Cell-fate plasticity, adhesion and cell sorting complementarily establish a sharp midbrain-hindbrain boundary

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First decision letter

MS ID#: DEVELOP/2019/186882

MS TITLE: Cell-fate plasticity, adhesion and cell sorting complementarily establish a sharp midbrain-hindbrain boundary

AUTHORS: Gokul Kesavan, Stefan Hans, and Michael Brand

My apologies for the unusually long time it has taken us to obtain the reports of one of the referees on your manuscript. I have now received the reports from three referees and I have reached a decision. The reports are appended below and you can access them online. For this, please go to and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, all the referees are enthusiastic in your work, but they also have some 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, I will be happy to receive a revised version of the manuscript. Your revised paper may 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

Overall, the study advances our understanding of how the gene expression boundary between midbrain and hindbrain, forming the mid-hindbrain boundary is established. The authors show that both cell fate plasticity and cell lineage restriction are involved. They continue to show that cell

lineage restriction is based on differential adhesion and mediated by N-cadherin and the Eph/Ephrin signalling pathway.
The experiments are well performed and the publication is well written, has good and convincing figures and the data are of high quality and clearly presented.
(I really like how clearly the cells can be identified along the MH area). The discussion is equivalent and covers the results.
The data show nicely how different cellular mechanisms work together to form a boundary between different areas.

Comments for the author

There are a few things, I did not understand or think are missing:
1. It puzzled me is that the authors describe nicely how many 'n' they performed in some experiments but others lack that information like in Fig 1, Fig. 4, Fig.5. Any specific reason?

2. Figure 7 legend explains nicely how often the different phenotype were observed but there is no mentioning in results or discussion that the outcome is not always 100%. However, I think, this is an nice indication that it is not only differential adhesion or only the Eph/Ephrin pathway that plays a role but rather that they work together.

3. The arrowheads in figure 1 are not mentioned in the figure legend.

4. Well, I do not see very sharp boundaries in fig.3A. Maybe just remove the ‘sharp’ from the text?

5. The authors talk about experiment they did but no figure is given. How about a (data not shown):
….4-hydroxytamoxifen induction at 24hpf… and low Fgf8 expression (data not shown)

6. Fig. 7 A-D are not indicated in the text.

Reviewer 2

Advance summary and potential significance to field

This study uses new tools to address the important question of how the sharp border at the mid-hindbrain boundary is established. Previous work from this lab and others had shown that cell intermingling is restricted across the MHB, but the mechanism was not known. It was possible that formation of the MHB also involves plasticity in cell identity, analogous to recent findings for hindbrain boundaries. By generating transgenic reporter lines for markers of the midbrain and hindbrain (Otx, Gbx genes), it is shown that there is some intermingling across the MHB at early stages. Since a sharp pattern of Otx and Gbx gene expression is formed, this implies that cell identity switching has occurred.
Lineage tracing with Cre-ERT Otx2 reporter confirms that there is mixing at early stages and later restriction of intermingling across the border. Analysis of time lapse movies suggests that cell segregation is involved in establishing the border. Measurements of cell cohesion find differences in homotypic and heterotypic adhesion of midbrain and hindbrain cells that are suggestive of differential adhesion across the border. Furthermore, knockout of Ncad leads to disruption of the border. Finally, the results of blocking Eph-ephrin signaling by overexpression of soluble ephrinB2a suggests that Ephs and ephrins may be involved in the restriction of cell intermingling across the MHB.

Comments for the author

Overall, this is an excellent study that significantly advances understanding of how the sharp border of gene expression at the MHB is formed. It is shown for the first time that cell intermingling and identity switching occurs at early stages, and that N-cadherin and Eph-ephrin signaling are involved in the later segregation. The data are clear and well presented. However, there is a significant problem with the interpretations of the findings for N-cadherin and Eph-ephrin signaling that needs to be addressed in a revised manuscript. Below are some suggestions to help with this.

