RET overactivation leads to concurrent Hirschsprung disease and intestinal ganglioneuromas

Nandor Nagy, Richard A. Guyer, Ryo Hotta, Dongcheng Zhang, Donald F. Newgreen, Viktoria Halasy, Tamas Kovacs and Allan M. Goldstein

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Original submission

First decision letter

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MS TITLE: RET overactivation leads to concurrent Hirschsprung disease and intestinal ganglioneuromas

AUTHORS: Nandor Nagy, Richard A. Guyer, Ryo Hotta, Dongcheng Zhang, Donald F. Newgreen, Viktoria Halasy, Tamas Kovacs, and Allan M. Goldstein

My apologies for the unusually long time it has taken us to receive the reports of three referees on your manuscript. I have now received the referees' reports 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 great interest in your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. In particular, Referee 2 asks that you examine the effect of concentration and time of action of GDNF on the gut cultures, and Referee 3 requests more information about the experimental approaches used in the study. 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.

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.

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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

This paper describes a culture system in which pieces of E7 chick or E11.5 mouse midgut and hindgut are cultured with GDNF, which results in large cellular aggregates forming on the surface of the ceca and post-umbilical gut. Analysis shows that these clusters contain Tuj1+ , Hu+, NF+, Synaptophysin+, BFABP+, and in some cases S100+ cells, but not SMA+ or GFAP+ cells. Transplantation of the aggregate containing guts onto the chick CAM and further culture showed these gut pieces had enlarged ENS plexus. Repeating chick experiments with younger stage (E6) chick mid/hindgut resulted in aggregates forming in the midgut only and a lack of ENCC in more caudal regions, described as distal colonic aganglionosis.

Dissociation of aggregates in culture with GDNF show that the cells proliferate in culture. Analysis of aggregates shows cells that are N-cadherin+, Sox10+ and can incorporate EdU, features of ENCC progenitors. The authors name the aggregates “ganglioneuromas”.

The origin of the aggregate cells is demonstrated using a strategy to label vagal NC cells or sacral NC cells with GFP and showing that only GFP+ vagal NC cells contribute to the aggregates, and can form Hu+ ENS neurons.

The potential of aggregate cells is tested by transplanting fluorescently labelled aggregates from either chick or mouse guts on to chick hindgut, then culturing on the CAM. An apparently normally colonised ENS is shown as evidence that aggregate cells have potential to form a complete ENS.

The manuscript appears to stretch to relate these findings to Janus mutations in RET, in which the same mutation is proposed to have differing impact depending on the cellular context (RET impairment leading to intestinal aganglionosis in and RET overactivity leading to MEN-associated tumours). The authors cite the presence of ganglioneuromas and gut colonisation phenotypes with the same GDNF treatment as evidence that RET overactivity can account for both phenotypes. The authors rightly cite numerous caveats with this interpretation, including the difference between ligand mediated responses in these experiments and gain of function responses in MEN2A mutations, or altered interactions and signalling in MEN2B. They also acknowledge the fact that ganglioneuromas and HSCR do not occur together in human patients (being associated with MEN2B and MEN2A, respectively).

The strongest significance drawn from these experiments appears to be that the organotypic cultures used can be used as a source of progenitor ENCCs for further studies.

Comments for the author

1) The presence of neurons in aggregates is well demonstrated. However, the evidence that glia are present relies on BFABP and S100, and no GFAP+ cells are seen. Since BFABP is a enteric glial progenitor marker, is it possible that aggregates contain largely glial progenitors and few mature glia (only limited S100b+ cells and no GFAP+ cells)?

2) The fact that ganglioneuromas only occur if generated during ENCC colonization of the gut, since none form in GDNF treatment of E9 guts, is not sufficiently discussed. How does this fact relate to the timing of appearance of neurogliomas in MEN2B patients?

3) The ‘reconstituted’ ENS in Figure 5 is deemed ‘normal’ based on the presence of regionally appropriate Hu staining. Do glial cells form also? Are the numbers of neurons and glial cells present equivalent to a normal ENS network? Is this network functional? Softer language is used in the figure

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legend than in the main text and abstract. Without further experimentation, the softer language is more appropriate.

