Avian ceca are indispensable for hindgut enteric nervous system development

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MS TITLE: Avian ceca are required for hindgut enteric nervous system development by promoting enteric neural crest cell proliferation and inhibiting neuronal differentiation via non-canonical Wnt signaling

AUTHORS: Nandor Nagy, Tamas Kovacs, Rhian Stavely, Viktoria Halasy, Adam Soos, Emoke Szocs, Ryo Hotta, Hannah Graham, and Allan Goldstein

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 express are enthusiastic about your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily all their major concerns. 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 authors show that the cecal appendages play a critical role in enabling the crest-derived population of cells that forms the enteric nervous system to colonize the avian handgut. The authors also implicate Wnt11 signaling as the critical process that occurs in the ceca.

Comments for the author

This is an elegant paper that clearly demonstrates that the cecal appendages play a critical role in enabling neural crest-derived cells to colonize the avian hindgut. The authors convincingly show that proliferation of crest-derived cells is at its highest in the wavefront of advancing cells in the ceca. They argue compellingly that neuronal differentiation is impaired within the ceca, which enables crest-derived cells to remain as precursors and build up sufficient numbers to fully colonize the hindgut. The ceca must thus contain one or more mitogens and an inhibitor of premature differentiation. The molecular data imply that Wnt11 might be a critical molecule for these purposes, and indeed, in vitro, Wnt11 does promote the proliferation of avian crest-derived cells and inhibit their differentiation into neurons. The data are highly dependent on the results of experiments with organotypic tissue culture, within which the nerves of Remak are missing. The authors argue that this is likely not to be a problem; however, it is a potential limitation of the study, and it is thus good that the absence of the nerves of Remak is mentioned as an admission.

It is interesting that much earlier mammalian data also suggest that the cecal region plays a role in enabling the descending crest-derived population of cells to enter the colon. Pachnis and colleagues have shown that GDNF expression moves proximo-distally during development and peaks in the cecum (1). GDNF is a chemoattractant for crest-derived cells. The descent of GDNF expression thus might help to guide migrating crest-derived cells as far as the cecum. If that were to be so, and nothing were to intervene in the cecum, the crest-derived cells would be trapped there. In fact, Kapur first demonstrated that the population hesitates in its migration when it reaches the cecum (2). Et3/Ednrb signaling opposes the attraction of crest-derived cells by GDFN, promotes the mitogenic effect of GDNF on crest-derived cells, and most importantly, inhibits GDNF-induced neuronal differentiation (3, 4). Some of the authors have shown a similar action of GDNF in avian development (5). Interestingly, in organotypic culture of mouse gut, the provision of Et3 rescues the colon of ls/ls mice and enables crest-derived cells to migrate into it. Et3/Ednrb signaling thus seems to play a role that is not dissimilar to that the authors postulate for Wnt11 and has previously been proposed to do so (6). Further discussion of the potential interactions between Et3/Ednrb signaling and the newly proposed one for Wnt11 would thus help readers to understand current observations.

In short, this very nice paper needs only a bit more amplification. It is a lovely piece of work.

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2. Kapur RP, Yost C, and Palmiter RD. A transgenic model for studying development of the enteric nervous system in normal and aganglionic mice. Development. 1992;116:167-75.
3. Wu JJ, Chen J-X, Rothman TP, and Gershon MD. Inhibition of in vitro enteric neuronal development by endothelin-3: mediation by endothelin B receptors. Development. 1999;126(6):1161-73.
4. Hearn CJ, Murphy M, and Newgreen D. GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons in vitro. Dev Biol. 1998;197:93-105.
5. Nagy N, and Goldstein AM. Endothelin-3 regulates neural crest cell proliferation and differentiation in the hindgut enteric nervous system. Dev Biol. 2006;293(1):203-17.

6. Gershon MD. Developmental determinants of the independence and complexity of the enteric nervous system. Trends in Neurosciences. 2010;33(10):446-56.

Reviewer 2

Advance summary and potential significance to field

This manuscript describes the necessity and sufficiency of the ceca to populate hindgut enteric nervous system (ENS) via enteric neural crest cell (ENCC) migration. Given the ease of ex-vivo maintenance of avian tissue and neural crest population, this study provides insightful information regarding ENS colonization and ENCC. To begin using the avian ceca as a source of hindgut population ENCCs, the authors establish that loss of the paired cecal tissue leads to a loss of ENS population in the post cecal colon from day E5-E8. The authors postulate the sequence of events that mediate hindgut ENS colonization by ENCCs: (1) ENCCs enter cecal region and proliferate (2) WNT11 expressed in the ceca promote stemness (3) ENCCs exit cecal tissue and populate hindgut tissue. Overall, the authors provide strong evidence of the necessity of the ceca for proper maintenance of ENCCs, thereby allowing for full population of the hindgut. They elucidate both a molecular (WNT11 signaling) and Cellular (increased proliferation and reduced differentiation) mechanism allowing for the proper colonization. This research is relevant to the study of enteric neuropathies and better enhances tissue-level genetic mechanisms that may drive enteric disease, namely HSCR.

