nature portfolio

Peer Review File

Common and divergent gene regulatory networks control injury-induced and developmental neurogenesis in zebrafish retina.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Müller glia in the zebrafish retina respond to injury by generating all retinal cell types. Different injury paradigms lead to the preferential loss of different retinal cell types. This offers the unique possibility to assess whether the reaction of Müller glia also preferentially replaces the lost cell type(s) over the others and which gene expression/regulatory elements are are responsible for any differences. Furthermore, they address whether regenerative neurogenesis recapitulates developmental neurogenesis and find it doesn't. The authors present a thorough analysis of the regulation of gene expression and regulatory elements and discover a number of interesting functional interactions. Most importantly, mmp9 is shown to regulate the generation of amacrine and ganglion cell relative to photoreceptors. A number of potential regulators of regenerative neurogenesis are touched upon, but not further investigated (e.g. Il1beta).

Overall, this is a carefully presented analysis that will be useful for a number of fields in which neurogenesis and cell fate decisions are investigated. However, the results are not always stringently bound to the biological model(s) and are overall a bit hard to follow because of that. This limits the broader accessibility and attractiveness of the data presented here.

In particular, a visualisation of the proposed cell fate transition and decision points (as first described from line 59) would aid understanding of the (in principal) strong conceptual framework.

major:

The author state that, different from development, neurons born after injury are not eliminated over time (e.g. in line 82, also line 534f). This is not conclusively shown or referenced. Furthermore, this statement seems to contradict their statement in line 132f that speculates that removal of "excess immature neurons" might be happening.

In the section line 127 - 141 it remains unclear whether NMDA significantly damages photoreceptors or not.

In line 146, no difference in the number of EdU labelled neurons is reported to occur between 7DR and 21DR - could this be a balance between cell proliferation and cell death? What is the evidence for/against this possibility?

The authors state that there they have not found genes for cilia in their expression profile, apart from FoxJ1a, which regulates neurogenesis in the retina in the present study. Cultured MGs have been described in mammals (PMID: 25504432), this should be discussed here.

The section around line 169 seems to suggest a replacement of lost neurons between 60 hours post light damage, when ONL shows the most pronounced loss of nuclei and 72 hours. Is 12 hours a reasonable time for the replenishment of ONL nuclei and what is the evidence for that?

Section starting line 332: there's a strong *down*regulation of several genes that are not discussed, e.g. TNFb, tnfsf12, stat1a, while Il11b is upregulated at the 36 hour time point - these should be discussed more clearly.

Discussion: Discussions of the results in the first section (page 13) should much more clearly be related back to the model. Schematics clearly illustrating which cell types are lost in each lesion model and which are replaced via which steps are needed to aid interpretation of the results (also would help statements in lines 530 ff).

lines 570ff: the authors speculate, based on their data from the mmp9 mutant that mmp9 might control the maturation of il1b and that this may mediate some of the observed phenotype. This is

a very testable hypothesis, since the activation of il1b can be inhibited by caspase 1 inhibition.

lines 582: the distinct progenitors described here should be highlighted in the figures.

minor:

line 6: Hitchcock (small i)
line 112: that HAD incorporated
line 115: define "extensive"
line 131: is the "slight decrease" reported here statistically significant?
line 307: "inhibiting GENERATION OF inner retinal neurons"
line 448: "with either uninjected or injected..." there's a grammar problem here
line 508: "that continue to proliferate"
line 529: "amacrines" - jargon

Reviewer #2 (Remarks to the Author):

Lyu et al., 2023

The work by Lyu et al., 2023 provides a significant advancement in our understanding of the molecular mechanisms regulating retina regeneration in zebrafish upon NMDA and light lesions. The authors use a combination of single cell-, single nucleus- and ATAC-sequencing to detect gene expression changes that underlie the two different lesion paradigms, and between regeneration and retinal development. All retinal neurons, with the exception of horizontal cells, are regenerated in both the light lesioned and the NMDA retinae, hence showing that the two paradigms are not as specific as previously thought in terms of ablating distinct retinal neuronal types. Nevertheless, a biased differentiation of MGPCs towards photoreceptors in the light lesioned retina and towards inner neurons in the NMDA-lesioned retina is apparent. Moreover, the authors suggest that matrix metalloprotease Mmp9 may promote generation of inner retinal neurons as compared to photoreceptors. They also point out shared and distinct gene expression between regeneration and development of the retina. Finally, they provide evidence that the transcription factor Foxj1a may be necessary for neuronal regeneration of the adult retina, but not during development.

This reviewer commends the authors for the huge effort to provide this overview of the sc-gene expression changes underlying retina regeneration using several, cutting-edge technologies. Specifically, the manuscript provides a thorough description of the later events of retina regeneration (up to 21 days post-lesion), which have so far been less well described and understood. This comprehensive study of retina regeneration in zebrafish is likely to be of lasting importance in the field, and will support genereation of future hypotheses and functional studies in the field. recommends some revision of this manuscript. I have the following comments and suggestions for revisions.

MAJOR COMMENTS
1. Although the manuscript is of a clear and significant importance for the understanding of retina regeneration in zebrafish, the flow of the text and figures is difficult to follow. The readability of the manuscript is likely to improve if the frequent mismatches between the main text and the figures, and with their captions, are corrected.

1a. Specifically, the flow of the text should better follow the order of the figures, to avoid frequent jumps between figure panels. For example, the authors describe first figure 2D, then jump back to describe figure 2C (compare line 208 to line 218). This applies to other figures in the manuscript: first figure S5C/D and then S5A, B, first figure 5E/H and then a jump to figure 6 G,H (lines 413-414).

1b. There are several mismatches between text and related figures, specifically in lines: 229-231; 234-237, 255-258, 258-260, 274-276; 295-295; also, what does the magenta color of the bar plots in figure 3E indicate? The plot looks unclear and difficult to relate to the text. In general, the authors may want to be more precise and exhaustive in their description of figure panels, at least for those mentioned in the highlighted lines. Line 304-figure panel S5H seems to support the opposite of what is stated in this line. There is a severe mismatch between the entire Figure 3 and its caption: panels described in the caption are not matched in the figure, which makes it very hard to understand.