4) The paper is thin on references, though well self-referenced. This fact might make the paper less accessible to a generalised audience. Moreover, insufficient attention is drawn to numerous other studies of model systems in which RET signalling is modulated to address MEN2 mechanisms.

5) Figure 3 is not correctly referenced in the text.

6) CN antibody does not appear to be shown in Figure 5I, though cited in the text and legend.

Reviewer 2

Advance summary and potential significance to field

The paper tests the hypothesis that over-stimulation of Ret can both cause tumor formation from crest-derived cells and the failure of crest-derived cells to migrate into and colonize the terminal bowel. Data are consistent with this hypothesis and also show that some cells in ganglioneuromas retain the potential to differentiate to form a normal-appearing ENS when the cells are transplanted into a normal embryonic environment.

Comments for the author

This is a fascinating manuscript. The authors use catenary cultures of developing chick and mouse gut to test the hypothesis that over-stimulation of Ret can result both in crest-derived cells tumor formation and failure of crest-derived cells to migrate into and colonize the terminal bowel. Results are consistent with the overarching hypothesis and further show that some cells from the resulting murine ganglioneuromas retain the potential to develop normally and differentiate to give rise to a normal-appearing ENS when the cells are transplanted into a normal embryonic environment in a chick hindgut explanted prior to its colonization by endogenous crest-derived cells. The ganglioneuromas retain within them precursor cells that have the capacity to differentiate normally. This interesting observation is not shocking and has previously been observed with other types of embryonic rest tumor, including neuroblastoma. The authors conclude that a Janus mutation in Ret is not necessary to explain both the gain-of-function induced tumorigenesis and loss-of-function induced aganglionosis of Hirschsprung disease. They do both with excessive stimulation of Ret (using the natural ligand) in developing crest-derived cells of the gut. The authors ought to note, however that although both types of effect occur in this system, they do not rule out the possibility that the occasional Hirschsprung phenotype in patients with MEN2A is due to a Janus mutation in Ret.

It is curious that the authors proceed with a single concentration of GDNF (40 ng/ml). There are no results exploring the concentration-effect relationship, either for tumor-formation or for colonization of the hindgut. The time-action curve for GDNF is also not given. These data are really necessary to understand the action of GDNF. The authors also present little information about the mechanism by which GDNF, which ought to enhance migration, stops it when applied in excess. Is apoptosis induced at the high concentration? Do neurites extend and get tangled? Are differentiation/migration incompatible with one another? Earlier papers have proposed such a mechanism in which premature differentiation of crest-derived precursors results in failure of crest-derived cells to colonize the hindgut. The earlier papers should be cited and discussed. The authors show tumors and demonstrate proliferation, but they do not show whether the tumors are simply exuberant growth that interferes with migration or really malignancies. In speaking of MEN2A, the authors should really demonstrate an ability of the ganglioneuromas that they generate to metastasize. Could a test of metastatic ability of the tumors be done?

In summary, this paper makes a good start. Additional work on the detail, especially concentration-effect and time-action experiments, is needed.

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Reviewer 3

Advance summary and potential significance to field

This study by Nagy et al., tackles the interesting question of how to explain co-existing Hirschsprung Disease (thought to be caused by RET deficiency) and multiple endocrine neoplasia (MEN) thought to be caused by role of RET overactivation. Using an intestinal catenary culture system, the authors propose that just RET overactivation – in this model done by the addition of GDNF to the culture – is sufficient to cause both phenotypes. Even though the study tackles an interesting question, it is a bit underdeveloped in its current form and lacks background and experimental information to make it understandable for the broad readership of Development as detailed in the comments below. The main concern is that it is not clear if the culture system accurately represents the disease phenotypes as they comprise limited RET overactivation, whereas in the disease RET overactivation would be present during the entire time of ENS development, which lowers the significance of the results.