1. The measurements of the cohesion of midbrain and hindbrain cells are interpreted as evidence for differential adhesion. However, there is an interplay between adhesion and tension, and at a mechanistic level the regulation of cortical tension may underlie the difference in cohesion. There is good evidence for this from studies of Eph receptor function. For example, see recent papers and reviews by Winklbauer, Fagotto and others on the roles of adhesion and tension in cell segregation.
2. A problem with N-cadherin as a mediator of differential adhesion is that there is no evidence that it has differential expression across the MHB. Recent studies of the relationships between Eph-ephrin signaling and cadherins have clarified how blocking of cadherin function can disrupt segregation that is driven by heterotypic tension/repulsion responses to Eph receptor activation.
Initial evidence came from Cortina et al, Nature Genetics 2007 who showed that cadherin knockdown disrupts Eph-ephrin mediated segergation in cell culture assays. A possible explanation is that Eph-ephrin signaling acts by decreasing cadherin-mediated adhesion, and there is evidence for such mechanisms. However,
recent work has shown that Eph receptor signaling drives cell segregation by increasing heterotypic tension or repulsion, and that this is a more efficient mechanism than differential adhesion; see Canty et al, Nature Communications 2017; Taylor et al, J Royal Soc Interface 2017. The latter study showed that knockdown of N-cadherin disrupts Eph-ephrin-mediated segregation in cell culture assays because it leads to a large increase in homotypic repulsion, and consequently a smaller difference between homotypic and heterotypic repulsion.
The homotypic repulsion is caused by the overlapping expression of low affinity Eph-ephrin pairs, as shown for the ectoderm/mesoderm border by Rohani et al,
PLOS Biol 2014, who also find that it is suppressed by cadherins, such that strong repulsion is limited to the heterotypic interface. To summarise: the authors should update their interpretations based on current understanding in the field, which is that Ephs and ephrins drive segregation through heterotypic tension / repulsion, and that there is an interplay with cadherins that is essential for border sharpening.
3. There is some evidence that the interplay discussed above is relevant to cell segregation in the developing brain, as work by Wizenmann and Lumsden, Mol Cell Neurosci 1997 found that blocking Ca(2+) dependent adhesion disrupts segregation of hindbrain rhombomere cells. Subsequent work found that this segregation is driven by Eph-ephrin signaling.
4. The authors correctly point out (p.11) that actomyosin and Yap based mechanisms have been implicated in maintaining rhombomere boundaries in zebrafish, whereas they find no evidence for this at the MHB. It can be further clarified that the actomyosin cables in the hindbrain are found several hours after border sharpening, and therefore do not seem to be involved in the initial segregation. Recent work (Cayuso et al, eLife 2019) has shown that actomyosin tension downstream of Eph forward signaling acts through Taz to induce boundary-specific gene expression in the hindbrain. Voltes et al, Development 2019 show that at later stages the actomyosin tension regulates the balance of proliferation and differentiation. The actomyosin cables may therefore be involved in these late properties of hindbrain boundaries that are not found at the MHB.

Reviewer 3

Advance summary and potential significance to field

This manuscript reports a set of gorgeous movies made on zebrafish sophisticated reporter lines (made using genome editing and insertion in endogenous ORFs) to elucidate the dynamics of cell sorting and gene regulation driving formation of the boundary between midbrain and hindbrain during early development. The authors exploit zebrafish genetics and imaging qualities to provide compelling datasets demonstrating the role of both cell sorting and gene regulation and confirming the importance of Eph/Ephrin and Cadherin-dependent adhesion in the process. Although the concepts supported by the findings are not new, the pieces of elegant biology demonstrating that our model of boundary formation are rare and therefore deserve publication. Some improvements are however needed, listed below.

