Gsx2 is required for specification of neurons in the inferior olivary nuclei from Ptf1a-expressing neural progenitors in zebrafish

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MS TITLE: Gsx2 is involved in specification of neurons in the inferior olivary nuclei from Ptf1a-expressing neuronal progenitors in zebrafish

AUTHORS: Tsubasa Itoh, Miki Takeuchi, Marina Sakagami, Kazuhide Asakawa, Koichi Kawakami, Takashi Shimizu, and Masahiko Hibi

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

As you will see, the referees express considerable interest in your work, but have some significant criticisms and suggestions for revisions to your manuscript. 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. 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.

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 is a nicely written and well-illustrated manuscript. The data are new, the rigor of the approaches and analyses, for the most part, are sufficient, and the conclusions are mostly supported by the data. The experiments do include a number of antisense morpholino knock downs but all of these are based on published work and genetic and pharmacological validations, so I am comfortable with those data as they are.
My specific comments, appearing below, can be addressed without additional experiments. Altogether, I think this manuscript provides new information about mechanisms of hindbrain patterning, inferior olivary neuron specification and hindbrain neural circuit formation as well as adding to our knowledge of Gsx2 function.

Comments for the author

1. The gene expression analysis is based largely on transgenic reporter gene expression without direct validation that the transgenes accurately represent endogenous gene expression. Are there available single-cell RNA-seq databases (for example, Tamblo et al, 2020, Development) the authors could examine to search for additional evidence of ptf1a and gsx2 co-expression?
2. The first subheading of the Results section and the heading of Figure legend 1 indicate that IO progenitors co-express gsx2 and ptf1a. However, no direct evidence of IO neurons, for example, expression of an independent, validated marker, is provided. Could the authors please be more explicit about the criteria they use to determine IO identity? This also is important for all the IO neuron quantitative data.
3. In Figure 1, panel 0, arrows are placed to indicate “CF neurons”. However, they don’t really seem to be pointing to anything. Are they intended to mark the CF axons? Perhaps it would be helpful to also mark these in panel P, where they appear more obvious.
4. In Figure 6 the authors measure the length of the hindbrain gsx2 expression domain in embryos with abnormal Fgf signaling. However, the relevance of this assay to IO neuron specification is not evident to me.
Gsx2 expression obviously is not limited to progenitors that only produce IO neurons, so how this phenotype relates to IO neuron formation is not clear. These data confuse matters without adding any benefit to the manuscript and I would recommend that the authors either provide a clear rationale for the assay or remove these data.
5. In Figure 6 the authors use IO region length as a proxy for IO neuron number and conclude at the top of page 10 that loss of fgf8/3 function increases the number of IO neurons. This is quite problematic because alterations of morphology or imaging plane could give a false sense of the relationship between area and cell number. Climbing fiber numbers do appear to be increased but, again, this appearance could result from abnormal axogenesis. If the authors have data for the number of IO neurons they should include them here. If not, the authors need to more clearly describe how they measured IO region length and to be a bit more cautious in their interpretation of the data.
6. Related to points 4 and 5, the authors make a concluding sentence at the top of page 10 that, “Our findings suggest that the Fgf signal suppressed gsx2 expression and thereby suppressed IO neuronal fate.”
Following my comments above, I do not think the data adequately support that conclusion. The authors may well be correct, but the data provided here are not sufficient to make that statement.
7. By contrast, for Figure 7 the authors provide the actual numbers of IO neurons, which provides them with better data. I think that if the authors are not able to obtain equivalent data for Figure 6 because of the current COVID-19 crisis, that they could easily modify their language in a way to suggest that expansion of size raises the possibility that there are more neurons.
8. As with Figure 6 (comment #4), the relevance of measuring the length of the hindbrain gsx2 expression domain in RA-manipulated embryos, shown in Figure 8, is not entirely clear to me.
9. Figure 9G shows numerous GFP+ cells scattered across the hindbrain of pbx2/4 deficient embryos. 9H shows a higher magnification view of putative IO neurons. In the text the authors state that “ectopic PCs were observed caudally but crest cells were present normally”. It is not clear to me how the authors know the identity of these GFP+ cells from these markers nor is it clear to me how they can accurately identify and count IO neurons to show that they are in deficit. This experiment and assay needs a much more careful and rigorous explanation.

10. I would like to see a description in the Methods of how the quantitative data, particularly IO neuron number, were collected. The authors should describe, for example, if cells were counted within 3 dimensional volumes and, if so, how this was done, and they should indicate if individuals collecting and analyzing the data were blinded to the embryo genotype.

Reviewer 2

Advance summary and potential significance to field

In this study, Itoh and colleagues explore the role of zebrafish Gsx2 in the development of Inferior Olivary (IO) neurons. Their primary finding is that Gsx2 function is required for development of the IO neurons. The authors additionally investigate the relationship between Fgf and RA signaling pathways and expression of the gsx2 gene. The work provides some novel insight into the mechanisms that control specification of a particular class of neurons, however it does not probe more deeply into the regulatory networks that underlie this process, and as a consequence is of limited significance.

Comments for the author

In this study, Itoh and colleagues explore the role of zebrafish Gsx2 in the development of Inferior Olivary (IO) neurons. The authors report co-expression of gsx2 with ptf1a, and generate CRISPR mutants to demonstrate a requirement for each gene in development of the IO neurons. The genes do not directly cross-regulate, suggesting they act independently, and consistent with this model the loss of ptf1a leads to IO apoptosis whereas loss of gsx2 leads to altered neuronal identity . The authors then go on to investigate the relationship between Fgf and RA signaling pathways and gsx2 expression.