Comments for the author

1. The discussion states that the cell culture approach can model the disease phenotypes of both MEN2A and 2B. There is very little detailed information on the type of RET mutations that are connected with both disease phenotypes. Thus, it is not quite clear how the approach described in the paper is an accurate representation of MEN, also because RET is only activated in a short period in time in the experimental model described in the paper, whereas presumably, the overactivation happens continuously in patients with MEN. It seems logical that distal colonic aganglionosis would occur when ENCCs differentiate precociously into neurons but is this how it would occur in human patients? More detail needs to be provided to make be convincing that this model is indeed a good representation of MEN phenotypes.
2. The paper would benefit from additional information on how the ganglioneuromas develop – how do cells migrate out of the gut and form these evenly spaced aggregates, as this culture system seems uniquely suited for this – even though the authors state that they'll leave this for future studies, I think that this would provide some exciting insights that are also relevant to explain the disease phenotype.
3. The results section needs to include much more experimental details to make it easier to follow the rationale and experimental design of experiments.
a. For example, it would be important to describe in more detail and provide an example figure to explain what the catenary culture model is and how it works even though it is published. It's the key technique for this paper and it needs to be clearer what exactly has been done.
b. It is not clear how the experiment that resulted in Fig. 1J-L relates to the previous experiment and how this shows what happens to the large ENCC aggregates over time. Is the gut prepared in the same way as in the previous experiment, just taken from E6 or is this just a gut treated with GDNF? What does transplanting it onto CAM do to the gut? Also, it would be helpful to see Hu staining in addition to Tuj1 staining to appreciate better that there are more neuronal cell bodies, which is hard to see in with the Tuj1 staining.
c. Please include how the distance of ENS migration was measured – from where to where, it is not clear what "extent of SOXE+ cells" in Fig.3I refers to.
d. Please include information on how ENS cells were quantified in Fig. 3J – what does the 40 degree arc refer to?
4. The composition of the cell aggregates is not completely clear. From Figure 1 & 2, it looks like basically all cells are positive for a neuronal marker, but then they also are described to express glial markers and progenitor cell markers – is there overlap between these markers or is it just a tight aggregate of these different cell types. This needs to be clarified with co-labeling with different markers.
5. It is not clear why the first set of experiments (Figure 1) required a catenary culture setting, when later in the paper cell aggregates form just after treatment with GDNF. Please explain how these two experimental setups relate to each other.
6. Please provide quantification of aggregates with and without GFP+ cells after treatment with GDNF. There are some GFP+ cells in the aggregate on the top of Figure 4, left panel. Please explain the presence of these GFP+ cells and also provide a quantification of how often they occur.

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7. Please include information on how many ganglioneuromas were transplanted for the mouse. As for the chick, only two were transplanted (in the same animal), the numbers for this experiment need to be increased.
8. More background needs to be provided on several points.
a. Please provide context what RET signaling is and what GDNF does and what it is – this is not explained well in the background section. It is also not described what is known about the function of GDNF in ENS development even though there is quite some body of literature on this already- does it result in ENCC proliferation or neuronal differentiation? This is important to add because it puts the results of this paper into context – is it to be expected that the addition of GDNF results in more neurons?
b. Please explain in the introduction what the hallmarks of ganglioneuroma are – additional information is necessary to determine that the cell aggregates indeed resemble ganglioneuroma.
c. Please include information on the nerve of Remak and what its origin when explaining the experimental set-up of Figure 4 – this will be helpful for the reader to follow the logic behind electroporating into either vagal or sacral level.
Minor comments:
Please include n for all experiments.

First revision

Author response to reviewers' comments

Reviewer 1
1) The presence of neurons in aggregates is well demonstrated. However, the evidence that glia are present relies on BFABP and S100, and no GFAP+ cells are seen. Since BFABP is a enteric glial progenitor marker, is it possible that aggregates contain largely glial progenitors and few mature glia (only limited S100b+ cells and no GFAP+ cells)?

In revised Fig. 1, we show expression of both Sox10 and BFABP in the aggregates in chick gut. While Sox10 is present in glia and progenitors, the expression of BFABP marks the glial lineage. This is confirmed in the inset in Fig. 1H, which shows both Sox10/BFABP co-expressing glial cells (arrows) as well as Sox10+/BFABP-negative progenitors (arrowheads). Revised Fig. 3 shows BFABP and S100 expression in the mouse gut, consistent with glia. The lack of GFAP expression in Fig. 3K likely reflects the fact that this marker of differentiated glia does not appear until E16.5 in mouse gut (Young et al, 2003), which is older than the E11.5 guts that we culture for 48 hours. This has been added to the text.

2) The fact that ganglioneuromas only occur if generated during ENCC colonization of the gut, since none form in GDNF treatment of E9 guts, is not sufficiently discussed. How does this fact relate to the timing of appearance of neurogliomas in MEN2B patients?