Comments for the author

- The first set of observations using the Otx/Gbx driver lines is only partially represented in the current Figure 1. The end of movie 1 shows a gorgeous intermediate landscape of a ~4 rows of cells double-labelled that should be represented in Figure 1. These may well be the population forming the midbrain and hindbrain halves of the boundary, population undergoing a massive morphogenetic reshaping.
- There is a problem with Figure 2. Panel F and G are not matching the expected pattern for Wnt1 nor the movie 3 provided.
- Figure 2 and 3 should be pulled into one figure.
- In Figure 5 the orientation of the field is very difficult to understand. Please add a schematic of the 2-som stage embryo with a box showing the area and orientation of the population shown. What is the proportion of the cells moving rapidly into the domain, from a distance? Quantification is needed to evaluate how rare is the long-distance sorting effect.
- More details are needed on the location of the dissection for the AFM-SCFS experiment. One would have liked to see measurement from midbrain, hindbrain and MHB. Is the Hb dataset from tissue near the anterior border of expression of Gbx? Wnt1 would have been great to add. Any possibility to do so in the timeframe of a revision?
- The statement that the differential adhesion between M and H is N-Cad dependent based on MO/mutant experiment is too strong. The MO (and mutant) affects adhesion extremely and the measurements in Fig 6I does not really address changes in differential adhesion.
- To support the differential adhesion model, the great conditional crispants experiments should also be done with a Gbx:CreER.
- Are the extopic Otx2+ cells in the conditional crispants also Gbx+? In other words, is it possible that adhesion code maintains Otx2 expression but does not prevent activation of the hindbrain programme?

First revision

Author response to reviewers' comments

List of figures and tables modified in the revised manuscript.

Main Figures

1. Figure 1: New figure panels (1C) is added based on Rev#3, comment1.
2. Figure 2: Figure 2 and 3 (old figure) were merged to generate a single figure (New Figure 2). This is based on comment Rev#3, comment 3.
3. Figure 3: This was old figure 4 and new figure 3.
4. Figure 4: This was old figure 5 and new figure 4. We have included a schematic representation of the field (New figure 4A) with the orientation of the embryo clearly marked and a box that encloses the area of interest based on Rev#3, comment 4.
5. Figure 5: This was old figure 6 and new figure 5. We have included additional data on panel H, to show Ca2+ dependent adhesion in hindbrain cells.
6. Figure 6: This was old figure 7 and new figure 6. Panels were rearranged for better representation.
7. Figure 7: This was old figure 8 and new figure 7. No change in content.

Supplementary Figures

1. Figure S1: New figure. We have provided new images from an independent transgenic line (*wnt1:venus*) which also show an expression pattern identical to that seen in the *wnt1:venus-NLS* line. We are confident that the *wnt1:venus-NLS* transgenic line recapitulates expected gene expression patterns (A.C. Lekven et al. / Developmental Biology 254 (2003) 172–187 , Figure 2C). This is based on Rev#3, comment 2.

Tables

1. Table 1: This is new data added as Table 1. We have quantified the proportion of *wnt-1* positive cells at a distance that sorted back to the midbrain domain (MB). Enumerating such cells from time lapse movies (n=5) showed that about 50% of cells sorted back to MB domain (51.6±11.9, Mean±SEM). This is based on Rev#3, comment 2.
2. Table 2: This is old table 1 and new table 2.
3. Table 3: This is old table 2 and new table 3.
4. Table 4: This is old table 3 and new table 4.

Detailed response to review comments:

Reviewer 1 Comments for the Author:

There are a few things, I did not understand, or think are missing:

1. It puzzled me is that the authors describe nicely how many 'n' they performed in some experiments, but others lack that information like in Fig 1, Fig. 4, Fig.5. Any specific reason?

Response: We have included the number of embryos analyzed (n) in the figure legends for Fig 1 (page 27), Fig 3 (page 29) and Fig 4(page 29) in the revised manuscript. Please note Fig 4 and 5 in the old MS is now 3 and 4.

2. Figure 7 legend explains nicely how often the different phenotype were observed but there is no mentioning in results or discussion that the outcome is not always 100%. However, I think, this is a nice indication that it is not only differential adhesion or only the Eph/Ephrin pathway that plays a role but rather that they work together.

Response: We have incorporated the above-mentioned point in the discussion section of the revised manuscript (page 16).

