Intrinsic positional memory guides target-specific axon regeneration in the zebrafish vagus nerve

Adam J Isabella, Jason A Stonick, Julien Dubrulle and Cecilia B Moens
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MS ID#: DEVELOP/2021/199706

MS TITLE: Intrinsic positional memory guides target-specific axon regeneration in the zebrafish vagus nerve

AUTHORS: Adam J Isabella, Jason A Stonick, and Cecilia B Moens

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some criticisms and suggestions for improving your manuscript; reviewer 1 in particular would like more detailed analysis of the phenotypes you describe. 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

In this manuscript by Isabella et al, the authors introduce the larval zebrafish vagus nerve as a model for target-selective regeneration. Target specificity during peripheral nerve regeneration is critical for functional recovery, yet how neurons achieve target-selective regeneration is largely unknown. Here, the authors apply both laser-mediated axotomy and a single-cell chimera model to conclude that guidance during regeneration is distinct from the developmental mechanism, that target selection is specified by the neuronÂ's initial position in the brain, and that targeting to a branch requires prior innervation. The experiments are well designed, the results are appropriately controlled and quantified, and for the most part the conclusions are very well supported by experimental evidence. While the authors do not identify molecules that mediate target specificity during vagus nerve regeneration, these models are amenable to both drug treatment (as demonstrated in this manuscript) and transcriptional analysis (as demonstrated by previous work from the Moens lab), making them valuable tools for future mechanistic work. Since this is the first description of this model, the manuscript would greatly benefit from the inclusion of additional information and experimental data as outlined below.

Comments for the author

Main points:
1. 53% of axons returning to their original branch suggests a somewhat random mechanism underlying regeneration rather than a specific mechanism. Also is it that of the 53% 89% exhibit topographic re-targeting? How do these numbers compare to other PNS regeneration paradigms (invertebrate and vertebrate)?

2.For the laser transection assay, it would be important to know what the degree of functional regeneration is. This might be difficult to achieve but at least some experimental evidence of synapses being re-established at axonal target sites, e.g. using pre and postsynaptic markers.

3. How often are all PA's reinnervated after injury? It would be helpful to quantify the number of arches that are reinnervated at all, could be a binary yes/no. Moreover, are the more posterior PA's more likely to be reinnervated? The authors note that recovery varies by branch, so additional, more qualitative data would be helpful.

4. Were mis-targeted axons more likely to end up in posterior PA's because they are closer to the nerve (Fig2)? The authors report that the majority of axons were topographically targeted, but additional information on any trends in the errors? Did any axons ever completely miss the PA's and grow to different locations/anatomy?

5. Additional information on the structure of the vagus at the time of injury seems critical for even the initial description of this system presented here, and for evaluating the utility of the model, but is missing, including the myelination status using available antibodies or at the ultrastructural level, presence/absence of glia cell types, how 'mature the nerve is, e.g. whether neurons/axons are been added at later stages etc.

6. For the transplant paradigm, did the transplanted neurons already extend an axon (prior to transplantation)? If so, did it have synaptic connections? This seems critical to know in order to evaluate the degree of regeneration, see above.

7. Similarly, not having encountered an injury gap, does the process of regrowth described here resemble more the process when 'late' born neurons whose axons join a nerve, a process that occurs e.g. during optic nerve development in teleost fish? It would be important to compare the neuron's injury response, e.g. the upregulation of injury response genes to the one in the transection assay, or at least to those commonly upregulated following injury.

8. For the cell transplant strategy the authors state that this strategy reveals target specificity. To further support this conclusion, it seems critical that the authors employ a complementary approach to confirm their finding, e.g. using their laser transection approach transecting individually labeled axons or small group of axons (labeled eg via photo conversion), and measuring the fidelity of growth towards to their original branch and targets. This would eliminate approach specific impacts on the regeneration process.

9. The authors state “we therefore conclude that single regenerating axons cannot rely solely on secreted chemical cues from the target tissue, and instead require pre-existing innervation of their target pharyngeal arch.” Yet, in the Crizo hosts, PA4 was never innervated and therefore by the time of transplantation might not be competent to secrete chemical cues to the regrowing transplanted axon. Indeed, as the authors note, this non-inervated PA might be missing critical support structures, like glia, that might normally secrete guidance cues. While we agree with the conclusion that prior innervation is necessary for reinneration, it is unclear whether the current experiments definitively prove secreted cues are not sufficient. We therefor suggest the authors modify their language regarding these conclusions.