We have added a paragraph to the Discussion to address this interesting question.

3) The 'reconstituted' ENS in Figure 5 is deemed 'normal' based on the presence of regionally appropriate Hu staining. Do glial cells form also? Are the numbers of neurons and glial cells present equivalent to a normal ENS network? Is this network functional? Softer language is used in the figure legend than in the main text and abstract. Without further experimentation, the softer language is more appropriate.

In response to this comment, we have revised Fig. 7 (previously Fig. 5) to show that the ENS generated by transplanted ganglioneuromas comprises a fully colonized hindgut organized into two plexuses and containing enteric neurons (Hu, PGP9.5, nNOS) and glia (S100, GFAP). We have softened the language and rather than describe it as a "normal" ENS, we now refer to this reconstituted ENS as "fully colonized," "normally patterned," and containing enteric neurons and glia.

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4) The paper is thin on references, though well self-referenced. This fact might make the paper less accessible to a generalised audience. Moreover, insufficient attention is drawn to numerous other studies of model systems in which RET signalling is modulated to address MEN2 mechanisms.

We have added additional citations and have added text in the Introduction and Discussion regarding MEN2 syndromes and other model systems to study them.

5) Figure 3 is not correctly referenced in the text.

We apologize for this error. It has been corrected.

6) CN antibody does not appear to be shown in Figure 5I, though cited in the text and legend.

Thank you for this comment. This has been corrected and is now shown in Fig. 7L.

Reviewer 2

1. It is curious that the authors proceed with a single concentration of GDNF (40 ng/ml). There are no results exploring the concentration-effect relationship, either for tumor-formation or for colonization of the hindgut.
The time-action curve for GDNF is also not given. These data are really necessary to understand the action of GDNF.

Thank you for this valuable recommendation. We performed a series of experiments to explore the effects of GDNF concentration and duration of treatment on ganglioneuroma formation, including size and number, and also on hindgut ENS colonization. The results are shown in a new Fig. 5 and summarized in the Results.

2. The authors also present little information about the mechanism by which GDNF, which ought to enhance migration, stops it when applied in excess. Is apoptosis induced at the high concentration? Do neurites extend and get tangled? Are differentiation/migration incompatible with one another? Earlier papers have proposed such a mechanism in which premature differentiation of crest-derived precursors results in failure of crest-derived cells to colonize the hindgut. The earlier papers should be cited and discussed.

Thank you for this comment. We have added a new first paragraph to the Discussion to address this important comment.

3. The authors show tumors and demonstrate proliferation, but they do not show whether the tumors are simply exuberant growth that interferes with migration or really malignancies. In speaking of MEN2A, the authors should really demonstrate an ability of the ganglioneuromas that they generate to metastasize. Could a test of metastatic ability of the tumors be done?

MEN2-associated ganglioneuromas are benign tumors without metastatic potential. We have now mentioned their benign nature in the Introduction, paragraph 1.

Reviewer 3

1. The discussion states that the cell culture approach can model the disease phenotypes of both MEN2A and 2B. There is very little detailed information on the type of RET mutations that are connected with both disease phenotypes. Thus, it is not quite clear how the approach described in the paper is an accurate representation of MEN, also because RET is only activated in a short period in time in the experimental model described in the paper, whereas presumably, the overactivation happens continuously in patients with MEN. It seems logical that distal colonic aganglionosis would occur when ENCCs differentiate precociously into neurons but is this how it would occur in human patients? More detail needs to be provided to be convincing that this model is indeed a good representation of MEN phenotypes.

Thank you for this comment. We have increased the detail provided, including additional citations, in the Introduction (paragraph 2), explaining the differences between mutations causing MEN2A and MEN2B. We have also added text to the Discussion (paragraph 1) that describes how GDNF-RET

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signaling functions during ENS development and how it may lead to tumor formation in our model and also mentions the limitations of our system.

2. The paper would benefit from additional information on how the ganglioneuromas develop – how do cells migrate out of the gut and form these evenly spaced aggregates, as this culture system seems uniquely suited for this – even though the authors state that they'll leave this for future studies, I think that this would provide some exciting insights that are also relevant to explain the disease phenotype.

Thank you for this comment. We have added additional text to the Discussion to address this.