1c. In Figure 1G there is no indication of the uninjured control, whereas in Figure 1J it is not clear what the control for NMDA injections is (uninjected? Vehicle injected?).The uninjured control is also missing from Figure S2.

1d. The nomenclature in Figure 2C does not match the nomenclature in Figure 2D. In Figure 2C the authors list retinal ganglion cells, amacrine cells, bipolar cells, cones, rods, while in Figure 2D the word "precursor" is added to the previous names. The authors should provide a clear definition of what they designate as a "precursor" (see point 2).

1e. Figure panels 4C and 4D are not clearly explained: there is no legend for the colors of the bar plots, as well as for the symbols (what does the circle stand for? And the square? What do E, G, P stand for? Please, specify. The same applies to Figure 6C, 6E, 6F). The way Figure 4F is described is highly unclear to me: lines 338-340. The same applies to figure 6F.

2. The nomenclature used in the manuscript is sometimes confusing. This applies specifically to the terms "resting" Müller glia, "reprogrammed" Müller glia, "activated" Müller glia and "precursor".

2a. How does a reprogrammed MG differ from an activated one? Activated MG is defined in Hoang et al., 2020, but in the present manuscript it seems to be used interchangeably with the term "reprogrammed" MG. How do the two cell populations differ (If they do)?

2b. Please, define what a precursor is in the context of retina regeneration: from the manuscript, it appears that "precursor" defines an immature or differentiating neuron. However, please note that in zebrafish retina development the word precursor refers to specific neuronal progenitors that undergo the last one or two mitoses to generate a specific class of neurons (e.g., horizontal cell precursors described in Godinho et al., 2007; cone precursors described in Suzuki et al., 2013; all reviewed in Amini et al., 2018).

3. Lack of evidence for statements in:

3a. Line 66: is there any published evidence that the excess of regenerated neurons does not integrate into the extant retinal circuit ?

3b. Lines 344-346: it is hard to find the info in the cited table;

3c. Lines 353-354: there is no evidence shown for the implication stated in these lines - maybe move to the discussion section ?

3d. Lines 348-354 significantly interrupt the flow of the text before and after, and the corresponding figure 4G does not fit well with the rest of the panels in figure 4.

4. The authors do not discuss the absence of horizontal cells and their precursors in their lesioned retinae, which are reported however by Lahne et al., 2021 and Celotto et al., 2023. onecut1, which is necessary for HC development and expressed strongly in HC precursors (Celotto et al., 2023), appears as a differentially upregulated motif in Figure 2H. How do the authors explain this upregulation, in light of the claimed absence of regenerated HCs and HC precursors upon injury?

5. Figure S7G and line 496: In the image shown for the foxj1a mutant, lamination of the retina

looks disrupted, in contrast to what is stated in the text (line: 496). The retinae in the mutant look smaller, and plexiform layers are hardly distinguishable, compared to controls. Also, why is lamination delayed in the foxj1a mutant, compared to the control: did the authors check whether the foxj1a mutant retina 'catches up' and has developed correctly at time points later than 96 hpf?

6. It is unclear what the birth order of retinal neurons is during development: why do lines 576-577 seem to contradict lines 209-2011?

7. Lines 606-607: Please, provide a different reference for these lines. The cited paper Nagashima et a., 2013 does not resolve the issue of symmetry or asymmetry of the MGPC division. Nagashima et al. showed that Müller glia likely undergo one asymmetric division within 42 hpl with respect to fate, generating a self-renewed MG and a MGPC. They did not examine whether the MGPC itself divides asymmetrically or symmetrically, nor does there appear to be any convincing evidence for an asymmetric division of MGPCs from the published literature.

MINOR COMMENTS

8. Please, kindly revise the font of the gene names throughout the manuscript. The gene names should be indicated in italics, which does not always seem to be the case in the current version of the manuscript. There is a slight typo in the Methods: z stacks are measured as μm and not as μM.

9. Did the authors perform a TUNEL staining to look for signs of unspecific cell death upon light lesion as well as upon NMDA lesion?

10. Please, kindly explain the time points chosen for the NMDA lesion (7DR and 14 DR) and those chosen for the light lesion (7DR, 14 DR and 21 DR) in figure 1A, 1E and 1G. Why did the authors examine also the 21 DR time point in the light lesion, but not in the NMDA lesion? Please, also revise the scheme in Figure 1E: there is no indication of the 21 DR time point.

11. Lines 287-293: The authors may want to expand the description of Figure 3. Please, clearly indicate that HuC/D is a label of RGCs and ACs: this might be obvious for a retina expert, but will be less obvious for readers who are not familiar with the distinct labels of retinal neurons.

12. Line 290: how do you know that they are EdU-positive neurons and not EdU-positive cells?

13. Line 304 appears to be redundant ("in the production of the generation…").

14. Line 306: the authors may want to revise the sentence. The way it is written it suggests that these "initial factors" inhibit the function of inner neurons, whereas they might inhibit the generation of inner neurons.

15. Lines 344-346: this information is not easily accessible in the current table format. In general, I suggest to revise the nomenclature of the tables (ST3, ST4…), because in the current manuscript version each "table" corresponds to an Excel file containing, in fact, several tables.

16. Line 350: Figure S3 is actually a general overview, and does not specifically refer to microglia only. Also, not all the microglia markers listed in the text are visible in the cited Figure S3, only mpeg1.1.