Minor Points:
1. Figure 1J: For J, the Red vs Blue labels (severed vs control) alternate from the left to the right side for each PA in the graph. It seems like the left side would correspond to the control as these values are consistently higher. Are the colors mis-labeled on the graph? If not, it seems like for some PA’s, such as 5 and 7, the fluorescence in severed conditions is higher than controls. Is this due to debris? Or additional defasciculation? Can the authors either correct the colors, or perhaps another quantification method is necessary if there is so much variability between conditions using this measure of fluorescence. Additionally, please specify what is being normalize for the Y axis. Given the amount of background or debris fluorescence, a valuable control might be to perform this severing experiment comparing an animal where the neurons themselves have been ablated or otherwise cannot regrow for the 6 dps to the control condition.

2. Figure 3H: Adding an arrow to denote end of green axon would be helpful.

Reviewer 2

Advance summary and potential significance to field

This manuscript describes with great care and exceptionally well illustrated, how neurons in a particular position in the vagus nucleus regenerate their axons into the different branches of the vagus nerve. The novelty described well is that the mechanism used is unlike the developmental mechanisms previously described by the same group. The findings lead to the assumption that neurons in the vagus have a memory of their previous position and the "appropriate" branch to re-innervate. The nature of that memory is not clarified.

Comments for the author

Overall, this paper is exceptionally systematically constructed and beautifully illustrated.
Major points:
I am missing discussions of Schwann cells as guidance structures, both as guiding channels and as origin of molecules promoting axonal repair. Only two papers have been cited, but there is insufficient information in the manuscript regarding Schwann cells in their system. Are there any at the time of lesion (3 or 4 dpl)? Do they linger?
Essential: describe Schwann cells at each time point. New experiments may not be needed
From the movies, it appears that the regenerating axons use the pre-existing axons to grow into the branches of the vagus nerve. Could the molecular "memory" simply be axons following other axons into their branch?
Could it be the first axon the encounter? This may be also mediated by a topography in the nerve, as has been described for the optic nerve. Have the authors managed to capture the actual point at which the axons make their decision (maybe similar to the horizontal myoseptum where CaP, MiP, and RoP axons choose their individual paths)?
Essential: Address questions above. This is briefly touched upon in lines 416ff, but not expanded enough.
New data from analysis of existing movies would significantly strengthen the paper. Specific experiments to clarify the two options discussed should be suggested.
The rhetorical questions in lines 360ff should be extended to suggest what kind of experimental approach would answer the alternatives.
Lines 380/81: is there precedent for such a mechanism?

Reviewer 3

Advance summary and potential significance to field

Isabella et al. presents a detailed investigation of how the axons of the zebrafish vagus nerve achieve target-specific axon regeneration after injury. The authors find that guidance of regenerating axons is distinct from guidance of developing axons and is dependent on the neuronsÂ' original location and innervation. How axons reach their targets post-developmentally is an open question, whose answer depends on cell-type and organism. The findings here are significant additions to our understanding of this process.

Comments for the author

The transplantation data in Figure 5A-H strongly indicate that the regenerating axons are mostly guided by intrinsic mechanisms. Which leaves the possibility open that some of the guidance is regulated by extrinsic signaling to Met and Hgf. It is not clear whether the absence of phenotype in figure 4 supports the strong conclusion that "the signaling mechanisms that guide vagus axon targeting during development…are not involved in the guidance of target specific axon regeneration". It seems possible that the negative results in Figure 4 could be caused by ineffective concentrations, spatial or temporal dynamics of RA and Crizo when they are supplied post-developmentally. Perhaps visualizing Met and Hgf (or temporally knocking them down) in regenerating axons and PA would confirm that developmental signaling mechanisms have been disrupted?
Alternatively, the conclusion could be reworded to a less strong statement.

First revision

Author response to reviewers' comments

We are grateful to the three reviewers for their enthusiasm for this work, and for their insightful and constructive comments. We have attempted to address all comments as best we can, including textual revisions as well as the addition of a substantial amount of new data to the manuscript. The relevant additions and revisions are highlighted in red in the manuscript, and are described below.