3. The results section needs to include much more experimental details to make it easier to follow the rationale and experimental design of experiments.

a. For example, it would be important to describe in more detail and provide an example figure to explain what the catenary culture model is and how it works even though it is published. It's the key technique for this paper and it needs to be clearer what exactly has been done.

Fig. 1A has been added to show the catenary culture system and a description of it has been added to Results, paragraph 1.

b. It is not clear how the experiment that resulted in Fig. 1J-L relates to the previous experiment and how this shows what happens to the large ENCC aggregates over time. Is the gut prepared in the same way as in the previous experiment, just taken from E6 or is this just a gut treated with GDNF? What does transplanting it onto CAM do to the gut? Also, it would be helpful to see Hu staining in addition to Tuj1 staining to appreciate better that there are more neuronal cell bodies, which is hard to see in with the Tuj1 staining.

In new Fig. 2, we take E6 chick gut treated for 48 hours with GDNF and transplant it onto the CAM of a host embryo for 7 days. This method allows a longer culture period so that we can assess the long-term effects of that initial 48-hour GDNF treatment on the ENS. As shown in Fig. 2, the enteric ganglia of both submucosal and myenteric plexuses are markedly hyperplastic and the interganglionic fibers are increased, as shown with Hu and Tuj1 staining. This has been added to Results, paragraph 1.

c. Please include how the distance of ENS migration was measured – from where to where, it is not clear what "extent of SOXE+ cells" in Fig.3I refers to.

Fig. 3I has been removed. ENCC migration is now shown in a new Fig. 5 and the methodology is described in the text.

d. Please include information on how ENS cells were quantified in Fig. 3J – what does the 40 degree arc refer to?

Quantification for Fig. 5L (previously Fig. 3J) was performed on cross-sections of the hindgut by dividing the circular sections into counting units of nine equal pie- segments, each comprising 40° of arc. The numbers of neurons (HuC/D+) and non- neurons (SoxE+) were counted in each segment.

4. The composition of the cell aggregates is not completely clear. From Figure 1 & 2, it looks like basically all cells are positive for a neuronal marker, but then they also are described to express glial markers and progenitor cell markers – is there overlap between these markers or is it just a tight aggregate of these different cell types. This needs to be clarified with co- labeling with different markers.

Thank you for this suggestion. Fig. 1 and Fig. 3 have been revised, including co- labeling, to clarify the cellular composition of the ganglioneuromas.

5. It is not clear why the first set of experiments (Figure 1) required a catenary culture setting, when later in the paper cell aggregates form just after treatment with GDNF. Please explain how these two experimental setups relate to each other.

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We apologize for this confusion. All experiments that show ganglioneuroma formation, whether they utilized chick or mouse intestine, were done using catenary culture. We have done our best to clarify this throughout the Results section.

6. Please provide quantification of aggregates with and without GFP+ cells after treatment with GDNF. There are some GFP+ cells in the aggregate on the top of Figure 4, left panel. Please explain the presence of these GFP+ cells and also provide a quantification of how often they occur.

A few GFP+ (and Hu+) cells in small outer aggregates in Fig. 6 (previously Fig. 4), left panel HG, are present in the sparse myenteric plexus. This image shows the difference in the size of the two ENS plexuses in the avian embryonic hindgut (SMP>MP; unlike the ENS of rodent hindgut). The presence and proportion of GFP+ cells after neural crest electroporation is highly and unpredictably variable due to innate variability of the electroporation process (Simkin et al., 2014) which is further amplified by stochastic “superstar cell” proliferation in the ENS (Cheeseman et al., 2014, J. Roy. Soc. Interface 11: 20130815).

7. Please include information on how many ganglioneuromas were transplanted for the mouse. As for the chick, only two were transplanted (in the same animal), the numbers for this experiment need to be increased.

When ganglioneuromas derived from either GFP-chick or Wnt1-tdT mouse were transplanted to the chick gut, we used the same method as follows: we used two ganglioneuromas and placed one in each of the chick ceca, as shown in Fig. 7A. This has been clarified in the Results section and in the Methods.

8. More background needs to be provided on several points.
a. Please provide context what RET signaling is and what GDNF does and what it is – this is not explained well in the background section. It is also not described what is known about the function of GDNF in ENS development even though there is quite some body of literature on this already– does it result in ENCC proliferation or neuronal differentiation? This is important to add because it puts the results of this paper into context – is it to be expected that the addition of GDNF results in more neurons?