3. The arrowheads in figure 1 are not mentioned in the figure legend.

Response: This has been included now in the revised version (page 27)

4. Well, I do not see very sharp boundaries in fig.3A. Maybe just remove the ‘sharp’ from the text?

Response: As the reviewer suggested, we have removed “sharp” in the Fig 2 legend (page 27) and the corresponding text in the results (page 6)

5. The authors talk about experiment they did but no figure is given. How about a (data not shown):
….4-hydroxytamoxifen induction at 24hpf… and low Fgf8 expression (data not shown)

Response: In the revised version, we have included the above-mentioned point (page 7)

6. Fig. 7 A-D are not indicated in the text.

Response: This is now included in the revised version, new figure 6 (page 10)

Reviewer 2 Comments for the Author:
Overall, this is an excellent study that significantly advances understanding of how the sharp border of gene expression at the MHB is formed. It is shown for the first time that cell intermingling and identity switching occurs at early stages, and that N-cadherin and Eph-ephrin signaling are involved in the later segregation. The data are clear and well presented. However, there is a significant problem with the interpretations of the findings for N-cadherin and Eph-ephrin signaling that needs

to be addressed in a revised manuscript. Below are some suggestions to help with this.

1. The measurements of the cohesion of midbrain and hindbrain cells are interpreted as evidence for differential adhesion. However, there is an interplay between adhesion and tension, and at a mechanistic level the regulation of cortical tension may underlie the difference in cohesion. There is good evidence for this from studies of Eph receptor function. For example, see recent papers and reviews by Winklbauer, Fagotto and others on the roles of adhesion and tension in cell segregation.

Response: We agree with the reviewer that there are multiple studies supporting cortical tension as a major force in establishing boundaries. It is possible that the differential adhesion and repulsion mechanism we observed at the MHB could be mediated by tensile forces; however, we have not tested this experimentally in this study. Nonetheless, we have incorporated this possibility in our discussion in the revised manuscript (page 16).

2. A problem with N-cadherin as a mediator of differential adhesion is that there is no evidence that it has differential expression across the MHB. Recent studies of the relationships between Eph-ephrin signaling and cadherins have clarified how blocking of cadherin function can disrupt segregation that is driven by heterotypic tension/repulsion responses to Eph receptor activation. Initial evidence came from Cortina et al, Nature Genetics 2007 who showed that cadherin knockdown disrupts Eph-ephrin mediated segergation in cell culture assays. A possible explanation is that Eph-ephrin signaling acts by decreasing cadherin-mediated adhesion, and there is evidence for such mechanisms. However, recent work has shown that Eph receptor signaling drives cell segregation by increasing heterotypic tension or repulsion, and that this is a more efficient mechanism than differential adhesion; see Canty et al, Nature Communications 2017; Taylor et al, J Royal Soc Interface 2017. The latter study showed that knockdown of N-cadherin disrupts Eph-ephrin-mediated segregation in cell culture assays because it leads to a large increase in homotypic repulsion, and consequently a smaller difference between homotypic and heterotypic repulsion. The homotypic repulsion is caused by the overlapping expression of low affinity Eph-ephrin pairs, as shown for the ectoderm/mesoderm border by Rohani et al, PLOS Biol 2014, who also find that it is suppressed by cadherins, such that strong repulsion is limited to the heterotypic interface. To summarise: the authors should update their interpretations based on current understanding in the field, which is that Ephs and ephrins drive segregation through heterotypic tension / repulsion, and that there is an interplay with cadherins that is essential for border sharpening.

Response: We agree with the reviewer that multiple studies have shown a link between Eph/Ephrin and cadherins. However, differential expression of N-cad is not necessary as changes in activity alone are sufficient, e.g., via Eph/ephrin signaling or heterotypic tension (as suggested by the reviewer). These aspects need to be further investigated during MHB formation and would form a part of an entirely mechanistic study, which has not been attempted in this manuscript. Additionally, we have incorporated the possibility that Eph-Ephrin signaling may drive cell segregation through heterotypic tension/repulsion through interplay with Ca2+ dependent cell adhesion molecules like cadherins. This has been added to the discussion along with relevant references (page 16).

