De novo enteric neurogenesis in post-embryonic zebrafish from Schwann cell precursors rather than resident cell types

Wael N El-Nachef and Marianne E. Bronner
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MS TITLE: De novo enteric neurogenesis in post-embryonic zebrafish from Schwann cell precursors rather than resident cell types

AUTHORS: Wael N El-Nachef and Marianne E. Bronner

I have now received the reports of three referees on your manuscript and I 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, all the referees are enthusiastic about your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, referees 2 and 3 request that you provide stronger evidence of the absence of resident glia in the zebrafish intestine. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.

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

Reviewer 1

Advance summary and potential significance to field

The study represents a solid and highly focused piece of experimental work aiming at understanding genesis of enteric nervous system with both medical and evolutionary biology tilts. The authors revealed that trunk nerves and associated progenitors contribute to the neurogenesis in a zebrafish gut, and prucalopride, an FDA-approved 5HT4 receptor agonist, is capable of enhancing the post-embryonic enteric neurogenesis in norm and after the injury in case of pre-injury exposure to the drug. Previously, trunk nerves were already investigated in a mouse model system as a source of a proportion of postnatal enteric neurons (the studies from Hideki Enomoto laboratory and others), and this study now extends the role of the trunk progenitors in a fish model system highlighting the importance and evolutionary conservation of this peripheral nerve-associated neurogenic pathway. Since the fish model is amenable to high-throughput screens and is an extraordinary popular and easy to handle experimental system, the reported discovery of SCP-derived enteric neurons will pave the way towards future discoveries of potent drugs facilitating neurogenesis in an array of human pathological conditions.
Generally, this timely and important piece of work should improve our future approaches to aganglionic gut problem.

Comments for the author

I have some comments that might help to improve the paper:
1. In the introduction, it might be good to mention that SCPs also give rise to neuroendocrine chromaffin cells (Dyachuk et al., Science 2017) and some sympathetic neurons in paraganglia (Kastriti et al., 2019) during post-neural crest developmental stages. Lines 97-99.
2. It would be really nice to add EdU experiments: injections at d3.5 and analysis at d5. I am curious if the newborn neurons will appear EdU positive.
This is not a necessary experiment since the paper contains enough proof, but this is easy and will make the whole story more convincing.
3. I wonder what exactly are the nerves that bring SCPs into a gut… Can those be visceral afferents? The genetic ablations of DRGs might be a good tool to try to investigate this question. Again, this a bit of a different question and I do not insist that the authors will follow it in the context of this study.
4. I think it is necessary to show which cells express 5HT4 receptor to serotonin in the gut and around. Are those SCPs of the trunk nerves or rather more committed progenitors after SCPs are entering the gut? Combining the drug treatment with EdU might be a good idea to clarify if the drug increases the recruitment of SCPs from trunk nerves or it does influence proliferation of SCP-derived cells after initiation of neurogenic differentiation…

Reviewer 2

Advance summary and potential significance to field

This paper shows that enteric neurons arise after the original crest-derived cohort colonizes the zebrafish fish bowel. The paper also shows that enteric neurons in the mature zebrafish ENS are derived from Schwann cell precursors that migrate into the bowel from outside. There is thus an extensive contribution from the truncal crest to the enteric nervous system. Strikingly, the authors maintain that there are no glia and no retained stem cells in the zebrafish intestine (although this point needs further verification). Finally, the authors show that an agonist that stimulates 5-HT4 receptors promotes enteric neurogenesis in zebrafish as in mammals and that it enhances neurogenesis after a defined injury to the ENS, if given prior to injury.

Comments for the author

The authors have written a lovely paper that suggests that Schwann cell precursors, which are derived from the truncal neural crest, are the source of enteric neurons that arise late in neurogenesis. Late neurogenesis in this sense, means formation of neurons occurring after the gut has been fully colonized by neuronal precursors derived from the vagal neural crest. The authors

further state that the ENS of the zebrafish is devoid of glial cells and also devoid of neural crest-derived stem cells. Because no glia or precursor cells thus exist within the zebrafish bowel, the authors argue that enteric glia and resident stem cells cannot be the source of new neurons arising after the initial wave of crest-derived cells has colonized the bowel.
Finally, the authors confirm that the 5-HT4 agonist, prucalopride, is effective as a prokinetic drug in the zebrafish intestine and is also effective in promoting enteric neurogenesis and the recovery of neurons after injury (if given before the injury). A variety of techniques, which appear to be unassailable, are used that take full advantage of the zebrafish as an experimental model.