Comments for the author

Prior to publication, I have several concerns/suggestions that the authors should address:
Comments:
1. The authors show an elevated percentage of EdU+ proliferative cells in the wavefront and undifferentiated cells in the cecal to intercecal tissue at E6 (Fig2) and make the conclusion that the wave front is at its highest proliferative rate during when it migrates through the ceca. However, to make this claim the authors would need to make a comparison between the wave front when it is in the ceca and also in the foregut/midgut, the midgut, as well as the hindgut. It could be that the proliferation increase observed is true within the context of the axial microenvironment of the ceca, but it could actually be higher while traveling through the more proximal or distal areas of the gut. Additionally, it would be worthwhile to see if the proliferative nature of the enteric crest varies between the two regions (ceca and interceca) at E7, which would suggest it's not solely the wave front, but a general feature of crest migrating through the ceca (which either result would still be fascinating).
2. Related to comment 1 above, it would help readers to understand more clearly, if the authors could explicitly define what the "migratory wave front" truly is, on first mention and in the methods. Do they mean the first 1-5 cells in the enteric chain wave front, or is it something encompassing a larger population of cells?
It is important to be clear about the distinction, as this will alter how data can be/is interpreted.
3. In the introduction, it would help the reader to understand the study better if some basic background on chicken enteric nervous system stages/time points were included briefly. As well, the references for vagal neural crest-derived cells in the second sentence requires a couple of primary citations, currently there are only review articles used.
4. The authors demonstrate that loss of cecal tissue at E5 prevents ENCCs from populating past the cecal proximal hindgut. However, it's also possible the cells are dying. I would suggest looking to see if there is a change in the cell death as opposed to just the aggregation of cells in the region.
5. Related to comment 4 above, it is possible that the ENCCs are prevented from populating past the cecal proximal gut due to a dramatic decrease in cellular proliferation. While the authors do show one image of some enteric cells with little overlap of EdU following cecal removal (Fig 3D), we don't see a control gut there to compare it to and I would suggest quantifying if there is a change in percentage of proliferation and/or a gross drop in the total number of wave front cells following cecal removal, when compared with cecal intact guts.
6. The RNA-seq experiment is very insightful. I would suggest that the authors provide a sentence or explanation for why the time point of E5 was chosen for analysis, as opposed to E6.

7. Did the authors observe a change in the expression of Ret/GDNF pathway in their RNA-seq experiments?
8. The explant experiments described in figure 8 are important to the study presented, however it took this reviewer quite some time to fully understand the conditions and schematize out the temporal elements of the explant incubations/conditions, etc. I suggest that the authors include a cartoon schematic to describe the timeline of explant GDNF/Wnt11 experiments, with conditions. It could possibly be included as a revised figure 8A.
9. The authors state in the results, "If GDNF is removed from the culture media after the first 24 hours migration over the next 24 hours is limited and cells aggregate into large ganglion-like structures with altered network morphology." Altered, when compared with what? in vivo gut? or images in 8D?
10. The experiments in figure 8 suggest that Wnt11 is sufficient to rescue enteric migration from the explant
(after initial GDNF priming), however is it also sufficient to rescue proliferation and cell numbers?
11. The authors state, "Interestingly, when compared to GDNF alone (Fig. 8D), Wnt11-treated cultures appear to have many more undifferentiated ENCCs (Fig. 8I)." To claim this, the authors need quantification here, or they should temper this claim.
12. The authors state, "…Wnt11 protein alone does not promote ENCC migration from the ceca (Fig. 8J-L)."
The image shown is not convincing, as presented. To be sure of this, the authors need to include a scale bar and/or show measurements of cells distance from explant to claim this, when compared with controls.
13. The authors should disclose and provide information for how the RNA-seq data sets will be made available, concerning data availability. Will they deposit this data into a public repository? Will they include a supplemental file with the top DE genes, to accompany figure 6? It is suggested to do so.
14. Globally, all microscopic images should ideally have a scale bar, if possible please. All gut images should have proximal distal axes clearly labeled or described in figure legends.
15. Could the authors briefly describe in their methods, or clarify, why they use a z score for the RNA-seq data presentation, as opposed to fold change in expression values?
16. In the reference list, the authors should double check that all are listed in alphabetical order. Saw one out of place.

Reviewer 3

Advance summary and potential significance to field

Hirschsprung disease is characterized by the absence of enteric ganglia in the distal intestine. The cecum marks the junction between small intestine and large intestine in both mammals and avians, ENCCs migrate through and then past the cecum to colonize the large intestine, and the cecum is a source of important ENCC signalling molecules (i.e. GDNF, ET3). The question is posed as to whether ENCCs receive specific signals from the cecum as they cross from midgut to hindgut that are essential for complete colonization of the gut.

