A 3D molecular map of the cavefish neural plate illuminates eye-field organization and its borders in vertebrates

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MS TITLE: A 3D molecular map of the cavefish neural plate illuminates eyefield organization and its borders in vertebrates

AUTHORS: Francois Agnes, Jorge Torres-Paz, Pauline Michel, and Sylvie Retaux

Apologies for the delay in obtaining reviews on your manuscript. However, 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 are mixed in their opinions on suitability of the study for publication in Development. Reviewer 1 has relatively minor comments whereas reviewers 2 and 3 have more significant concerns about the data and its interpretation. Although reviewer 3 thinks that the study is not suitable for publication in Development, he/she makes various suggestions for improvements, some of which align with the suggestions from reviewer 2. Consequently, if you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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

By using imaging and cell sorting techniques, the authors perform a detailed quantitative study of the expression patterns of barhl2, cxcr4b, emx3, lhx2 nkx2.1a, pax6, rx3 and zic1. This exceptional and comprehensive analysis of the forebrain territory at tailbud stage in Astyanax will hopefully set the standard for future research in forebrain and eye field gene expression studies.

The authors open the paper by analysing the expression of rx3 in the eye field. 3D rendering of confocal images enables visualising a ventral midline indentation that cannot be seen by 2D images. They also observe that the rx3 expression domain is smaller in cavefish embryos. They tackle the problem of sample developmental stage dispersion that is normally observed by grouping embryos of a similar morphological moment by fitting the anterior curvature of the eyefield to the shape of a parabola (Coefficient ‘d’). This enables a more accurate description of the phenomena by comparing experimental samples going through a similar developmental moment. This is a nice step forward for the analysis and quantification of eye field development and gene expression patterns. A histogram depicting the distribution of eye field stages show that cavefish eye fields have a clear bias towards later stages morphology suggesting heterochrony compared to surface fish eyefield. This kind of analysis also enabled finding that the cavefish eye field does not condensate at later stages like the surface fish. A detailed analysis of expression levels of rx3 confirms previous findings that cavefish shows lower levels of gene expression in the eye field.

The authors cloned Astyanax cxcr4 and found that only cxcr4b was expressed at 10hpf in the eyefield in a subdomain within the rx3 expression area and that its expression in cavefish is reduced by 25% compared to surface fish. They also observe that cavefish cells expressing lower levels rx3 show higher expression of cxcr4b and vice versa.

By performing a battery of imaging analysis, the authors conclude that the expression of pax6 is in line with what was previously described. The volume of pax6 expression and the size of the overlapping region between surface and cave fish is no different. They do mention that the “pax6+/rx3- posterior region was slightly smaller in cavefish (not shown)” in line 212. However, when comparing panels C and D in figure 3 it looks like this region is bigger in Cavefish because there is less rx3 posterior expression in these samples. Could the authors please comment on this discrepancy?

The authors confirm that nkx2.1a hypothalamic marker expression limits the ventral medial eye field domain and that cavefish has a broader and longer nkx2.1a expression that shows overlap with the rx3 domain. The more anterior position of the hypothalamus in cavefish may also be indicative of the suggested heterochrony in cavefish compared to surface fish.

The authors then add the markers emx3, lhx2, zic1 and barhl2 to their analysis to further elaborate and delineate new subdomains of the eye field. Overall, they show that the eyefield has three AP and three mediolateral subdivisions; and reveal new putative subdivisions in the presumptive telencephalon.

In the discussion, the authors nicely make parallels between their findings of forebrain sub-territories, and changes in the size and cell type overlapping in such domains, between surface and cave fish, to previously documented reports that predict and back up these observations.

Overall a great manuscript that will significantly contribute to research in the field of forebrain and eyefield specification, and eye development in general.

Comments for the author

Minor Suggestions:
1. Line 212: The authors mention that the “pax6+/rx3- posterior region was slightly smaller in cavefish (not shown)”. However, when comparing panels C and D in figure 3 it looks like this region is bigger in Cavefish because there is less rx3 posterior expression in these samples. Could the authors please comment on this discrepancy?
2. Line 228: “rx3/pax6 non-expressing domain”. Is not very clear, can the authors please find a better way to describe this.
3. Line 229: “(M-N) Maximum intensity projections (3 µm, mid-stack)”. Maximum intensity projections of what?
4. Line 231: “…lines, Q)”. Lines in O?
5. Line 231: “Shaded rectangle indicate eyefield posterior limit”. Ambiguous maybe indicated the rx3 expression domain? Otherwise, which end of the ends of the shaded rectangle indicated the posterior limit of rx3?
6. Line 236 and 253: gradient o graded expression of pax6? I think the latter as pax6 is not a diffusible molecule. This also applies to other parts of the manuscript.
7. Line 238: “…intensity in posterior rx3+ cells was six times higher than in anterior rx3+ cells”. Is that limit defined by expression 0.2 in Fig 3F?. Please clarify?
8. Line 239: The description for the expression of Pax6 “Pax6 levels in the intermediate region were also significantly higher in cavefish, suggesting subtle variations in the control of its expression between the two morphotypes (Figure 3F, right).” Is not very accurate and does not make justice to the amount of work performed. Maybe explain what you define by intermediate region? It is also ambiguous as you use the word ‘also’ when in the previous sentence you argue that there is no difference between both conditions.
9. It would be helpful in Fig 3G to better define what we are looking at in the figure legend. The analysis is focused on the expression of pax6, but you look at the anterior, medium, and posterior region in the expression limits of rx3, not pax6.
10. In general the figure legends would benefit by making them more descriptive so the reader does not have to guess about what the panels are showing.
11. As in cavefish, the expression of rx3 has is much reduced in zebrafish tcf7l1a mutants. However, the eye size phenotype seems to be regulated by compensatory growth mechanisms in the zebrafish retina. Hence, the reduction of rx3 on its own may not explain the eye phenotypes observed in cavefish. Could the authors speculate on this discrepancy?
12. Considering the extensive depth of the discussion, I miss the authors elaborating on the fate and aspects of the posterior eye field domain that is only pax6+.
13. The altered AP position of the hypothalamus in cavefish was mentioned in the results section but not in the discussion, which only comments on the condensation of the eye field. Could there be a relationship between both phenomena?

Reviewer 2

Advance summary and potential significance to field

The manuscript entitled “A 3D molecular map of the cavefish neural plate illuminates eyefield organization and its borders in vertebrates “ reports a descriptive study based on imaging and quantification of a set of mRNA markers of anterior neural plate territories in cavefish, aiming to understand better the complexity of territories and boundaries in and around the eyefield and their variations between cave and surface variants. The study is very meticulously done and leads to a couple of interesting conceptual hypotheses, the most prominent being that the eyefield is already molecularly subdivided into antero-posterior medio-lateral and ‘dorso-ventral’ domains. However, I find that there is often a rather substantial leap from observations to claims/conceptual conclusions. A more careful set of conclusions is advisable and some additional staining needed to support the conclusions of this work.

