Live imaging of retinotectal mapping reveals topographic map dynamics and a previously undescribed role for Contactin 2 in map sharpening.

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Original submission

First decision letter

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MS TITLE: Live imaging of retinotectal mapping reveals topographic map dynamics and a novel role for Contactin-2 in map sharpening.

AUTHORS: Olivia Spead and Fabienne E Poulain

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 appreciate the quality of much of the data you present but have two major concerns. The first is the relative lack of novel biological insights into map formation and the second is that the analysis of the role of Contactin is preliminary and needs both better validation (for instance through analysis of a stable mutant line) and more refined phenotypic analysis. It seems that if you can address the second concern then this may also address the first concern. Consequently, ifyou 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

The manuscript describes the development of the retino-tectal projection in zebrafish using a new fluorescent tool allowing to follow map development of nasal and temporal axons over time in good detail, therefore having access to parameters such as initial mistargeting, tectal coverage of nasal and temporal axons, refinement index and importantly also the size of the arborisation field.
The study is very well carried out and is technically of high standard. The findings are overall a confirmation of principles of map development in the mouse retino-collicular system, but here now taking full advantage of the zebrafish as a model system.

Comments for the author

Also very interesting is a later part of the manuscript where another player in retino-tectal mapping, cntn2, is introduced. This molecule is shown to be expressed in the nasal half of the zebrafish retina at relevant times of map development. Two independent crispr approaches were used to target the cntn2 locus, while the manuscript appears to lack a characterization of a successful knockout of its protein expression/function.

The phenotype presented appears clear that is the reduced refinement step, i.e. the retraction of mistargeting nasal axons from the anterior tectum appears to occur later, or not at all (did the authors check later time points?). Interestingly, in consequence it would be expected that temporal axons now show also targeting defects since they are now in competition with nasal axons in the anterior tectum.
This is at least a lesson from work in the mouse retino-collicular system, and one would essentially expect the same for zebrafish.

The discussion of the phenotype of cntn2 appears unclear. The interpretation focusses very much on the known cntn1 function, but is it known that cntn2 – like cntn1 – interacts with sodium channels? In the results section, it is introduced as an adhesion molecule promoting axon growth and fasciculation, maybe it is a reduced preferential adhesion in the posterior tectum? What is known about the 'ligand' expression in the posterior tectum?

Reviewer 2

Advance summary and potential significance to field

This manuscript by Spead and Poulain reports the generation of a novel transgenic zebrafish model that allows visualization of the retinotectal topographic map development in live animals. The authors identified regulatory elements from the hmx1 gene that drive expression specifically in nasal retinal ganglion cells. They generated a transgenic line with the hmx1 regulatory element driving Cre recombinase, and the isl2b promoter (which drives expression in all RGCs) driving a lox-red-lox-green colorswitch construct. The resulting transgenics have GFP expressed in the nasal RGCs and TagRFP expressed in temporal RGCs. The authors use this line to carry out a detailed characterization of topographic map formation, and define quantitative parameters to analyze axon arborization, topographic targeting, and map refinement over time. They discover new information about the dynamics of map formation, showing that while the rough map is formed at early stages, it remains dynamic as the retina and tectum grow. The find that nasal RGCs initially arborize in the anterior half of the tectum and then later prune these branches and refine their arbors to the posterior tectum. The authors then go on to use their transgenic model to identify a novel role for Contactin-2 in topographic map refinement.

This transgenic line is an exciting and important resource for the study of topographic map formation. It represents a major improvement over previous lipophilic dye-based approaches, which suffer from inconsistencies in number of cells labeled from one embryo to another. The role for Contactin-2 in map refinement would have been very challenging to reveal with prior methodologies.

This study in itself represents an advance to our understanding of development and the transgenic tool developed by the authors is very likely to lead to further advances in our understanding of mechanisms of topographic map formation.

Comments for the author

A few concerns that the authors should address are detailed below.
1. The authors use CRISPR F0 analysis to assess the function of cntn2. While the effect they see is convincing, it is a fairly subtle effect. The embryos are mosaic for the mutations and the extent of cntn2 loss-of-function is unknown.