The study is carefully and thoroughly done, with strengths being the high quality of the images provided, each of which nicely supports the authors conclusions, and the clear logical style of writing. However, a significant weakness of the study is the lack of mechanistic understanding regarding the levels at which Fgf (via Mafb) and RA function to regulate gsx2 expression and thus IO development. Both the MafB/Val mutant, and loss of RA signaling, lead to very significant hindbrain pattering changes – as revealed by altered Hox expression. If tissue is missing, as in the case of loss of RA signaling, then the neurons which the tissue normally gives rise to will necessarily also be missing. Reciprocal arguments can be made for MafB. While the inclusion of Hox manipulation goes a small way to filling this gap the multiple question marks in the schematic supplied in the final figure serve to highlight the problem. Other experiments, such as analysis of regulatory sequences and their function, would be needed to fill this gap.

In the absence of a more in depth understanding of gsx2 regulation, this manuscript does not represent a sufficiently significant advance to be is suitable for publication at Development.

Reviewer 3

Advance summary and potential significance to field

In all vertebrates, inferior olivary (IO) neurons provide input into cerebellar circuits in the form of climbing fibers. IO neurons are derived from ptf1a+ progenitors in the hindbrain ventricular zone, as are cerebellar Purkinje cells and other neuron types; the type of neuron generated by a ptf1a+ progenitor depends on its anterior-posterior location in the hindbrain. This manuscript identifies hindbrain signals and transcription factors involved in the specification of IO neurons, using the transparent zebrafish model and new transgenic lines that mark IO neurons and their progenitors under a range of genetic and pharmacological perturbations.

In the most significant novel finding in the manuscript, the authors discover that the transcription factor gsx2 previously implicated mainly in cortical development in mammals, is expressed in IO progenitors and is essential for IO formation.

The paper also defines the IO progenitor domain along the hindbrain anterior-posterior axis and shows that its sharp anterior limit at the rhombomere 6/7 boundary is limited by the well-known rhombomere 6 regulatory cascade involving Fgf signals and MafB. They also show that the gsx2+ IO progenitor domain (and the IO) requires trunk-derived retinoic acid signals for its early specification. This is also anticipated by the fact that this entire region of the posterior hindbrain depends on RA for its initial specification.

Overall, the work is clearly presented and fairly well supported by the data although in a number of places n's are not provided or are too low (see below). Although it might be described as being largely descriptive of a mutant phenotype, in identifying gsx2 as being essential for IO specification I feel that the work represents an important addition to the developmental neurobiology literature. As such I am supportive of publication in Development. However a number of weaknesses should first be addressed.

Comments for the author

Major criticisms:

1) Identity of gsx2+ neurons: are they just the IO? Based on the absence of IO neurons in gsx2 mutants, it seems likely that the gsx2+, ptf1a+ neurons in the ventral hindbrain in Fig1R-T are the same as the IO neurons labeled by the 28C enhancer trap line and the mnx2b:GFF lines. However, this should be shown directly. Is the overlap precise? Or does gsx2 mark more than just IO neurons in this region? What other neurons does it mark? It is not until the discussion that a larger role for gsx2 in the development of neuron contributing mossy fibers is mentioned—this is important information that should be mentioned up-front. Along the same lines there appear to be mnx2b+ neurons beyond the IO that are missing in gsx2 mutants (Fig. 2I-L). What are these additional gsx2-dependent neurons? While the paper focuses appropriately on the IO, as currently presented it gives the impression that this function is more exclusive than it is.

2) Sufficiency of gsx2: The sufficiency experiment whose negative result is presented in Fig. S6 is important for the model and, assuming it is supported by appropriate n's, it should be included in the main results section (perhaps in place of Fig. 9, see below). The pou4f1 in situ readout is fine if combining these transgenes with the 28C reporter for IO cell quantification is not possible in a timely manner.

3) Function of gsx2: In the discussion and corresponding supplemental data the authors hint at alternative fates adopted by ventricular progenitors in the absence of gsx2 function. If any further insights could be obtained about these alternative identities it would increase the impact of the paper.

4) Data quantification: There are a number of places where data is shown without quantification or with insufficient quantification.
• The number of 28C+ IO neurons in ptf1a mutants is not quantified.
• n=4 larvae for counting caspase+ cells in ptf1a and wildtype controls. If the four wildtype larvae in Fig. 5Q (avg. 1 caspase+ cell) had instead been the five wildtype larvae in Fig. 5R (avg. 3.5 caspase+ cells), the difference between wildtype and ptf1a mutants (avg. 8 caspase+ cells) may not have been significant. This experiment should be repeated (and scored blind, given the challenge of distinguishing caspase+ cells from background, see Fig. 5P) with at least n=10 per experiment given the low numbers of caspase+ cells and the evident variability in caspase staining from experiment to experiment.
• The length of the gsx2 domain in mafB mutants in Fig. 7A-F is not quantified.
• n=4-5 larvae for mafb mutant phenotype in Fig. 7O (given the very bimodal distribution in mutants.
• the length of the gsx2 domain in pbx morphants is not quantified (Fig. 9A-D)
• the length of the gsx2 domain in the hoxb4a rescue experiment is not quantified (Fig. 9L-O)
• scale bars are lacking throughout.

5) Statistical analysis: With regard to Fig. 6M and 6AA, the text says that "inhibition of fgf8a and fgf3 further increased gsx2:RFP and the number of IO neurons" (beyond that caused by fgf8a or fgf3 inhibition alone). However, this is not the comparison that is made in the graph. Is the

difference between single fgf3 or fgf8 and double fgf3; fgf8 inhibition really significant? If not, the text should be revised. Otherwise the correct interpretation of the data in Fig. 6AA is that fgf8 inhibition causes an increase in the length of the IO and that fgf3 does not significantly affect it. Furthermore, it is not clear why, whereas in all other figures the assay was to count 28C+ IO neurons, in Fig. 6 the authors chose instead to measure the length of the IO region. Counting IO neurons is a more quantitative way to assess the phenotype. Note that in reference to Fig. 6AA the authors say "the number of IO neurons" (page 9) which is an inaccurate representation of the data.

