas, and decreased with sulfasalazine-treatment. We have added those results throughout the Results section in the manuscript and added representative images and histograms to a new Figure 3.

2. The reviewer states "*NMDA is used at low (63 nM) and high (1 microMolar) concentrations for different experiments, yet Muller cells responded similarly to both concentrations. It seems to this reviewer that the lower concentration that stimulates Muller cell proliferation is preferable to minimize potential off target effects and for consistency.*"
- There is a positive correlation with NMDA dose, increased retinal damage/cell death, and

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

increased numbers of proliferating MGPCs (Fischer et al., 2008). Thus, applying drugs that increase proliferation of MGPCs with 1000 nmol NMDA will result in little or no further increase in numbers of proliferating cells. The lower dose of NMDA appears to "prime" the Muller glia for reprogramming or render the Muller glia receptive to activation (Fischer et al 2009a), and thereby "leaving room" for further increases in numbers of proliferating MGPCs. To test for possible decreases in proliferation we use a higher concentration of NMDA to create a larger pool of proliferating MGPCs to better distinguish changes. For example, data in the current manuscript indicate about 30 EdU+ MGCPs per field of view (~14,000 μm^2) for the lower dose of NMDA and about 100 EdU+ MGPCs for the higher dose of NMDA. We have added text to the Results to better explain the rational for using the different doses of NMDA.

3. The reviewer states "*The distinction between different labels all seemingly marking proliferating Müller glia is not clear. Why are Sox2+/EdU+ cells and Sox2+/NF+/pHisH3+ cells mentioned separately? The authors seem to want to make the data more complete, but clarification would help.*"
-This concern is addressed above, according to similar comments from reviewer #1.

4. The reviewer states "*Fig. 3h: Please provide the % of Sox2+ cells that are also Pax6+ in NMDA-treated retina +/- sulfasalazine.*"
-All of the Sox2+ Muller glia nuclei express some level of Pax6, thus measurements of Pax6 represent increases across all Muller glia. Text has been added to the Results to better describe that all Sox2+ Muller glia up-regulate Pax6 with inhibition of NFkB.

5. The reviewer states "*There is no TUNEL labeling with Colchicine, why? This should be included.*"
-In our paradigm for colchicine damage, we wait 10 days after treatment to assess numbers of surviving ganglion cells. Stanke and Fischer (2010) showed that after colchicine- treatment of the chick retina, cell death is scattered during the first 5 days following treatment and there is no cell death after day 6. Thus, delayed or accelerated cell death would be difficult to pin-point given the relatively large "time-window" where death could occur. Thus, determination of end-point neuronal survival is more reliable and simpler than assaying for numbers TUNEL+ cells collectively across the first 5 days after treatment. We have added this rationale to the Results.

6. The reviewer asks and states "*Is pax6 the only reprogramming factor induced in MGPCs? It seems reasonable to conclude that Muller cell reprogramming precedes proliferation and thus measuring the induction of reprogramming factors prior to proliferation would be a better indicator of reprogramming. Does sulfasalazine have an effect on expression of other reprogramming factors? Assaying one factor after Muller cells enter the cell cycle is not sufficient to conclude that Nf-kB inhibition promotes Muller glia reprogramming. Also, how does PCG2, SC757, and prostratin affect these genes?*"
-Proliferation of MGPCs is considered to be the culminative read-out of reprogramming. scRNA-seq provide compelling data for a comprehensive correlation of progenitor-related transcription factors and markers of proliferation in MGPCs in both fish and chick model system. We have added text to the Results to clearly state this rationale. Accordingly, we have focused on proliferation assays to best assess reprogramming of Muller glia into MGPCs.

7. "Investigating how NF-kB signaling fits into the network of cell-signaling pathways that regulate the formation of MGPCs was limited to Notch signaling, but this group has also shown involvement of MAPK, glucocorticoid, Jak/Stat, and retinoic acid. Is NF-kB signaling acting downstream of all these pathways? Also, in the experiments with DAPT and sulfasalazine, a DAPT control should be included. Finally, it is not clear if the pharmacological reagents used in this study also affect Notch signaling; this should be tested."
-The question as to where NFkB fits into the hierarchy of cell signaling networks that regulate the reprogramming of Muller glia is good. However, we feel this is beyond the current scope of the paper. We have plans for these studies and plans to establish scRNA-seq (and possibly scATAC-seq) to determine comprehensive, cell-level changes in gene expression that are downstream of changes in NFkB- and Notch-signaling. These studies are expected to take many months and significant resources to complete, and, thus, remains beyond the scope of the current study. We have removed the DAPT data because of the preliminary nature of these findings.

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

8. With respect to the data in Figure 7, the reviewer asks *"How can the authors be sure chlodrinate liposomes don't act on both microglia and Muller cells? Just because Muller cells survive does not mean they do not phagocytose the liposomes and are unaffected by the drug. In fact, the Hyde lab has shown Muller cells have phagocytic activity. Finally, the authors have sited an article for >99% microglia ablation within two days. But the following experiments are performed within 24 hours. In this context, the percentage of microglia ablated should be shown for confirmation."*
-We have reported previously (Zelinka et al 2012; Fischer et al., 2014) that DiI-labeled clodronate-liposomes accumulate at the vitread surface of the retina, are only taken-up by reactive microglia, and do not deplete numbers of Muller glia. We have added the rationale that Muller glia in normal and damaged retinas are not affected by clodronate-liposomes. We have clarified the description of the paradigm that the clodronate-liposome are applied at P6 and other experimental procedures begin 3 days later at P9.