There appear to be substantial wild type HRMA peaks in many of the injected embryos. Indeed, the authors acknowledge that they may be missing potential phenotypes on other processes such as axon growth because of incomplete knock-down. Others have found that cocktails of 2-4 guide RNAs can be highly effective at causing widespread mutation in F0 embryos. Have the authors tried injecting both gRNAs together into the same embryos, or cocktails of 4 gRNAs? Also, an antibody to Cntn2 is available at ZFIN (http://zfin.org/ZDB-ATB-091105-1). It would be helpful to know the extent of protein loss in their injected embryos.

2. The images in Figure 6C-F appear to be of lower resolution/quality than the images in previous figures (which are beautiful). As this figure is making an important mechanistic point for the study, it would be best to have images that match the quality of their other images.

3. How did the authors determine that their experiments were sufficiently powered to avoid type I and type II errors?

Reviewer 3

Advance summary and potential significance to field

This study advances the field in two main areas. The first one is technical, with the description of an innovative color-switch transgenic construct to visualize the arborization patterns of different retinal ganglion cell populations. The second advance is to apply this method to uncover a previously unrecognized role of Contactin-2 in retinotopic map sharpening.

Comments for the author

The paper by Spead and Poulain describes the clever use of color switch reporter line to visualize the formation and refinement of the retinotectal map in zebrafish. This is an important new tool that will prove useful in future studies on retinotectal map formation. With these methods, they nicely demonstrate the ongoing refinement of the retinotectal projection with nasal projections initially arborizing anterior before extending to their posterior targets. Finally, the authors describe the involvement of the cell adhesion molecule Contactin-2 (alias Tag1) in this process.

Overall this is a very thorough study with beautiful images and careful statistical evaluations. Nevertheless, I see the paper as more of a technical advance than gaining much insight into the process of retinotectal mapping. It is well known from a number of studies in various species that the map is initially formed in a less precise way and sharpens up over time. This works in parallel to tectal growth and is most likely influenced by neuronal activity. A limitation of the technique is that axons can not easily be labeled sparsely to timelapse single axons during the refinement process.

The demonstration that Contactin-2 is involved in the refinement is a new result, but mechanistically somewhat preliminary.

First revision

Author response to reviewers' comments

We would like to thank the reviewers and reviewing editor for the time spent in evaluating our manuscript. We appreciate their positive comments and remarkably helpful suggestions for improving our study. We have performed a number of additional experiments to address reviewers' queries and have revised our manuscript accordingly (changes in manuscript have been highlighted in yellow). We provide a point-by-point response to the reviewers' comments (shown in italics) below. Our point-by-point response is also available as a pdf document provided as supplementary information.

Reviewer 1
The manuscript describes the development of the retino-tectal projection in zebrafish using a new fluorescent tool allowing to follow map development of nasal and temporal axons over time in good de-tail, therefore having access to parameters such as initial mistargeting, tectal coverage of nasal and temporal axons, refinement index and importantly also the size of the arborisation field. The study is very well carried out and is technically of high standard. The findings are overall a confirmation of principles of map development in the mouse retino-collicular system, but here now taking full advantage of the zebrafish as a model system.

> We thank reviewer 1 for this very positive evaluation of our analysis.

Also very interesting is a later part of the manuscript where another player in retino-tectal mapping, cntn2, is introduced. This molecule is shown to be expressed in the nasal half of the zebrafish retina at relevant times of map development. Two independent crispr approaches were used to target the cntn2 locus, while the manuscript appears to lack a characterization of a successful knockout of its protein
expression/function.