6) Hox function: a likely role for Hox genes is based on a severe reduction of IO neurons in pbx2/4 morphants and an expanded gsx2:RFP domain in embryos injected with hoxb4a mRNA at the 1-cell stage. Neither of these are very compelling results given that pbx2/4 mutants are known to lack posterior hindbrain identity, and ectopic Hox expression (any Hox gene) from the 1-cell stage is likewise expected to have massive consequences on hindbrain development. The model in Fig.9 would be better supported if gsx2 expression/IO neurons could be rescued in RA-inhibited embryos by hoxb4a mRNA injection. If not then for this reviewer, the data in Fig. 9 does not strengthen the paper and could be removed.

7) Gsx2 function in mammals: In the discussion, the authors mention the known functions for mammalian Gsx2 in the specification of neurons in the telencephalon, olfactory bulb and spinal cord. Is the IO/climbing fibers normal in Gsx2 mutants or has this just never been assessed?

Minor points:

Labeling of Fig. 1 is confusing: the transgene contains Tomato, is called RFP in the text but is labeled dsRED in the figures.

In the text (results and discussion) regarding Fig. 4, it says that the fact that gsx2 and ptf1a mRNA expression persists in ptf1a and gsx2 mutants, respectively, means that the two genes are regulated independently. This is in accurate: they could still be regulated by a common upstream factor(s). The text should be revised to say that the two genes are regulated independent of each other; or that they are not interdependent.

The sentence on page 10: "Inhibition of the RA signal with both MO and DEAB resulted in reduced gsx2:RFP expression but higher mafbaGFF;UASGFP expression". Is poorly phrased. Does it mean that mafbaGFP expression is increased?

In the discussion (page 16) it says that reducing Fgf signaling decreases the number of IO neurons, but should say that it increases the number of IO neurons.

First revision

Author response to reviewers' comments

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AUTHORS: Tsubasa Itoh, Miki Takeuchi, Marina Sakagami, Kazuhide Asakawa, Koichi Kawakami, Takashi Shimizu, and Masahiko Hibi

> We appreciate the positive opinions from the Reviewers regarding the significance and novelty of our findings, and value their criticisms and suggestions. We have carefully considered all the issues that were raised by the Reviewers and carried out additional experiments to address each of them. Point-by-point detailed responses are provided below.

Reviewer 1 Advance Summary and Potential Significance to Field:
This is a nicely written and well-illustrated manuscript. The data are new, the rigor of the approaches

and analyses, for the most part, are sufficient, and the conclusions are mostly supported by the data. The experiments do include a number of antisense morpholino knock downs but all of these are based on published work and genetic and pharmacological validations, so I am comfortable with those data as they are. My specific comments, appearing below, can be addressed without additional experiments. Altogether, I think this manuscript provides new information about mechanisms of hindbrain patterning, inferior olivary neuron specification and hindbrain neural circuit formation as well as adding to our knowledge of Gsx2 function.
> We are again grateful for the comments from the Reviewer 1. Although we were not asked by the reviewer to perform additional experiments, we have done some relevant experiments, and revised figures and text to respond the reviewer's comments.

Reviewer 1 Comments for the Author:
1. The gene expression analysis is based largely on transgenic reporter gene expression without direct validation that the transgenes accurately represent endogenous gene expression. Are there available single-cell RNA-seq databases (for example, Tambalo et al, 2020, Development) the authors could examine to search for additional evidence of ptf1a and gsx2 co-expression?
> We carefully looked at the data from Tambalo et al., Development (2020) 147, dev184243. In this paper that *gsx2*-expressing cells were detected in the medial and the dorsal progenitors at 24 hpf, whereas *ptf1a*-expressing cells were not observed at 24 hpf. At 44 hpf, both *gsx2* and *ptf1a* were expressed in cells in the dorsomedial progenitor cluster (DMP, cluster 4 in Fig. 4A Tambalo et al). Therefore, the single-cell RNA-seq data also support the presence of neuronal progenitors expressing *ptf1a* and *gsx2* in the hindbrain. We cited the paper and described this issue in page 13, line 287-289 in the revised manuscript.

2. The first subheading of the Results section and the heading of Figure legend 1 indicate that IO progenitors co-express gsx2 and ptf1a. However, no direct evidence of IO neurons, for example, expression of an independent, validated marker, is provided. Could the authors please be more explicit about the criteria they use to determine IO identity? This also is important for all the IO neuron quantitative data.
> We considered neurons that locate in the ventral part of the caudal hindbrain and extend axons rostrally to Purkinje cells in the cerebellum as IO neurons. Most gsx2:RFP+ cells located in the caudoventral hindbrain extended axons to the cerebellum (Fig. 1O, V). We further examined localization of gsx2:RFP+ and 28C;UAS:GFP+ cells that project axons to the cerebellum, and found that gsx2:RFP and 28C;UAS:GFP signals were co-localized in the ventral part of the caudal hindbrain. All the data consistently support that *gsx2* and *ptf1a*-expressing cells give rise to the IO neurons. Although there were gsx2:RFP+ 28C;UAS:GFP- cells, it is possibly due to mosaic expression of UAS:GFP in the 28C;UAS:GFP fish as expression of UAS-dependent reporters is known to subjected to silencing in transgenic zebrafish (Akitake et al., 2011). We replaced the data for gsx2:RFP with new ones (Fig. 1O, P, V, W) and added the data of the co-localization of gsx2:RFP and 28C;UAS:GFP in Fig. S2. We also explained this issue in page 7, line 142-149 in the revised manuscript.