9. p25, top paragraph, last sentence: States "…. signals provided by microglia are required to initiate Muller glial reactivity as a first step toward reprogramming….." This sentence does not reflect the finding that NF-kB acts downstream of Notch signaling in the injured chick retina as suggested in Fig. 3i (it does not appear to be an initiator or a first step).
-We have edited this statement as follows "these findings suggest that pro-inflammatory signals provided by microglia are required to stimulate Müller glial reactivity as a step toward reprogramming into MGPCs."

10. The reviewer states "*Overall the error bars in most figures are very large with significant overlap in control and experimental conditions, which reduces confidence in data interpretation.*"
-It must be noted that the statistical analyses were paired t-tests to compared differences between treated and control eyes from the same individual to account for inter-individual differences. To better convince the reader we have added P values to each histogram.

Minor comments.
1. The reviewer states "*Names of genes are sometimes listed without much explanation and some of them are listed twice with different names and roles. A table detailing everything would have been great.*"
-We have created a new figure and table (new Figure 1) to define genes, proteins, drugs and cites of action.

2. The reviewer states "*Please expand on the value of the pseudotime analysis. It seems to not add much and we already know Muller glia change over time with injury."*
-As stated on Monocle's website: "*Monocle introduced the strategy of ordering single cells in pseudotime, placing them along a trajectory corresponding to a biological process.*
Monocle orders cells by learning an explicit principal graph from the single cell genomics data with advanced machine learning techniques (Reversed Graph Embedding), which robustly and accurately resolves complicated biological processes." We performed pseudotime analysis to order the resting MG, reactive MG, MGPCs, and MG transition from reactivity back to resting in an unbiased manner according to the large changes in gene expression, which fortuitously corresponds to ordering resting Muller glia opposite proliferating MGPCs across pseudotime, with reactive Muller glia spanning pseudotime. This is reminiscent of zebrafish Muller glia, but very different to mouse Muller glia ordered across pseudotime (Hoang et al., BioRxiv). We believe it is informative to assess levels of expression of NFkB components across pseudotime to implicate NFkB components in resting/reactive Muller glia.

3. "*p19, top paragraph: change "P112" to P12.*"
-This error has been corrected in the text.

4. "p20, top paragraph, penultimate sentence should end with …..retinas missing reactive microglia (Fig. 7c). "microglia" was missing from original."
-This error has been corrected in the text.

5. "p26, top paragraph, lines 7-9: Please add Ramachandran et al., PNAS, 2011 reference to sentence referring to "Wnt signaling promotes formation of proliferating MGPCs in….. which is consistent with findings in mouse and fish retinas…."

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

-This citation has been added to the text.

6. Text states: "Tnfsf15 was detected only in microglia (Fig. 8c)". But the fig does not show that".
-The reviewer is correct and we have omitted this statement and reference to the figure.

7. "Fig 6d, co-localization of GS+EDU does not seem to be increasing 2-fold in prostratin treated retinae as claimed in Fig 6f."
-The number of EdU+ cells is, in fact, not different (P>0.05) but the proportion of EdU+ cells that are GS+ is increased with prostratin-treatment following NMDA+sulfaslazine.

Reviewer 3
1a. "*The appearance of tSNE clustering plots is known to be highly dependent on both the algorithm and the chosen parameters but no information is provided how the process was performed or on what parameters were used. Sufficient info should be provided so that the analysis may be repeated using the deposited data.*"
-We have added information to the Methods describing the parameters used to establish tSNE plots. tSNE plots were generated and probed using Cell Ranger and Cell Browser software (10X Genomics) using the following parameters: perplexity = 30, float = 0.5, max dimensions = 2, and max iterations = 1000. This information has been added to the Methods.

1b. "*It should be described what known cell-type specific markers were employed to identity the generated tSNE clusters (Fig 1).*"
-We have added lists of gene that were used to identify different types of retinal cells that were clustered together in tSNE plots.

2a. The reviewer states "*It was concluded in the result part that: "Nfkbia was prominently expressed in microglia, bipolar cells and NIRG cells, and had scattered expression, at relatively high levels, in Müller glia, MGPCs, amacrine cells and photoreceptors (Fig. 1d,h)". It is not clear from the text or figs how this conclusion was reached and what the quantitative thresholds were used for the statement. Just by watching fig 1d it is not obvious that the NIRG cell cluster have more prominent expression than several of the other clusters. The quantitative assessment should be explained and objectively supported.*"
-We have softened this interpretation to suggest that NFKBIA had scattered expression in clusters of cells identified as microglia, NIRG cells, Muller glia, MGPCs, bipolar cells, amacrine cells and photoreceptors.