Reviewer 1 Advance Summary and Potential Significance to Field:
In this manuscript by Isabella et al, the authors introduce the larval zebrafish vagus nerve as a model for target-selective regeneration. Target specificity during peripheral nerve regeneration is critical for functional recovery, yet how neurons achieve target-selective regeneration is largely unknown. Here, the authors apply both laser-mediated axotomy and a single-cell chimera model to conclude that guidance during regeneration is distinct from the developmental mechanism, that target selection is specified by the neuron's initial position in the brain, and that targeting to a branch requires prior innervation. The experiments are well designed, the results are appropriately controlled and quantified, and for the most part the conclusions are very well supported by experimental evidence.
While the authors do not identify molecules that mediate target specificity during vagus nerve regeneration, these models are amenable to both drug treatment (as demonstrated in this manuscript) and transcriptional analysis (as demonstrated by previous work from the Moens lab), making them valuable tools for future mechanistic work. Since this is the first description of this model, the manuscript would greatly benefit from the inclusion of additional information and experimental data as outlined below.

Reviewer 1 Comments for the Author:
Main points:

1. 53% of axons returning to their original branch suggests a somewhat random mechanism underlying regeneration rather than a specific mechanism. Also is it that of the 53% 89% exhibit topographic re-targeting? How do these numbers compare to other PNS regeneration paradigms (invertebrate and vertebrate)?

1. Our data show a statistically non-random distribution (Fig. 2E), indicative of a target-specific guidance mechanism. However, because the number of axons showing topographically correct re-targeting (89% of all regenerating axons) is greater than the number of axons returning to the exact same branch (53% of all regenerating axons), we propose that target-specificity in this case means ensuring that the axon returns to a branch that would be appropriate for that neuron's position, and choosing between multiple topographically correct branches may indeed be random (e.g. if branches 4 and 5 would both be appropriate targets given that neuron's position in the brain, it will be limited to these two branches but may not discriminate between them). We have clarified these points in both the results (lines 177-187) and discussion (lines 367-370).

2. To further quantify the target-specificity of axon regeneration, we have added new quantitative analysis of our branch backfill data (Fig. 2P-R) showing very little difference in the gross topographic map between uninjured and regenerated vagus nerves.

3. We have included discussion of how our data compare to other studies that measured the extent of target-specificity during regeneration (lines 86-89 & 186-189).

2. For the laser transection assay, it would be important to know what the degree of functional regeneration is. This might be difficult to achieve but at least some experimental evidence of synapses being re-established at axonal target sites, e.g. using pre and postsynaptic markers.

We have addressed this point in two ways. 1) we have included new functional data showing that the larva's ability to swallow, which requires vagus motor activity, is completely lost 1 day after severing, but largely recovers by 6dps (Fig. 1K-L). 2. We have included new data showing that synapses are re-established at axonal target sites during regeneration and are grossly indistinguishable from unsevered controls (Fig. S1E). These data make a strong argument for substantial functional recovery.

3. How often are all PA's reinnervated after injury? It would be helpful to quantify the number of arches that are reinnervated at all, could be a binary yes/no. Moreover, are the more posterior PA's more likely to be reinnervated? The authors note that recovery varies by branch, so additional, more qualitative data would be helpful.

Qualitatively, we always see at least some axons innervating all branches by 6dps. The variability between branches that we noted regards the quantitative degree of reinnervation (Fig. 1J). We have updated the text to clarify that all branches show some degree of reinnervation in all animals (lines 155-157).

4. Were mis-targeted axons more likely to end up in posterior PA's because they are closer to the nerve (Fig2)? The authors report that the majority of axons were topographically targeted, but additional information on any trends in the errors? Did any axons ever completely miss the PA's and grow to different locations/anatomy?

1. To address trends in errors, we have added a new analysis of the target specificity data presented in Figure 2E, in which we graph the degree of correct and incorrect targeting for axons originating in each branch (Fig. S3A). We observe no trends in this data (eg anterior axons were not more or less likely to mis-target than posterior axons).

2. In our transplant experiments, we did rarely observe axons that grew to completely aberrant positions – usually posterior neurons extending axons posteriorly out of the vagus nucleus.~2.5% of our transplanted cells (5/188 wt à wt transplants examined) show this behavior. We have made note of this observation in the text (Lines 222-223).

5. Additional information on the structure of the vagus at the time of injury seems critical for even the initial description of this system presented here, and for evaluating the utility of the

model, but is missing, including the myelination status using available antibodies or at the ultrastructural level, presence/absence of glia cell types, how 'mature the nerve is, e.g. whether neurons/axons are been added at later stages etc.