3. In Figure 1, panel 0, arrows are placed to indicate "CF neurons". However, they don't really seem to be pointing to anything. Are they intended to mark the CF axons? Perhaps it would be helpful to also mark these in panel P, where they appear more obvious.
> We replaced Fig. 1O and 1P with new ones (Fig. 1O, P, V, W). The gsx2:RFP signals marked two types of axons; one CFs and the other axons from the nucleus commissure of Wallenberg, which are indicated by arrows and asterisks in the Fig. 1.

4. In Figure 6 the authors measure the length of the hindbrain gsx2 expression domain in embryos with abnormal Fgf signaling. However, the relevance of this assay to IO neuron specification is not evident to me. Gsx2 expression obviously is not limited to progenitors that only produce IO neurons, so how this phenotype relates to IO neuron formation is not clear. These data confuse matters without adding any benefit to the manuscript and I would recommend that the authors either provide a clear rationale for the assay or remove these data.
> As the reviewer mentioned, *gsx2*-expressing progenitors generate neurons other than IO neurons, but only a limited number of cell types cells as *gsx2* expression domain is limited to the dorsal region. We would like to show that the rostral expansion of *gsx2*- expression with the inhibition of Fgf signaling led to rostral expansion of IO progenitors and subsequent increase in IO neurons. We carefully examined gsx2:RFP expression and the number of 28C;UAS;GFP+ IO neurons in *fgf8a* mutant *fgf3* morphant larvae, and larvae treated with different concentration of the Fgf receptor inhibitor

SU5402 (Fig. 7 and S7). We found clear correlation between strength of Fgf signal inhibition, and increase in gsx2:RFP expression and the number of 28C;UAS;GFP+ IO neurons. Our findings suggest the Fgf signal suppresses rostral (r5/6) expansion of *gsx2* expression, thereby limiting *gsx2*-expressing IO progenitor domain to the caudal-most hindbrain (r7). We would like to keep the data. We explained this issue in page 10, line 225-line 237 in the revised manuscript.

5. In Figure 6 the authors use IO region length as a proxy for IO neuron number and conclude at the top of page 10 that loss of fgf8/3 function increases the number of IO neurons. This is quite problematic because alterations of morphology or imaging plane could give a false sense of the relationship between area and cell number. Climbing fiber numbers do appear to be increased but, again, this appearance could result from abnormal axogenesis. If the authors have data for the number of IO neurons they should include them here. If not, the authors need to more clearly describe how they measured IO region length and to be a bit more cautious in their interpretation of the data.
> We counted the number of 28C;UAS:GFP+ IO neurons when *fgf8a* and *fgf3* function was inhibited by genetic mutation, morpholino, and/or chemical (Fig. 7). We found that IO neurons were significantly increased in *fgf8ati282a/ti282a*; *fgf3* MO and SU5402-treated larvae, compared to control larvae. Although we observed some increase in *fgf8*a mutants and *fgf3* morphants, compared to control, the difference was not statistically significant. It is likely due to slight difference, low numbers of analyzed samples, and/or mosaic expression of 28C;UAS:GFP. We showed the number of 28C;UAS:GFP+ IO neurons in *fgf8ati282a/ti282a*; *fgf3* MO and SU5402-treated larvae with appropriate controls in revised Fig. 7 and S7, and described this issue in page 10, line 225-237 in the revised manuscript.

6. Related to points 4 and 5, the authors make a concluding sentence at the top of page 10 that, "Our findings suggest that the Fgf signal suppressed gsx2 expression and thereby suppressed IO neuronal fate." Following my comments above, I do not think the data adequately support that conclusion. The authors may well be correct, but the data provided here are not sufficient to make that statement.
> As we described above, the Fgf signal suppressed *gsx2* expression in r5/6. We revised the sentence as "Our findings suggest that the Fgf signal suppresses rostral expansion of *gsx2* expression, thereby limiting *gsx2*-expressing IO progenitor domain to r7". We described this issue in page 10, line 234-237 and page 17, line 399-page 18, line 407 in the revised manuscript. If the reviewer thinks that our data do not provide sufficient information for this statement, we will revise or remove relevant data or description.

7. By contrast, for Figure 7 the authors provide the actual numbers of IO neurons, which provides them with better data. I think that if the authors are not able to obtain equivalent data for Figure 6 because of the current COVID-19 crisis, that they could easily modify their language in a way to suggest that expansion of size raises the possibility that there are more neurons.
> We counted IO neurons in larvae having inhibition of the Fgf signal. We revised Fig. 7 as described above (please see our response to comment 5).

8. As with Figure 6 (comment #4), the relevance of measuring the length of the hindbrain gsx2 expression domain in RA-manipulated embryos, shown in Figure 8, is not entirely clear to me.
> Although *gsx2*-expressing progenitors generate neurons other than IO neurons, they are limited types of cells. In the manuscript, we found that *gsx2*-expressing progenitors give rise to IO neurons and the neurons sending the axons to the ventral part of the cerebellum (Fig. 1O, P, V and S6), which are potentially the nucleus commissure of Wallenberg. We think that Gsx2 determines the progenitor domain for those neurons. We measured the length of gsx2:RFP+ hindbrain region to quantify the expression level of *gsx2*. There was correlation between reduction of gsx2:RFP expression and IO neurons in larvae having inhibition of the RA signal. Therefore, our data suggest that the RA signal regulates *gsx2* and thereby controls generation of IO progenitors. We added the sentence "The reduction of *gsx2* expression by the RA signal inhibition likely led to reduction of IO progenitors and subsequent reduction in IO neurons" in page 11, line 259-261 in the revised manuscript. If our rationale is not acceptable by the reviewer, we are willing to revise it or remove relevant data and description.