2a (continued) "*On the same line: It is stated that: "By comparison, Nfkbib was predominantly expressed by Müller glia in normal and damaged retinas, and by MGPCs, but was less abundant in Müller glia at 48 and 72 hrs after NMDA-treatment (Fig. 1e,h). Similarly, Nfkbiz was predominantly expressed by Müller glia in control and damaged retinas, and was less abundant in MGPCs (Fig. 1f,h)." In Fig. 1e and 1f, the expression pattern is obviously different, although similar. From Fig. 1e, Nfkbib does not seem to be expressed in most Müller glia but only part of the Müller glia. In Fig. 1e, the expression of Nfkbib is higher at the edge of the cluster, while in Fig. 1f the expression of Nfkbiz is more evenly distributed. It is not clear how the result and conclusion was reached based on the patterns presented in the clusters.*
-We have performed statistical analyses (Wilcoxon rank sum with Bonferroni correction) and included the statistically significant changes to the violin plots in the revised figure. Further, we have edited the text in the Results to better describe the significant changes in gene expression.

b. "Part of the methods as well as results is presented in the figure legends and only in the figure legends. This organization makes it very difficult to follow and to find information. This reviewer prefer that relevant methods should be in the methods section or as a supplementary info and results should be in the results part/figures."

-We have move the details regarding numbers of cells captured in each scRNA-seq library from the legends to the Methods. In addition, we have included details of markers used to identify clusters of cells to the Methods.

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

c. "*Page 12. It is stated that: Among Müller glia, the number of Nfkbia-expressing cells were most abundant at 24 hrs after NMDA-treatment (Fig. 1h). This statement should be supported by a statistical analysis between the groups. The violin/cluster plots may look different but it is not clear what makes the 24 hs time point different from the MGPCs. This comment is valid for several of the claimed differences.*"
-We have performed statistical analyses (Wilcoxon rank sum and Bonferroni correction), edited the figures to include significance, and adjusted text in the Results to represent the statistically significant changes in expression.

d. "*The combined violin/cluster plots may be explained to facilitate for the reader.*"
-Explanations have been added to the Methods: "The violin/scatter plots provide the probability density distribution and relative expression level of genes within an identified cluster of cells. The width of the violins provides a measure of the relative abundance of cells within a cluster that are present at a relative expression level. Wilcoxon rank sum with Bonferoni correction was used to determine whether there are significant differences in relative expression levels."

e. "*Fig. 1h shows relative expression level in Müller cells /MGPCs. It should be clarified what the relative expression level is.*"
-Relative expression level refers to Log Transcripts Per Million (TPM) normalized across scRNA-seq libraries. This has been added to the Methods.

3. The reviewer states "*Two different doses of NMDA was used to inflict damage (1 µmole and 63 nmol) without explanation. Should be motivated. Does the two quite different doses inflict a similar damage.*"
-We have addressed this issue above (Reviewer 2 point #2).

4. The reviewer states "*Page 15 2d para. "Treatment of NMDA-damaged retinas with PGJ2 or SC757, but not sulfasalazine, resulted in decreased proliferation of microglia (Fig S1). Data for sulfasalazine is not presented so it is not clear how the comparison/conclusion can be made. PGJ2 has both immune and cell count. SC757 has only cells counted.*"
-These differences may occur because the drugs are acting at different cites of the pathway and perhaps the effects on microglia are secondary to changes in the Muller glia (which are directly affected by the drugs). We have added text to the Results and histograms to supplemental figure 1.

5a. The reviewer states "*Page 16 mid para. "There was no significant difference in the number of Sox2+/Edu+ cells in damaged retinas treated with sulfasalazine and DAPT compared to damaged retinas treated with sulfasalazine alone (Fig. 3k)." Figure 3k shows number of Sox9+ cells, not Sox2+ cells.*"
-This error has been corrected in the text.

b. "*Figure 4a+b shows number of Sox9+ cells, not Sox2+ cells as claimed in results.*"
-This error has been corrected in the text.

6. "Page 18, 1st para: It is stated. "Consistent with these findings, numbers of TUNEL-positive cells were significantly reduced in NMDA-damaged retinas treated with PGJ2 compared to controls (Fig 5.e) TUNEL data is shown for 72h but for sulfasalazine its 4h +24h + 72h. The conclusion may be overstated. Same applies to Fig 5f+g."
-The abundance of TUNEL+ cells peaks at 24hrs after treatment (Fischer et al., 1998), and the relative abundance of TUNEL+ cells that appear after NMDA-treatment appears to be consistent at different times after treatment up to 72hrs, regardless of treatment. Nevertheless, we have softened the interpretation to "suggesting that PGJ2 may be neuroprotective".

7. The reviewer states "*Page 22, mid para. "Tnfsf15 was detected only in microglia (Fig. 8c)". Figure 8c only show Nfkbia, Nfkbib, Nfkbiz, Tnfsf10, Tnfrsf1a, Tnfrsf21.*"
-We have omitted mention of TNFSF15 with regard to Figure 8.

8. "Page 23 mid para. "Four consecutive daily treatments with NF-kB inhibitors (sulfasalazine, PGJ2, or SC757) in combination with FGF2 significantly increased the numbers of Edu+/Sox9+ proliferating MGPCs compared to treatment with FGF2 alone (Fig 9a-c)" Data for SC757 is not

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

shown."
-The text has been edited to correctly reflect the data presented in the figure.

9. Minor comments:
a. "*Introduction, row 12, reference Gallina et all., 2014a, 201 ? Truncated reference?*"
-This error has been corrected.

b. "*Page 22/23. Sentence is truncated. "Similar to trends seen in damaged retinas, levels of Nfkbib and Mfkbiz were relatively high in resting MG, decreased in transitional glia, and Nfkbib increased while Nfkbiz decreased toward (Fig. 8e*)."
-We have edited this text.

c. "*Fig 3. The dose NMDA is indicated in a) (63 nmol) but not in i). Should be clarified. See also comment 3.*"
-We have added 63nmol to figure 3i.

d. "*The use of figure abbreviation should be congruent. Fig vs Figs (Ex. Figs 7, Fig 6-f).*"
-This has been corrected.

e. "*The use of reagent nomenclature should be congruent. pHisH3 (Fig 4) vs pHH3 (fig 3).*"
-This has corrected to use pHH3 for consistency.