This work provides a novel working model to explain distal colonic aganglionosis, describing migration through the ceca as essential for onwards colonization of the gut by ENCCs and proposes differentiation-inhibiting influences of cecal-expressed Wnt11 as a mediator this effect.

Using chick as a model, experiments show that guts explanted at E5 (when colonization has extended to distal midgut) and then cultured for 3 days will go on to have a gut fully colonized by ENCCs. Experiments demonstrate that proliferation of migratory wavefront ENCCs is high when these cells are in the cecal region. Ceca removal performed at E5 and followed by 3 days in culture results in guts with only the proximal hindgut colonized by ENCCs. Ceca removal at E6 (when ENCCs are found within the ceca), followed by replacement with age-matched GFP-expressing ceca by transplantation, shows that after 3 days of culture the hindgut is colonised by ENCCs, and that all of these cells are GFP+. DiI labelling of cells in the E5.5 cecal vs intercecal areas is used to determine the relative contribution of ENCCs from these regions to the hindgut ENS, with the conclusion that only cecal ENCCs migrate to the hindgut. RNA-seq of E5 ceca and intercecal region was performed and select enriched GO terms and DEGs are described, along with presentation of

these overlayed onto an interactome display, with Wnt signalling associated genes being highlighted. The expression of Wnt pathway genes is then explored, with Wnt5a and Wnt11 shown as expressed in the E5 ceca and Fzd7 absent from the E5 ceca, but expressed in the E6 ceca and in ENCCs. In vitro culture experiments to test the influence of Wnt11 show that culture of ceca in the presence of GDNF for 24 hours and then Wnt11 for 24 hours shows a reduction in the differentiation of migrating ENCCs relative to those in culture of ceca in the presence of GDNF for 48 hours. Finally, E5 guts are cultured in the presence of Wnt11 and these show an absence of nNOS+ neurons in the distal gut, whereas control guts contain nNOS+ neurons, leading to the suggestion that Wnt11 keeps cells in a more undifferentiated state.

Comments for the author

1) The statement “wavefront ENCCs are most proliferative as they migrate through the cecal buds” (p. 4, and similar statement on p. 6) is not accurate, since comparisons were not made to wavefront ENCCs at earlier timepoints when they are found in more rostral regions (foregut, rostral midgut).

2) In the Discussion, the effect of cecal removal is said to cause ENCCs to cease migrating and “form large clusters of differentiated neurons”. This presumably refers to Fig3C and/or 3D, however 3C does not show TuJ1+ clusters and 3D shows only Ncadh+EdU- cells, and these are not necessarily differentiated neurons. To make this statement, it would be necessary to actually show clusters of differentiated neurons.

3) It isn’t clear why no GFP- cells are found in the hindgut in Fig4D, since in Fig1C it appears that E6 GFP- cells have moved past the ceca and into the rostral hindgut. Therefore prior to ceca removal and transplantation, GFP- cells would be in the rostral hindgut (not just the intercecal region). Either the image in Fig1C is misleading, or there is a problem with the experimental design/interpretation/model. This should be further clarified.

4) Fig 5B described as showing DiI labelled cells “streaming” into the hindgut. However, only a small number of cells are seen, despite being 72 hours after DiI injection. Why aren’t more cells seen? Why aren’t they also found in the hindgut? The relatively small numbers of cells makes the comparison between DiI injections in the ceca (5B) vs intercecal (5F) region less convincing. Can this be quantified from the n=9 vs n=7 replicates described in the methods?

5) The RNA-seq analysis is very superficially presented, without supporting data and documentation. For example, are the GO terms shown in Fig6B the top GO terms associated with ceca vs interceca? Or are these hand-picked? The methods suggest that “enriched GO terms associated with neural crest colonization are summarized” or “data for selected enriched GO terms were presented”. A list of all associated GO terms should be included, in rank order with corresponding p-values. In general, the RNA-seq results should be presented in a complete way, i.e. the data for DEGs should be presented (as a supplementary table) , and not just represented with a volcano plot (Fig6A). This full reporting is important since the full table of DEGs would reveal whether known genes behave in the expected manner (i.e. with Gdnf and Et3 being more abundant in the ceca). This would allow the success of the experiment to be evaluated. And demonstrating a successful experiment would lend support to following up on novel DEGs.

6) In Fig 7A-C, Wnt5a and Wnt11 appear to show regional restricted expression within the ceca. If this is true, then it should be described, and the significance discussed. In Fig7K,K’, Fzd7 appears in only a proportion of HNK1+ ENCCs migrating in response to GDNF co-express Fzd7. If this is true, then it should be described and this observation included in models for Wnt11/Fzd7 action (see below).

7) In Fig8M, to properly assess the effect of Wnt11 on neuronal differentiation, the samples used for comparison should include Gdnf (24h) + no add (24h).