9. Figure 9G shows numerous GFP+ cells scattered across the hindbrain of pbx2/4 deficient embryos. 9H shows a higher magnification view of putative IO neurons. In the text the authors state that

"ectopic PCs were observed caudally but crest cells were present normally". It is not clear to me how the authors know the identity of these GFP+ cells from these markers nor is it clear to me how they can accurately identify and count IO neurons to show that they are in deficit. This experiment and assay need a much more careful and rigorous explanation.
> As the reviewer mentioned, the 28C;UAS:GFP+ cells does not necessary mean IO neurons since 28C:UAS:GFP was expressed in other neurons. We considered 28C;UAS:GFP+ neurons that were located in the ventral part of caudal hindbrain and extended axons rostrally as IO neurons. These neurons are significantly reduced in the *pbx2/4* morphant larvae. In addition, we found absent expression of *pou4f1*, a marker of IO neurons in the *pbx2/4* morphant larvae (Fig. 10J). We replaced the 28C;UAS:GFP data with new one, and described this issue in page 12, line 269-272 in the revised manuscript.

10. I would like to see a description in the Methods of how the quantitative data, particularly IO neuron number, were collected. The authors should describe, for example, if cells were counted within 3 dimensional volumes and, if so, how this was done, and they should indicate if individuals collecting and analyzing the data were blinded to the embryo genotype.
> We carefully looked at optical sections from the confocal images and manually counted IO neurons (28C;UAS:GFP+ cells) in a 120 μm x 120 μm x 52 μm region of the ventral part of caudal hindbrain, since these neurons were located in that region and extended CFs. We agree that it is ideal to analyze the data that were blinded to larval genotypes. As we examined so many different conditions and treatments for zebrafish larvae, it was not easy to do genotype-blind examination. We did not intentionally pick up the data. We carefully examined statistical analyses. We hope that the reviewer understands it. We described the method for counting IO neurons in page 25, line 593-597.

Reviewer 2 Advance Summary and Potential Significance to Field:
In this study, Itoh and colleagues explore the role of zebrafish Gsx2 in the development of Inferior Olivary (IO) neurons. Their primary finding is that Gsx2 function is required for development of the IO neurons. The authors additionally investigate the relationship between Fgf and RA signaling pathways and expression of the gsx2 gene. The work provides some novel insight into the mechanisms that control specification of a particular class of neurons, however it does not probe more deeply into the regulatory networks that underlie this process, and as a consequence is of limited significance.

Reviewer 2 Comments for the Author:
In this study, Itoh and colleagues explore the role of zebrafish Gsx2 in the development of Inferior Olivary (IO) neurons. The authors report co-expression of gsx2 with ptf1a, and generate CRISPR mutants to demonstrate a requirement for each gene in development of the IO neurons. The genes do not directly cross-regulate, suggesting they act independently, and consistent with this model the loss of ptf1a leads to IO apoptosis whereas loss of gsx2 leads to altered neuronal identity. The authors then go on to investigate the relationship between Fgf and RA signaling pathways and gsx2 expression.

The study is carefully and thoroughly done, with strengths being the high quality of the images provided, each of which nicely supports the authors conclusions, and the clear logical style of writing. However, a significant weakness of the study is the lack of mechanistic understanding regarding the levels at which Fgf (via Mafb) and RA function to regulate gsx2 expression and thus IO development. Both the MafB/Val mutant, and loss of RA signaling, lead to very significant hindbrain pattering changes – as revealed by altered Hox expression. If tissue is missing, as in the case of loss of RA signaling, then the neurons which the tissue normally gives rise to will necessarily also be missing. Reciprocal arguments can be made for MafB. While the inclusion of Hox manipulation goes a small way to filling this gap, the multiple question marks in the schematic supplied in the final figure serve to highlight the problem. Other experiments, such as analysis of regulatory sequences and their function, would be needed to fill this gap.

In the absence of a more in depth understanding of gsx2 regulation, this manuscript does not represent a sufficiently significant advance to be is suitable for publication at Development.

> We agree with the reviewer's comments that our data do not provide detailed molecular mechanisms by which Fgf (via Mafba) and RA signals regulate the *gsx2* expression in the caudal hindbrain. As the reviewer mentioned, overexpression and/or loss of function of Hox and Mafba do

not address whether the effects on *gsx2* expression are direct or indirect. To address this, we collaborated with Dr. Kenta Sumiyama in RIKEN BDR, who identified a core enhancer element which is located at 3' downstream of mouse *Gsx2* open reading frame (ORF) and can drive a reporter expression in ventral telencephalon and hindbrain in transgenic mice (unpublished results). We found that zebrafish *gsx2* has an about 800-bp enhancer element on the 3' side of the ORF that displays a partial sequence homology to the mouse *Gsx2* enhancer. This enhancer contains putative binding motifs of MafBa and Hox proteins. We injected a Venus reporter construct having the zebrafish enhancer and the promoter of heat shock protein *hsp70l* gene into zebrafish embryos with Tol1 or Tol2 transposon system. We found that the zebrafish *gsx2* enhancer also recapitulated the *gsx2* enhancer activity for the expression in the telencephalon and the caudal hindbrain (Reference Fig. 1). However, transient reporter assays in zebrafish are always mosaic (even when we used transposon systems) and the data were not conclusive. We need to generate Tg lines (in progress) to obtain conclusive results although it takes more than three months. Furthermore, detailed analysis of the enhancer (e.g. identification of transcription factor-binding sites, ChIP assay) takes a long time to obtain comprehensive data. It is beyond the scope of a single paper. We decided not to include the reporter assays in the revised manuscript. We mentioned the *gsx2* enhancer in Discussion. We hope that future analysis on the *gsx2* enhancer will clarify the molecular mechanisms controlling *gsx2* expression in IO progenitors. We described this issue in page 19, line 445-452 in the Discussion.