Comments for the author

- When referring to pax6 in the introduction, the authors need to be more specific. Pax6a is expressed in future posterior retina and diencephalon and Pax6b is expressed in the whole eyefield, nasal placode territory and anterior diencephalon.
- In the intro. what is the evidence supporting the statement that the reduction on volume of rx3 in cave fish is due to lack of posterior medial parts?
The anterior part is narrower and stumpy therefore reduced too.
- The authors rightly state that the shape of the anterior neural plate is highly dynamic at a 'same' stage and therefore it is extremely important to make sure that the stage is as close as being the same when comparing surface with cave ANP. They also correctly observed that the curvature of the anterior border of RX3 is more pronounced in cave fish at the same stage. Given this observation, in a couple of cases the experiments do not manage to show convincing matching ages (curvature of cave domain equal or lower than their compared surface embryos:
Cxcr4b (Figure 2) and emx3 (Figure 7) expression domains.
- The absence of eyefield condensation over time in cave fish is a rushed conclusion. It may be as likely that neurulation itself is overall slower in cave embryos. This needs to be checked by measuring medio-lateral 'shrinkage' of Pax6 domain over time in surface and cave during the same time interval to distinguish between neurulation and eyefield condensation.
- The finding in Fig 1 that the rx3 domain has higher curvature in cave fish support my opinion that movie 3 is done at a younger stage in cave fish than movie 2 (surface fish) it is compared to. Moreover, movie 3 is made differently. Movie 2 and 3 needs to be more comparable. In zebrafish rx3 intensity changes from weak and patchy to high and homogenous from expression onset to bud stage. Is the surface embryo displaying more heterogenous rx3 (and complementary cxcr4) expression levels at earlier stage, suggesting that cave eyefield is 'stuck' in its early developmental stage? This needs to be checked.
- The measurement of rx3/cxcr4 expression level is possibly mis-interpreted.
Fig 2J shows that the cave fish distribution of expression levels of cxcr4 is the same as surface, just shifted by overall lower expression of rx3 in the cave fish
(counting also showing lower number of eyefield cells in the cave fish). The bar chosen to define low and high for cxcr4 skews the results shown in 2 K, L. There does not seem to be an enrichment of cells of a given cxcr4 expression level at a specific intensity of rx3 expression for any of the two populations but rather an interesting bell curve in the level of rx3 expression leading to lower level of cxcr4 in both populations. If you plot the intensity of dots independently of their genetic identity, you see majority of cells have low cxcr4 expression and there is a peak bias for low cxcr4 at 0.6 of rx3 expression. The fact that the cave fish rarely reaches this level creates the bias in apparent 'regulation'.
- The possibility that there is a posterior part of the eyefield that is rx3 negative is potentially very interesting. However, the rx3:GFP transgenics described by Witbrodt and Wilson labs do not seem to show any GFP-negative cells in the eye vesicle (only heterogeneity in expression levels), suggesting that all retinal cells are rx3 positive. Staudt et al. also had fezf2 overlapping with barhl2 and fate mapped to hypothalamus and prethalamus. Is this marker also having a gap with Rx3. Double in situ with rx3+barhl2 and rx3+fezf2 are needed to support the statement, with AP and DV intensity quantification as the boundaries are likely to vary across tissue depth. Moreover, emx3 has been described as having small latero-posterior 'arms' surrounding the eyefield (similar to zic 1 in Fig5A). Would this territory be eye then? Or is this area a dorso-posterior telencephalic population? Staudt fate map rather supports this latter possibility with fate of this population being dorsal posterior telencephalon and epithalamus.
- The statement of a rx3-negative domain in the eyefield needs therefore to be more carefully worded. Only a fate mapping of this population specifically would ascertain their eye identity.
- Rax in mouse and rx3 in fish is expressed in both eyefield and anterior-most hypothalamus, therefore expecting a population expressing both rx3 and nkx2.1a. The section needs to be re-written according to this published knowledge.
The finding that there are more anterior hypothalamic cells in the cave embryos is very interesting and indeed fits with the increase in sHH.
- The more anterior position of the nkx2.1 rostral tip in cave fish may well be due to a faster convergence/extension during gastrulation, already suggested in previous publications.
- The medial rx3+,zic1-negative population much more prominent in cave embryos and located ventrally is likely to be hypothalamic.

- The subdivision of the ‘telencephalic population into three domains is very interesting. However, emx3 overlaps with dlx3a and therefore the emx3 only domain may well be placodal instead of telencephalic. A series of double in situs with dlx3a in surface and cave embryos is needed.
- Finally, the map on Fig. 8A is misleading. The rx3- territory is shown much bigger than the expected narrow gap between rx3 and barhl2 published previously (example Young et al. 2019). Also, the authors do not consider the emx3/rx3 on this map. This gap is expected to be lhx2+. Could the author provide a double in situ lhx2+rx3 in cave embryos? Moreover, the data in Fig. 6N, O is not clean enough. Rx3 seems very fuzzy/backgroundy in cave embryo, making it difficult to understand what is the identity of the zic1 gap in the cave embryos. The status of the three ‘telencephalic’ territories need to be clarified better in cave embryos.

Reviewer 3

Advance summary and potential significance to field

In this paper the authors aim to define a molecular portrait of the eyefield. To do so, they perform in situ hybridisation on embryos of the dimorphic fish, Astyanax mexicanus (which comes in a wildtype and a troglodytic form) comparing the two for subtle differences. The key advances are the detail in which the authors have analysed gene expression profiles. The authors have performed the work in 3-d – so have performed high quality imaging and image analyses. They have performed quantitative analyses on the 3d images, including measurements of area, volume, fluorescence levels in individual cells – to provide quantitative analyses of gene expression profiles in 3d, and a quantitative comparison of the wild type and troglodytic fish. By taking into account the parabolic shape of Rx3 expression, they have subclassified the 10h fish according to subtle morphogenetic advances.
The detailed description of the different gene expression patterns is a step-forward in the field.

Comments for the author

However, I do have concerns. Although a very thorough piece of work, the manuscript is descriptive.
A key aim is to distinguish the eyefield from adjacent territories – the posterior limits of the eyefield are currently not well-defined. A second aim is to describe sub-territories, within the eyefield and at its borders with adjacent territories. I am not convinced that the work has added significantly to what we already know through other studies.

Comments
The manuscript is written as a series of careful observations on different genes, but comes across in a disjointed manner that makes it hard to see the overall picture, especially when it comes to border regions. This makes it difficult to judge a key aim of the paper (ie to define the eyefield). Specific examples are provided below:
It is not clear whether some of the territories described as eye field are uniquely such. Rx3 is introduced as an eyefield-specific marker (with references to previous studies in zebrafish), but Rx3 additionally marks the hypothalamus (eg Tessmar Raible et al 2007). The 25% reduction in posterior-medial parts of the Rx3 domain in the cavefish (a key conclusion of Figure 1) could include changes to the hypothalamus (an idea supported through Fig 4).
Likewise - in Fig3, an outstanding question is whether the posterior-medial Pax6-Rx3-domain(expanded in the cavefish) likely harbours diencephalic/hypothalamic midline cells that are expanded in the cavefish. This was, in fact, addressed in Fig 4 (they are NKx2-1+). But I struggled to understand an overarching model. Do the authors think that there are changes to cell migration , proliferation and/or morphogenesis? How do these subtle changes really help to define the eye-field as distinct from the nascent hypothalamus? Previous studies (including Staudt and Houart 2007) in zebrafish have combined analyses of gene expression patterns with fate-mapping studies. These highlight the difficulties in establishing the difference between eye-field and hypothalamic territories, especially in ventral regions. I hoped that the current careful analyses would help to address this, but was not convinced that it did.

On a similar note, the authors define the hypothalamus through Nkx2-1 expression, but in most species Nkx2-1 defines the basal, not the alar hypothamus (for instance the paraventricular region).
This is not addressed at all.

The authors need to explain which strain of cavefish was analysed: this is not my area of expertise but I believe there are numerous strains – which have undergone convergent evolution – so the findings here may be particular to one strain?
For instance - the different correlations in Rx3-cxcr4b between surface and cavefish (Figure 2) are interesting – but is this seen in other cavefish strains? And is it mechanistically important?
The authors overlook/fail to discuss, morphogenetic processes that might contribute to the patterns they observe: I realise this would be speculative, but it would add weight to the discussion and help to frame a model.