3. There is some evidence that the interplay discussed above is relevant to cell segregation in the developing brain, as work by Wizenmann and Lumsden, Mol Cell Neurosci 1997 found that blocking Ca(2+) dependent adhesion disrupts segregation of hindbrain rhombomere cells. Subsequent work found that this segregation is driven by Eph-ephrin signaling.

Response: Please see our response to comment no: 2

4. The authors correctly point out (p.11) that actomyosin and Yap based mechanisms have been implicated in maintaining rhombomere boundaries in zebrafish, whereas they find no evidence for this at the MHB. It can be further clarified that the actomyosin cables in the hindbrain are found several hours after border sharpening, and therefore do not seem to be involved in the initial segregation. Recent work (Cayuso et al, eLife 2019) has shown that actomyosin tension downstream of Eph forward signaling acts through Taz to induce boundary-specific gene expression in the hindbrain. Voltes et al, Development 2019 show that at later stages the actomyosin tension

regulates the balance of proliferation and differentiation. The actomyosin cables may therefore be involved in these late properties of hindbrain boundaries that are not found at the MHB.

Response: We have added the phrase “Both actomyosin and Yap appear much later at the hindbrain boundaries” in the pertinent results section to provide a possible explanation for why we did not find these pathways playing a role in early cell segregation mechanisms or in late boundary maintenance (page 12).

Reviewer 3

1. The first set of observations using the Otx/Gbx driver lines is only partially represented in the current Figure 1. The end of movie 1 shows a gorgeous intermediate landscape of a ~4 rows of cells double labelled that should be represented in Figure 1. These may well be the population forming the midbrain and hindbrain halves of the boundary, population undergoing a massive morphogenetic reshaping.

Response: We agree with the suggestion of the reviewer and have included snapshots from movie 1 in Figure 1, panel C. Additionally, the *otx2b-gbx1* overlapping region has been highlighted (dotted rectangle).

2. There is a problem with Figure 2. Panel F and G are not matching the expected pattern for Wnt1 nor the movie 3 provided.

Response: The reason for the discrepancy between *in situ* pattern of *wnt1* and fluorescent reporter expression is explained below.
Venus-NLS fluorescence presence is broader than that of *wnt1 in situ* expression at 24hpf because of the perdurance of the fluorescent protein (i.e., Venus). This perdurance also suggests that several midbrain cells had previously expressed *wnt1* (at earlier time points), for e.g., at late gastrulation and/or early segmentation stages.
We have provided new images (new supplementary figure S1) from an different transgenic line (*wnt1:venus*) which shows an expression pattern identical to that seen in the *wnt1:venus- NLS* line. Further, both dorsal and lateral views are provided for better clarity. Additionally, lateral views of the embryos in the *wnt1:venus* transgenic line can be compared to those previously described by Lekven et al (A.C. Lekven et al. / Developmental Biology 254 (2003) 172–187 , Figure 2C). Taken together, we are confident that the *wnt1:venus-NLS* transgenic line fully recapitulates the endogenous *wnt1* expression pattern.

3. Figure 2 and 3 should be pulled into one figure.

Response: We have merged Figs 2 and 3 into a single figure (New figure 2).

4. In Figure 5 the orientation of the field is very difficult to understand. Please add a schematic of the 2-som stage embryo with a box showing the area and orientation of the population shown. What is the proportion of the cells moving rapidly into the domain, from a distance? Quantification is needed to evaluate how rare is the long-distance sorting effect.

Response: We have included a schematic representation of the field (New figure 4A) with the orientation of the embryo clearly marked and a box that encloses the area of interest. We have also quantified the proportion of *wnt-1* positive cells at a distance that sorted back to the midbrain domain (MB). Enumerating such cells from time lapse movies (n=5) showed that about 50% of cells sorted back to MB domain (51.6±11.9, Mean±SEM). This data is provided in a new table (Table 1).

5. More details are needed on the location of the dissection for the AFM-SCFS experiment. One would have liked to see measurement from midbrain, hindbrain and MHB. Is the Hb dataset from tissue near the anterior border of expression of Gbx? Wnt1 would have been great to add. Any possibility to do so in the timeframe of a revision?