Thank you for this comment. We have added a new first paragraph to the Discussion to address this important comment.

b. Please explain in the introduction what the hallmarks of ganglioneuroma are – additional information is necessary to determine that the cell aggregates indeed resemble ganglioneuroma.

Thank you for this suggestion. We have added a sentence regarding hallmarks of ganglioneuromas to paragraph 1 of the Introduction.

c. Please include information on the nerve of Remak and what its origin when explaining the experimental set-up of Figure 4 – this will be helpful for the reader to follow the logic behind electroporating into either vagal or sacral level.

A brief explanation about the nerve of Remak has been added to the Results section where Fig. 6 (previously Fig. 4) is explained.

Minor comments:
Please include n for all experiments.

We have added the “n” for each set of experiments in the Methods section.

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Second decision letter

MS ID#: DEVELOP/2020/190900

MS TITLE: RET overactivation leads to concurrent Hirschsprung disease and intestinal ganglioneuromas

AUTHORS: Nandor Nagy, Richard A. Guyer, Ryo Hotta, Dongcheng Zhang, Donald F. Newgreen, Viktoria Halasy, Tamas Kovacs, and Allan M. Goldstein

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 positive and we would like to publish a revised manuscript in Development, provided that you satisfactorily address the remaining couple of suggestions of referee 1. Please attend to these 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.

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

This paper describes a culture system in which pieces of E7 chick or E11.5 mouse midgut and hindgut are cultured with GDNF, which results in large cellular aggregates forming on the surface of the ceca and post-umbilical gut. Analysis shows that these clusters contain Tuj1+ , Hu+, NF+, Synaptophysin+, BFABP+, and in some cases S100+ cells, but not SMA+ or GFAP+ cells. Transplantation of the aggregate containing guts onto the chick CAM and further culture showed these gut pieces had enlarged ENS plexus. Repeating chick experiments with younger stage (E6) chick mid/hindgut resulted in aggregates forming in the midgut only and a lack of ENCC in more caudal regions.

Dissociation of aggregates in culture with GDNF show that the cells proliferate in culture. Analysis of aggregates shows cells that are N-cadherin+, Sox10+ and can incorporate EdU, features of ENCC progenitors. The authors name the aggregates “ganglioneuromas”. The origin of the aggregate cells is demonstrated using a strategy to label vagal NC cells or sacral NC cells with GFP and showing that only GFP+ vagal NC cells contribute to the aggregates, and can form Hu+ ENS neurons. The potential of aggregate cells is tested by transplanting fluorescently labelled aggregates from either chick or mouse guts on to chick hindgut, then culturing on the CAM. An apparently normally colonised ENS is shown as evidence that aggregate cells have potential to form a complete ENS.

The manuscript appears to stretch to relate these findings to Janus mutations in Ret, in which the same mutation is proposed to have differing impact depending on the cellular context (RET impairment leading to intestinal aganglionosis in and RET overactivity leading to MEN-associated tumours). The authors cite the presence of ganglioneuromas and gut colonisation phenotypes with the same GDNF treatment as evidence that RET overactivity can account for both phenotypes. The authors rightly cite numerous caveats with this interpretation, including the difference between ligand mediated responses in these experiments and gain of function responses in MEN2A mutations, or altered interactions and signalling in MEN2B. They also acknowledge the fact that ganglioneuromas and HSCR do not occur together in human patients (being associated with MEN2B

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and MEN2A, respectively). The strongest significance drawn from these experiments appears to be that the organotypic cultures used can be used as a source of progenitor ENCCs for further studies.

Comments for the author

The authors have addressed many reviewer comments and improved the manuscript. However one significant issue remains:

A significant issue is the still incomplete introduction to the question at hand, which is still inaccessible to a generalized audience. A reader in the field will know that RET is a receptor tyrosine kinase, that GNDF is a RET ligand, that GDNF-RET signalling can stimulated by binding of GDNF to dimerized RET and GFRA co-receptors, and that MEN2 mutations affect different domains of the RET receptor tyrosine kinase. However, a generalised audience will struggle to understand much of the paper with the current introduction. The word "receptor" first appears in the Discussion and “GDNF” is not mentioned in the Introduction. A reader is therefore left to guess how Ret might become activated (or constitutively active) or how GDNF exposure might influence RET signalling. In the Introduction, the extracellular or cysteine residues or tyrosine kinase domains of RET are obliquely mentioned, but this does not help to understand how RET signalling occurs. Placing key background in the Discussion is not appropriate. The Introduction should provide all the information that is needed to understand the questions and the results.