8) In Fig9C, it appears that ENCCs do not appear in clear migratory streams as seen in Fig9A (and Fig1H). Is this true in all n=28 guts? If so, then this should be described. In Fig 9Bvs9D, nNOS differentiation should be quantified in the n=28 guts to support statements on differentiation. According to the model of Wnt11 in the ceca leading to ENCC proliferation, exogenous Wnt11

should lead to increased proliferation. Is there an increase in proliferating ENCCs? If so, this should be described. If not, then this should also be described and discussed.

9) In the Discussion, a hypothesis is put forth that Wnt11 and GDNF have opposing roles to inhibit or promote differentiation, respectively. The role of GDNF to promote ENCC migration is known and demonstrated again here in Fig8A-C. Other experiments presented in Fig 8 show that while Wnt11 does not promote ENCC migration (Fig8J,K), it can "restore" migration after GDNF withdrawal (Fig8H). The Discussion should be expanded to describe this result and the roles of Wnt11 and GDNF on ENCC migration, since this would constitute another important influence on the ENCC population in the cecal region.

10) Although the work is presented as exploring the etiology of HSCR, the Discussion does not explicitly return to HSCR. Can the Discussion be expanded on this point?

Minor comments
-- Fig 1A inset the ceca cannot be seen (as spatial reference) and should be made visible or outlined with dotted lines
-- Fig6C should be broken up into 4 or 5 separately designated panels for clarity in the text
-- Fig6 inter-ceca (vs interceca in text)

First revision

Author response to reviewers' comments

Thank you for the very helpful reviews and the opportunity to resubmit our manuscript. We have carefully read the excellent critiques of the Reviewers and have made significant revisions to the manuscript in accordance with their recommendations. The Reviewer comments are listed below and our responses follow.

Reviewer 1
1. Further discussion of the potential interactions between Et3/Ednrb signaling and the newly proposed one for Wnt11 would thus help readers to understand current observations.

Thank you for this suggestion. We have added a discussion of known and potential interactions among ET3/EDNRB, GDNF/RET, and Wnt signaling to the Discussion, paragraph 3.

Reviewer 2
1. The authors show an elevated percentage of EdU+ proliferative cells in the wavefront and undifferentiated cells in the cecal to intercecal tissue at E6 (Fig2) and make the conclusion that wavefront is at its highest proliferative rate during when it migrates through the ceca. However, to make this claim the authors would need to make a comparison between the wavefront when it is in the ceca and also in the foregut/midgut, the midgut, as well as the hindgut. It could be that the proliferation increase observed is true within the context of the axial microenvironment of the ceca, but it could actually be higher while traveling through the more proximal or distal areas of the gut.

Additionally, it would be worthwhile to see if the proliferative nature of the enteric crest varies between the two regions (ceca and interceca) at E7, which would suggest it's not solely the wavefront , but a general feature of crest migrating through the ceca (which either result would still be fascinating).

We appreciate this comment and performed more detailed quantitative analysis of ENCC proliferation at E5 through E8, as shown in a new Fig. 2B. Our results show that proliferation is highest in the ceca at E6, when the wavefront is located there, and this is significantly higher than when the wavefront is in the distal midgut (E5) or in the hindgut (E7). While the rate appears higher in the ceca as compared to the interceca, this did not reach statistical significance. The Results section (paragraph 2) has been modified accordingly.

2. Related to comment 1 above, it would help readers to understand more clearly, if the authors could explicitly define what the “migratory wavefront” truly is, on the first mention and in the methods. Do they mean the first 1-5 cells in the enteric chain wavefront, or is it something encompassing a larger population of cells? It is important to be clear about the distinction, as this will alter how data can be/is interpreted.

We have clarified the definition of “migratory wavefront” in the first paragraph of the Results, defining it as “the migratory and undifferentiated neural crest-derived cells at or near the leading edge of migration.”

3. In the introduction, it would help the reader to understand the study better if some basic background on chicken enteric nervous system stages/time points were included briefly. As well, the references for vagal neural crest-derived cells in the second sentence requires a couple of primary citations, currently there are only review articles.

We have added this background, including primary references, to the Introduction to briefly summarize ENS development in the chicken embryo.

4. The authors demonstrate that loss of cecal tissue at E5 prevents ENCCs from populating past the cecal proximal hindgut. However, it’s also possible the cells are dying. I would suggest looking to see if there is a change in the cell death, as opposed to just the aggregation of cells in the region.

Thank you for this comment. We have added panel F to Fig. 3 and find no evidence of apoptotic cell death of the ENCCs. This has been added to the Results (paragraph 3) and figure legend.

5. Related to comment 4 above, it is possible that the ENCCs are prevented from populating past the cecal proximal gut due to a dramatic decrease in cellular proliferation. While the authors do show one image of some enteric cells with little overlap of EdU following cecal removal (Fig 3D), we don’t see a control gut there to compare it to and I would suggest quantifying if there is a change in percentage of proliferation and/or a gross drop in the total number of wavefront cells following cecal removal, when compared with cecal intact guts.