The conclusion of the authors that the zebrafish ENS lacks enteric glia is heavily influenced by the authors' failure to find Sox10 in the zebrafish ENS. Sox10 is retained in mammalian enteric glia and is a reliable enteric glial marker in the mammalian ENS. More strikingly, all of the enteric cells derived from Sox10-expressing progenitors appear to develop as Phox2b-expressing enteric neurons as the zebrafish mature. The possibility that there is a neural crest-derived precursor in the bowel that does not express Sox10, which has been postulated by Kulkarni et al, (Kulkarni et al., 2017) has not been ruled out, but the migration of Schwann cell precursors into the gut has been ruled in. During mammalian development, enteric glia arise later than enteric neurons; therefore, it is not surprising that glia are not found during early development in zebrafish. It would be good to see a discussion in the manuscript that is a little more definitive about the possibilities that zebrafish have enteric glia that do not express Sox10 or that they have retained enteric neuronal precursors that are able to give rise to enteric neurons but which never expressed Sox10. It would also be useful to present evidence of what holds the zebrafish ENS together if it totally lacks glial cells. It is true that enteric glia can be ablated from the mouse ENS for short periods of time without significant consequences but the experiments in which enteric glia were ablated could not determine long-term effects of glial ablations because the method involved PLP-1-directed toxicity, which affects the brain (Rao et al., 2017). The gut had to be examined within the window of time that preceded manifestation of symptoms of CNS toxicity. GFAP has been found in the zebrafish ENS, which the authors acknowledge but dismiss as fibrillary in appearance.

The authors point out that GFAP immunostaining does not reveal the presence of GFAP in cell bodies within the zebrafish ENS. GFAP, however, is an intermediate filament protein and thus its fibrillary appearance is to be expected. If intermediate filaments were to run through cell bodies and extend into processes, the immunostaining of GFAP might not reveal the locations of cell bodies, which in any case, are probably very small. The GFAP-containing processes have to come from cells that are located somewhere. The authors seem to imply that the cell bodies are located outside of the gut. That seems bizarre. Even if Schwann cells enter the bowel in the sheaths of extrinsic nerves, they usually do so as small cells, not at exceedingly long processes. Some additional evidence that supports the authors' contention that the zebrafish ENS lacks enteric glia is needed. Perhaps EM might help.

The authors maintain that they are the first to employ prucalopride to look at neurogenesis. This is not correct. The authors might want to consult (Margolis et al., 2016), a manuscript that showed that prucalopride effectively promotes enteric neurogenesis in mice. This paper provides a nice background for the authors' observations. It would also be nice if the authors were to test the idea that 5-HT affects enteric neurogenesis in zebrafish as it has been shown to do in mammals. Prucalopride is a great agonist but it is not the natural ligand for the 5-HT4 receptor in the zebrafish intestine. Presumably, the authors work suggests that 5-HT, which is the natural ligand, acts as a growth factor during ENS development in zebrafish. 5-HT is a neurotransmitter in the teleost ENS. Could the authors determine the effects of an inhibitor of SERT (like fluoxetine) on the zebrafish ENS? In the mammalian gut SERT is the primary means of inactivating 5-HT and SERT inhibitors potentiate its activity. SSRI treatment and genetic SERT ablation enhance enteric neurogenesis and lead to hyperplasia of the ENS in mice (Margolis et al., 2016).

In summary, this outstanding manuscript needs just a little more attention to become a truly outstanding contribution.

Citations

Kulkarni, S., Micci, M.A., Leser, J., Shin, C., Tang, S.C., Fu, Y.Y., Liu, L., Li, Q., Saha, M., Li, C., Enikolopov, G., Becker, L., Rakhilin, N., Anderson, M., Shen, X., Dong, X., Butte, M.J., Song, H.,

Southard-Smith, E.M., Kapur R.P., Bogunovic, M., Pasricha, P.J., 2017. Adult enteric nervous system in health is maintained by a dynamic balance between neuronal apoptosis and neurogenesis. Proc Natl Acad Sci U S A 114, E3709-E3718.

Margolis, K.G., Li, Z.S., Stevanovic, K., Saurman, V., Israelyan, N., Anderson, G.M., Snyder, I., Veenstra-VanderWeele, J., Blakely, R.D., Gershon, M.D., 2016. Serotonin transporter variant drives preventable gastrointestinal abnormalities in development and function. Journal of Clinical Investigation 126, 2221-2235.