17. It is not clear why the authors sequenced the whole embryo heads (line 366) – please explain.

Reviewer #3 (Remarks to the Author):

In the manuscript, the authors investigated the molecular process of retina regeneration in zebrafish in both light damage and NMDA models at single cell resolution. By comparing the two damage models and normal development process, the authors observed that the regeneration process is similar but distinct between the two damage models and also distinct from normal development process. In addition, the impact of two key factors, Mmp9 and foxj1a, on the regeneration have been examined. The results suggest that Mmp9 plays an important role in repressing regeneration of AC and RGC. In addition, knock down of Foxj1a reduces the number of regenerative neurons. I would like to congratulate the authors for generating such a significant resource and the new insights of the molecular process of retina regeneration in zebrafish. The design of the study is very thorough, and the manuscript is well written. My specific comments are the following:

1. No regeneration of horizontal cells are observed in this study. I am wondering if this is due to no degeneration of horizontal cell in the LD and NMDA damage model.
2. Does Mmp9 affect the normal development of the retina? Is higher AC/RGC to photoreceptor ratio observed in the Mmp9 mutant?
3. It seems that MG activation is faster in the LD model than the NMDA model. Furthermore, given the heterogeneity of MG and derived cells in any given time points, it might be useful to try to take this into account by calculating and correcting pseudotime during DEG and DAR analysis to exclude DEGs between two models due to phase shift.
4. "When examining the MG-to-MGPC branch across the two damage models, noticeable similarities are present. However, unique DEGs and DARs exist between them. The LD model exhibits a heightened neurogenic signal, while the NMDA model emphasizes a more robust inflammatory response pathway. From these observations, the authors suggest a hypothesis: MGPCs from these two damage models are in unique states, leading to varying proportions of retinal neurons being produced. Yet, this difference might merely be a reflection of environmental variations due to different cell type degenerations, rather than distinct MG regeneration pathway's choice. How can we differentiate between these two models?
5. During typical development, the cell type that a progenitor cell differentiates into is predominantly governed intrinsically. In the context of regeneration, MGPCs differentiate into cell types degenerated. How might MGPCs detect environmental cues and make corresponding differentiation choices? Could the author provide some speculation on this?
6. Based on the data, is foxj1a required for MG activation or subsequent MGPC proliferation and differentiation?
7. Considering the extensive and complex dataset presented, a summarizing model figure highlighting the key findings at the end would be beneficial.

1 We thank the Reviewers for their detailed and constructive comments. We have
2 addressed each individual point raised. Our responses are listed below in blue font:
3
4
5 **REVIEWER COMMENTS**
6
7 Reviewer #1 (Remarks to the Author):
8
9 Müller glia in the zebrafish retina respond to injury by generating all retinal cell types.
10 Different injury paradigms lead to the preferential loss of different retinal cell types. This
11 offers the unique possibility to assess whether the reaction of Müller glia also
12 preferentially replaces the lost cell type(s) over the others and which gene
13 expression/regulatory elements are are responsible for any differences. Furthermore,
14 they address whether regenerative neurogenesis recapitulates developmental
15 neurogenesis and find it doesn't. The authors present a thorough analysis of the
16 regulation of gene expression and regulatory elements and discover a number of
17 interesting functional interactions. Most importantly, mmp9 is shown to regulate the
18 generation of amacrine and ganglion cell relative to photoreceptors. A number of
19 potential regulators of regenerative neurogenesis are touched upon, but not further
20 investigated (e.g. Il1beta).
21
22 Overall, this is a carefully presented analysis that will be useful for a number of fields in
23 which neurogenesis and cell fate decisions are investigated. However, the results are
24 not always stringently bound to the biological model(s) and are overall a bit hard to
25 follow because of that. This limits the broader accessibility and attractiveness of the
26 data presented here.
27
28 In particular, a visualisation of the proposed cell fate transition and decision points (as
29 first described from line 59) would aid understanding of the (in principal) strong
30 conceptual framework.
31
32 We thank the Reviewer for his/her overall positive assessment of the manuscript.
33
34 major:
35
36 The author state that, different from development, neurons born after injury are not
37 eliminated over time (e.g. in line 82, also line 534f). This is not conclusively shown or
38 referenced. Furthermore, this statement seems to contradict their statement in line 132f
39 that speculates that removal of "excess immature neurons" might be happening.
40
41 This conclusion is based on the lack of change in the number of EdU-positive neurons
42 over time, following labeling from 60-108 hours post-injury, which corresponds closely to
43 the observed peak in MGPC proliferation observed by both scRNA-Seq and
44 snRNA/ATAC-Seq analysis (Fig. 2). With one exception, we observe no statistically
45 significant decrease in EdU incorporation through 14-21 days of recovery post-injury for
46 any cell type in any injury model, implying that no EdU-positive cells are eliminated

47 through apoptosis. The one exception is the rather modest reduction in the number of
48 EdU-labeled rod photoreceptors between 7 and 14 days post-injury, which is referenced
49 on line 132f. We have modified the text to make this point clearer.
50
51 In the section line 127 - 141 it remains unclear whether NMDA significantly damages
52 photoreceptors or not.
53
54 We identify both TUNEL-positive cells and DAPI-positive cells with pyknotic nuclei in the
55 INL with light damage, as well as in the photoreceptor layer following NMDA (Fig. S3).
56 This, combined with the corresponding reduction in the number of DAPI-positive nuclei,
57 supports the conclusion that these cells are dying.
58
59 In line 146, no difference in the number of EdU labelled neurons is reported to occur
60 between 7DR and 21DR - could this be a balance between cell proliferation and cell
61 death? What is the evidence for/against this possibility?
62
63 We have quantified the number of brightly-labeled EdU-positive cells in the section.
64 EdU was administered between 60 and 108 hours (2.5-4.5 days) post-injury. While
65 both scRNA-Seq and snRNA/ATAC-Seq showed only very low levels of MGPC
66 proliferation after this time, a small number of neurons are definitely still being
67 generated between 7 and 21 days post-injury. The Reviewer is correct that we did not
68 do EdU labeling between 7-21 days, and while new cells may be generated, the finding
69 that the number of EdU-labeled cells does not change in this period implies that no
70 substantial removal of newly-generated neurons is taking place.
71
72 The authors state that there they have not found genes for cilia in their expression
73 profile, apart from FoxJ1a, which regulates neurogenesis in the retina in the present
74 study. Cultured MGs have been described in mammals (PMID: 25504432), this should
75 be discussed here.
76
77 Muller glia, like virtually every cell type, do indeed possess primary cilia, and we now
78 cite this reference to emphasize this point. However, Foxj1 and its zebrafish
79 homologues have been most extensively studied as master transcriptional regulators of
80 motile multiciliated cells (Stubbs, et al. 2008; Hellman, et al. 2010). Neither Muller glia
81 nor MGPCs have been observed to be multiciliated, and we likewise do not observe
82 molecular markers that would suggest that this is the case. This point is now discussed
83 in more detail in the revised text.
84
85 The section around line 169 seems to suggest a replacement of lost neurons between
86 60 hours post light damage, when ONL shows the most pronounced loss of nuclei and
87 72 hours. Is 12 hours a reasonable time for the replenishment of ONL nuclei and what is
88 the evidence for that?
89
90 While some of these DAPI-positive nuclei may indeed be regenerated photoreceptors
91 cells at 72 hours, it is likely that many of them instead represent progenitors undergoing
92 interkinetic nuclear migration (Lahne and Hyde 2016), in which progenitors transitioning