Thank you for considering this manuscript for publication.

Sincerely,
Andy Fischer

Second decision letter

MS ID#: DEVELOP/2019/183418

MS TITLE: NF-κB signaling regulates the formation of proliferating Muller glia-derived progenitor cells in the avian retina.

AUTHORS: Isabella Palazzo, Kyle Deistler, Thanh V. Hoang, Seth Blackshaw, and Andy J. Fischer

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 BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees remain interested in your work, but continue to have some significant criticisms and recommend a substantial revision. All of the referees request clarifications and additional information to help them navigate the data. I think these are helpful suggestions given the complexity of some of the figures. In addition, there appear to be 3 substantive issues that need to be addressed:
- Referee 1 highlights that experiments in Figure 6 do not have PBS injection controls for comparison.
- Referee 2, in particular, questions the specificity of the pharmacological reagents used to manipulate NfKB signaling. Providing evidence of specificity or acknowledging potential off-target effects would address this.
- Referee 2 also indicates that this is no direct evidence demonstrates that NFkB signaling in Muller glia is responsible for regulating the response to retinal injury. Discussing this issue seems appropriate.

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

I also found the paper to be a long read and if you can condense the text, perhaps by keeping the Discussion focussed on the issues directly related to the Results, I think it will help readers.

If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

I commend the authors for their revised manuscript. It is much improved. I have just a few minor comments/suggestions.

Comments for the author

Figure 1. I still take issue with Figure 1 as it is difficult to follow. At the end of the figure description, the authors conclude, “Taken together, these findings indicate that essential components of the NF-κB pathway are dynamically expressed in Müller glia after damage and during the process of reprogramming into MGPCs”.
After all these data, is this the only thing we have learned? I suggest the authors summarize the findings beyond a vague term like “dynamically expressed” as this will better set up the remainder of the paper.

Figure 6. Why did the authors not analyze a PBS injected control for the experiments in this figure? It seems important to know whether the level of rescue approaches an undamaged retina.
The magnification of Fig. 6H (bottom right) appears to be a bit lower than the other 3 panels. Please double check this.

Figure 7. In the text “EdU+/HuD+; Fig. 6c or EdU+/Otx2+; not shown” should be Fig. 7C. Also, Fig. 7 should be ordered with the description in the text.

Figure 9. When describing the expression data in Figure 9, the authors should end with a brief conclusion statement.

Reviewer 2

Advance summary and potential significance to field

Palazzo et al., use scRNAseq and pharmacological reagents to suggest that microglia activate an NFkB signaling pathway in Muller glia that regulates their reprogramming and proliferation in the NMDA damaged chick retina.

Comments for the author

The revised paper by Palazzo et al., suggests a role of NFkB signaling in generation of MGPCs in the injured chick retina. They used scRNAseq data to group retinal neurons and glia into distinct cell types. NFkB signaling components are expressed widely in the retina with expression detected in all MG populations. NFkB signaling is often engaged by TNF signaling, and their scRNAseq data suggests TNF ligands and receptors are expressed by MG/MGPCs and microglia. Their scRNAseq