Rao, M., Rastelli, D., Dong, L., Chiu, S., Setlik, W., Gershon, M.D., Corfas, G., 2017. Enteric Glia Regulate Gastrointestinal Motility but Are Not Required for Maintenance of the Epithelium in Mice. Gastroenterology 153, 1068-1081 e1067.

Reviewer 3

Advance summary and potential significance to field

Recent genetic studies have identified Schwann cell precursors (SCPs) as a novel cellular source of the enteric nervous system (ENS). However, biological roles of SCP-derived enteric neurogenesis in the maintenance of the ENS have remained unclear. In this manuscript, El-Nachef et al. addressed this issue using zebrafish as a model organism. Using various genetic tools and time-lapse imaging analyses, the authors found that enteric neurogenesis persists during the postnatal period in the zebrafish. By DiI labeling, they also found that cells at the trunk level contribute to the postnatal neurogenesis. Genetic cell labeling using Sox10-CreERT2 revealed that cells with a history of Sox10 expression contribute to postnatal enteric neurogenesis. Because the authors found no contribution of Sox10-lineage cells to enteric glia, they concluded that SCPs at the trunk level are the prime cellular source for the postnatal neurogenesis. The authors also demonstrated that SCP-derived neurogenesis is enhanced by neuronal loss or by 5HT4R agonist prucalopride. Overall this is an interesting study, one which will deepen our understanding of the biological significance of SCP-derived neurogenesis.

Comments for the author

However, I have several concerns with regard to the methodologies and interpretation of the data. The authors should address these issues before the manuscript is suitable for publication.

1. By GFAP staining, the authors observed signals in the cellular processes but not in the cell body. Based on this observation, they concluded that enteric glia are absent in the zebrafish ENS and speculated that GFAP-positive cellular processes are of SCP origin. This is a premature conclusion. It is well known that GFAP mainly stains cell processes, not cell bodies, and the cell body of enteric glia can be very small in size, which would also make it difficult to identify their presence by GFAP immunohistochemistry. To prove the authors' hypothesis in a more convincing manner, they should perform in situ hybridization using GFAP riboprobes to show that GFAP signals are absent in the gut mesenchyme but present in the cells (SCPs) surrounding the extrinsic nerve fibers. The authors should also demonstrate that these GFAP signals overalp with Sox10 expression. This confirmation is crucial for the later experiments. Moreover, if GFAP turns out to be a reliable and specific marker for SCPs, the authors should use the GFAP-CreERT2 driver line to show SCP-derived neurogenesis.

2. The authors used Sox10-Cre, Sox10-Cre ERT2 driver lines to identify or track the fate of Sox10-expressing cells. Although both are reported to recapitulate endogenous expression of Sox10 (Development 2014, Dev Biol 2015), they are also both transgenic lines using only ~5kb fragments of the Sox10 promoter, and not knockin animals. This suggests that we may not be able to visualize all of cells with a history of Sox10 expression using these driver lines. In Figure 2A, there are many Hu-positive cells that are not labeled by Sox10-Cre, which clearly indicates that there are enteric neurons whose origin cannot be identified by these Sox10-Cre drivers. The same scenario

applies to the enteric glia, and there remains a possibility that enteric glia are present but cannot be visualized simply by these Sox10-Cre driver lines.

3. The authors demonstrated SCP-derived enteric neurogenesis using the Sox10-CreERT2 driver, however, the exact location and differentiation states of the SCPs during the course of this experiment are not shown. The authors should show that, immediately after the tamoxifen-mediated Cre recombination, the labeled cells are only identified outside of the gut. Later, these cells should enter the gut mesenchyme and acquire neuronal phenotype. This type of time sequence analysis should be conducted and included in the manuscript to demonstrate SCP-derived neurogenesis in a more convincing manner.

First revision

Author response to reviewers' comments

Reviewer 1:

1. In the introduction, it might be good to mention that SCPs also give rise to neuroendocrine chromaffin cells (Dyachuk et al., Science 2017) and some sympathetic neurons in paraganglia (Kastriti et al., 2019) during post-neural crest developmental stages. Lines 97-99.

Thank you very much for the suggestion, we agree that these references are highly appropriate. We have now expanded the discussion of derivatives of SCPs and added appropriate references in the introduction as suggested.