Our main conclusion in this manuscript is that Gsx2 functions downstream of the positional information (e.g. Fgf and RA signaling) to control specification of IO neurons from Ptf1a-expressing progenitors in zebrafish. We hope that without detailed mechanism of *gsx2* regulation, the manuscript provides significant information in the field of neural development.

Reviewer 3 Advance Summary and Potential Significance to Field:
In all vertebrates, inferior olivary (IO) neurons provide input into cerebellar circuits in the form of climbing fibers. IO neurons are derived from ptf1a+ progenitors in the hindbrain ventricular zone, as are cerebellar Purkinje cells and other neuron types; the type of neuron generated by a ptf1a+ progenitor depends on its anterior-posterior location in the hindbrain. This manuscript identifies hindbrain signals and transcription factors involved in the specification of IO neurons, using the transparent zebrafish model and new transgenic lines that mark IO neurons and their progenitors under a range of genetic and pharmacological perturbations.

In the most significant novel finding in the manuscript, the authors discover that the transcription factor gsx2, previously implicated mainly in cortical development in mammals, is expressed in IO progenitors and is essential for IO formation.

The paper also defines the IO progenitor domain along the hindbrain anterior-posterior axis and shows that its sharp anterior limit at the rhombomere 6/7 boundary is limited by the well-known rhombomere 6 regulatory cascade involving Fgf signals and MafB. They also show that the gsx2+ IO progenitor domain (and the IO) requires trunk-derived retinoic acid signals for its early, specification. This is also anticipated by the fact that this entire region of the posterior hindbrain depends on RA for its initial specification.

Overall, the work is clearly presented and fairly well supported by the data although in a number of places n's are not provided or are too low (see below). Although it might be described as being largely descriptive of a mutant phenotype, in identifying gsx2 as being essential for IO specification I feel that the work represents an important addition to the developmental neurobiology literature. As such I am supportive of publication in Development. However a number of weaknesses should first be addressed.

> Again, we would like to thank the reviewer for positive comments. For the experiments involving a relatively small number of samples, we examined more samples. We described the number of examined samples (*n*) and revised the manuscript at the reviewer suggestions.

Reviewer 3 Comments for the Author:
Major criticisms:
1) Identity of gsx2+ neurons: are they just the IO? Based on the absence of IO neurons in gsx2 mutants, it seems likely that the gsx2+, ptf1a+ neurons in the ventral hindbrain in Fig1R-T are the

same as the IO neurons labeled by the 28C enhancer trap line and the mnx2b:GFF lines. However, this should be shown directly. Is the overlap precise? Or does gsx2 mark more than just IO neurons in this region? What other neurons does it mark? It is not until the discussion that a larger role for gsx2 in the development of neuron contributing mossy fibers is mentioned—this is important information that should be mentioned up-front. Along the same lines, there appear to be mnx2b+ neurons beyond the IO that are missing in gsx2 mutants (Fig. 2I-L). What are these additional gsx2-dependent neurons? While the paper focuses appropriately on the IO, as currently presented it gives the impression that this function is more exclusive than it is.
> We crossed gsx2:RFP and 28C;UAS:GFP, or gsx2:RFP and mnx2b:GFF, UAS:GFP Tg fish to examine their expression in the ventral hindbrain (Fig. S2). The gsx2:RFP signals overlapped with 28C;UAS:GFP and mnx2b:GFF;UAS GFP signals in the ventral part of the caudal hindbrain. Although there were gsx2:RFP+ 28C;UAS:GFP- and gsx2:RFP+ mnx2b;UAS:GFP- cells in that region, these cells extend axons and likely IO neurons. The absence of 28C;UAS:GFP or mnx2b;UAS:GFP signals in gsx2:RFP+ cells in the ventral part of the caudal hindbrain is likely due to mosaic expression of UAS:GFP (Akitake et al., 2011). Our findings suggest that ventral hindbrain neurons derived from *gsx2*-expressing progenitors are IO neurons. We described this issue in page 7, line 142- 149 and page 8, line 168-170 in the revised manuscript.

As shown in Fig. 1, the gsx2:RFP signal marks dorsal neuronal progenitors, IO neurons (located ventrally), and ventro-lateral neurons that extend their axons to the cerebellum region and potentially the nucleus commissure of Wallenberg. The data do not exclude the possibility that gsx2:RFP progenitors give rise other types of neurons. We described this issue in page 7, line 150-154 in the Result section of the revised manuscript. As the reviewer mentioned, mnx2b:GFF;UAS:GFP+ non-IO neurons that were located laterally to IOs were also reduced in *gsx2* and *ptf1a* mutant larvae. The data suggest that IO neurons are not sole neurons derived from the *gsx2*- and *ptf1a*-expressing neuronal progenitors. Currently we do not know the identity of these neurons. We carefully described this issue in page 8, line 179-180; page 9, line 191-195; and page 16, line 380- page 17, line 383 in the revised manuscript. Although *gsx2*- and *ptf1a*-expressing neuronal progenitors generate multiple types of neurons, they are limited-types of neurons as *gsx2* is expressed in the restricted area of the dorsal hindbrain.