> We agree with reviewer 1 that the new function of Cntn2 is very interesting, and have now started the generation of a stable cntn2 mutant line to conduct in-depth characterization of Cntn2 in future stud-ies. As suggested by both reviewers 1 and 2, we have attempted to measure the loss of Cntn2 protein in cntn2 crispants. However, we have been unable to find an antibody that recognizes zebrafish Cntn2 (see response to reviewer 2 for more details). As an alternative, we have repeated our experiments using a combination of two gRNAs in order to ensure efficient mutagenesis in F0 embryos. Our new results (shown in a new Fig. S8) show a similar phenotype in embryos injected with one or a combination of gRNAs, suggesting that the function of Cntn2 is strongly invalidated in cntn2 crispants.

The phenotype presented appears clear that is the reduced refinement step, i.e. the retraction of mis-targeting nasal axons from the anterior tectum appears to occur later, or not at all (did the authors check later time points?). Interestingly, in consequence it would be expected that temporal axons now show also targeting defects since they are now in competition with nasal axons in the anterior tectum. This is at least a lesson from work in the mouse retino-collicular system, and one would essentially ex-pect the same for zebrafish.

> We thank reviewer 1 for bringing up these excellent points. We have analyzed cntn2 crispants at a later time point (6 dpf) and did not observe any refinement at that stage either, indicating that refinement does not occur in cntn2 crispants. This new data is now presented in a revised Fig. S5. We agree with the reviewer's excellent comment that temporal axons should also be mistargeted in cntn2 crispants due to increased competition with nasal axons. We have however not been able to detect any differences in the TagRFP anterior tectal coverage (Fig. 6H) or the temporal arborization field (Fig. 6G), likely because temporal projections have a very dense topography in the anterior tectal half. We tried to analyze single temporal arbors in cntn2 crispants but have not obtained conclusive results, maybe because of the mosaicity of mutations in crispants. Detecting potential subtle phenotypes will likely require an extensive analysis of stable cntn2 mutants that we will initiate in six to eight months.

The discussion of the phenotype of cntn2 appears unclear. The interpretation focusses very much on the known cntn1 function, but is it known that cntn2 – like cntn1 – interacts with sodium channels? In the results section, it is introduced as an adhesion molecule promoting axon growth and fasciculation, maybe it is a reduced preferential adhesion in the posterior tectum? What is known about the 'ligand' expression in the posterior tectum?

> We thank reviewer 1 for raising these interesting questions and apologize for not having discussed Cntn2's function in more detail in our initial submission. We have now modified our discussion to clarify the different modes of action that could underlie Cntn2 function at the tectum. Our initial discussion focused on the possible link between Cntn2 and neuronal activity because we observed a similar absence of tectal refinement in macho mutants that lack sodium current in RGCs (data not shown). While it remains unknown whether Cntn2 interacts with sodium channels, Cntn2 is highly homologous to Cntn1. In particular, its fibronectin type III-like domains share 48 % amino acid identity (66 % homology) with that of Cntn1, which are known to mediate Cntn1's interaction with voltage-gated sodium channels (Mc Ewen et al., 2004). It is thus possible that Cntn2 might interact with sodium channels as well and regulate activity-dependent refinement at the tectum. As an adhesion molecule, Cntn2 is the only member of the Contactin family known to participate in homophilic interactions (Mohebiany et al., 2014), which raises the possibility that the lack of refinement observed in crispants might be due to a lack of fasciculation among nasal axons resulting in their delayed outgrowth to the posterior tectum. However, we did not observe any fasciculation defect in crispants, and the lack of refinement persists at later stages (new Fig. S5), which argues against a delayed phenotype. Cntn2 also engages in heterophilic interactions with other adhesion molecules including L1CAM, NCAM and NrCAM (Kuhn et al., 1991; Kunz et al., 1998; Suter et al., 1995; Buchstaller et al., 1996; Fitzli et al., 2000), but none of these have so far been shown to be preferentially expressed in the posterior tectum. Finally, Cntn2 is known to regulate other guidance signaling pathways (Sema3A for instance) and could modulate the responsiveness of nasal axons to a yet uncharacterized repulsive cue present in the anterior tectum.