2. It would be really nice to add EdU experiments: injections at d3.5 and analysis at d5. I am curious if the newborn neurons will appear EdU positive. This is not a necessary experiment since the paper contains enough proof, but this is easy and will make the whole story more convincing.

We agree. To this end, we have performed EdU pulse labeling in larvae from 4.5 – 5 d dpf (the time frame corresponding with our photoconversion experiments). The results showed that only a minority of enteric neurons were marked during this post-embryonic stage. This is consistent with our initial findings that enteric neurogenesis persists during the post-embryonic stage and strengthens that claim. These data are now included as Supplement 4. Many thanks for this excellent suggestion.

3. I wonder what exactly are the nerves that bring SCPs into a gut… Can those be visceral afferents? The genetic ablations of DRGs might be a good tool to try to investigate this question. Again, this a bit of a different question, and I do not insist that the authors will follow it in the context of this study.

We agree that this is an interesting question. When initially performing DiI injections, we used an Hb9-GFP transgenic line which marks motor neurons and their axons and failed to find DiI-labelled cells along motor neuron axons. To address this further, we have combined DiI labeling with immunohistochemistry. The results suggest that DiI-labelled cell bodies (i.e. SCPs) are associated with nerve fibers marked by acetylated tubulin (Supplement 7D). Taken together, these early experiments suggest that SCPs migrate along non-motor axon nerves from the neural tube to the intestine, likely emanating to/from the DRG as the reviewer suggests. Further study, however, will be required to fully define this.

4. I think it is necessary to show which cells express 5HT4 receptor to serotonin in the gut and around. Are those SCPs of the trunk nerves or rather more committed progenitors after SCPs are entering the gut? Combining the drug treatment with EdU might be a good idea to clarify if the drug increases the recruitment of SCPs from trunk nerves or it does influence proliferation of SCP-derived cells after initiation of neurogenic differentiation…

Thank you for this helpful suggestion. To address this, we have performed the recommended 5HT4 receptor staining and observed expression throughout the mucosa as well as within some enteric neurons, similar to what has been reported in murine studies. These data have been added to Figure 6.

Reviewer 2:

1. The conclusion of the authors that the zebrafish ENS lacks enteric glia is heavily influenced by the authors' failure to find Sox10 in the zebrafish ENS. Sox10 is retained in mammalian enteric glia and is a reliable enteric glial marker in the mammalian ENS. More strikingly, all of the enteric cells derived from Sox10-expressing progenitors appear to develop as Phox2b-expressing enteric neurons as the zebrafish mature. The possibility that there is a neural crest-derived precursor in the bowel that does not express Sox10, which has been postulated by Kulkarni et al, (Kulkarni et al., 2017) has not been ruled out, but the migration of Schwann cell precursors into the gut has been ruled in. During mammalian development, enteric glia arise later than enteric neurons; therefore, it is not surprising that glia are not found during early development in zebrafish. It would be good to see a discussion in the manuscript that is a little more definitive about the possibilities that zebrafish have enteric glia that do not express Sox10 or that they have retained enteric neuronal precursors that are able to give rise to enteric neurons but which never expressed Sox10. It would also be useful to present evidence of what holds the zebrafish ENS together if it totally lacks glial cells.

We thank the reviewer for this comment which made us realize that we did not sufficiently explain the evidence for "lack" of glia and agree that it is important to put forward alternative narratives. We first noticed what we will refer to as the "apparent lack" of glia in the basal vertebrate lamprey when we stained with an antibody to GFAP and found no expression in the ENS while there was expression in glia in other parts of the animal. This led us to perform GFAP staining in zebrafish and again found no evidence of its presence in cell bodies, but only fibrillar staining (similar to what we had seen in lamprey). We then noted the complete absence of Sox10+ cells in the ENS by 5dpf, which was intriguing and made us speculate that perhaps enteric glia were missing or at least molecularly different than in amniotes. To address the reviewer's comment, we have expanded our repertoire with live imaging of a GFAP transgenic line (Supplement 7B-C) and immunostaining for an additional glial marker, S100 (Supplement 7D-E). The results of these additional assays further support our claim that enteric glia with canonical markers appear to be absent in the zebrafish intestine.

We acknowledge that the "apparent lack" of a cell type is in many ways a negative result and therefore we cannot rule out the possibility that some glia-like cells exist but are undetectable by current methods in the larvae and the adult. We have amended the discussion to clarify this point and completely agree with the reviewer that it is important to be circumspect in this regard.