2) Sufficiency of gsx2: The sufficiency experiment whose negative result is presented in Fig. S6 is important for the model and, assuming it is supported by appropriate n's, it should be included in the main results section (perhaps in place of Fig. 9, see below). The pou4f1 in situ readout is fine if combining these transgenes with the 28C reporter for IO cell quantification is not possible in a timely manner.
> We repeated mis-expression of *gsx2* in all *ptf1a*-expressing cells (*n*=5). We showed the data in Fig. 6 and described the data in page 10, line 216-220 in the Result section and page 17, line 391-392 in the Discussion of the revised manuscript.

3) Function of gsx2: In the discussion and corresponding supplemental data the authors hint at alternative fates adopted by ventricular progenitors in the absence of gsx2 function. If any further insights could be obtained about these alternative identities it would increase the impact of the paper.
> We carefully re-examined gsx2:RFP+ neurons in wild-type and *gsx2* mutant larvae. Although we observed gsx2:RFP+ commissure axons in the ventral midline of the mutants, we also observed similar commissure axons in wild-type larvae. We think that the presence of gsx2:RFP signals in the IO neurons perturbed detection of commissure axons in wild-type (Fig. S6 C, G). Thus, it is unlikely that *gsx2*-lineage cells became the commissure neurons in the *gsx2* mutants. We decided to remove the description on the fate change to the commissure neurons. Currently we do not know what types of neurons *gsx2*-lineages differentiated to in the *gsx2* mutants. We carefully described this issue in page 17, line, 382-383.

4) Data quantification: There are a number of places where data is shown without quantification or with insufficient quantification.
- The number of 28C+ IO neurons in ptf1a mutants is not quantified.

> We counted 28C;UAS:GFP+ cells in *ptf1a* mutant larvae and show the data in Fig. 3M. 28C;UAS:GFP+ IO neurons were significantly reduced in *ptf1a* mutants, compared to wild-type larvae.

- n=4 larvae for counting caspase+ cells in ptf1a and wildtype controls. If the four wildtype larvae in Fig. 5Q (avg. 1 caspase+ cell) had instead been the five wildtype larvae in Fig. 5R (avg. 3.5 caspase+ cells), the difference between wildtype and ptf1a mutants (avg. 8 caspase+ cells) may not have been significant. This experiment should be repeated (and scored blind, given the challenge of distinguishing caspase+ cells from background, see Fig. 5P) with at least n=10 per experiment given the low numbers of caspase+ cells and the evident variability in caspase staining from experiment to experiment.

> We increased the number of examined larvae and revised the graphs (Fig. 5Q, R). We analyzed active caspase3+ cells in 10 *ptf1a*+/+ and 10 *ptf1a*-/- larvae (Fig. 5Q) and in 9 *gsx2*+/+ and 11 *gsx2*-/- larvae (Fig. 5R).

- The length of the gsx2 domain in mafB mutants in Fig. 7A-F is not quantified.

> We quantified the length of the gsx2:RFP expression in control (*mafba*+/*GFF*, *n*=9) and *mafbaGFF*/*GFF* (*n*=8) larvae in Fig. 8O.

- n=4-5 larvae for mafb mutant phenotype in Fig. 7O (given the very bimodal distribution in mutants.

> We counted 28C;UAS:GFP+ IO neurons in more WT (*n*=10) and *mafba* mutant (*n*=11) larvae and revised the graph (Fig. 8P in the revised manuscript).

- the length of the gsx2 domain in pbx morphants is not quantified (Fig. 9A-D)

> We quantified the length of the gsx2:RFP expression in control (*n*=5) and *pbx2/4* morphant larvae (*n*=4) larvae in Fig. 10K.

- the length of the gsx2 domain in the hoxb4a rescue experiment is not quantified (Fig. 9L-O)

> We quantified the length of the gsx2:RFP+ hindbrain region and 28C;UAS:GFP+ IO neurons in control (*n*=5) and larvae having injection of *hoxb4a* mRNA (*n*=4) larvae in Fig. 10Q and R. We performed rescue of *aldh1a2* morphant or DEAB-treated larvae by injection of *hoxb4a* mRNA. We quantified the length of gsx2:RFP+ hindbrain region and the number of 28C;UAS:GFP+ IO neurons and showed graphs (Fig. 10S-V).

- scale bars are lacking throughout.

> We put scale bars in all figures where scale bars are necessary.

5) Statistical analysis: With regard to Fig. 6M and 6AA, the text says that “inhibition of fgf8a and fgf3 further increased gsx2:RFP and the number of IO neurons” (beyond that caused by fgf8a or fgf3 inhibition alone). However, this is not the comparison that is made in the graph. Is the difference between single fgf3 or fgf8 and double fgf3; fgf8 inhibition really significant? If not, the text should be revised. Otherwise the correct interpretation of the data in Fig. 6AA is that fgf8 inhibition causes an increase in the length of the IO, and that fgf3 does not significantly affect it. Furthermore, it is not clear why, whereas in all other figures the assay was to count 28C+ IO neurons, in Fig. 6 the authors chose instead to measure the length of the IO region. Counting IO neurons is a more quantitative way to assess the phenotype. Note that in reference to Fig. 6AA the authors say “the number of IO neurons” (page 9) which is an inaccurate representation of the data.