Reviewer 2
This transgenic line is an exciting and important resource for the study of topographic map formation. It represents a major improvement over previous lipophilic dye-based approaches, which suffer from inconsistencies in number of cells labeled from one embryo to another. The role for Contactin-2 in map refinement would have been very challenging to reveal with prior methodologies. This study in itself represents an advance to our understanding of development, and the transgenic tool developed by the authors is very likely to lead to further advances in our understanding of mechanisms of topo-graphic map formation.

> We thank reviewer 2 for his/her positive evaluation of our study and enthusiasm for this new trans-genic resource for the community.

A few concerns that the authors should address are detailed below.

1. The authors use CRISPR F0 analysis to assess the function of cntn2. While the effect they see is convincing, it is a fairly subtle effect. The embryos are mosaic for the mutations and the extent of cntn2 loss-of-function is unknown. There appear to be substantial wild type HRMA peaks in many of the injected embryos. Indeed, the authors acknowledge that they may be missing potential phenotypes on other processes such as axon growth because of incomplete knockdown. Others have found that cocktails of 2-4 guide RNAs can be highly effective at causing widespread mutation in F0 embryos. Have the authors tried injecting both gRNAs together into the same embryos, or cocktails of 4 gRNAs? Also, an antibody to Cntn2 is available at ZFIN (http://zfin.org/ZDB-ATB-091105-1). It would be helpful to know the extent of protein loss in their injected embryos.

> We agree with reviewer 2 that measuring the loss of Cntn2 protein in crispants would be ideal. Unfortunately, we have been unable to obtain the antibody listed on Zfin, as this antibody is not a commercial one. Dr. Claudia Stuermer, whose lab generated this antibody in 2001, indicated that the antibody was exhausted and no longer available. Her former collaborators did not have any antibody left either. As an alternative, we tested five different commercial antibodies known to recognize conserved epitopes in human or rat Cntn2 (R & D Systems AF4439, Invitrogen PA5-86701, Developmental Studies Hybridoma Bank 4D7 and 3.1C12, and Aviva Systems Biology ARP32085). None of them detected zebrafish Cntn2 in western blot assays. We thus followed reviewer 2's

suggestion and repeated our experiments using a combination of two gRNAs to ensure efficient mutagenesis in F0 embryos. Results show a similar phenotype in embryos injected with one or a combination of gRNAs, suggesting that the function of Cntn2 is strongly invalidated in cntn2 crispants. This new data is presented in a new Fig. S8 and mentioned in the discussion.

2. The images in Figure 6C-F appear to be of lower resolution/quality than the images in previous fig-ures (which are beautiful). As this figure is making an important mechanistic point for the study, it would be best to have images that match the quality of their other images.

> We thank reviewer 2 for pointing that out and have included higher resolution pictures in Fig. 6.

3. How did the authors determine that their experiments were sufficiently powered to avoid type I and type II errors?

> We initially conducted a pilot study on 22 crispant and control larvae to estimate the effect size for the comparisons with mixed effects one-way ANOVA. We then conducted a statistical power analysis to determine the sample size needed for our experiments. To minimize the probability of a type I error, we minimized the significance level of our hypothesis tests to 1% (a = 0.01). We minimized the probability of a type II error by increasing the level of significance to 0.1. With an estimated effect size of 0.25, alpha of 0.01 and power of 0.90, the projected sample size needed was 64 for within and be-tween group comparisons. We therefore analyzed approximately 20 larvae per group in two biological replicates (n=19+21+19=59).

Reviewer 3
The paper by Spead and Poulain describes the clever use of color switch reporter line to visualize the formation and refinement of the retinotectal map in zebrafish. This is an important new tool that will prove useful in future studies on retinotectal map formation. With these methods, they nicely demon-strate the ongoing refinement of the retinotectal projection with nasal projections initially arborizing anterior before extending to their posterior targets. Finally, the authors describe the involvement of the cell adhesion molecule Contactin-2 (alias Tag1) in this process.
Overall this is a very thorough study with beautiful images and careful statistical evaluations. Nevertheless, I see the paper as more of a technical advance than gaining much insight into the process of retinotectal mapping. It is well known from a number of studies in various species that the map is initially formed in a less precise way and sharpens up over time. This works in parallel to tectal growth and is most likely influenced by neuronal activity. A limitation of the technique is that axons can not easily be labeled sparsely to timelapse single axons during the refinement process. The demonstration that Contactin-2 is involved in the refinement is a new result, but mechanistically somewhat preliminary.