93 through the cell cycle migrate apically, divide, and then undergo radial migration in the
94 basal direction. This is now discussed in the text.
95
96 Section starting line 332: there's a strong *down*regulation of several genes that are not
97 discussed, e.g. TNFb, tnfsf12, stat1a, while Il11b is upregulated at the 36 hour time
98 point - these should be discussed more clearly.
99
100 Tnfb is actually upregulated at 36hrs with similar kinetics to il1b/il11b (Fig. 4G). Tnfsf12
101 and stat1a are, however, are indeed transiently downregulated following LD at 36hrs,
102 but upregulated following NMDA injury. This point is now discussed in the text.
103
104 Discussion: Discussions of the results in the first section (page 13) should much more
105 clearly be related back to the model. Schematics clearly illustrating which cell types are
106 lost in each lesion model and which are replaced via which steps are needed to aid
107 interpretation of the results (also would help statements in lines 530 ff).
108
109 We now include schematic Figure S9 to summarize the overall findings and address
110 these points.
111
112 lines 570ff: the authors speculate, based on their data from the mmp9 mutant that
113 mmp9 might control the maturation of il1b and that this may mediate some of the
114 observed phenotype. This is a very testable hypothesis, since the activation of il1b can
115 be inhibited by caspase 1 inhibition.
116
117 This is an excellent suggestion, but we believe it is beyond the scope of the current
118 study. There are four different Caspase-1 paralogues in zebrafish, and both their
119 substrate specificity and specificity of known Caspase-1 inhibitors in zebrafish remain
120 uncertain, so even if partial functional rescue was observed, these results would be
121 difficult to interpret without further functional analysis of IL-1beta processing and
122 signaling.
123
124 lines 582: the distinct progenitors described here should be highlighted in the figures.
125
126 The distinct progenitors described here correspond to postmitotic rod and cone
127 precursors which has been labeled in red arrow in Figure S4A .
128
129 minor:
130
131 line 6: Hitchcock (small i)
132 line 112: that HAD incorporated
133 line 115: define "extensive"
134 line 131: is the "slight decrease" reported here statistically significant?
135 line 307: "inhibiting GENERATION OF inner retinal neurons"
136 line 448: "with either uninjected or injected..." there's a grammar problem here
137 line 508: "that continue to proliferate"
138 line 529: "amacrines" – jargon

139
140 These have been corrected.
141
142
143 Reviewer #2 (Remarks to the Author):
144
145 Lyu et al., 2023
146
147 The work by Lyu et al., 2023 provides a significant advancement in our understanding of
148 the molecular mechanisms regulating retina regeneration in zebrafish upon NMDA and
149 light lesions. The authors use a combination of single cell-, single nucleus- and ATAC-
150 sequencing to detect gene expression changes that underlie the two different lesion
151 paradigms, and between regeneration and retinal development. All retinal neurons, with
152 the exception of horizontal cells, are regenerated in both the light lesioned and the
153 NMDA retinae, hence showing that the two paradigms are not as specific as previously
154 thought in terms of ablating distinct retinal neuronal types. Nevertheless, a biased
155 differentiation of MGPCs towards photoreceptors in the light lesioned retina and towards
156 inner neurons in the NMDA-lesioned retina is apparent. Moreover, the authors suggest
157 that matrix metalloprotease Mmp9 may promote generation of inner retinal neurons as
158 compared to photoreceptors. They also point out shared and distinct gene expression
159 between regeneration and development of the retina. Finally, they provide evidence that
160 the transcription factor Foxj1a may be necessary for neuronal regeneration of the adult
161 retina, but not during development.
162
163 This reviewer commends the authors for the huge effort to provide this overview of the
164 sc-gene expression changes underlying retina regeneration using several, cutting-edge
165 technologies. Specifically, the manuscript provides a thorough description of the later
166 events of retina regeneration (up to 21 days post-lesion), which have so far been less
167 well described and understood. This comprehensive study of retina regeneration in
168 zebrafish is likely to be of lasting importance in the field, and will support genereation of
169 future hypotheses and functional studies in the field. recommends some revision of this
170 manuscript. I have the following comments and suggestions for revisions.
171
172 We thank the Reviewer for his/her positive assessment of the manuscript.
173
174 MAJOR COMMENTS
175 1. Although the manuscript is of a clear and significant importance for the understanding
176 of retina regeneration in zebrafish, the flow of the text and figures is difficult to follow.
177 The readability of the manuscript is likely to improve if the frequent mismatches
178 between the main text and the figures, and with their captions, are corrected.
179
180 We thank the Reviewer for his/her careful reading of the text. Please see below:
181
182 1a. Specifically, the flow of the text should better follow the order of the figures, to avoid
183 frequent jumps between figure panels. For example, the authors describe first figure 2D,
184 then jump back to describe figure 2C (compare line 208 to line 218). This applies to