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

data suggests small, but statistically significant changes in expression of many of these signaling components in MG/MGPCs after NMDA treatment. The authors use a variety of pharmacological reagents to manipulate NFkB signaling in the injured retina but do not show specificity of action and thus leave open the possibility that these reagents are acting independent of their effects on NfkB; indeed many of these inhibitors are known to act on multiple signaling systems.
Finally, the authors report that MG reprogramming and proliferation is regulated by microglia and propose that microglia are responsible for activating NFkB signaling in Muller glia. Although the revised paper by Palazzo has been improved, the data is mostly descriptive and correlative with no direct evidence that NFkB signaling in Muller glia is responsible for regulating MG response to retinal injury.
Detail of concerns:
1. Fig. 2A,B tSNE plots: I do not understand why control reps 1 and 2 (Fig. 2A) do not overlap better. It seems like a bipolar cell subset were highly enriched in rep1, while all retinal cell types were represented in rep2.
Also, I don't understand why NMDA-treated retinas appear to lack retinal neurons and are almost exclusively MG or MGPCs - what happened to retinal neurons in these samples? I think this data would be clearer if presented in different tSNE plots - one with control and identification of the cell types and others with NMDA treatment showing how these glial and neuron populations change with NMDA treatment.
2. Fig. 2C,C': These panels add little to the paper and the unique microglia expression of markers to identify microglia could just be mentioned in text as done for retinal neurons; this would allow them to enlarge panels 2A, B so the writing in panels could be read.
3. Fig. 2D-G: it is not clear where this data comes from - are these plots showing increased expression after 72hrs of NMDA-treatment? This should be clearly stated in the legend or on the panels. Also, it is important to know which retinal cell types are expressing these NFkB signaling components in the uninjured retina. This data should be added. It is not provided in the violin plots that only show changes in MG/MGPCs. Finally, the significance of the heterogeneous induction of these genes in MG/MGPCs as indicated in panels D-G should be added.
4. IkBa Western blot suggests a role for NFkB signaling in the retina, but the gene is expressed in most retinal cell types with expression being most predominant in bipolar cells and microglia (Fig. 2D) so a Western blot with whole retina does not help in evaluating if this signaling pathway is specifically regulated in Muller glia following retinal injury.
Furthermore, the protection of IkBa from degradation by sulfasalazine is quite modest (Fig. 3A,B). Evaluating the effect of additional regulators of NFkB signaling like PGJ2, prostratin, and TNFSF15 would help lend credence to the idea that IkBa is reporting NFkB signaling in the injured retina. Also a time course analysis for IkBa expression would help determine when this pathway is activated/repressed after NMDA treatment.
5. Sulfasalazine, PGJ2 and SC757 are inhibitors of NFkB signaling as shown in Fig. 1; however, only PGJ2 and SC757 suppress microglia proliferation while sulfasalazine had no effect on microglia proliferation in the NMDA-treated retina (supplemental Fig. 1). Furthermore, it was stated that prostratin had no effect on microglia proliferation in the NMDA treated retina. When assaying cell death, it is reported that sulfasalazine and PGJ2 inhibit cell death, TNFSF15 stimulates cell death while prostratin has no effect on cell death. In contrast, all these drugs, except TNFSF15 regulate Muller glia proliferation in the NMDA damaged retina. Together these data suggest that these pharmacological reagents are acting on multiple signaling systems and make it difficult to accept the authors conclusion that NFkB signaling is solely responsible for their effects on MG/MGPCs.
6. The authors suggest that ligands from invading immune cells are responsible for activating NFkB signaling in Muller glia after retinal injury with NMDA. Support for this could be obtained by ablating microglia and assaying NFkB levels by Western blot +/- NFkB inhibition in the injured retina. Although being able to show NFkB regulation in Muller glia remains a concern.

Reviewer 3

Advance summary and potential significance to field

The data contributes to the understanding of what signalling pathways regulate the glioses and dedifferentiation of Müller glia cells into Müller cell-derived progenitors after an excitotoxic lesion to the retina. It may be important to understand how the Müller cells are regulated either in order to keep them from dedifferentiating or to stimulate them to become retinal progenitors with

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

capacity to contribute to cell regeneration. Müller cells are important for maintaining retinal homeostasis and this may be essential during a lesion and thus keeping them from becoming retinal progenitors may be as important.

Comments for the author

The ms is without page numbers and it is hard to refer to specific parts. This contributes to the complexity.
Citations from the manuscript text is therefore copied from the text in order to indicate the sentences or parts adressed in the comments.

Essential revisions 1-1 The presentation of the results in old figure 1; new figure 2 is more comprehensible, however it is still very complex and not easy to digest. It contains too much information and some parts are made so small in order to fit the graphs into the figure, that they are hard to read. The relative expression plot with data from pseudotime analysis Fig 2Q is too small to read.

1-2 All time- and data points are not referred to in the results. Fig 2H shows a more pronounced down-regulated expression of Nfkbib at the 24hrs NMDA treatment group compared to the 48&72 hrs group. Still the 48&72 hr timepjoint is only one mentioned. A similar situation is for Nfkbiz expression. Only the 48&72 hrs time point and the MGPC were compared and explained an in the text. Comparison of violin-plots in 2H, the 24hs time points should therefore be motivated more clearly.
Cited from the revised manuscript: “By comparison, NFKBIB was predominantly expressed by Müller glia in normal and damaged retinas, and by MGPCs but was significantly less abundant in Müller glia at 48 and 72 hrs after NMDA treatment, and further down-regulated in MGPCs (Fig. 2E,H).”

2-1 The NMDA dose was commented on in the initial review. However, not all relevant aspects were revised.
Because the dose of NMDA is affecting the level of proliferation of MGPCs, it should be specified when presenting the results in Figure 2 and not just referred to as in previous work.
Cited from the revised manuscript: 3. “The proliferation of MGPCs in the chick retina is maximal with higher (1000 ≥nmol) doses of NMDA, and numbers of proliferating MGPCs are diminished with lower (≤80 nmol) doses of NMDA (Fischer et al., 2004).”

3-1 -Prostratin and TNFSF15 are both used to activate NF-κB pathway. Please provide in the text an explanation what are the effects?
Citation: 5. “By comparison, application of prostratin following a NMDA (1 µmol) had no effect upon numbers of dying cells (Fig. 6F), whereas application of TNFSF15 following NMDA resulted in a modest, but significant, increase in numbers of dying cells (Fig. 6G).”

4 -In Fig. 8C, the expression level of Nfkbia is displayed but it is not mentioned in the text. Include in text and discussion or remove from the figure.
Citation: “Although the absence of microglia had no effects upon the relative expression of NF-κB components in Müller glia in saline-treated retinas, levels of expression of NFKBIB and TNFRSF21 were reduced in NMDA-damaged retinas missing reactive microglia (Fig. 8C).”

5 - There is still confusion if parts of the results are about Sox9 or Sox2. Sox2 is mentioned in the text and the Sox9 in the figure 8I for the same data set.
Citation: 8. “Interestingly, treatment with prostratin or TNFSF15 caused significant increases in numbers Sox2+ /EdU+ cells in microglia-depleted NMDAdamaged retinas (Fig. 8I-K).”