Some additional minor comments:
From the outset, please define the two fish forms – ie 'surface' and 'cavefish'.
I cannot follow the figure legend in Figure 1 – in particular I-I"'. The authors suggest these show evidence for variation in Rx3 expression between samples (line 128) – but it was not clear from the legend that these were different samples.
Figure 1Q – the 'homogeneity' vs 'heterogeneity' in surface vs cavefish in 1Q is subtle – please highlight ROI in graphs.
Figure 4D, D' – do not match the text, which refer to 4D, E. The subtle gap between Rx3 and Nkx2-1 needs to be pointed out with an arrowhead
In general there are a number of instances where figure and the graphs have been incorrectly labelled – ie figures and plots that do not match the text.

First revision

Author response to reviewers' comments

Reviewer 1

By using imaging and cell sorting techniques, the authors perform a detailed quantitative study of the expression patterns of barhl2, cxcr4b, emx3, lhx2, nkx2.1a, pax6, rx3 and zic1. This exceptional and comprehensive analysis of the forebrain territory at tailbud stage in Astyanax will hopefully set the standard for future research in forebrain and eye field gene expression studies.
> Thank you for this nice appreciation of our work.

They do mention that the "pax6+/rx3- posterior region was slightly smaller in cavefish (not shown)" in line 212. However, when comparing panels C and D in figure 3 it looks like this region is bigger in cavefish because there is less rx3 posterior expression in these samples. Could the authors please comment on this discrepancy?

> The reviewer is right; this was an error in the text. It now reads "the pax6+/rx3- posterior region was slightly bigger in cavefish (Figure 3L)" (line 221). The quantification of this phenotype has been added and is now shown in Fig 3L.

The authors confirm that nkx2.1a hypothalamic marker expression limits the ventral medial eye field domain and that cavefish has a broader and longer nkx2.1a expression that shows overlap with the rx3 domain. The more anterior position of the hypothalamus in cavefish may also be indicative of the suggested heterochrony in cavefish compared to surface fish.

> We agree with this remark. We slightly modified the section heterochronic cavefish in the discussion and included this point (line 654).

Reviewer 1 Comments for the Author:

Minor Suggestions:

1. Line 212: The authors mention that the “pax6+/rx3- posterior region was slightly smaller in cavefish (not shown)”. However, when comparing panels C and D in figure 3 it looks like this region is bigger in Cavefish because there is less rx3 posterior expression in these samples. Could the authors please comment on this discrepancy?

> Lines 194-196 - As written above, it was a mistake. The pax6+/rx3- domain lying posterior to rx3, whose volume corresponds to the difference between [pax6 volume] and [coloc vol] for each sample, is significantly larger in cavefish (p value < 0.0001). See modified Figure 3L.

2. Line 228: “rx3/pax6 non-expressing domain”. Is not very clear, can the authors please find a better way to describe this.

> OK. We replaced “rx3/pax6 non-expressing domain” by “the rx3-/pax6- medial domain” (line 955-56)

3. Line 229: “(M-N) Maximum intensity projections (3 µm, mid-stack)”. Maximum intensity projections of what?
> In the legend of Figure 3, “Maximum intensity projections” was changed to “Maximum intensity projections of intermediate substack (3 µm).” (line 957)

4. Line 231: “…lines, Q)”. Lines in O?
> Mistake corrected in legend to Figure 3 (line 958)

5. Line 231: “Shaded rectangle indicate eyefield posterior limit”. Ambiguous, maybe indicated the rx3 expression domain? Otherwise, which end of the ends of the shaded rectangle indicated the posterior limit of rx3?
> These shaded rectangles were indeed confusing, so we decided to remove them.

6. Line 236 and 253: gradient o graded expression of pax6? I think the latter as pax6 is not a diffusible molecule. This also applies to other parts of the manuscript.
> Totally right, we changed to “graded expression” throughout the manuscript.

7. Line 238: “…intensity in posterior rx3+ cells was six times higher than in anterior rx3+ cells”. Is that limit defined by expression 0.2 in Fig 3F?. Please clarify?
> In A-P plot profiles, Pax6 average levels between A-P positions 0.1-0.3 in the x-axis are 0.109 in surface fish and 0.146 in cavefish. Between A-P positions 0.7-0.9, Pax6 average levels reach 0.768 in surface fish and 0.780 in cavefish. It is now indicated in text. (lines 251-252)

8. Line 239: The description for the expression of Pax6 “Pax6 levels in the intermediate region were also significantly higher in cavefish, suggesting subtle variations in the control of its expression between the two morphotypes (Figure 3F, right).” Is not very accurate and does not make justice to the amount of work performed. Maybe explain what you define by intermediate region? It is also ambiguous as you use the word ‘also’ when in the previous sentence you argue that there is no difference between both conditions.
> OK. We now defined the intermediate zone [0.4-0.6 in the x-axis]. (Line 212-213). It corresponds to the region where the graded expressions of rx3 and pax6 show opposite trends (i.e., decreasing for rx3 and increasing for pax6 along the x-axis). The adverb “also” was removed.

9. It would be helpful in Fig 3G to better define what we are looking at in the figure legend. The analysis is focused on the expression of pax6, but you look at the anterior, medium, and posterior region in the expression limits of rx3, not pax6.
> Line 952-954 - We better defined Fig 3G legend and the ROI used for the measurements in 3H. We added pax6 and rx3 labels in the corresponding panels of Figure 3.

10. In general the figure legends would benefit by making them more descriptive, so the reader does not have to guess about what the panels are showing.

> We faced the huge problem of space to fit the authorized word number limit. We have now added some descriptions and improvements of the legends and we will concomitantly ask the editor for a possible 300 words increase in the manuscript length.

11. As in cavefish, the expression of rx3 is much reduced in zebrafish tcf7l1a mutants. However, the eye size phenotype seems to be regulated by compensatory growth mechanisms in the zebrafish retina. Hence, the reduction of rx3 on its own may not explain the eye phenotypes observed in cavefish. Could the authors speculate on this discrepancy?
> Lines 529-530. In cavefish, the optic vesicles and the eyes formed are always of smaller size than in surface fish. In a recent paper we have examined the proliferation patterns in the optic vesicles of cavefish and surface fish and observed that cavefish optic tissues proliferate at least at the same rate as surface fish, proportionately to their size (Devos et al., Biology Open 2021). Between 11hpf and 13.5hpf, there was a potential slight compensatory effect (no statistical significance) that was reminiscent of the tcf7l1a mutants described in Young et al, 2019. However, in cavefish, and contrary to the tcf7l1a mutants, the small and apoptotic lens soon will trigger eye degeneration, which most probably impairs any attempts of compensatory growth. Thus, the reduction of rx3 expression and domain size on its own explains well the eye phenotype observed in cavefish.

12. Considering the extensive depth of the discussion, I miss the authors elaborating on the fate and aspects of the posterior eye field domain that is only pax6+.
> Lines 441-443 – Indeed, this important information was missing from the discussion. We added a sentence: “The posterior rx3-/pax6+/barhl2-/emx3- eyefield cells, subdivided in at least two populations (zic1+ and zic1-), are potentially fated to become posterior retinas and RPE.”

13. The altered AP position of the hypothalamus in cavefish was mentioned in the results section but not in the discussion, which only comments on the condensation of the eye field. Could there be a relationship between both phenomena?
> A functional experiment to potentially link altered position of hypothalamus in cavefish and lack of eyefield condensation would be great to perform, for instance by manipulating Shh levels. For reasons of space and since it is not the scope of this paper, we preferred not to include such hypothesis in the discussion. We commented only the second facet of heterochrony (condensation) in the discussion section.

Reviewer 2

The manuscript entitled “A 3D molecular map of the cavefish neural plate illuminates eyefield organization and its borders in vertebrates “ reports a descriptive study based on imaging and quantification of a set of mRNA markers of anterior neural plate territories in cavefish, aiming to understand better the complexity of territories and boundaries in and around the eyefield and their variations between cave and surface variants. The study is very meticulously done and leads to a couple of interesting conceptual hypotheses, the most prominent being that the eyefield is already molecularly subdivided into antero-posterior, medio-lateral and ‘dorso-ventral’ domains. However, I find that there is often a rather substantial leap from observations to claims/conceptual conclusions. A more careful set of conclusions is advisable and some additional staining needed to support the conclusions of this work.
> We have carefully addressed all the points, see below.