185 other figures in the manuscript: first figure S5C/D and then S5A, B, first figure 5E/H and
186 then a jump to figure 6 G,H (lines 413-414).
187
188 We have reorganized the figures to better reflect the order of description.
189
190 1b. There are several mismatches between text and related figures, specifically in lines:
191 229-231; 234-237, 255-258, 258-260, 274-276; 295-295; also, what does the magenta
192 color of the bar plots in figure 3E indicate? The plot looks unclear and difficult to relate
193 to the text. In general, the authors may want to be more precise and exhaustive in their
194 description of figure panels, at least for those mentioned in the highlighted lines. Line
195 304-figure panel S5H seems to support the opposite of what is stated in this line. There
196 is a severe mismatch between the entire Figure 3 and its caption: panels described in
197 the caption are not matched in the figure, which makes it very hard to understand.
198
199 We have carefully revised both the figures and legends to greater clarity and to correct
200 any mismatches. We now clearly state that the magenta bar indicates data from *mmp9*
201 mutant animals.
202
203 1c. In Figure 1G there is no indication of the uninjured control, whereas in Figure 1J it is
204 not clear what the control for NMDA injections is (uninjected? Vehicle injected?).The
205 uninjured control is also missing from Figure S2.
206
207 Figure 1B represents the PBS-injected, uninjured control for both the light-damage and
208 NMDA-damage experiments. The purpose of the experiment is to demonstrate how the
209 number of EdU-labeled cells changes, or not, at different timepoints of recovery from
210 either LD or NMDA-mediated injury, not compared to the undamaged control. This is
211 also the case for Fig. S2.
212
213 1d. The nomenclature in Figure 2C does not match the nomenclature in Figure 2D. In
214 Figure 2C the authors list retinal ganglion cells, amacrine cells, bipolar cells, cones,
215 rods, while in Figure 2D the word “precursor” is added to the previous names. The
216 authors should provide a clear definition of what they designate as a “precursor” (see
217 point 2).
218
219 A precursor is an immature postmitotic neuron, as opposed to a progenitor, which is
220 mitotic. This has now been defined explicitly. Figure 2 has been revised for greater
221 clarity, as requested.
222
223 1e. Figure panels 4C and 4D are not clearly explained: there is no legend for the colors
224 of the bar plots, as well as for the symbols (what does the circle stand for? And the
225 square? What do E, G, P stand for? Please, specify. The same applies to Figure 6C,
226 6E, 6F). The way Figure 4F is described is highly unclear to me: lines 338-340. The
227 same applies to figure 6F.
228
229 These figures and the corresponding legends and manuscript text have been revised for
230 greater clarity, as requested.

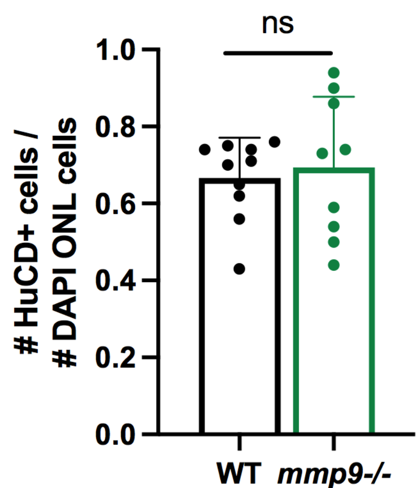
231
232 2. The nomenclature used in the manuscript is sometimes confusing. This applies
233 specifically to the terms “resting” Müller glia, “reprogrammed” Müller glia, “activated”
234 Müller glia and “precursor”.
235
236 2a. How does a reprogrammed MG differ from an activated one? Activated MG is
237 defined in Hoang et al., 2020, but in the present manuscript it seems to be used
238 interchangeably with the term “reprogrammed” MG. How do the two cell populations
239 differ (If they do)?
240
241 A “reprogrammed MG” in this context represents a Muller glia-derived progenitor cell
242 (MGPCs). We have altered the text throughout to reflect this.
243
244 2b. Please, define what a precursor is in the context of retina regeneration: from the
245 manuscript, it appears that “precursor” defines an immature or differentiating neuron.
246 However, please note that in zebrafish retina development the word precursor refers to
247 specific neuronal progenitors that undergo the last one or two mitoses to generate a
248 specific class of neurons (e.g., horizontal cell precursors described in Godinho et al.,
249 2007; cone precursors described in Suzuki et al., 2013; all reviewed in Amini et al.,
250 2018).
251
252 In this context, a precursor is an immature postmitotic neuron, while a progenitor (or
253 MGPC) is mitotic. This has been defined explicitly in the text.
254
255 3. Lack of evidence for statements in:
256
257 3a. Line 66: is there any published evidence that the excess of regenerated neurons
258 does not integrate into the extant retinal circuit ?
259
260 There is no direct evidence to this effect. This fact is now stated explicitly.
261
262 3b. Lines 344-346: it is hard to find the info in the cited table;
263
264 The relevant supplemental dataset (formerly table) has been revised for clarity.
265
266 3c. Lines 353-354: there is no evidence shown for the implication stated in these lines -
267 maybe move to the discussion section ?
268
269 This has been done.
270
271 3d. Lines 348-354 significantly interrupt the flow of the text before and after, and the
272 corresponding figure 4G does not fit well with the rest of the panels in figure 4.
273
274 We have revised the text for better clarity and smoother narrative flow.
275
276 4. The authors do not discuss the absence of horizontal cells and their precursors in