6. The results for TNFRSF21 described in the cited text are not obvious in the expression data plots in Fig 9F.
Citation "Relative expression of TNFRSF21 was high in resting Müller glia, decreased in activated Müller glia and increased in MGPCs (Fig. 9E,F).”

Minor comments:
An extra comma between “By” and “comparison”. Citation: “By, comparison, in NMDA damaged retinas treated with sulfasalazine...."

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

A sentence misses an essential part between “Müller glia” and “the absence of microglia”, making the sentence obscure.
Citation: “Expression levels of VIM were significantly increased as a result of damage, but levels were not different in saline-treated Müller glia the absence of microglia, and increased in Müller glia in NMDA-damaged retinas where microglia were ablated (Fig. 8B,C).”

Second revision

Author response to reviewers' comments

Please find attached the revised research article titled “*NF-kB signaling regulates the formation of proliferating Muller glia-derived progenitor cells in the avian retina*” authored by Isabella Palazzo, Kyle Deistler, Thanh V. Hoang, Seth Blackshaw and Andy Fischer to be considered for publication in *Development*.

We have carefully revised the manuscript according to the comments of the editor and reviewers as follows:

Comments from Editor

(1) Referee 1 highlights that experiments in Figure 6 do not have PBS injection controls for comparison.

-We have added saline-injected controls providing data for normal numbers of ganglion cells per retinal area (0.34 mm^2) to Figures 6I and 6J.

(2) Referee 2, in particular, questions the specificity of the pharmacological reagents used to manipulate NfKB signaling. Providing evidence of specificity or acknowledging potential off- target effects would address this.

-We have modified interpretations and we add text to the Results to discuss that we cannot exclude the possibility of off-target effects with the agents used in our studies.

(3) *Referee 2 also indicates that this is no direct evidence demonstrates that NFkB signaling in Muller glia is responsible for regulating the response to retinal injury. Discussing this issue seems appropriate.*
-We have added text to the Discussion regarding the possibility that NFkB-signaling in retinal cells, in addition to Muller glia, may secondarily impact neuronal survival and the formation of proliferating MGPCs.

(4) I also found the paper to be a long read and if you can condense the text, perhaps by keeping the Discussion focussed on the issues directly related to the Results, I think it will help readers.

-We have carefully edited the text in the Discussion to better focus on Results.

Comments from Reviewer 1:
(5) Figure 1. I still take issue with Figure 1 as it is difficult to follow. At the end of the figure description, the authors conclude, “Taken together, these findings indicate that essential components of the NF-κB pathway are dynamically expressed in Müller glia after damage and during the process of reprogramming into MGPCs”. After all these data, is this the only thing we have learned? I suggest the authors summarize the findings beyond a vague term like “dynamically expressed” as this will better set up the remainder of the paper.

-We have simplified the scRNA-seq data, included a supplemental data, and included text to

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

better describe the findings from scRNA-seq in Figure 2 (formerly Figure 1). We have added the following text to better summarize the seq data: Damage-induced changes in expression of components of the NFkB pathway and TNF-ligands/receptors in Muller glia implies that these signals are involved in the responses of Muller glia to retinal damage, and may be involved in the reprogramming of Muller glia into MGPCs.

(6) Figure 6. Why did the authors not analyze a PBS injected control for the experiments in this figure? It seems important to know whether the level of rescue approaches an undamaged retina. The magnification of Fig. 6H (bottom right) appears to be a bit lower than the other 3 panels. Please double check this.

-We have included counts of Brn3+ ganglion cells per 0.34 mm2 from whole mounted undamaged retinas. We have added these data to Figure 6. Additionally, we double checked and have corrected the magnification error of the bottom right panel of Figure 6H.

(7) Figure 7. In the text "EdU+/HuD+; Fig. 6c or EdU+/Otx2+; not shown" should be Fig. 7C. Also, Fig. 7 should be ordered with the description in the text.

-We have edited this within the text.

(8) Figure 9. When describing the expression data in Figure 9, the authors should end with a brief conclusion statement.

-We have added the following statement to wrap-up the results presented in Figure 9:

"Taken together, these findings indicate that in retinas treated with insulin and FGF2 components of the NF-kB pathway and TNF-receptors are differentially expressed by Müller glia, thereby suggesting that NF-kB- and TNF-signaling may influence the formation of MGPCs in the absence of neuronal damage."

Comments from Reviewer 2:
(9) The authors use a variety of pharmacological reagents to manipulate NFkB signaling in the injured retina, but do not show specificity of action and thus leave open the possibility that these reagents are acting independent of their effects on NFkB; indeed many of these inhibitors are known to act on multiple signaling systems.

-As discussed above, we have added text to indicate that we cannot exclude off-target effects for the pharmacological agents.

(10) Fig. 2A,B tSNE plots: I do not understand why control reps 1 and 2 (Fig. 2A) do not overlap better. It seems like a bipolar cell subset were highly enriched in rep1, while all retinal cell types were represented in rep2. Also, I don't understand why NMDA-treated retinas appear to lack retinal neurons and are almost exclusively MG or MGPCs - what happened to retinal neurons in these samples? I think this data would be clearer if presented in different tSNE plots - one with control and identification of the cell types and others with NMDA treatment showing how these glial and neuron populations change with NMDA treatment.