In response to this comment, we added panel G to Fig. 3 (note that the control is Fig. 1I). Fig. 3G shows that ENCC proliferation is significantly reduced following cecal ablation. This has been added to the Results (paragraph 3) and figure legend.

6. The RNA-seq experiment is very insightful. I would suggest that the authors provide a sentence or explanation for why the time point of E5 was chosen for analysis, as opposed to E6.

E5 is the earliest stage we can isolate the ceca from the rest of the gut as there are no cecal buds prior to this stage. In addition, we chose E5 instead of E6 because we wanted to exclude the presence of ENCCs from our analysis and focus on the cecal microenvironment that is being primed for the arrival of the ENCCs. This explanation has been added to the Results, paragraph 4.

7. Did the authors observe a change in the expression of Ret/GDNF pathway in their RNA-seq experiments?

Figure 6 has been revised to include additional analysis of the RET-GDNF pathway and other HSCR-related genes (Gui, et al. 2017) which are likely to be involved in ENCC colonization of the hindgut. From these data we observe increased RET expression in the interceca and GDNF in the ceca, consistent with previous observations in the chick at this developmental stage (Nagy and Goldstein, 2006). This finding is interesting as we previously showed that GDNF overexpression promotes enteric neuronal differentiation and also, given the chemoattractive role of GDNF to ENCCs, prevents their further migration. This has been added to the Results (paragraph 4) and to the Discussion (paragraph 2).

8. The explant experiments described in figure 8 are important to the study presented, however it took this reviewer quite some time to fully understand the conditions and schematize out the temporal elements of the explant incubations/conditions, etc. I suggest that the authors include a

cartoon schematic to describe the timeline of explant GDNF/Wnt11 experiments, with conditions. It could possibly be included as a revised Figure 8A.

Thank you for this suggestion. We have made a schematic diagram of the experiment described in Fig. 8 and added this as a new Supplemental Figure 1.

9. The authors state in the results, “If GDNF is removed from the culture media after the first 24 hours, migration over the next 24 hours is limited and cells aggregate into large ganglion-like structures with altered network morphology.” Altered when compared with what? in vivo gut? or images in 3D?

This has been clarified in the Results, paragraph 7.

10. The experiments in Figure 8 suggest that Wnt11 is sufficient to rescue enteric migration from the explant (after initial GDNF priming), however is it also sufficient to rescue proliferation and cell numbers?

We tested the effect of Wnt11 on ENCC proliferation in E6 gut cultures and found that it has an inhibitory effect on proliferation. This has been added as a new Supplemental Figure 2 and to the end of the Results section.

11. The authors state “Interestingly, when compared to GDNF alone (Fig. 8D), Wnt11-treated cultures appear to have many more undifferentiated ENCCs (Fig. 8I). To claim this, the authors need quantification here, or they should temper this claim.

Thank you for this comment. We have added quantification of neuronal differentiation (see Fig. 8M) to support this statement.

12. The authors state, “ … Wnt11 protein alone does not promote ENCC migration from the ceca (Fig. 8J-L).” The image shown is not convincing, as presented. To be sure of this, the authors need to include a scale bar and/or show measurements of cells distance from explant to claim this, when compared with controls.

Thank you for this suggestion. We have added scale bars to the figure.

13. The authors should disclose and provide information for how the RNA-seq data sets will be made available, concerning data availability. Will they deposit this data into a public repository? Will they include a supplemental file with the top DE genes, to accompany figure 6? It is suggested to do so.

We have added a complete analysis of the differentially expressed genes in supplementary data (see response to Reviewer 3, comment 5). We have also submitted the data to the GEO repository. They have been assigned GEO accession numbers and will be publicly available on Nov. 1, 2021.
The following secure token has been created to allow review of record GSE182783 while it remains in private status: inebasscxhknncd
To review, go to https://secure-web.cisco.com/18mJ3Tc3C2NJqWs94YGhiC3P1O1qGJCDzp1cgqifVjhsVCOxvS382h1-vggyt0mZ7Ixleql0lp8dYwyX45chm0-dl9wujaJXZDO6AVdWt2l8N8RIEZAL_45zMVWa_aNwYRX4Dp8eNI-odgH59HsVZmcO7C--J2Dl1kw2SQoTUxLGtswQMnsu1h5cgnwm3VcZZTeoeDtSKLYEbNYUJYZuapYj99bZ2gE-N6dExHFtkH1tuL4RS8NP7KFgNIHrVPq2m/https%3A%2F%2Fwww.ncbi.nlm.nih.gov%2Fgeo%2Fquery%2Facc.cgi%3Facc%3DGSE182783

14. Globally, all microscopic images should ideally have a scale bar, if possible please. All gut images should have proximal distal axes clearly labeled or described in figure legends.

Thank you for this suggestion. We have added scale bars and labels as requested.

15. Could the authors briefly describe in their methods, or clarify, why they use a z score for the RNA-seq data presentation, as opposed to fold change in expression values?