277 their lesioned retinae, which are reported however by Lahne et al., 2021 and Celotto et
278 al., 2023. onecut1, which is necessary for HC development and expressed strongly in
279 HC precursors (Celotto et al., 2023), appears as a differentially upregulated motif in
280 Figure 2H. How do the authors explain this upregulation, in light of the claimed absence
281 of regenerated HCs and HC precursors upon injury?
282
283 Onecut family transcription factors have an essential role in promoting cone
284 photoreceptor specification in mammals (Lonfat et al. 2021; Emerson et al. 2013),
285 although this has not been directly shown in zebrafish. Mammalian cone photoreceptors
286 arise from Onecut/Otx2-positive neurogenic progenitors that have the potential to
287 generate either cones or horizontal cells. Interestingly, we observe neurogenic MGPCs
288 in zebrafish that express both Onecut1/2 and Otx2, but do not generate horizontal cells,
289 raising the possibility that horizontal cell generation might be actively inhibited through
290 unknown mechanisms in these cells. We discuss this point in the revised manuscript.
291
292 5. Figure S7G and line 496: In the image shown for the foxj1a mutant, lamination of the
293 retina looks disrupted, in contrast to what is stated in the text (line: 496). The retinae in
294 the mutant look smaller, and plexiform layers are hardly distinguishable, compared to
295 controls. Also, why is lamination delayed in the foxj1a mutant, compared to the control:
296 did the authors check whether the foxj1a mutant retina 'catches up' and has developed
297 correctly at time points later than 96 hpf?
298
299 Because the morpholino effect is transient, looking at later timepoints will not be a true
300 indication of the loss of foxj1a expression, as it is likely to increase after the morpholinos
301 are lost. The size of the embryos was also much smaller in the morphant than the
302 control and we are unable to separate the issue of the embryo size and the retina size.
303
304 More generally, foxj1a is broadly expressed in the early embryo, and the morphant
305 affects the overall size of many organs, including the eye. However, we do not observe
306 expression of foxj1a in retinal progenitors or neural precursors in the developing retina,
307 and no clear effects on overall levels of retinal neurogenesis in morphants.
308
309 6. It is unclear what the birth order of retinal neurons is during development: why do
310 lines 576-577 seem to contradict lines 209-2011?
311
312 This has been clarified in the revised text.
313
314 7. Lines 606-607: Please, provide a different reference for these lines. The cited paper
315 Nagashima et a., 2013 does not resolve the issue of symmetry or asymmetry of the
316 MGPC division. Nagashima et al. showed that Müller glia likely undergo one asymmetric
317 division within 42 hpl with respect to fate, generating a self-renewed MG and a MGPC.
318 They did not examine whether the MGPC itself divides asymmetrically or symmetrically,
319 nor does there appear to be any convincing evidence for an asymmetric division of
320 MGPCs from the published literature.
321

322 This is absolutely correct. There is no direct evidence to support either symmetric or
323 asymmetric patterns of cell division by the MGPCs themselves. We revised the text
324 accordingly.
325
326 MINOR COMMENTS
327 8. Please, kindly revise the font of the gene names throughout the manuscript. The
328 gene names should be indicated in italics, which does not always seem to be the case
329 in the current version of the manuscript. There is a slight typo in the Methods: z stacks
330 are measured as µm and not as µM.
331
332 This has been corrected.
333
334 9. Did the authors perform a TUNEL staining to look for signs of unspecific cell death
335 upon light lesion as well as upon NMDA lesion?
336
337 As stated in the response to Reviewer 1, we observe pyknotic nuclei in all cell layers
338 following both LD and NMDA injury. While these often overlap with TUNEL-positive
339 cells, we observe substantially more pyknotic cells than TUNEL-positive cells. This is
340 now shown in Figure S3.
341
342 10. Please, kindly explain the time points chosen for the NMDA lesion (7DR and 14 DR)
343 and those chosen for the light lesion (7DR, 14 DR and 21 DR) in figure 1A, 1E and 1G.
344 Why did the authors examine also the 21 DR time point in the light lesion, but not in the
345 NMDA lesion? Please, also revise the scheme in Figure 1E: there is no indication of the
346 21 DR time point.
347
348 The 21 DR timepoint was also examined in LD simply because we had more LD-treated
349 animals available, owing to the fact that this procedure does not involve any direct
350 manipulation of the animals. In any case, no significant difference is observed between
351 the 14 DR and 21 DR samples for any of the parameters tested. We have revised the
352 schematic in Figure 1E to include the 21 DR timepoint.
353
354 11. Lines 287-293: The authors may want to expand the description of Figure 3. Please,
355 clearly indicate that HuC/D is a label of RGCs and ACs: this might be obvious for a
356 retina expert, but will be less obvious for readers who are not familiar with the distinct
357 labels of retinal neurons.
358
359 This has been done.
360
361 12. Line 290: how do you know that they are EdU-positive neurons and not EdU-
362 positive cells?
363
364 As stated in Figures 1 and S1 and S2 we have stained for markers for Muller glia and
365 microglia, and observe little EdU incorporation, while we observe extensive EdU
366 incorporation in rods, cones, and HuC/D-positive amacrine and ganglion cells. We
367 likewise do not observe substantial numbers of any other non-neuronal cell type in our

368 scRNA-Seq or snRNA/ATAC-Seq analysis. We therefore feel confident in referring to
369 these as overwhelmingly EdU-positive neurons in this case.
370
371 13. Line 304 appears to be redundant ("in the production of the generation…").
372
373 This has been corrected.
374
375 14. Line 306: the authors may want to revise the sentence. The way it is written it
376 suggests that these "initial factors" inhibit the function of inner neurons, whereas they
377 might inhibit the generation of inner neurons.
378
379 This has been corrected.
380
381 15. Lines 344-346: this information is not easily accessible in the current table format. In
382 general, I suggest to revise the nomenclature of the tables (ST3, ST4…), because in the
383 current manuscript version each "table" corresponds to an Excel file containing, in fact,
384 several tables.
385
386 We now specifically cite these files as Supplemental Datasets rather than Tables to
387 reduce confusion, and also specifically cite relevant tabs within the datasets in the
388 following format: Supplemental Dataset X, T(ab) Y.
389
390 16. Line 350: Figure S3 is actually a general overview, and does not specifically refer to
391 microglia only. Also, not all the microglia markers listed in the text are visible in the cited
392 Figure S3, only mpeg1.1.
393
394 We now include a reference to Supplemental Dataset 5, which lists the full complement
395 of microglial markers.
396
397
398 17. It is not clear why the authors sequenced the whole embryo heads (line 366) –
399 please explain.
400
401 Prior to 36 hpf, it was simply not possible to cleanly dissect retinas. This is now
402 explained in the text.
403
404
405 Reviewer #3 (Remarks to the Author):
406
407 In the manuscript, the authors investigated the molecular process of retina regeneration
408 in zebrafish in both light damage and NMDA models at single cell resolution. By
409 comparing the two damage models and normal development process, the authors
410 observed that the regeneration process is similar but distinct between the two damage
411 models and also distinct from normal development process. In addition, the impact of
412 two key factors, Mmp9 and foxj1a, on the regeneration have been examined. The
413 results suggest that Mmp9 plays an important role in repressing regeneration of AC and