-NMDA-treatment damages or destroys amacrine and bipolar cells in the chick retina. Thus, many of these neurons are absent or fail to be captured because they are stressed and may not survive the capture process. Accordingly, diminished numbers of inner retinal neurons are present in the scRNA-seq libraries from NMDA-treated retinas. The point, in part, of the tSNE plots was to demonstrate that unsupervised PCA and embedding clearly distinguishes the Muller glia from different treatments into distinct clusters of cells whereas the embedding of neuronal cell types does not result in distinct clustering.

-The distinct clustering of bipolar cells in control rep1 appears to be a bi-product of filtering and normalization through 10X Cell Ranger. More stringent filtering and normalization through Seurat V3 followed by tSNE or UMAP embedding eliminates the inconsistency in bipolar cells, yet maintains distinct clustering of Muller glia from different times after damage. Since the focus of this study is the glial cells. We have incorporated the newly generated UMAPs in Figure 2.

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

(11) Fig. 2C,C': These panels add little to the paper and the unique microglia expression of markers to identify microglia could just be mentioned in text as done for retinal neurons; this would allow them to enlarge panels 2A, B so the writing in panels could be read.

-We have removed the inset panels and enlarged panels 2A and 2B to improve readability.

(12) Fig. 2D-G: it is not clear where this data comes from - are these plots showing increased expression after 72hrs of NMDA-treatment? This should be clearly stated in the legend or on the panels.

-These data represent patterns of expression in control and NMDA at 24, 48 and 72 hrs after treatment. The tSNE plots in 2D-G and 2I-L have been revised and simplified to UMAP plots 2C-E. We have added text to legend to improve clarity.

(13) Also, it is important to know which retinal cell types are expressing these NFkB signaling components in the uninjured retina. This data should be added. It is not provided in the violin plots that only show changes in MG/MGPCs.

-We have generated a new supplemental figure to provide data for expression of NFkB components in control retina.

(14) Finally, the significance of the heterogeneous induction of these genes in MG/MGPCs as indicated in panels D-G should be added.

-The significance is calculated and presented for the violin plots which specifically analyze levels of expression in the MG and MGPCs.

(15) IkBa Western blot suggests a role for NFkB signaling in the retina, but the gene is expressed in most retinal cell types with expression being most predominant in bipolar cells and microglia (Fig. 2D) so a Western blot with whole retina does not help in evaluating if this signaling pathway is specifically regulated in Muller glia following retinal injury. Furthermore, the protection of IkBa from degradation by sulfasalazine is quite modest (Fig. 3A,B). Evaluating the effect of additional regulators of NFkB signaling like PGJ2, prostratin, and TNFSF15 would help lend credence to the idea that IkBa is reporting NFkB signaling in the injured retina.
Also a time course analysis for IkBa expression would help determine when this pathway is activated/repressed after NMDA treatment.
-Unfortunately, the western blot data is limited given that levels are measured across all retinal cells in homogenates. Ideally, we would have phospho-specific antibodies to different NFkB components to immunofluorescently label cells in tissue sections as a read-out of active cell signaling. However, we have screened nearly 20 different antibodies, paired with different methods for antigen retrieval, and failed to produce convincing patterns of labeling. The phosphorylation sites for many NFkB components are not conserved from mammal to chicken, thus the phospho-specific antibodies were unsuccessful. Indeed, only one of these antibodies worked well for western blots. We feel that running additional experiments to obtain triplicate samples and running numerous additional western blots is beyond the current scope of the paper.

(16) *Sulfasalazine, PGJ2 and SC757 are inhibitors of NFkB signaling as shown in Fig. 1; however, only PGJ2 and SC757 suppress microglia proliferation, while sulfasalazine had no effect on microglia proliferation in the NMDA-treated retina (supplemental Fig. 1). Furthermore, it was* stated that prostratin had no effect on microglia proliferation in the NMDA treated retina. When assaying cell death, it is reported that sulfasalazine and PGJ2 inhibit cell death, TNFSF15 stimulates cell death while prostratin has no effect on cell death. In contrast, all these drugs, except TNFSF15, regulate Muller glia proliferation in the NMDA damaged retina. Together these data suggest that these pharmacological reagents are acting on multiple signaling systems and make it difficult to accept the authors conclusion that NFkB signaling is solely responsible for their effects on MG/MGPCs.

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

-We have adjusted our interpretations to address the possibility that pharmacological agents may be acting on other retinal cells, in addition to Muller glia, to manifest changes in the formation of MGPCs. Text has been added to the Results and Discussion to address this issue.

(17) The authors suggest that ligands from invading immune cells are responsible for activating NFkB signaling in Muller glia after retinal injury with NMDA. Support for this could be obtained by ablating microglia and assaying NFkB levels by Western blot +/- NFkB inhibition in the injured retina. Although being able to show NFkB regulation in Muller glia remains a concern.

-Western blot data for NFkB levels +/- microglia would not provide cell-level context and fail to provide unambiguous evidence for changes in NFkB-signaling in Muller glia. The purpose of the western blots was to indicate an increase in NFkB in the retina following damage, and indicate efficacy of NFkB-inhibition. We do have an NFkB-reporter line of mice and we have very preliminary data indicating that NFkB-signaling is exclusively active in Muller glia following NMDA-damage and this activity is absent when the microglia are ablated with PLX5622.
However, these data are very preliminary and are intended to form the foundation of a separate publication centered on the mouse model with combinations of targeted genetic manipulations and pharmacological manipulations.