We have more thoroughly reported the structure/maturity of the nerve at the time of injury by 1) adding a reference reporting that neurogenesis is complete by 3dpf ; 2) including new data showing that vagus axons have begun to establish synapses by 3dpf; 3) including new data reporting the presence/absence of glial cells and the myelination status of the nerve at 3-9dpf (Lines 139-148 and Figs. S1 & S2). Please see our response to reviewer #2, comment #2 below for a more thorough description of our findings regarding glia/myelination status.

6. For the transplant paradigm, did the transplanted neurons already extend an axon (prior to transplantation)? If so, did it have synaptic connections? This seems critical to know in order to evaluate the degree of regeneration, see above.

Transplanted neurons had already extended axons, although in this paradigm we cannot measure the branch to which a transplanted neuron had extended prior to its removal and must infer this from the neuron's A-P position. We have amended the text to clarify these points (lines 216-218 & 237-241).
We have also added new data showing that neuromuscular synapses are present at 3dpf (Fig. S1A-D).

7. Similarly, not having encountered an injury gap, does the process of regrowth described here resemble more the process when 'late' born neurons whose axons join a nerve, a process that occurs e.g. during optic nerve development in teleost fish? It would be important to compare the neuron's injury response, e.g. the upregulation of injury response genes to the one in the transection assay, or at least to those commonly upregulated following injury.

The process of single-cell regeneration is similar in some ways to cases of late neurogenesis (either natural or stimulated) in that new axons are being added to an existing nerve, as we discuss in lines 406-417. However, these events are fundamentally different in that the transplanted neuron had previously innervated a target and suffered an injury (a previously-formed axon and dendrite have been ripped from the cell body), whereas a newly born neuron would be naïve and uninjured. These points are discussed in lines 406-422.

We agree that examining the transcriptional response of neurons to injury will constitute important future work to gain further mechanistic insight into how they regenerate, but we feel that this is a sufficiently large undertaking that it is best left to be addressed in a future study.

8. For the cell transplant strategy the authors state that this strategy reveals target specificity. To further support this conclusion, it seems critical that the authors employ a complementary approach to confirm their finding, e.g. using their laser transection approach transecting individually labeled axons or small group of axons (labeled eg via photo conversion), and measuring the fidelity of growth towards to their original branch and targets. This would eliminate approach specific impacts on the regeneration process.

We agree that the suggested experiment would be a good complementary approach. We have tried to sever individual axons and groups of axons in the brain before all axons condense into a single fascicle, but we have found that this level of precision is not feasible, likely due to a combination of a smaller target and the fact that the axons are much deeper in the body at this position. Because we have shown that vagus axons can regrow to topographically correct targets under two injury paradigms at opposite ends of the severity spectrum – a severe whole-nerve injury and a less severe single-cell injury – we are confident that our results are not specific to the experimental approaches used.

9. The authors state "we therefore conclude that single regenerating axons cannot rely solely on secreted chemical cues from the target tissue, and instead require pre-existing innervation of their target pharyngeal arch." Yet, in the Crizo hosts, PA4 was never innervated and therefore by the time of transplantation might not be competent to secrete chemical cues to the regrowing transplanted axon. Indeed, as the authors note, this non-inervated PA might be missing critical

support structures, like glia, that might normally secrete guidance cues. While we agree with the conclusion that prior innervation is necessary for reinneration, it is unclear whether the current experiments definitively prove secreted cues are not sufficient. We therefore suggest the authors modify their language regarding these conclusions.

We agree with the reviewer's comments on this point and see that we did not clearly communicate this. We have revised this conclusion (Lines 343-344) and enhanced our discussion of this point to make clear that secreted guidance cues are still a viable mechanism, and to more clearly explain what the nature of those cues might be considering the requirement for pre-existing innervation (lines 382-394).

Minor Points:

1. Figure 1J: For J, the Red vs Blue labels (severed vs control) alternate from the left to the right side for each PA in the graph. It seems like the left side would correspond to the control as these values are consistently higher. Are the colors mis-labeled on the graph? If not, it seems like for some PA's, such as 5 and 7, the fluorescence in severed conditions is higher than controls. Is this due to debris? Or additional defasciculation? Can the authors either correct the colors, or perhaps another quantification method is necessary if there is so much variability between conditions using this measure of fluorescence. Additionally, please specify what is being normalize for the Y axis. Given the amount of background or debris fluorescence, a valuable control might be to perform this severing experiment comparing an animal where the neurons themselves have been ablated or otherwise cannot regrow for the 6 dps to the control condition.