Reviewer 2 Comments for the Author:

-When referring to pax6 in the introduction, the authors need to be more specific. Pax6a is expressed in future posterior retina and diencephalon and Pax6b is expressed in the whole eyefield, nasal placode territory and anterior diencephalon.
> We refer to pax6a, as indicated in the phylogenetic tree shown in a new Fig. S4. We changed the text accordingly (line 211-214).

-In the intro. what is the evidence supporting the statement that the reduction on volume of rx3 in cave fish is due to lack of posterior medial parts?

The anterior part is narrower and stumpy therefore reduced too.

> Lines 106-107 – Indeed, formally, at this step of the study, no strict evidence support this statement. We changed the text to “The rx3 domain showed a 25% reduction of volume in cavefish (Figure 1M), likely corresponding to a lack of posterior rx3 expression or a more isotropic size reduction (Figure 1J-J’’’).” It is also mentioned in discussion (lines 603-604).

-The authors rightly state that the shape of the anterior neural plate is highly dynamic at a ‘same’ stage and therefore it is extremely important to make sure that the stage is as close as being the same when comparing surface with cave ANP. They also correctly observed that the curvature of the anterior border of rx3 is more pronounced in cave fish at the same stage. Given this observation, in a couple of cases the experiments do not manage to show convincing matching ages (curvature of cave domain equal or lower than their compared surface embryos: Cxcr4b (Figure 2) and emx3 (Figure 7) expression domains.
> For figure 2, we disagree with the reviewer since the D factors for the two samples (calculated based on their rx3 expression profile) shown were 2.25 and 2.5, respectively. Therefore, they are comparable. Moreover, when comparing Cxcr4b, one sees the same dorsal (A” vs B”) and ventral (C” vs D”) patterns.
In Figure 7B, we changed the panel for CF to be more consistent regarding D factors. We now present samples with very similar D factors (calculated on rx3: 4.5 for cave and 4.75 for surface). Of note, it is important to say that the gap between rx3 and emx3 and its absence in CF were very penetrant phenotypes, seen at all D factors.

-The absence of eyefield condensation over time in cave fish is a rushed conclusion. It may be as likely that neurulation itself is overall slower in cave embryos. This needs to be checked by measuring medio-lateral ‘shrinkage’ of Pax6 domain over time in surface and cave during the same time interval to distinguish between neurulation and eyefield condensation.

> We performed these additional measurements, now shown in Fig. S5. We measured medio-lateral ‘shrinkage’ of the pax6 domain, for the same d factor intervals in both morphs. The results show that similar slopes are observed in SF and CF when medio-lateral widths are plotted according to D factor. We changed the text accordingly (Lines 196-199). This new piece of data rules out the possibility that neurulation itself is slower in cavefish. It thus confirms our interpretation that condensation over time is compromised in CF. We produced a new Supplementary Figure (Fig. S5) to illustrate our conclusion.

-The finding in Fig 1 that the rx3 domain has higher curvature in cave fish support my opinion that movie 3 is done at a younger stage in cave fish than movie 2 (surface fish) it is compared to. Moreover, movie 3 is made differently. Movie 2 and 3 needs to be more comparable.
> We changed Movie 2 and Movie 3 to allow 3D comparisons between morphs.

In zebrafish rx3 intensity changes from weak and patchy to high and homogenous from expression onset to bud stage. Is the surface embryo displaying more heterogenous rx3 (and complementary cxcr4) expression levels at earlier stage, suggesting that cave eyefield is ‘stuck’ in its early developmental stage? This needs to be checked.
> The reviewer raises a very important aspect of rx3 expression pattern description. We analyzed rx3 expression pattern at an earlier stage during gastrulation in both morphs to compare it with that of tail bud stage. These new results are now presented in modified Figure 1 (panels W-Y) and show quantified rx3 dynamics at “single cell” level. They allow distinguishing patchiness at the onset of expression from true expression heterogeneity. At 80% of epiboly, we observed embryos with either patchy or homo/heterogeneous pattern, which allowed discriminating patterns visually and finding quantitative differences (single cell quantification).
We also slightly changed the discussion, deleting the “Swiss cheese” analogy, which lets think that there are on-off levels in the rx3 domain in cavefish, which is not the case.

-The measurement of rx3/cxcr4 expression level is possibly mis-interpreted. Fig 2J shows that the cave fish distribution of expression levels of cxcr4 is the same as surface, just shifted by overall lower expression of rx3 in the cave fish (counting also showing lower number of eyefield cells in the cave fish). The bar chosen to define low and high for cxcr4 skews the results shown in 2 K, L. There
does not seem to be an enrichment of cells of a given cxcr4 expression level at a specific intensity of rx3 expression for any of the two populations but rather an interesting bell curve in the level of

rx3 expression leading to lower level of cxcr4 in both populations. If you plot the intensity of dots independently of their genetic identity, you see majority of cells have low cxcr4 expression and there is a peak bias for low cxcr4 at 0.6 of rx3 expression. The fact that the cave fish rarely reaches this level creates the bias in apparent 'regulation'.
> We produced a new set of complementary histograms to facilitate reader's interpretations (Figure 2K and Fig. S3B-D).

As suggested, we first plotted cell distributions independently of their genetic identity. The reviewer is right, cxcr4 levels in cxcr4-expressing cells are similarly distributed in surface fish and cavefish (Fig. S3, 2nd line, green). Then, if we plot cxcr4b levels according to rx3 levels, the distributions are different in surface and cavefish because of the globally lower rx3 expression levels in cavefish (Fig. S3, 3rd and 4th lines, grey and pale green).

This is represented in another manner in Figure 2K: the bell shaped curve of the distribution of rx3 levels is shifted to the left and asymmetrical in cavefish whereas it is symmetrical and centered on 0.65 in surface fish. Within these rx3-expressing cells, the distribution of cxcr4+ cells (green) follows the same shape/curve, which might suggest that cxcr4 expression level is independent from rx3 expression level.

Then, in the Fig. S3A, we now provide violin plots showing the levels of expression of one gene (rx3 or cxcr4) as a function of the level of expression in the other (cxcr4 or rx3). In surface fish (blue) the two genes vary together, i.e., rx3 expression is higher in cxcr4-high cells and cxcr4 expression is higher in rx3-high cells. It is not the case in cavefish (red): there, the levels of the two genes vary in opposing manner, i.e., rx3 expression is lower in cxcr4-high cells and cxcr4 expression is lower in rx3-high cells. The same data are plotted, as % of cells in cumulated histograms, in Figure 2L. This is what, we think, suggests a difference in the fine regulation of gene expression in the two morphs.
The text has been modified accordingly (lines 171-180).

-The possibility that there is a posterior part of the eyefield that is rx3 negative is potentially very interesting. However, the rx3:GFP transgenics described by Witbrodt and Wilson labs do not seem to show any GFP-negative cells in the eye vesicle (only heterogeneity in expression levels), suggesting that all retinal cells are rx3 positive.
> Indeed, rx3:GFP patterns described by these labs do not seem to show any GFP-negative cells

in the optic vesicles. However, expression of the GFP reporter is very weak at 1ss (Petersen and Morris 2021) and really shows up after that stage (Rembold et al 2006, Cavodeassi et al 2013, Petersen and Morris, 2021). Interestingly, in medaka, Loosli et al, 2003 (Fig 3C) show using colorimetric ISH at 14 hpf that rx3 expression does not occupy the whole volume of the optic vesicles, whereas the paralogous genes rx1 and rx2 (to a lesser extent) or vsx2 do. Tg(rx3:GFP) also shows expression in the lens as revealed in Figure 7CD (Hocking et al, 2018), whereas no endogenous rx3 is detected in the placode at tail bud stage or in the lens during optic vesicle morphogenesis. Importantly, GFP expression is fully lost in chk/rx3 mutant (Cavodeassi et al, 2013). Altogether, these data suggest that endogenous rx3 transcriptional regulation in optic vesicles might be controlled by DNA elements not included in the rx3:GFP construct (which contains "only 4kb" of rx3 promoter). Also, the expression of rx3 in the whole optic vesicle of transgenic embryos does not necessarily reflect expression at the end of gastrulation/onset of neurulation and all these results are not mutually incompatible. Another tempting unifying hypothesis would be that posterior eyefield cells initially do not express rx3, which would then be turned on as off 1SS-3SS in the posterior region.
For the sake of space constraints in the manuscript, we have not included these pieces of discussion in the revised version.