Z-scores are commonly used to visualize gene expression data and can be viewed as the standard deviation of the samples from the average expression. This is a good way perceive the inter-sample variability which is more heavily skewed by fold change values. Similarly, the use of fold change values makes it difficult to visualize genes that are differentially expressed with a low fold change (even though a p value may indicate they are equally/more important). Nonetheless, we have now added RPKM values and fold change values in the supplementary data which could be useful for comparisons between genes if required.

16. In the reference list, the authors should double check that all are listed in alphabeticel order. Saw one out of place.

We apologize for this error and have corrected it.

Reviewer 3
1) The statement “wavefront ENCCs are most proliferative as they migrate through the cecal buds.” (p. 4, and similar statement on p. 6) is not accurate, since comparisons were not made to wavefront ENCCs at earlier timepoints when they are found in more rostral regions (foregut, rostral midgut).

Thank you for this comment. Please see our response to Reviewer 2, comment 1.

2) In the Discussion, the effect of cecal removal is said to cause ENCCs to cease migrating and “form large clusters of differentiated neurons”. This presumably refers to Fig3C and/or 3D, however 3C does not show TuJ1+ clusters and 3D shows only Ncadh+EdU- cells, and these are not necessarily differentiated neurons. To make this statement, it would be necessary to actually show clusters of differentiated neurons.

We appreciate this recommendation from the Reviewer. We added a new panel E to Fig. 3, showing that the large ENCC clusters that form following cecal ablation are largely neuronally differentiated and expressed nNOS.

3) It isn’t clear why no GFP- cells are found in the hindgut in Fig4D, since in Fig1C it appears that E6 GFP- cells have moved past the ceca and into the rostral hindgut. Therefore prior to ceca removal and transplantation, GFP- cells would be in the rostral hindgut (not just the intercecal region). Either the image in Fig1C is misleading, or there is a problem with the experimental design/interpretation/model. This should be further clarified.

We apologize for this confusion and have now clearly marked the boundaries of the cecal buds in Fig. 1A-C. At E6, ENCCs have not yet entered the hindgut; they have only colonized the ceca and interceca. We have confirmed this using Sox10 immunostains in many embryos. ENCCs enter the proximal hindgut at E6.5.

4) Fig 5B described as showing DiI labelled cells “streaming” into the hindgut. However, only a small number of cells are seen, despite being 72 hours after DiI injection. Why aren’t more cells seen? Why aren’t they also found in the hindgut? The relatively small numbers of cells makes the comparison between DiI injections in the ceca (5B) vs intercecal (5F) region less convincing. Can this be quantified from the n=9 vs n=7 replicates described in the methods?

Thank you for this comment. The DiI that was injected into the cecal buds is progressively diluted by tissue growth and cell proliferation, accounting for why more cells aren’t seen. We quantified the NCadh+ ENCCs that contain DiI crystals under high power magnification and found a significant difference: 31% of ENCCs were DiI+ after cecal injection, whereas none were labelled after injection of the interceca. This has been added to the Results, paragraph 3.

5) The RNA-seq analysis is very superficially presented, without supporting data and documentation. For example, are the GO terms shown in Fig6B the top GO terms associated with ceca vs interceca? Or are these hand-picked? The methods suggest that “enriched GO terms associated with neural crest colonization are summarized” or “data for selected enriched GO terms were presented”. A list of all associated GO terms should be included, in rank order with corresponding p-values. In general, the RNA-seq results should be presented in a complete way, i.e.

the data for DEGs should be presented (as a supplementary table), and not just represented with a volcano plot (Fig6A). This full reporting is important since the full table of DEGs would reveal whether known genes behave in the expected manner (i.e. with Gdnf and Et3 being more abundant in the ceca). This would allow the success of the experiment to be evaluated. And demonstrating a successful experiment would lend support to following up on novel DEGs.

We appreciate this comment and agree that readers can benefit from the inclusion of our complete data in the supplement of the manuscript and thi will help validate our results. We have now included gene expression counts and DEG analysis (Supplementary table 1), overrepresentation analysis of these DEGs (Supplementary table 2), and data to recreate our PPI network and module analysis (Supplementary table 3). For the semantic similarity plot, as many ontologies as possible were labeled without compromising the legibility of the figure. The supplementary data can now assist the reader in determining which ontologies are important based on p-value or enrichment scores. From the added supplementary data, GDNF and EDN3 are observed to be upregulated in the ceca, as previously reported, lending credence to the accuracy of our dataset. In addition, we have included a heatmap of HSCR-related genes (Gui, et al. 2017) to Figure 6B which highlights the expression of Gdnf and Et3 and brings this to attention in the main manuscript.

6) In Fig 7A-C, Wnt5a and Wnt11 appear to show regional restricted expression within the ceca. If this is true, then it should be described, and the significance discussed. In Fig7K,K', Fzd7 appears in only a proportion of HNK1+ ENCCs migrating in response to GDNF co-express Fzd7. If this is true, then it should be described and this observation included in models for Wnt11/Fzd7 action (see below).