The Red and Blue labeling was erroneous in this graph, thank you for bringing this error to our attention. We have corrected the colors, and you will now see that fluorescence intensity of severed conditions (red, right side for each PA) is never higher than controls (blue, left side). Fluorescence values are normalized to make the mean control value for each branch equal to one, so that relative differences between control and severed conditions for each branch are readily evident; this information has been added to the methods (Line 590).

2. Figure 3H: Adding an arrow to denote end of green axon would be helpful.

The arrow has been added as requested.

Reviewer 2 Advance Summary and Potential Significance to Field:
This manuscript describes with great care and exceptionally well illustrated, how neurons in a particular position in the vagus nucleus regenerate their axons into the different branches of the vagus nerve. The novelty described well is that the mechanism used is unlike the developmental mechanisms previously described by the same group. The findings lead to the assumption that neurons in the vagus have a memory of their previous position and the "appropriate" branch to re-innervate. The nature of that memory is not clarified.

Reviewer 2 Comments for the Author:
Overall, this paper is exceptionally systematically constructed and beautifully illustrated.

Major points:
1) I am missing discussions of Schwann cells as guidance structures, both as guiding channels and as origin of molecules promoting axonal repair. Only two papers have been cited, but there is insufficient information in the manuscript regarding Schwann cells in their system. Are there any at the time of lesion (3 or 4 dpl)? Do they linger?
Essential: describe Schwann cells at each time point. New experiments may not be needed

We have examined Schwann cells from 3-9dpf in unsevered and severed larvae using two transgenic marker lines: *Tg(Sox10:mRFP)*, an early Schwann cell marker, and *Tg(mbp:EGFP)*, a marker of myelinating glia. Consistent with published data (Cox et al., 2011), we saw that Schwann cells were present along the vagus fascicle at 3dpf. However, from 4-9dpf this signal is gone except for around the most proximal region of the fascicle before the nerve turns ventrally into the periphery, suggesting only a transient association of Schwann cells with the nerve. Likewise, by 9dpf we only see evidence of myelination in this most proximal region. We thus conclude that the vagus nerve is

unmyelinated and generally not associated with Schwann cells during the period when regeneration is occurring.
Accordingly, we see no Schwann cells associated with the regenerating nerve at any point during regeneration. We have included these findings in the paper (Fig. S2) and amended the discussion to acknowledge that Schwann cells are important contributors to axon regeneration in other contexts but do not appear to be present in the right time and place to play a role in vagus regeneration (Lines 390-393).

2) From the movies, it appears that the regenerating axons use the pre-existing axons to grow into the branches of the vagus nerve. Could the molecular "memory" simply be axons following other axons into their branch? Could it be the first axon the encounter? This may be also mediated by a topography in the nerve, as has been described for the optic nerve. Have the authors managed to capture the actual point at which the axons make their decision (maybe similar to the horizontal myoseptum where CaP, MiP, and RoP axons choose their individual paths)?
Essential: Address questions above. This is briefly touched upon in lines 416ff, but not expanded enough. New data from analysis of existing movies would significantly strengthen the paper. Specific experiments to clarify the two options discussed should be suggested.

We have not identified the point at which axons make their decision, but we agree that following other axons into their branch might be the mechanism by which regenerating axons find their targets. We do not believe that an axon follows the first axon it encounters, as that would be inconsistent with the findings of our heterotopic transplant data; rather, we think that axons that innervate different branches may possess distinct molecular identities which could be recognized, likely via homophilic interactions, by regenerating axons. We intend to investigate this further, but believe that experimentally addressing this hypothesis will be a significant and lengthy undertaking best left for a second paper. For this paper, we have included an expanded discussion of this hypothesis and how it might be addressed in the future (lines 395-403).

3) The rhetorical questions in lines 360ff should be extended to suggest what kind of experimental approach would answer the alternatives.

We have included a description of the experimental paradigms that would be helpful in addressing these questions (lines 377-379).

4) Lines 380/81: is there precedent for such a mechanism?

Spead & Poulain 2020 is a nice review, and Treubert-Zimmerman et al. 2002 and Raper et al. 1983 are exemplary studies, of how axon-axon interactions can guide axon targeting in this manner. We have included references to these papers in the discussion (lines 397-398).