Staudt et al. also had fezf2 overlapping with barhl2 and fate mapped to hypothalamus and prethalamus. Is this marker also having a gap with Rx3. Double in situ with rx3+barhl2 and rx3+fezf2 are needed to support the statement, with AP and DV intensity quantification as the boundaries
are likely to vary across tissue depth. The statement of a rx3-negative domain in the eyefield needs therefore to be more carefully worded. Only a fate mapping of this population specifically would ascertain their eye identity.

> As suggested, we performed double in situ for barhl2/rx3. Image analysis and quantification confirmed the existence of large gap between rx3 and barhl2 domain, as already described for zebrafish at the same stage in Young et al, 2019. This result reinforces the model in which a posterior eyefield domain initially composed of pax6 cells lies posterior to rx3. We included a new Fig. S6 to show these data and slightly changed the text (lines 231-234) and discussion (lines 530-531).

Moreover, emx3 has been described as having small latero-posterior 'arms' surrounding the eyefield (similar to zic1 in Fig. 5A). Would this territory be eye then? Or is this area a dorso-posterior telencephalic population? Staudt fate map rather supports this latter possibility with fate of this population being dorsal posterior telencephalon and epithalamus.

> To answer this point we performed pax6/emx3 double stainings, and indeed found a small posterior territory co-expressing both markers (shown in a new Fig. S12). This result suggests that a small subset of emx3+ cells could be posterior eyefield cells or alternatively that this region, fated to become prospective telencephalon and/or epithalamus by Staudt and colleagues, contains another subdomain composed of pax6 positive cells.
We modified the corresponding section accordingly (lines 347-350).

-Rax in mouse and rx3 in fish is expressed in both eyefield and anterior-most hypothalamus, therefore expecting a population expressing both rx3 and nkx2.1a. The section needs to be re-written according to this published knowledge. The finding that there are more anterior hypothalamic cells in the cave embryos is very interesting and indeed fits with the increase in sHH.
> We modified the section according to the published knowledge, and with the aim of distinguishing prospective hypothalamus and hypothalamus, basal and alar hypothalamus.
lines 262-265.

-The more anterior position of the nkx2.1 rostral tip in cave fish may well be due to a faster convergence/extension during gastrulation, already suggested in previous publications.
> We took in consideration the hypothesis that the more anterior positioning of the prospective hypothalamus could be due to the earlier/faster convergence and extension previously described in our team (Torres-Paz et al., 2019). However, when we compared the rostral limit of the hypothalamus relative to the rx3 expression domain at different stages of neurulation, by comparing embryos with different D factors, we did not find that hypothalamus position changed significantly over time during the stages analyzed (Fig. S7). We propose that the differences found in the prospective hypothalamus position between cave and surface embryos may rather be due to the expanded specification of the hypothalamus, as a consequence of enhanced SHH signaling.
Lines 250-255.

-The medial rx3+,zic1-negative population much more prominent in cave embryos and located ventrally is likely to be hypothalamic.
> See modification Line 443-445. Indeed, the rx3+/zic1-/nkx2.1- population could either be part of the prospective alar hypothalamus and/or the ORR.

-The subdivision of the 'telencephalic population into three domains is very interesting. However, emx3 overlaps with dlx3a and therefore the emx3 only domain may well be placodal instead of telencephalic. A series of double in situs with dlx3a in surface and cave embryos is needed.

> We performed dlx3b/emx3 fluorescent double in situ as requested and confirmed that these two markers overlap, as already shown in Toro and Varga 2007. We would like to respectfully oppose to reviewer 2, that a emx3+/dlx3+ territory could be interpreted either prospective placode (based on dlx3) or prospective telencephalon (based on emx3), as suggested by Toro and Varga 2007. We added a new Fig. S11 to show the existence of yet another subdivision at the telencephalic border based on these dlx3 stainings. Given that subdivisions in the prospective telencephalon is not the major topic of our manuscript, we did not analyze in further detail this forebrain territory.
We also gave tribute to Toro and Varga (2007) for the zic1/emx3 overlapping domain already described as well as the dlx3/emx3 territory fate mapping.

-Finally, the map on Fig. 8A is misleading. The rx3- territory is shown much bigger than the expected narrow gap between rx3 and barhl2 published previously (example Young et al. 2019).
> We disagree on this point. The gap between rx3 and barhl2 described in zebrafish (Young et al, 2019) looks narrow. In our new Fig. S6, a full-stack maximum intensity projection is presented, which clearly shows the actual large space lying between the two markers.

Also, the authors do not consider the emx3/rx3 on this map. This gap is expected to be lhx2+. Could the author provide a double in situ lhx2+rx3 in cave embryos?
> In order to show the conservation of the slight anterior extension of lhx2 in cavefish, we produced a new supplementary Figure 10 and an animation comparing two entire SF and CF stacks in the z axis (Movie 8).

Moreover, the data in Fig. 6N, O is not clean enough. Rx3 seems very fuzzy/backgroundy in cave embryo, making it difficult to understand what is the identity of the zic1 gap in the cave embryos.
> A new panel was made for Fig 6N-O (also including panels without DAPI staining for clarity) to better illustrate the zic1-depleted phenotype and to facilitate the understanding of the identity of zic1 gap cells in the cave embryos.

The status of the three ‘telencephalic’ territories need to be clarified better in cave embryos.
> See above

Reviewer 3

In this paper the authors aim to define a molecular portrait of the eyefield. To do so, they perform in situ hybridisation on embryos of the dimorphic fish, Astyanax mexicanus (which comes in a wildtype and a troglodytic form) comparing the two for subtle differences. The key advances are the detail in which the authors have analysed gene expression profiles. The authors have performed the work in 3-d – so have performed high quality imaging and image analyses. They have performed quantitative analyses on the 3d images, including measurements of area, volume, fluorescence levels in individual cells – to provide quantitative analyses of gene expression profiles in 3d, and a quantitative comparison of the wild type and troglodytic fish. By taking into account the parabolic shape of Rx3 expression, they have subclassified the 10h fish according to subtle morphogenetic advances.
The detailed description of the different gene expression patterns is a step-forward in the field.
> Thank you.

Reviewer 3 Comments for the Author:

However, I do have concerns. Although a very thorough piece of work, the manuscript is descriptive.
A key aim is to distinguish the eyefield from adjacent territories – the posterior limits of the eyefield are currently not well-defined. A second aim is to describe sub-territories, within the eyefield and at its borders with adjacent territories. I am not convinced that the work has added significantly to what we already know through other studies.

Comments

The manuscript is written as a series of careful observations on different genes, but comes across in
a disjointed manner that makes it hard to see the overall picture, especially when it comes to border regions. This makes it difficult to judge a key aim of the paper (ie to define the eyefield). Specific examples are provided below:
It is not clear whether some of the territories described as eye field are uniquely such. Rx3 is introduced as an eyefield-specific marker (with references to previous studies in zebrafish), but Rx3 additionally marks the hypothalamus (eg Tessmar Raible et al 2007).
> Rx3 has been shown to be expressed in hypothalamus by Loosli et al, (2003) and by later studies using transgenics. This expression is detectable at 14hpf but not at neural plate stage. Interestingly, at tail bud, Tessmar Raible et al. (2007) showed a double rx3/nkx2.1a staining using colorimetric ISH, suggesting that some cells might express the two markers. However, without any confocal imaging, it is impossible to assess which ones. In our study, we were able to determine

that some ventral rx3-expressing cells are also positive for nkx2.1, and to observe differences in the size of this cell population between the two morphotypes.