Response: We have dissected the hindbrain cells (Hb) close to the MHB in the *gbx1:venus*

transgenic line, and as gbx1 is expressed in the entire hindbrain domain, additional care was taken to dissect close to the prospective MHB.
Next, we have used the *dusp6:d2eGFP* transgenic line for AFM-SCFS experiments as eGFP expression is restricted to anterior HB (in the 1-2 somite stages) in these fish. AFM-SCFS data from both *gbx1:venus* and *dusp6:d2eGFP* lines showed similar adhesive strength, suggesting that they represent similar cell populations. Further, heterotypic adhesion experiments were performed on cells isolated from the *dusp6:d2eGFP* line so that only anterior hindbrain cells were tested. Based on concurring data from both transgenic fish lines, we are confident that our measurements are representative of cells in the anterior hindbrain (close to MHB).

With respect to using cells from the *wnt1:venus* and *wnt1:venus-NLS* lines, multiple attempts to perform AFM-SCFS were made. Unfortunately none were successful due to the low (weak) expression of the venus fluorescent protein at 1-2 somite stage in the dissociated cells. Therefore, we were unable to identify *wnt1:venus*-positive cells with high confidence. New Tg lines expressing a fluorescent protein with a stronger signal than venus are required to perform these experiments. Currently, we do not have such lines and are, therefore, not in a position to perform these experiments.

6. The statement that the differential adhesion between M and H is N-Cad dependent based on MO/mutant experiment is too strong. The MO (and mutant) affects adhesion extremely and the measurements in Fig 6I does not really address changes in differential adhesion. To support the differential adhesion model, the great conditional crispants experiments should also be done with a Gbx:CreER.

Response: In response to similar concerns from other reviewers as well (Please see our response to Rev#1, point 2 and Rev#2, points 1 and 2), we have modified the discussion and have included the possibility of multiple mechanisms, such as cortical tension, differential adhesion and Eph-Ephrin mediated signaling, contributing towards the establishment of a sharp boundary at the MHB (page 16). Thus, the differential adhesion observed between M and H cells could be due to multiple reasons (Tension/Eph-Ephrin) with N-cad being one possible molecular mechanism; however, it is not the only one (See discussion page 16).
Currently, we do not have a Gbx:CreER transgenic line to perform conditional ablation experiments for N-cad in the Hb domain. The generation of such a line will take time, on an average 6-8 months, and therefore, currently, we are not in a position to perform these experiments.

7. Are the ectopic Otx2+ cells in the conditional crispants also Gbx+? In other words, is it possible that adhesion code maintains Otx2 expression but does not prevent activation of the hindbrain program?

Response: This is an interesting question and the suggested experiments may provide extra details. However, given the current situation with the Covid-19 associated shutdown and uncertainty surrounding the breeding capability of lab animals under emergency care, we are unable to perform relevant experiments in a timely manner. Further, the results of these experiments are unlikely to change the conclusions made in our study. Therefore, we kindly request the reviewer to consider an exception from performing these experiments.

Second decision letter

MS ID#: DEVELOP/2019/186882

MS TITLE: Cell-fate plasticity, adhesion and cell sorting complementarily establish a sharp midbrain-hindbrain boundary

AUTHORS: Gokul Kesavan, Anja Machate, Stefan Hans, and Michael Brand
ARTICLE TYPE: Research Article

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

Reviewer 2

Advance summary and potential significance to field

The significance is described in my review of version 1.

Comments for the author

The authors have satisfactorily addressed all of the points raised in my review of version 1 by modifications to the text. The main issue was to discuss the relationship between Eph signialing and cadherins, and this has now been presented.

Reviewer 3

Advance summary and potential significance to field

I am now fully satisfied with the responses given and the new version of the manuscript. I would have pushed a bit more for a couple of small experiments but under the pandemic situation, I find completely reasonable to accept this version for publication.

Comments for the author

No suggestion needed