We agree that Wnt5a and Wnt11 are regionally restricted to the cecal mesenchyme and have added this to the Results. We also agree that only a subset of HNK1+ ENCCs express Fzd7, suggesting that the ENCCs represent a heterogeneous group wherein Fzd7 expression is variable, although further studies will be needed to clarify this. This has been added to the Results, paragraph 5.

7) In Fig8M, to properly assess the effect of Wnt11 on neuronal differentiation, the samples used for comparison should include Gdnf (24h) + no add (24h).

Thank you for this suggestion. We have added this and revised Fig. 8 accordingly.

8) In Fig9C, it appears that ENCCs do not appear in clear migratory streams as seen in Fig9A (and Fig1H). Is this true in all n=28 guts? If so, then this should be described. In Fig 9B vs 9D, nNOS differentiation should be quantified in the n=28 guts to support statements on differentiation. According to the model of Wnt11 in the ceca leading to ENCC proliferation, exogenous Wnt11 should lead to increased proliferation. Is there an increase in proliferating ENCCs? If so, this should be described. If not, then this should also be described and discussed.

As the Reviewer notes, we generally see "migratory streams" of ENCCs, but in Fig9C we believe the lack of these streams is due to the sagittal section plane of the hindgut. Analyzing consecutive longitudinal sections stained with N-cadherin demonstrate that "streams" are better seen when the section is parasagittal or slightly oblique (see Fig 1H, wavefront). When the section is perfectly sagittal and the gut lumen is visible, the migratory ENCC population looks more dispersed.
To quantify nNOS differentiation, we measured the distance between the most distal Ncadh+ wavefront cell and the most distal nNOS+ cell in the hindgut, wince differentiation is known to proceed in a craniocaudal fashion. Those results are shown in Fig. 9E and confirm that Wnt11 delays neuronal differentiation. This has been added to Results (2nd to last paragraph).

Regarding the effect of Wnt11 on ENCC proliferation, we quantified the proportion of Ncadh+ cells that incorporate EdU. The results, described in the Results (last paragraph), show that Wnt11 reduces the rate of ENCC proliferation. We repeated this in explanted guts in the presence of GDNF so promote ENCC migration away from the gut as shown in a new Supplemental Fig. 2. Once again, Wnt11 proved to have an anti-mitogenic effect on the ENCCs. This has been added to the Results (last paragraph) and to the Discussion (paragraph 4).

9) In the Discussion, a hypothesis is put forth that Wnt11 and GDNF have opposing roles to inhibit or promote differentiation, respectively. The role of GDNF to promote ENCC migration is known and

demonstrated again here in Fig8A-C. Other experiments presented in Fig 8 show that while Wnt11 does not promote ENCC migration (Fig8J,K), it can “restore” migration after GDNF withdrawal (Fig8H). The Discussion should be expanded to describe this result and the roles of Wnt11 and GDNF on ENCC migration, since this would constitute another important influence on the ENCC population in the cecal region.

We appreciate this important point. Based on our results, we conclude that while Wnt11 alone is not chemoattractive for ENCCs, it is permissive, allowing the cells to respond to the pro-migratory effect of GDNF. We have added this to the Discussion, paragraph 4.

10) Although the work is presented as exploring the etiology of HSCR, the Discussion does not explicitly return to HSCR. Can the Discussion be expanded on this point?

We appreciate this suggestion and have added a discussion of HSCR as it relates to the current findings to the Discussion, beginning of paragraph 2.

Minor comments:
- Fig 1A inset the ceca cannot be seen (as spatial reference) and should be made visible or outlined with dotted lines
- Fig6C should be broken up into 4 or 5 separately designated panels for clarity in the text
- Fig6 inter-ceca (vs interceca in text)

Thank you for these suggestions. Fig. 1 and Fig. 6 were edited as suggested.

Second decision letter

MS ID#: DEVELOP/2021/199825

MS TITLE: Avian ceca are required for hindgut enteric nervous system development by inhibiting neuronal differentiation via non-canonical Wnt signaling and by promoting enteric neural crest cell proliferation

AUTHORS: Nandor Nagy, Tamas Kovacs, Rhian Stavely, Viktoria Halasy, Adam Soos, Emoke Szocs, Ryo Hotta, Hannah Graham, and Allan Goldstein

I have now received the reports of two of the 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 positive and we would like to publish a revised manuscript in Development, provided that you satisfactorily address the remaining minor suggestions of referee 3. Please attend to all of the reviewer's comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 2