The 25% reduction in posterior-medial parts of the Rx3 domain in the cavefish (a key conclusion of Figure 1) could include changes to the hypothalamus (an idea supported through Fig 4).
> The data presented in Figure 4 show that the rx3+/zic1- posterior/medial domain of the ANP, which is larger in cavefish, encompasses a thin layer of nkx2.1a+/rx3+ cells at its ventral border. We think that the nkx2.1 marker strongly suggests a basal hypothalamic fate for these cells. Conversely, the rx3+/zic1-/nkx2.1- cells might be oriented towards a prospective alar hypothalamic or ORR fate. This is illustrated in Figure 8B (schematics). Finally, we proposed that the posterior rx3-/pax6+/barhl2-/emx3- eyefield cells, subdivided in at least two populations (zic1+ and zic1-), are potentially fated to become posterior retinas and RPE (see lines 441-445).

Likewise - in Fig3, an outstanding question is whether the posterior-medial Pax6-Rx3- domain (expanded in the cavefish) likely harbours diencephalic/hypothalamic midline cells that are expanded in the cavefish. This was, in fact, addressed in Fig 4 (they are NKx2-1+).
But I struggled to understand an overarching model. Do the authors think that there are changes to cell migration, proliferation and/or morphogenesis?

> We think that the changes observed at the midline are due to changes in cell specification, locally (probably under the influence of variations of signaling), rather than migration or proliferation and morphogenesis. This would be sufficient to explain the discrepancy between SF and CF neural plate at the rx3/nkx2.1 border.

How do these subtle changes really help to define the eye-field as distinct from the nascent hypothalamus? Previous studies (including Staudt and Houart 2007) in zebrafish have combined analyses of gene expression patterns with fate-mapping studies. These highlight the difficulties in establishing the difference between eye-field and hypothalamic territories, especially in ventral regions. I hoped that the current careful analyses would help to address this, but was not convinced that it did.
>Indeed these subtle changes do not help per se to define eyefield as distinct from nascent hypothalamus in this specific and tiny area, but rather show micro-evolutionary variations of great interest in the evo-devo field, with potential interest in human health.

On a similar note, the authors define the hypothalamus through Nkx2-1 expression, but in most species Nkx2-1 defines the basal, not the alar hypothamus (for instance the paraventricular region).
This is not addressed at all.
> Thanks to reviewer 3, we tackled this question at two different levels, distinguishing the dorsal and lateral borders of the nkx2.1+ prospective hypothalamus with the eyefield, respectively. Figure 4 shows the analysis of the dorsal border (nkx2.1 <-> rx3 boundary) and a new Fig. S8 shows the lateral borders (nkx2.1 <-> pax6).
To better map the border between prospective hypothalamus and eyefield, we performed double ISH pax6/nkx2.1. Our results show that neither anteriorly nor posteriorly do rx3 and pax6a overlap with the lateral side of the nkx2.1 domain, even in cavefish where the nkx2.1 medial domain is expanded (Fig. S8, arrows in Fig 4D'; lines 322-326). This indicates that only a tiny population of cells at the dorsal interface of nkx2.1 with rx3 express both markers. And this population is more important in cavefish. However, at the lateral interface of nkx2.1 with rx3 or pax6, no double-labelled cells are detected. This new piece of data demonstrates that, in this position, there is a thin layer of cells that are neither pax6 (=eyefield) nor nkx2.1a (=basal hypothalamus) positive. These cells likely correspond to the prospective alar hypothalamus, which can be distinguished from basal prospective hypothalamic based on absence of nkx2.1a expression.

The authors need to explain which strain of cavefish was analysed: this is not my area of expertise, but I believe there are numerous strains – which have undergone convergent evolution – so the findings here may be particular to one strain?

> The Pachón cavefish population was used for the whole study, see material and methods section (line 684).

For instance - the different correlations in Rx3-cxcr4b between surface and cavefish (Figure 2) are interesting – but is this seen in other cavefish strains? And is it mechanistically important?
> The comparative analysis of rx3/cxcr4b pattern, as well as all the other markers we used in this study would be very interesting to compare between different, sometimes independently-evolved, cavefish populations. However, in our lab we only have Pachón fish. It is also the cavefish population that has been the best studied in developmental biology analyses, and for which some information is available regarding gastrulation, eye morphogenesis and degeneration processes. We agree that the comparison with other cavefish populations would be interesting, but doing it with the depth of analysis used in our study seems like a monumental task.

The authors overlook/fail to discuss, morphogenetic processes that might contribute to the patterns they observe: I realise this would be speculative, but it would add weight to the discussion and help to frame a model.
> The proposed teleost ANP molecular portrait was deduced only from fixed tissue analyses, at a single stage (10 hpf). As mentioned in the introduction, the extent of cell migrations and tissue rearrangements at that stage is important. However, by looking at hundreds of samples that were classified according to their neurulation progression (d factor), we were able to find logic and order behind inter-individual variations. And this was true for all markers analyzed. This shows that cavefish and surface fish share the same ANP Bauplan, which vary subtly. We think that true variations in morphogenetic processes occur later, during the formation of the eye vesicle (see Devos et al., Biology Open 2021). We prefer not engaging in hypothetical scenarios that our data could not rigorously support. Moreover, we desperately lack space for the manuscript.

Some additional minor comments:

From the outset, please define the two fish forms – ie 'surface' and 'cavefish'.
> The two fish forms are now defined in the introduction (lines 90-91).

I cannot follow the figure legend in Figure 1 – in particular I-I"'. The authors suggest these show evidence for variation in Rx3 expression between samples (line 128) – but it was not clear from the legend that these were different samples.
> We distinguished (sub)stages of neurulation using the "d factor" and inter-individual variability between samples of same d factor. In Figure 1I-I''', what is shown is the classification of different 10hpf samples according to "neurulation advancement" categories, to base sample comparison on a more objective parameter than the experimenter's eye. Within these categories, there is also inter-individual variation, e.g. in terms of size and shape (Figure 1P).

Figure 1Q – the 'homogeneity' vs 'heterogeneity' in surface vs cavefish in 1Q is subtle – please highlight ROI in graphs.
> The heterogeneous expression pattern of rx3 in cavefish is visible to the eye under a binocular loupe. Using confocal microscope, each cavefish rx3 expression pattern shows heterogeneity, revealed at individual level (Figure 1Q, left and 1V).
We included ROI = line in C,D above the graph.

Figure 4D, D' – do not match the text, which refer to 4D, E.
> The correction was done.

The subtle gap between Rx3 and Nkx2-1 needs to be pointed out with an arrowhead
> Arrows were added to Figure 4D'.

In general there are a number of instances where figure and the graphs have been incorrectly labelled – ie figures and plots that do not match the text.
> We made the necessary corrections all along the text.

Second decision letter

MS ID#: DEVELOP/2021/199966

MS TITLE: A 3D molecular map of the cavefish neural plate illuminates eyefield organization and its borders in vertebrates

AUTHORS: Francois Agnes, Jorge Torres-Paz, Pauline Michel, and Sylvie Retaux

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the remaining concerns of reviewer 2 can be satisfactorily addressed. This reviewer is concerned that your data does not distinguish whether cells are destined for posterior eye field or diencephalon and that your interpretation of expression patterns may consequently be incorrect. I agree with the reviewer on this point and, more generally, toacknowledge the limitations of using gene expression patterns to infer later fates without tracking cells. Please attend to the referee's comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors have acknowledged and addressed all suggestions I made to the original manuscript and have made changes accordingly.