Regarding "what holds the zebrafish ENS together"—the role of enteric glia in the structural integrity of the ENS of amniotes is not clear. Furthermore, even if enteric glia have a structural role in the ENS of other species, it may not be required in earlier vertebrates such as agnathans and teleosts. The zebrafish ENS is closely apposed to several mesenchymal-derived cell types, including smooth muscle cells and a network of interstitial cells of Cajal, which could potentially serve as structural support for enteric neurons.

2. It is true that enteric glia can be ablated from the mouse ENS for short periods of time without significant consequences but the experiments in which enteric glia were ablated could not determine long-term effects of glial ablations because the method involved PLP-1-directed toxicity, which affects the brain (Rao et al., 2017). The gut had to be examined within the window of time that preceded manifestation of symptoms of CNS toxicity. GFAP has been found in the zebrafish ENS, which the authors acknowledge but dismiss as fibrillary in appearance. The authors point out that GFAP immunostaining does not reveal the presence of GFAP in cell bodies within the zebrafish ENS. GFAP, however, is an intermediate filament protein and thus its fibrillary appearance is to be expected. If intermediate filaments were to run through cell bodies and extend into processes, the immunostaining of GFAP might not reveal the locations of cell bodies, which in any case, are probably very small. The GFAP-containing processes have to come from cells that are located

somewhere. The authors seem to imply that the cell bodies are located outside of the gut. That seems bizarre. Even if Schwann cells enter the bowel in the sheaths of extrinsic nerves, they usually do so as small cells, not at exceedingly long processes. Some additional evidence that supports the authors' contention that the zebrafish ENS lacks enteric glia is needed. Perhaps EM might help.

Thank you for this input. We have performed further immunohistochemistry using the GFAP antibody to demonstrate that GFAP-positive processes extend throughout the body, with extensions into the intestinal wall, and these data have been included as Supplement 3A. Furthermore, we suspect this antibody may have nonspecific binding to other fibrillary structures such as smooth muscle. To further test this, we have now performed additional assays for enteric glia including using an antibody to S100 (a predominantly nuclear protein) which stained glia in the CNS but exhibited no staining in the intestine, consistent with prior work by others.

Regarding electron microscopy: this method is ultimately a subjective assay that in our opinion is best utilized to describe subcellular features of a known cell type rather than to posit the existence or absence of a controversial cell type.

3. The authors maintain that they are the first to employ prucalopride to look at neurogenesis. This is not correct. The authors might want to consult (Margolis et al., 2016), a manuscript that showed that prucalopride effectively promotes enteric neurogenesis in mice. This paper provides a nice background for the authors' observations.

Thank you very much for pointing out this important study which we inadvertently missed. We have corrected the manuscript accordingly and apologize for the omission.

4. It would also be nice if the authors were to test the idea that 5-HT affects enteric neurogenesis in zebrafish as it has been shown to do in mammals. Prucalopride is a great agonist but it is not the natural ligand for the 5-HT4 receptor in the zebrafish intestine. Presumably, the authors work suggests that 5-HT, which is the natural ligand, acts as a growth factor during ENS development in zebrafish. 5-HT is a neurotransmitter in the teleost ENS. Could the authors determine the effects of an inhibitor of SERT (like fluoxetine) on the zebrafish ENS? In the mammalian gut SERT is the primary means of inactivating 5-HT and SERT inhibitors potentiate its activity. SSRI treatment and genetic SERT ablation enhance enteric neurogenesis and lead to hyperplasia of the ENS in mice (Margolis et al., 2016).

Thank you for this excellent suggestion. Accordingly, we have now performed experiments to explore the effects of 5HT4R antagonism on development and regeneration of the ENS. Using the same experimental design as the prucalopride experiments, we exposed the zebrafish to the 5HT4R antagonist, GR 113808. We found a significant decrease in enteric neuron number during post-embryonic development as well as in regeneration after injury, and these data have been included in Figure 7B and 7E. We greatly appreciate this suggestion, as it has strengthened this work.

5. In summary, this outstanding manuscript needs just a little more attention to become a truly outstanding contribution.

Thank you for helpful comments which have greatly improved the paper.