414 RGC. In addition, knock down of Foxj1a reduces the number of regenerative neurons. I
415 would like to congratulate the authors for generating such a significant resource and the
416 new insights of the molecular process of retina regeneration in zebrafish. The design of
417 the study is very thorough, and the manuscript is well written.
418
419 We thank the Reviewer for his/her positive assessment of the manuscript.
420
421 My specific comments are the following:
422 1. No regeneration of horizontal cells are observed in this study. I am wondering if this is
423 due to no degeneration of horizontal cell in the LD and NMDA damage model.
424
425 While we observe no evidence for injury-induced loss of horizontal cells in either injury
426 model, we lack a cell specific marker to label them, so it is only based on their unique
427 location (which shifts upon the loss of the ONL) and their morphology. Following injury,
428 it is often not straightforward to distinguish horizontal cells because of interkinetic
429 nuclear migration of MGPC nuclei, which enter the OPL and obscure the horizontal
430 cells. We do not observe evidence for immature MGPC-derived horizontal cell
431 precursors at any timepoint in either injury model. It is likely that selective ablation of
432 horizontal cells using techniques such as cell-specific NTR transgenic lines would
433 indeed lead to selective horizontal cell regeneration. Whether this would also lead to
434 indirect death and regeneration of photoreceptors and AC/RGC is an interesting topic
435 for future research.
436
437 2. Does Mmp9 affect the normal development of the retina? Is higher AC/RGC to
438 photoreceptor ratio observed in the Mmp9 mutant?
439
440 We now quantify the relative ratio of HuC/D-positive AC/RGC relative to total DAPI-
441 positive cells and DAPI-positive ONL cells in control samples for both wildtype and
442 mmp9-deficient fish. We do not observe any changes in the relative ratio of AC/RGC to
443 photoreceptors. This is included below as Reviewer Figure 1:
444



445

446 **Reviewer Figure 1**: The ratio of the total number of HuC/D and DAPI-positive inner
447 retinal neurons to the total number of DAPI-positive ONL cells is shown for wildtype and
448 mmp9-deficient adult retina. Each point represents data from a single animal.
449
450 3. It seems that MG activation is faster in the LD model than the NMDA model.
451 Furthermore, given the heterogeneity of MG and derived cells in any given time points,
452 it might be useful to try to take this into account by calculating and correcting
453 pseudotime during DEG and DAR analysis to exclude DEGs between two models due
454 to phase shift.
455
456 We thank the reviewer for this suggestion. In the differential analysis (Figure 2G) of
457 Rest MG, Act MG, and MGPCs, we did not utilize pseudotime to identify DEGs and
458 DARs; instead, to eliminate the phase shift effect, we used combined cells from all time
459 points within a single cell type to call DEGs and DARs. Pseudotime (Figure 2B) was
460 only employed for visualizing and clustering DEGs and DARs. This methodology is now
461 detailed in the methods section.
462
463 4. "When examining the MG-to-MGPC branch across the two damage models,
464 noticeable similarities are present. However, unique DEGs and DARs exist between
465 them. The LD model exhibits a heightened neurogenic signal, while the NMDA model
466 emphasizes a more robust inflammatory response pathway. From these observations,
467 the authors suggest a hypothesis: MGPCs from these two damage models are in
468 unique states, leading to varying proportions of retinal neurons being produced. Yet, this
469 difference might merely be a reflection of environmental variations due to different cell
470 type degenerations, rather than distinct MG regeneration pathway's choice. How can we
471 differentiate between these two models?
472
473 It is undoubtedly true that extrinsic factors that are differentially induced by LD vs.
474 NMDA injury control the formation of fate-biased MGPCs. The text has been revised to
475 make this point explicitly clear.
476
477 5. During typical development, the cell type that a progenitor cell differentiates into is
478 predominantly governed intrinsically. In the context of regeneration, MGPCs
479 differentiate into cell types degenerated. How might MGPCs detect environmental cues
480 and make corresponding differentiation choices? Could the author provide some
481 speculation on this?
482
483 A broad range of extrinsic cues could potentially signal to MGPCs to confer fate biases.
484 These might include signals differentially released by dying neurons, signals
485 differentially released from MG or microglia, the loss of contact-dependents signals from
486 the dying neurons to MG, etc. This is now discussed in the text.
487
488 6. Based on the data, is foxj1a required for MG activation or subsequent MGPC
489 proliferation and differentiation?
490