Comments for the author

I have no further observations and think the current manuscript is ready for publication.

Reviewer 2

Advance summary and potential significance to field

The new version of the manuscript is much improved. However, the core difficulty in interpreting expression maps resides in the assumption that genes are expressed in same domains across species. This assumption is not always correct introducing a danger of misinterpretation of data.
I am still very puzzled by the interpretation of cell identity of the pax6 domain.
I can't find any compelling evidence from the descriptive data presented that supports labelling THE WHOLE of this region posterior eye field. If true, the eye field in astyanax is taking a much bigger portion of the neural plate than in other fish studied so far. This needs to be further elucidated as it is the one original finding in an otherwise mostly confirmatory set of observations.

Comments for the author

In fish, chick and mouse, the neural plate domain expressing pax6 has been found to cover most forebrain territories, including diencephalon, based on fate mapping data as well as descriptive gene expression studies. The interpretation of the authors is solely based on their assumption of pax6 being a marker of eye identity. As they say: 'the prominent eye-gene marker pax6'.
If the authors want to convince the reader that the pax6 cells behind the rx3+
field are eye field cells, they will need some experiments of labelling these cells and follow them over-time, tracing them to the brain or eye area they end up in after neural plate closure. Any other marker-based argument is always going to be weak.

What is the domain expressing other than pax6 and zic1? zic1 has been published as pan-forebrain neural plate marker in frog and 'eye-field' in fish. Across species and across time gene expression is highly dynamic for all markers we ever consider. It is really very weak to base strong conclusion on gene expression without backing them up by cell labelling or genetic manipulations.

Reviewer 3

Advance summary and potential significance to field

In this paper the authors aim to define a molecular portrait of the eyefield. To do so, they perform in situ hybridisation on embryos of the dimorphic fish, Astyanax mexicanus (which comes in a wildtype and a troglodytic form) comparing the two for subtle differences. The key advances are the detail in which the authors have analysed gene expression profiles. The authors have performed the work in 3-d – so have performed high quality imaging and image analyses. They have performed quantitative analyses on the 3d images, including measurements of area, volume, fluorescence levels in individual cells – to provide quantitative analyses of gene expression profiles in 3d, and a quantitative comparison of the wild type and troglodytic fish. By taking into account the parabolic shape of Rx3 expression, they have subclassified the 10h fish according to subtle morphogenetic advances.

The detailed description of the different gene expression patterns is a step-forward in the field, and the revised manuscript provides additional data where it is easier to judge how borders between eyefield and adjacent territories have been defined, as well as sub-territories within the eyefield.

Comments for the author

No further revisions required; I still think that some of the changes could reflect differences in morphogenesis, rather than patterning - but I do accept that there is a huge amount of careful work here, and that it is a careful descriptive analysis that can provide a reference for future studies

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Second revision

Author response to reviewers' comments

Responses to the Editor and Reviewer2

Editor's remark:

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the remaining concerns of reviewer 2 can be satisfactorily addressed. This reviewer is concerned that your data does not distinguish whether cells are destined for posterior eye field or diencephalon and that your interpretation of expression patterns may consequently be incorrect. I agree with the reviewer on this point and, more generally, to acknowledge the limitations of using gene expression patterns to infer later fates without tracking cells. Please attend to the referee's comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

> Thank you for your positive appreciation of our work.

It is, practically and currently, impossible for us to provide a proper fate-mapping study to support our interpretations. This would constitute a completely new project, largely beyond the scope of the revision of this manuscript (but we are indeed very interested in doing so in the future as we think the current proposed teleost map will serve this aim).

We are aware and agree on the limitations of using gene expression patterns to infer later fates without tracking cells, so to be rigorous and honest we have now added a statement clearly acknowledging this limitation, in the Discussion section: *"… thanks to the comparative and quantitative observations made in our comparative model. These hypotheses are based solely on expression patterns and will need further tracking and lineage analyses for confirmation."*

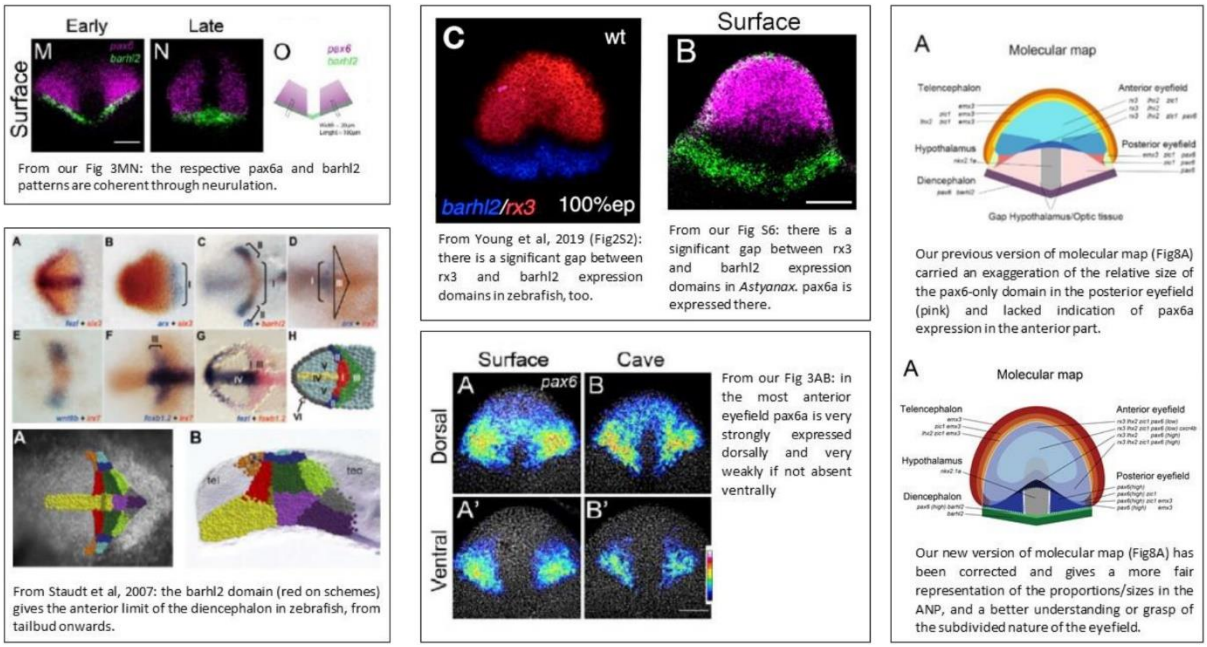
Nevertheless, as a point of interesting scientific debate with you and reviewer2, we would like to stress the following: no, we do not have cell and lineage tracking; but yes, we do have evo-devo/ intraspecies comparative indication that the tentative fates we suggest for the subdivisions of the eyefield probably make sense (i.e. variation in relative size of sub-territories of same identity between the two morphs). This was the very goal of our systematic *quantitative* comparison of the organization of the neural plate in the two morphs, taking advantage of the cavefish/surface fish comparison:

" The rx3+/zic1+/pax6a+ cells would represent the anterior eyefield potentially fated to become retinas, which are smaller in cavefish (Alunni et al., 2007; Devos et al., 2021; Yamamoto and Jeffery, 2000). The posterior rx3-/pax6a+/barhl2-/emx3- eyefield cells, subdivided in at least two populations (zic1+ and zic1-), are potentially fated to become posterior retinas and RPE. By contrast, the medial rx3+/zic1- cells, which occupy a larger territory in cavefish, could prefigure the medial ORR/alar hypothalamus and the optic stalk region, both larger in cavefish at 24hpf (Torres-Paz et al., 2019) ».

Further, detailed responses to reviewer 2 are below.