Comments from Reviewer 3:
(18) The presentation of the results in old figure 1; new figure 2 is more comprehensible, however it is still very complex and not easy to digest. It contains too much information and some parts are made so small in order to fit the graphs into the figure, that they are hard to read. The relative expression plot with data from pseudotime analysis Fig 2Q is too small to read.

-Figure 2 has been revised to further simplify the data and improved readability, particularly panels for the legends.

(19) All time- and data points are not referred to in the results. Fig 2H shows a more pronounced down-regulated expression of Nfkbib at the 24hrs NMDA treatment group compared to the 48&72 hrs group. Still the 48&72 hr timepjoint is only one mentioned. A similar situation is for Nfkbiz expression. Only the 48&72 hrs time point and the MGPC were compared and explained an in the text. Comparison of violin-plots in 2H, the 24hs time points should therefore be motivated more clearly. *Cited from the revised manuscript: "By comparison, NFKBIB was predominantly expressed by Müller glia in normal and damaged retinas, and by MGPCs, but was significantly less abundant in Müller glia at 48 and 72 hrs after NMDA treatment, and further down-regulated in MGPCs (Fig. 2E,H)."*
-This portion of Results has been edited to describe all portions of the data presented in Figure 2.

(20)The NMDA dose was commented on in the initial review. However, not all relevant aspects were revised. Because the dose of NMDA is affecting the level of proliferation of MGPCs, it should be specified when presenting the results in Figure 2 and not just referred to as in previous work. Cited from the revised manuscript: 3."The proliferation of MGPCs in the chick retina is maximal with higher (1000 ≥nmol) doses of NMDA, and numbers of proliferating MGPCs are diminished with lower (≤80 nmol) doses of NMDA (Fischer et al., 2004)."

-We have added information regarding the high dose (1000 nmol) of NMDA used in Figure 2.

(21) Prostratin and TNFSF15 are both used to activate NF-κB pathway. Please provide in the text an explanation what are the effects?
Citation: 5. "By comparison, application of prostratin following a NMDA (1 µmol) had no effect upon numbers of dying cells (Fig. 6F), whereas application of TNFSF15 following NMDA resulted in a modest, but significant, increase in numbers of dying cells (Fig. 6G)."

-The TNFSF15 likely activates different isoforms of TNF receptors and may activate signaling pathways in addition to NFkB, and thereby elicit different effects upon cell death when compared to those of prostratin. We have added text to the Results to better explain these results.

(22) In Fig. 8C, the expression level of Nfkbia is displayed but it is not mentioned in the text. Include in text and discussion or remove from the figure. Citation: "Although the absence of microglia had

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

no effects upon the relative expression of NF-κB components in Müller glia in saline-treated retinas, levels of expression of NFKBIB and TNFRSF21 were reduced in NMDA- damaged retinas missing reactive microglia (Fig. 8C)."

-We have added text to describe expression of NFKBIA to the Results: "Although damage induced a significant decrease in *NFKBIA*, the presence of microglia had no significant effect upon levels in normal and damaged retinas (Fig. 8C)."

(23) There is still confusion if parts of the results are about Sox9 or Sox2. Sox2 is mentioned in the text and the Sox9 in the figure 8I for the same data set. Citation: 8. "Interestingly, treatment with prostratin or TNFSF15 caused significant increases in numbers Sox2+ /EdU+ cells in microglia-depleted, NMDA damaged retinas (Fig. 8I-K)."

-We have corrected the text to indicate that the label is Sox9.

(24) The results for TNFRSF21 described in the cited text are not obvious in the expression data plots in Fig 9F. Citation "Relative expression of TNFRSF21 was high in resting Müller glia, decreased in activated Müller glia, and increased in MGPCs (Fig. 9E,F)."

-We have edited the text to better describe the changes in expression of TNFRSF21 that are illustrated by the pseudotime and violin plots in Fig. 9.

(25) An extra comma between "By" and "comparison". Citation: "By, comparison, in NMDA damaged retinas treated with sulfasalazine...."

-This has been corrected.

(26) A sentence misses an essential part between "Müller glia" and "the absence of microglia", making the sentence obscure.
Citation: "Expression levels of VIM were significantly increased as a result of damage, but levels were not different in saline-treated Müller glia the absence of microglia, and increased in Müller glia in NMDA-damaged retinas where microglia were ablated (Fig. 8B,C)."

-This sentence has been edited to improve clarity.

Thank you for considering this manuscript for publication.

Sincerely,
Andy Fischer

Third decision letter

MS ID#: DEVELOP/2019/183418

MS TITLE: NF-κB signaling regulates the formation of proliferating Muller glia-derived progenitor cells in the avian retina.

AUTHORS: Isabella Palazzo, Kyle Deistler, Thanh V. Hoang, Seth Blackshaw, and Andy J Fischer
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.

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Reviewer 1

Advance summary and potential significance to field

This manuscript provides evidence that NF-κB signaling normally suppresses Müller glial cell reprogramming to a progenitor-like identity. This finding may eventually inform strategies to promote Müller glial mediated retinal regeneration in mammals.

Comments for the author

I feel the manuscript is much improved. This is especially true for Figure 1.

Reviewer 3

Advance summary and potential significance to field

Already stated

Comments for the author

No further comments

© 2020. Published by The Company of Biologists under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).