> We thank reviewer 3 for his/her positive comments on the quality of our work. As Reviewer 3 mentions, we demonstrate a novel function for Cntn2 in mediating the refinement of nasal projections. Although we recognize that the mechanism underlying Cntn2 function remains to be dissected out, this new function was quite unexpected and as such, is of high interest and worth reporting. Since cntn2 was shown to modulate neurogenesis at early stages of nervous system development (Ma et al., 2008) and is expressed as early as 24 hpf in the retina (Thisse et al., 2008 and data not shown), we have tested whether the loss of cntn2 might affect retinal patterning along the A-P axis. Our quantification of hmx1 expression by ISH at 4 dpf revealed a normal patterning of the retina in cntn2 crispants (new Fig. S7), suggesting that Cntn2 acts directly on retinal axons to regulate their refinement. Thoroughly determining whether Cntn2 acts cell-autonomously in nasal axons and how it does so will be the focus of our next study using stable cntn2 mutant and transgenic lines that we will have in hands in about a year.

Second decision letter

MS ID#: DEVELOP/2021/199584

MS TITLE: Live imaging of retinotectal mapping reveals topographic map dynamics and a novel role for Contactin-2 in map sharpening.

AUTHORS: Olivia Spead, Cory J. Weaver, Trevor Moreland, and Fabienne E Poulain
ARTICLE TYPE: Research Article

I am happy to tell you that the referees are happy with your revisions and your manuscript has been accepted for publication in Development, pending our standard ethics checks. The referee reports on this version are appended below.

Reviewer 1

Advance summary and potential significance to field

I am fully satisfied with the response of the authors to my suggestions

Comments for the author

no further suggestions to authors

Reviewer 2

Advance summary and potential significance to field

This manuscript by Spead and Poulain reports the generation of a novel transgenic zebrafish model that allows visualization of the retinotectal topographic map development in live animals. The authors identified regulatory elements from the hmx1 gene that drive expression specifically in nasal retinal ganglion cells. They generated a transgenic line with the hmx1 regulatory element driving Cre recombinase, and the isl2b promoter (which drives expression in all RGCs) driving a lox-red-lox-green colorswitch construct. The resulting transgenics have GFP expressed in the nasal RGCs and TagRFP expressed in temporal RGCs. The authors use this line to carry out a detailed characterization of topographic map formation, and define quantitative parameters to analyze axon arborization, topographic targeting, and map refinement over time. They discover new information about the dynamics of map formation, showing that while the rough map is formed at early stages, it remains dynamic as the retina and tectum grow. The find that nasal RGCs initially arborize in the anterior half of the tectum and then later prune these branches and refine their arbors to the posterior tectum. The authors then go on to use their transgenic model to identify a novel role for Contactin-2 in topographic map refinement.

This transgenic line is an exciting and important resource for the study of topographic map formation. It represents a major improvement over previous lipophilic dye-based approaches, which suffer from inconsistencies in number of cells labeled from one embryo to another. The role for Contactin-2 in map refinement would have been very challenging to reveal with prior methodologies.
This study in itself represents an advance to our understanding of development and the transgenic tool developed by the authors is very likely to lead to further advances in our understanding of mechanisms of topographic map formation.

Comments for the author

The authors include new data in the revised manuscript that strengthen the study and sufficiently address the reviewer concerns.

Reviewer 3

Advance summary and potential significance to field

The authors used state of the art genetic and imaging tools to study retinotectal map formation and identified a previously unknown key player in this process.

Comments for the author

I am very happy with the few adjustments the authors made in the revisions. Congratulations on a really exciting and rigorous study.