Reviewer 2

Please, check the figure assembled below, which contains the elements found in the literature and in our manuscript to support our responses:



The new version of the manuscript is much improved.

> Thank you

However, the core difficulty in interpreting expression maps resides in the assumption that genes are expressed in same domains across species. This assumption is not always correct introducing a danger of misinterpretation of data.

> It is true that the risk of mistakes based on gene expression comparisons is not zero. However, the whole field of evo-devo and its subfield aiming at defining homologies from orthologous genes

expression patterns is based on this assumption. There is always a risk of paralog inversion, but it is not the case for the crucial genes that are discussed here. *Rx3*, *pax6a* and *barhl2* in zebrafish and *Astyanax* are expressed in the same manner topographically, with *pax6a* domain extending more posteriorly than *rx3*, and abutting posteriorly on the transverse band of the *barhl2* domain. Moreover, these patterns are coherent over the time window studied and across the different D factors (our readout of the advancement of neurulation), which reinforces the idea that they can be used at these stages as *bona fide* markers of homologous domains in the ANP.

I am still very puzzled by the interpretation of cell identity of the pax6 domain. I can't find any compelling evidence from the descriptive data presented that supports labelling THE WHOLE of this region posterior eye field. If true, the eye field in astyanax is taking a much bigger portion of the neural plate than in other fish studied so far. This needs to be further elucidated as it is the one original finding in an otherwise mostly confirmatory set of observations.

> We respectfully disagree with this statement.

First, we believe this is not the only novel finding of our paper. All our data on the subdivisions and graded expressions, their respective organization in 3D using double fluorescent ISH, their size quantification, and their intraspecies comparisons in the two morphs of *Astyanax* are novel. We have no knowledge of previous work that have hypothesized and tackled the idea of a highly regionalized eyefield at the end of gastrulation, neither its evolution. In fact, at the beginning of our project, from published data, we had the idea of a single entity, with some degree of overlapping hypothesized based on colorimetric ISH (Zuber et al, 2003).

Second, we think the eyefield in *Astyanax* and zebrafish are totally comparable, in proportion in the ANP. We suspect that the reviewer got this impression from our previous Fig8A (model schema), in which, for sake of illustration and schematization, the relative sizes of the different eyefield subdivisions were not respected. Moreover, the anterior-most part of the *pax6a* expression domain was mistakenly not represented and legended, because it is dorsal and we did not want to give the impression to the reader that *pax6a* was in the whole eyefield along the D/V axis. Indeed, in the *Astyanax* ANP, the *pax6a*-only posterior domain (*rx3* negative) is not that large, especially considering that the posterior-most rows of *pax6a* cells co-express *barhl2*, hence they should belong to the prospective diencephalon. This situation is fully comparable to zebrafish, where there is also a significant gap between the *rx3* posterior limit and the *barhl2* expression band. To correct these problems, we have now generated a new Fig8A/scheme that is more representative of the actual data in terms of sizes and proportions in the ANP, and that will more helpfully serve as comparison with the zebrafish.

Comments for the Author:

In fish, chick and mouse, the neural plate domain expressing pax6 has been found to cover most forebrain territories, including diencephalon, based on fate mapping data as well as descriptive gene expression studies. The interpretation of the authors is solely based on their assumption of pax6 being a marker of eye identity. As they say: 'the prominent eye-gene marker pax6'. If the authors want to convince the reader that the pax6 cells behind the rx3+ field are eye field cells, they will need some experiments of labelling these cells and follow them over-time, tracing them to the brain or eye area they end up in after neural plate closure. Any other marker-based argument is always going to be weak.
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acknowledging this limitation, in the Discussion section: *"… thanks to the comparative and quantitative observations made in our comparative model. These hypotheses are based solely on expression patterns and will need further tracking and lineage analyses for confirmation."*

Nevertheless, as a point of interesting scientific debate with reviewer2, we would like to stress the following: no, we do not have cell and lineage tracking; but yes, we do have evo-devo/ intraspecies comparative indication that the tentative fates we suggest for the subdivisions of the eyefield probably make sense (i.e. variation in relative size of sub-territories of same identity between the two morphs). This was the very goal of our systematic *quantitative* comparison of the organization of the neural plate in the two morphs, taking advantage of the cavefish/surface fish comparison:

" The rx3+/zic1+/pax6a+ cells would represent the anterior eyefield potentially fated to become retinas, which are smaller in cavefish (Alunni et al., 2007; Devos et al., 2021; Yamamoto and Jeffery, 2000). The posterior rx3-/pax6a+/barhl2-/emx3- eyefield cells, subdivided in at least two populations (zic1+ and zic1-), are potentially fated to become posterior retinas and RPE. By contrast, the medial rx3+/zic1- cells, which occupy a larger territory in cavefish, could prefigure the medial ORR/alar hypothalamus and the optic stalk region, both larger in cavefish at 24hpf (Torres-Paz et al., 2019) ».

2> Then, the reviewer argues that our interpretation is solely based on the assumption of *pax6* being a marker of eye identity, because we write: 'the prominent eye-gene marker pax6'. To avoid conveying this (wrong) impression we have changed the text and removed the word "prominent". It now reads *"We next used the eye-gene marker pax6, which forms together with rx3 and six3 the eyefield specific transcription factor network (Sinn and Wittbrodt, 2013). Specifically, we used here pax6a (Fig. S4), which is expressed in the eyefield (Staudt and Houart, 2007) and the future posterior retina and diencephalon in zebrafish at 8 somites (Macdonald et al., 1994)»*
Moreover, please note that we hypothesize a diencephalic fate for the posterior-most rows of *pax6* cells that co-express *barhl2*, therefore we do not consider *pax6a* as an exclusive and specific eye marker.

Much more importantly, our interpretation is based on the anterior limit of the diencephalon given by *barhl2*. Staudt et al (2007) have shown in zebrafish, including using fate tracing experiments, that *barlh2* marks the anterior limit of the diencephalon, from tailbud to larval stage. Given the 3D organization of the prospective forebrain territories (telencephalon, hypothalamus, diencephalon and eyefield), we do not see any other possibility than an eye fate for this small *rx3- pax6a+ barhl2-* domain (intense blue in our new Fig8A). Of course, this interpretation all depends on *barhl2* marking for good the anterior limit of the diencephalon. If not, we cannot rule out that the *pax6a* cells anterior to *barhl2* could end up being diencephalic, but the current elements of literature reject this possibility. Also, note that the situation is identical in zebrafish, as Young et al (2019) also found a significant gap between the posterior *rx3* border and the *barlh2* expression domain.

Third decision letter

MS ID#: DEVELOP/2021/199966

MS TITLE: A 3D molecular map of the cavefish neural plate illuminates eyefield organization and its borders in vertebrates

AUTHORS: Francois Agnes, Jorge Torres-Paz, Pauline Michel, and Sylvie Retaux

Apologies for the delay in reading your revised manuscript and response to the previous round of reviews. I am sure you consider me to be too pedantic, but I still consider that some of the statements you make are too dogmatic when drawing conclusions about fate from expression patterns. Prior to publication, I would like you to do a final check of the text and moderate wording where this happens. For instance, from the results:

These results *show suggest* that the eyefield is not a single territory but a composite tissue.
... potentially had a prospective telencephalic identity in surface fish

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Third revision

Author response to reviewers' comments

Dear Steve,

we apologize for the delay in responding to the rather simpler revision you were asking. However we have been traveling for field work + meeting for quite a long time.
Here we have very seriously and stringently tracked and corrected (in red in the text) all conclusions and formulations that you may have found too affirmative in the absence of fate mapping evidence. We thank you for your "pickiness", which definitively increases the quality and impact of our manuscript.

Best
François Agnès and Sylvie Rétaux

Fourth decision letter

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ARTICLE TYPE: Research Article

Thanks for making the final text changes and I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.