491 This is a good question, and difficult to address without a systematic analysis of
492 molecular markers specific to both activated MG and MGPCs using techniques such as
493 scRNA-Seq. We plan to investigate this in future studies.
494
495 7. Considering the extensive and complex dataset presented, a summarizing model
496 figure highlighting the key findings at the end would be beneficial.
497
498 A schematic summarizing the findings is now included as Figure S9.
499
500 Reviewer #4 comments to the authors:
501
502 In the present manuscript, Lyu et al. performed single-nuclear and single-cell RNA-seq and
503 ATAC-Seq of developing and regenerating retinas to investigate the molecular mechanisms
504 controlling injury-induced neurogenesis. The results indicated that retinal regeneration was
505 similar to retinal development, but the regeneration process did not precisely recapitulate retinal
506 development. The authors also displayed the similarities and differences in gene expression
507 and gene regulatory networks in both retina damage models. In addition, the authors claimed
508 that mmp9 was a selective inhibitor of amacrine and ganglion cell formation and foxj1a was
509 essential for injury-induced neurogenesis. Overall, this study depicted the major differences
510 between gene regulatory networks between retinal regeneration and development.
511
512 Some comments for consideration for the authors are listed below in hope the authors will find
513 them useful.
514 1. There are inconsistencies in the content and legends of Figures 1 and 3. For instance, in
515 Figure 3, the panels C and D in the legend does not present in the actual figure.
516
517 This has been corrected.
518
519 2. In Figures 1J and 1L, after NMDA and constant light damage, the lowest cell numbers were
520 seen at 60 hours in the different three layers. Why do different types of injuries lead to the same
521 results? Can the authors explain the possible reasons for this result?
522
523 Both forms of injury are severe, and lead to the loss of the majority of cells directly affected by
524 the injury in question (photoreceptors following light damage and amacrine/ganglion cells
525 following NMDA treatment). We do not know why 60 hours represents the peak time for both
526 direct and indirect neuronal death, although we would predict that if we were to conduct a finer
527 resolution temporal analysis of cell loss (e.g. sampling every 4 hours between 48 and 72hrs
528 post-injury), we would observe that the peak of indirect neuronal death (e.g. loss of
529 amacrine/ganglion cells following light damage) would lag that seen for direct neuronal death.
530
531 3. “Both Gene Ontology and KEGG pathway analysis identified functional differences in
532 differentially expressed genes between the two injury conditions”, 1791 genes highly expressed
533 in the light damage, so how many genes were utilized for this analysis? Did the authors check
534 whether these genes have statistical significance?
535
536 We identified LD-enriched DEGs between LD and NMDA using the following criteria: logfc >
537 0.25 and an adjusted p-value < 0.05. We enumerated the number of DEGs for RestMG, ActMG,
538 and MGPCs separately in the text and table (Supplemental Dataset 3: T5). Before conducting
539 GO analysis, we combined the DEGs from RestMG, ActMG, and MGPCs and removed
540 redundant genes. From this, we obtained 890 and 571 non-overlapping genes enriched in LD or

541 NMDA, respectively. These genes were re-clustered based on their expression profile along the
542 trajectory. Subsequently, GO and KEGG analyses were conducted for each cluster of genes.
543 We've revised the text, tables, and Methods sections to clarify this.
544
545 4. For scRNA-seq or multi-omics data, expression of marker genes of individual cell types was
546 not shown, which make it impossible to validate the cell type annotation.
547
548 Marker genes for each cell cluster are shown on the x-axis of Figure S3D, demonstrating the
549 accuracy of cell type annotation. Also, a list of all the marker genes that are selectively
550 expressed in each cell type is now included in Supplemental Dataset 5.
551
552 5. The authors utilized CCA (canonical correlation analysis) to integrate the single cell RNA-seq
553 data and single nuclear RNA-seq data, during the process of data integration, did the authors
554 observe any differences from the two different sequencing methods? Are there variations in the
555 genes detected through each sequencing approach?
556
557 We appreciate the reviewer's question. In this paper, the injury or development samples
558 from different sequencing methods are processed and analyzed separately. We only
559 used CCA to integrate the datasets which derived from the same sequencing method
560 (Figure 2A, 5A, snRNA-seq). Our group has systematically investigated both cell
561 representation and gene expression patterns in scRNA- and snRNA-Seq datasets
562 prepared from the same starting material (Santiago et al. 2023). In this study, which
563 closely reflects our findings in the current study, we found that while both methods can
564 accurately profile gene expression in major retina cell types, but also observed
565 differences in cell type proportions captured by snRNA-seq and scRNA-seq.
566 Specifically, single-cell RNA sequencing overrepresented Müller glia and microglia, but
567 captured fewer amacrine and retinal ganglion cells (Fig S4A). Cell type-specific scRNA-
568 Seq profiles generally show higher levels of cross-contamination with transcripts
569 enriched in other cell types. This is especially so for photoreceptor-specific markers,
570 which frequently contaminate other cell types in scRNA-Seq datasets, but do so much
571 less in snRNA-Seq datasets. The number of transcripts and genes detected in scRNA-
572 Seq samples, as expected, considerably higher than observed with snRNA-seq analysis
573 of the same cell types. Finally, scRNA-Seq data is significantly enriched for genes
574 encoding ribosomal proteins, while snRNA-Seq data is enriched for RNA-binding
575 proteins.
576
577 We hope that these changes have addressed any outstanding concerns, and look
578 forward to hearing your response to the revised manuscript.
579
580

581 **References cited:**

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585 Lahne, Manuela, and David R. Hyde. 2016. "Interkinetic Nuclear Migration in the Regenerating Retina."
586 *Advances in Experimental Medicine and Biology* 854: 587–93.
587 Lonfat, Nicolas, Su Wang, Changhee Lee, Mauricio Garcia, Jiho Choi, Peter J. Park, and Connie Cepko.

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589 Transcription Factors.” *Development* 148 (9). https://doi.org/10.1242/dev.198549.
590 Santiago, Clayton P., Megan Y. Gimmen, Yuchen Lu, Minda M. McNally, Leighton H. Duncan, Tyler J.
591 Creamer, Linda D. Orzolek, Seth Blackshaw, and Mandeep S. Singh. 2023. “Comparative Analysis of
592 Single-Cell and Single-Nucleus RNA-Sequencing in a Rabbit Model of Retinal Detachment-Related
593 Proliferative Vitreoretinopathy.” *Ophthalmology Science* 3 (4): 100335.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have carefully considered my comments and answered most. The added specifications, discussions and figure S9 significantly aid the understanding of the complex results.

I still think an experimental validation of the Il1beta hypothesis would have strengthened the manuscript, but I am looking forward to these results in a follow up manuscript.

Reviewer #2 (Remarks to the Author):

The authors have substantially revised the ms along the lines suggested by the reviewers. They have convincingly adressed all the points that I have raised and fixed the various smaller issues, and the revised version of the ms is therefore now fine in my opinion. Congratulations on a nice piece of work !

Reviewer #3 (Remarks to the Author):

The author had adequately addressed issues and questions raised by the reviewers by modifying/correcting the text and add additional supplement figures. The study is well designed and clearly written. I believe the manuscript is ready to be accepted for publications.

Reviewer #4 (Remarks to the Author):

The authors addressed all my concerns, no further question.