Reviewer 3:

1. By GFAP staining, the authors observed signals in the cellular processes but not in the cell body. Based on this observation, they concluded that enteric glia are absent in the zebrafish ENS and speculated that GFAP-positive cellular processes are of SCP origin. This is a premature conclusion. It is well known that GFAP mainly stains cell processes, not cell bodies, and the cell body of enteric glia can be very small in size, which would also make it difficult to identify their presence by GFAP immunohistochemistry. To prove the authors' hypothesis in a more convincing manner, they should perform in situ hybridization using GFAP riboprobes to show that GFAP signals are absent in the gut mesenchyme but present in the cells (SCPs) surrounding the extrinsic nerve fibers. The authors should also demonstrate that these GFAP signals overlap with Sox10 expression. This confirmation is

crucial for the later experiments. Moreover, if GFAP turns out to be a reliable and specific marker for SCPs, the authors should use the GFAP-CreERT2 driver line to show SCP-derived neurogenesis.

Thank you very much for this helpful feedback. We feel that ISH would not resolve this question as the mRNA for GFAP will be cytosolic and thus will not clearly differentiate between cell body and cellular projections. To address these concerns, we have instead performed immunostaining with S100, a glial marker that is predominantly nuclear. Using this nuclear marker, we found an absence of S100-positive glia in the zebrafish intestine, and these data are included in Supplement 3E.

To clarify, we do not believe GFAP or any other canonical marker of differentiated glia are present in SCPs. We have reworded the text to make this more clear. The SCP nomenclature (which precedes our study) can be somewhat confusing, but SCPs may be alternatively referred to as neural crest-derived stem cells in that they represent cells with progenitor potential for multiple downstream derivatives, including glia.

2. The authors used Sox10-Cre, Sox10-Cre ERT2 driver lines to identify or track the fate of Sox10-expressing cells. Although both are reported to recapitulate endogenous expression of Sox10 (Development 2014, Dev Biol 2015), they are also both transgenic lines using only ~5kb fragments of the Sox10 promoter, and not knockin animals. This suggests that we may not be able to visualize all of cells with a history of Sox10 expression using these driver lines. In Figure 2A, there are many Hu-positive cells that are not labeled by Sox10-Cre, which clearly indicates that there are enteric neurons whose origin cannot be identified by these Sox10-Cre drivers. The same scenario applies to the enteric glia, and there remains a possibility that enteric glia are present but cannot be visualized simply by these Sox10-Cre driver lines.

Thank you for bringing up this point, making us realize that we did not adequately describe the Sox10-Cre ERT2 line. Indeed, inducible Cre lines are often very patchy and mark only a subset of Sox10 expressing cells, making it difficult to conclusively prove the absence of a cell type using this technique. We use this as an alternative approach to DiI labeling to show the origin of some enteric neurons from SCPs since this is a positive result. We now clarify the limitations of the approach in the manuscript (line 334-6). That said, we did detect a significant number of cells derived from Sox10 expressing precursors per fish but never any enteric glia using this type of labeling approach.

3. The authors demonstrated SCP-derived enteric neurogenesis using the Sox10-CreERT2 driver, however, the exact location and differentiation states of the SCPs during the course of this experiment are not shown. The authors should show that, immediately after the tamoxifen-mediated Cre recombination, the labeled cells are only identified outside of the gut. Later, these cells should enter the gut mesenchyme and acquire neuronal phenotype. This type of time sequence analysis should be conducted and included in the manuscript to demonstrate SCP-derived neurogenesis in a more convincing manner

We appreciate this suggestion. Accordingly, we have now performed tamoxifen induction at 24 hpf and found many Cre-labelled cells outside of the intestine, including within DRGs and along nerve fibers, consistent with them being SCPs. These data have been added to Supplement 8.

Second decision letter

MS ID#: DEVELOP/2019/186619

MS TITLE: De novo enteric neurogenesis in post-embryonic zebrafish from Schwann cell precursors rather than resident cell types

AUTHORS: Wael N El-Nachef and Marianne E. Bronner

I have now received the reports of the three referees who reviewed the earlier version of your manuscript and I 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 reviewers' evaluation is very positive and we would like to publish a revised manuscript in Development, provided that you satisfactorily address the remaining minor comment of referee 2 regarding a citation in your manuscript. Please attend to this comment in your revised manuscript and your point-by-point response. If you do not agree with it, explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 1

Advance summary and potential significance to field

The authors addressed all my comments. The paper is ready to be published.

Comments for the author

No more revision are necessary, all my comments were answered inlding new experiments.