Advance summary and potential significance to field

This manuscript describes the necessity and sufficiency of the ceca to populate hindgut enteric nervous system (ENS) via enteric neural crest cell (ENCC) migration. Given the ease of ex-vivo maintenance of avian tissue and neural crest population, this study provides insightful information regarding ENS colonization and ENCC. To begin using the avian ceca as a source of hindgut population ENCCs, the authors establish that loss of the paired cecal tissue leads to a loss of ENS population in the post cecal colon from day E5-E8. The authors postulate the sequence of events

that mediate hindgut ENS colonization by ENCCs: (1) ENCCs enter cecal region and proliferate (2) WNT11 expressed in the ceca promote stemness (3) ENCCs exit cecal tissue and populate hindgut tissue. Overall, the authors provide strong evidence of the necessity of the ceca for proper maintenance of ENCCs for full population of the hindgut, and elucidate both a molecular (WNT11 signaling) and Cellular (increased proliferation and reduced differentiation) allowing for the proper colonization of the hindgut. This research is relevant to the study of enteric neuropathies and better elucidates tissue-level genetic mechanisms that may drive enteric disease, namely HSCR

Comments for the author

the authors have addressed my prior concerns.

Reviewer 3

Advance summary and potential significance to field

Hirschsprung disease is characterized by the absence of enteric ganglia in the distal intestine. The cecum marks the junction between small intestine and large intestine in both mammals and avians, ENCCs migrate through and then past the cecum to colonize the large intestine, and the cecum is a source of important ENCC signalling molecules (i.e. GDNF, ET3). The question is posed as to whether ENCCs receive specific signals from the cecum as they cross from midgut to hindgut that are essential for complete colonization of the gut.

This work provides a novel working model to explain distal colonic aganglionosis, describing migration through the ceca as essential for onwards colonization of the gut by ENCCs and proposes differentiation-inhibiting influences of cecal-expressed Wnt11 as a mediator this effect.

Using chick as a model, experiments show that guts explanted at E5 (when colonization has extended to distal midgut) and then cultured for 3 days will go on to have a gut fully colonized by ENCCs. Experiments demonstrate that proliferation of migratory wavefront ENCCs is high when these cells are in the cecal region. Ceca removal performed at E5 and followed by 3 days in culture results in guts with only the proximal hindgut colonized by ENCCs. Ceca removal at E6 (when ENCCs are found within the ceca), followed by replacement with age-matched GFP-expressing ceca by transplantation, shows that after 3 days of culture the hindgut is colonised by ENCCs, and that all of these cells are GFP+. DiI labelling of cells in the E5.5 cecal vs intercecal areas is used to determine the relative contribution of ENCCs from these regions to the hindgut ENS, with the conclusion that only cecal ENCCs migrate to the hindgut. RNA-seq of E5 ceca and intercecal region was performed and select enriched GO terms and DEGs are described, along with presentation of these overlayed onto an interactome display, with Wnt signalling associated genes being highlighted. The expression of Wnt pathway genes is then explored, with Wnt5a and Wnt11 shown as expressed in the E5 ceca and Fzd7 absent from the E5 ceca, but expressed in the E6 ceca and in ENCCs. In vitro culture experiments to test the influence of Wnt11 show that culture of ceca in the presence of GDNF for 24 hours and then Wnt11 for 24 hours shows a reduction in the differentiation of migrating ENCCs relative to those in culture of ceca in the presence of GDNF for 48 hours. Finally, E5 guts are cultured in the presence of Wnt11 and these show an absence of nNOS+ neurons in the distal gut, whereas control guts contain nNOS+ neurons, leading to the suggestion that Wnt11 keeps cells in a more undifferentiated state.

Comments for the author

The authors have performed new experiments and analysis, supplied additional supporting data and modified the text and figures to satisfactorily address previous comments.

Three further minor comments are:

1) To remove the word “streaming” in the description of the results in Fig5B,C, since as the authors acknowledge, the incomplete labelling of cells means that a mere small number of cells are observed. It may be more appropriate to described these cells as having “migrated into” or “entered into” the hindgut.

2) To supply a reference for the list of “HSCR-related genes” analysed in Fig6B.
3) To note that multiple Wnt-related biological processes are associated with DEGs up-regulated in the ceca. The ones cited in the current MS “cell-cell Wnt signaling” (actually should be “cell-cell signaling by Wnt”) and “negative regulation of canonical Wnt signaling pathway” are 278 and 273, respectively, on the list ranked by fold-enrichment supplied in Supplementary table 2. Yet other Wnt-related biological processes show greater fold enrichment, such as “regulation of non-canonical Wnt signaling pathway” (17) and “canonical Wnt signaling” (177).

Second revision

Author response to reviewers' comments

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Third decision letter

MS ID#: DEVELOP/2021/199825

MS TITLE: Avian ceca are required for hindgut enteric nervous system development by inhibiting neuronal differentiation via non-canonical Wnt signaling and by promoting enteric neural crest cell proliferation

AUTHORS: Nandor Nagy, Tamas Kovacs, Rhian Stavely, Viktoria Halasy, Adam Soos, Emoke Szocs, Ryo Hotta, Hannah Graham, and Allan Goldstein
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.