Reviewer 3 Advance Summary and Potential Significance to Field:
Isabella et al. presents a detailed investigation of how the axons of the zebrafish vagus nerve achieve target- specific axon regeneration after injury. The authors find that guidance of regenerating axons is distinct from guidance of developing axons and is dependent on the neurons' original location and innervation. How axons reach their targets post-developmentally is an open question, whose answer depends on cell-type and organism. The findings here are significant additions to our understanding of this process.

Reviewer 3 Comments for the Author:
The transplantation data in Figure 5A-H strongly indicate that the regenerating axons are mostly guided by intrinsic mechanisms. Which leaves the possibility open that some of the guidance is regulated by extrinsic signaling to Met and Hgf. It is not clear whether the absence of phenotype in figure 4 supports the strong conclusion that "the signaling mechanisms that guide vagus axon targeting during development…are not involved in the guidance of target specific axon regeneration". It seems possible that the negative results in Figure 4 could be caused by ineffective concentrations, spatial or temporal dynamics of RA and Crizo when they are supplied post-developmentally. Perhaps visualizing Met and Hgf (or temporally knocking them down) in regenerating axons and PA would confirm that developmental signaling mechanisms have been disrupted? Alternatively, the conclusion could be reworded to a less strong statement.

To confirm that Hgf/Met signaling is not required for axon targeting during regeneration, we have added a second experiment in which we performed A ⟶ A transplants from *met*^{fh533} mutant donors into wild-type hosts. In this case we can be sure that the transplanted neurons lack Met function during their regeneration. As with the Crizotinib experiments, we observed that loss of Met function has no effect on axon growth or guidance (Fig 4I-P). We believe that the combined Crizotinib and Met mutant data now support the conclusion that Hgf/Met signaling is not required for axon guidance during regeneration. Incidentally, these new data also provide additional support for our conclusion that targeting history does not impact target selection during regeneration (Fig. 5I-P)

We tried two approaches to confirm that our RA treatment was increasing RA-dependent gene expression in larval vagus neurons: we performed *in situ* hybridization for *cyp26a1*, and we visualized a RARE:GFP transgene, both of which are known to increase their expression levels and domains upon increased RA signaling. We failed to see a major effect of our RA treatment on expression of either of these genes, although our RA-treated larvae do show a dorsal curvature phenotype that we generally observe with RA treatment. We conclude that, although this RA treatment regimen works well to induce RA-dependent gene expression in the embryo, it is less effective in larvae. We were able to induce strong reporter gene upregulation by increasing the RA concentration 10-fold, but this made the animals sufficiently sick that we did not consider it a reliable condition in which to assess axon behavior. Because we could not find a RA treatment condition that clearly increases RA- dependent gene expression but does not make the animals very sick, and because the Met/Hgf data provides a more clear and direct test of the question we were trying to address (do regenerating axons rely on the developmental guidance signals?), we have chosen to remove the RA data from the paper. As the lack of a role for RA in target-specific regeneration was a minor point in the paper, we do not believe that omitting this data affects the overall conclusions of the paper.

Second decision letter

MS ID#: DEVELOP/2021/199706

MS TITLE: Intrinsic positional memory guides target-specific axon regeneration in the zebrafish vagus nerve

AUTHORS: Adam J Isabella, Jason A Stonick, Julien Dubrulle, and Cecilia B Moens
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 are appended below.

Reviewer 1

Advance summary and potential significance to field

Target specificity during peripheral nerve regeneration is critical for functional recovery, yet how neurons achieve target-selective regeneration is largely unknown. The manuscript introduces the larval zebrafish vagus nerve as a powerful model to study target-selective regeneration.

Comments for the author

The authors have fully addressed this reviewers experiment and textual concerns.

Reviewer 2

Advance summary and potential significance to field

This manuscript describes with great care and exceptionally well illustrated, how neurons in a particular position in the vagus nucleus regenerate their axons into the different branches of the vagus nerve. The novelty described well is that the mechanism used is unlike the developmental mechanisms previously described by the same group. The findings lead to the assumption that neurons in the vagus have a memory of their previous position and the "appropriate" branch to re-innervate. Particularly exciting is the finding that the injury causes a functional phenotype - a reduction of swallowing - that is corrected after regeneration of the nerve.

Comments for the author

Thank you very much for addressing all reviewers' comments so carefully with really beautiful and impactful new experiments. This is a really interesting model, which I am confident will find traction in the community based on your thorough establishment of the system.

Reviewer 3

Advance summary and potential significance to field

The authors have addressed all of my comments.

Comments for the author

The authors have sufficiently addressed my concerns.