Reviewer 2

Advance summary and potential significance to field

The authors document the very important contribution to ENS development that Schwann Cell precursors make and especially their importance in postnatal eNS development and regeneration. The authors also highlight the paucity (or absence) of glia in the teleost ENS, which is surprising. Finally, the authors confirm the importance of 5-HT and the 5-HT4 receptor in ENS development. The latter observation is a confirmation of earlier observations made in mice.

Comments for the author

The authors have basically done a good job of revising their manuscript. One problem, however, is that they incorrectly cite a manuscript to which this reviewer referred them.(Margolis et al., 2016). The authors state:
"Our study is the first to demonstrate that 415 prucalopride, a highly specific 5HT4R agonist that has recently been approved for use in the United 416 States ("Drug Approval Package," n.d.; Wong et al., 2010), promotes enteric neurogenesis during normal development, in contrast to a previous study (Margolis et al., 2016)."
Actually, the Magolis et al paper showed the following the abstract is quoted verbatim and explanations are added in brackets: "…Ala56-expressing mice [animals that express an overly active mutant version of SERT] display GI defects that resemble those seen in mice lacking neuronal 5-HT [animals in which TPH2 has been deleted. The excessively rapid removal of 5-HT does not permit that neurotransmitter to work.] "These defects included enteric nervous system hypoplasia [enteric neurogenesis is deficient when 5-HT is removed too rapidly so that it cannot work], slow GI transit, diminished peristaltic reflex activity, and proliferation of crypt epithelial cells [effects of the ENS are deficient because it has too few neurons to function normally].
An opposite phenotype was seen in SERT-deficient mice [removal of 5-HT is too slow, thereby potentiating its action] and in progeny of WT dams given the SERT antagonist fluoxetine. The reciprocal phenotypes that resulted from increased or decreased SERT activity support the idea that 5-HT signaling regulates enteric neuronal development and can, when disturbed, cause long-lasting abnormalities of GI function.
Administration of a 5-HT4 agonist to Ala56 mice during development prevented Ala56-associated GI perturbations [that is, the 5-HT4 agonist stimulated enteric neurogenesis and could not, like 5-HT,

be removed by the hyperactive SERT because prucalopride is not a substrate for SERT], suggesting that excessive SERT activity leads to inadequate 5-HT4-mediated neurogenesis." The paper thus shows that 5-HT normally promotes enteric neurogenesis through the 5-HT4 receptor and that prucalopride can substitute for the normal action of 5-HT. The authors seem to think that the paper showed the opposite effect on line 417. The authors' manuscript is thus not the first to show that prucalopride stimulates enteric neurogenesis.
They should write something like "Our study CONFIRMS that prucalopride…"In fact the Margolis et al manuscript should also be cited on line 86, when the authors introduce the concept of 5-HT4 stimulation of neurogenesis, and again on line 343 in results.

The remainder of the comments have been revised very well; however, prior work should not only be cited but cited correctly. Once that is done, this will be an important and interesting contribution to the understanding of ENS development and evolution.

Citation
Margolis KG, Li ZS, Stevanovic K, Saurman V, Israelyan N, Anderson GM, Snyder I, Veenstra-VanderWeele J Blakely RD, Gershon MD (2016) Serotonin transporter variant drives preventable gastrointestinal abnormalities in development and function. Journal of Clinical Investigation 126:2221-2235.

Reviewer 3

Advance summary and potential significance to field

Biological roles of SCP-derived enteric neurogenesis in the maintenance of the ENS have remained unclear. In this manuscript, El-Nachef et al. addressed this issue using zebrafish as a model organism. The authors found that SCPs at the trunk level are the prime cellular source for the postnatal neurogenesis. The authors also demonstrated that SCP-derived neurogenesis is enhanced by neuronal loss or by 5HT4R agonist prucalopride. This is an interesting study, one which will deepen our understanding of the biological significance of SCP-derived neurogenesis.

Comments for the author

The authors have addressed most of the concerns, and the revised manuscript is more complete and suitable for publication.

Second revision

Author response to reviewers' comments

We have revised the discussion of the Margolis et al citation per the comments of Reviewer 2 and have also referenced this article on line 86, per Reviewer 2's suggestion.

Third decision letter

MS ID#: DEVELOP/2019/186619

MS TITLE: De novo enteric neurogenesis in post-embryonic zebrafish from Schwann cell precursors rather than resident cell types

AUTHORS: Wael N El-Nachef and Marianne E. Bronner
ARTICLE TYPE: Research Article

I am delighted to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.