This point was not mentioned in the first review, but can be addressed simply:

Figure F,G,&H the GDNF aggregate is apparently the same sample, but F is not at the same magnification as G&H. Therefore it is not easy to determine whether the SOX10+ cells are EdU+ (though they appear to be). Images in the same magnification with reference arrows in all panels (F-H) will facilitate interpretation. Also SOX10 only and BFABP only images should be shown to clearly demonstrate: “EdU+ cells are undifferentiated ENCCs that express Sox10, but not BFABP.”

Reviewer 2

Advance summary and potential significance to field

This paper illustrates the importance of balance in the critical action of Ret in ENS development. The authors employ a catenary culture system to show that GDNF overstimulation leads both to the formation of ganglionuromas and to distal aganglionosis. Ret overstimulation thus includes aspects of the phenotype associated with Ret deficiency. A Janus mutation in Ret is not necessary to explain the syndrome.

Comments for the author

The authors have satisfied all of my questions.

Reviewer 3

Advance summary and potential significance to field

This study by Nagy et al., tackles the interesting question of how to explain co-existing Hirschsprung Disease (thought to be caused by RET deficiency) and multiple endocrine neoplasia (MEN) thought to be caused by role of RET overactivation. Using an intestinal catenary culture system, the authors propose that just RET overactivation – in this model done by the addition of GDNF to the culture – is sufficient to cause both phenotypes. Even though the study tackles an interesting question, it is a bit underdeveloped in its current form and lacks background and experimental information to make it understandable for the broad readership of Development as detailed in the comments below. The main concern is that it is not clear if the culture system accurately represents the disease phenotypes as they comprise limited RET overactivation, whereas in the disease RET overactivation would be present during the entire time of ENS development, which lowers the significance of the results.

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Comments for the author

The author have addressed all my previous comments.

Second revision

Author response to reviewers' comments

Response to Reviewers:

We appreciate the Reviewer comments. In response to the two concerns raised by Reviewer 1:

1) A significant issue is the still incomplete introduction to the question at hand, which is still inaccessible to a generalized audience. A reader in the field will know that RET is a receptor tyrosine kinase, that GNDF is a RET ligand, that GDNF-RET signalling can stimulated by binding of GDNF to dimerized RET and GFRA co-receptors, and that MEN2 mutations affect different domains of the RET receptor tyrosine kinase. However, a generalised audience will struggle to understand much of the paper with the current introduction. The word "receptor" first appears in the Discussion and “GDNF” is not mentioned in the Introduction. A reader is therefore left to guess how Ret might become activated (or constitutively active) or how GDNF exposure might influence RET signalling. In the Introduction, the extracellular or cysteine residues or tyrosine kinase domains of RET are obliquely mentioned, but this does not help to understand how RET signalling occurs. Placing key background in the Discussion is not appropriate. The Introduction should provide all the information that is needed to understand the questions and the results.

Thank you for this suggestion. We have revised the Introduction (paragraph 1) and the Discussion (paragraph 1) as recommend by the Reviewer and agree that this should make it more accessible to a general audience.

2) This point was not mentioned in the first review, but can be addressed simply:
Figure F,G,&H the GDNF aggregate is apparently the same sample, but F is not at the same magnification as G&H. Therefore it is not easy to determine whether the SOX10+ cells are EdU+ (though they appear to be). Images in the same magnification with reference arrows in all panels (F-H) will facilitate interpretation. Also SOX10 only and BFABP only images should be shown to clearly demonstrate: “EdU+ cells are undifferentiated ENCCs that express Sox10, but not BFABP.”

Thank you for this comment. We have revised Fig. 4 and modified the figure legend and Results section accordingly.

Third decision letter

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MS TITLE: RET overactivation leads to concurrent Hirschsprung disease and intestinal ganglioneuromas

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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.

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