> We counted 28C;UAS:RFP+ neurons in larvae receiving inhibition of the Fgf signal and carefully performed statistical analysis again. We found statistical difference in the gsx2:RFP expression and the number of IO neurons only between *fgf8a*+/+;Ctrl MO (control) and *fgf8ati282a*/*ti282a*;*fgf3* MO larvae. We revised Fig. 7 (Fig. 6 in the original manuscript) and described the data in page 10, line 226-229: “Inhibition of *fgf8a* and *fgf3* by injection of antisense morpholino (MO) into *fgf8a* mutants (*fgf8ati282a*/*ti282a*;*fgf3* MO) significantly increased gsx2:RFP expression and the number of 28C;UAS:GFP+ IO neurons, compared to control MO-injected WT larvae (Fig. 7A-D, I, K-P, W)”. Whereas both gsx2:RFP expression and the number of 28C;UAS:GFP+ IO neurons were increased in the *fgf8a* mutant compared to WT, the increase in gsx2:RFP expression was not statistically significant. Thus, the difference was not conclusive. We decided not to discuss a redundant role of *fgf8a* and *fgf3* in the revised manuscript.

6) Hox function: a likely role for Hox genes is based on a severe reduction of IO neurons in pbx2/4 morphants and an expanded gsx2:RFP domain in embryos injected with hoxb4a mRNA at the 1-cell stage. Neither of these are very compelling results given that pbx2/4 mutants are known to lack posterior hindbrain identity, and ectopic Hox expression (any Hox gene) from the 1-cell stage is

likewise expected to have massive consequences on hindbrain development. The model in Fig.9 would be better supported if gsx2 expression/IO neurons could be rescued in RA-inhibited embryos by hoxb4a mRNA injection. If not then for this reviewer, the data in Fig. 9 does not strengthen the paper and could be removed.

> At the reviewer's suggestion, we examined the effect of *hoxb4a* mRNA injection on the RA-inhibited larvae. We found that injection of *hoxb4a* mRNA rescued gsx2:RFP expression and 28C;UAS:GFP+ IO neurons in *aldh1a2* morphant and DEAB-treated larvae. The data support our conclusion. We added the data in Fig. 10S-V and described this issue in page 12, line 275-277 and page 19, line 435-437 in the revised manuscript.

7) Gsx2 function in mammals: In the discussion, the authors mention the known functions for mammalian Gsx2 in the specification of neurons in the telencephalon, olfactory bulb and spinal cord. Is the IO/climbing fibers normal in Gsx2 mutants or has this just never been assessed?
> Histological analysis of hindbrain of *Gsx2* mutant mice were previously reported (Szucsik et al., 1997). Although there was no description of IOs in the text, IOs were present in the *Gsx2* mutant hindbrain. However, it is not feasible to discuss whether or not IO neurons were reduced in the mutants. Although the IO phenotype in *Gsx2* mouse mutants was milder than that in *gsx2* zebrafish mutants, it may be due to redundant roles of *Gsx1* and *Gsx2*, and expansion of *Gsx1* expression in *Gsx2* mutants, which were reported for development of mouse LGE (Toresson and Campbell, 2001). The compensation for *gsx2* deficiency by *gsx1* may be weak in zebrafish, compared to mice. We discussed this issue in page 15, line 352-357 in the revised manuscript.

Minor points:
Labeling of Fig. 1 is confusing: the transgene contains Tomato, is called RFP in the text but is labeled dsRED in the figures.
> We corrected DsRed to RFP.

In the text (results and discussion) regarding Fig. 4, it says that the fact that gsx2 and ptf1a mRNA expression persists in ptf1a and gsx2 mutants, respectively, means that the two genes are regulated independently. This is in accurate: they could still be regulated by a common upstream factor(s). The text should be revised to say that the two genes are regulated independent of each other; or that they are not interdependent.
> We changed the title of the section to "Non-interdependent regulation and distinct role of *gsx2* and *ptf1a* in IO progenitors" and described "These results indicate that *ptf1a* and *gsx2* are regulated independent to each other in the caudal hindbrain" in page 9, line 205-206. Accordingly, we also changed the sentence in the Abstract (page 2, line 38).

The sentence on page 10: "Inhibition of the RA signal with both MO and DEAB resulted in reduced gsx2:RFP expression but higher mafbaGFF;UASGFP expression". Is poorly phrased. Does it mean that mafbaGFP expression is increased?
> We measured the length of mafbaGFF;UAS:GFP expression domain and showed the data in Fig. 8. We observed significant increase in mafbaGFF;UAS:GFP expression in DEAB-treated larvae (*n*=10) compared to control larvae (*n*=10); in *aldh1a2* morphant larvae (*n*=5) compared to control larvae (*n*=5). We corrected the sentence to "Inhibition of the RA signal with both MO and DEAB resulted in reduced gsx2:RFP expression but increased mafbaGFF;UAS:GFP expression (5/6, Fig. 9A-J, F, L)" in page 11, line 251-253.

In the discussion (page 16) it says that reducing Fgf signaling decreases the number of IO neurons, but should say that it increases the number of IO neurons.
> We corrected the sentence to "Inhibition of the signal of *fgf3* and *fgf8a*…..led to reduced *mafba* expression, increased *gsx2* expression, and an increase in the number of IO neurons".

Second decision letter

MS ID#: DEVELOP/2020/190603

MS TITLE: Gsx2 is involved in specification of neurons in the inferior olivary nuclei from Ptf1a-expressing neural progenitors in zebrafish

AUTHORS: Tsubasa Itoh, Miki Takeuchi, Marina Sakagami, Kazuhide Asakawa, Kenta Sumiyama, Koichi Kawakami, Takashi Shimizu, and Masahiko Hibi

The reviewers are happy with your revisions and there are now just a few minor suggestions for you to consider before we proceed to publication. 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.

Reviewer 1

Advance summary and potential significance to field

This manuscript provides new information about mechanisms of hindbrain patterning, inferior olivary neuron specification and hindbrain neural circuit formation as well as adding to our knowledge of Gsx2 function.

Comments for the author

The authors responded nicely to the first round of reviews and made numerous improvements to the manuscript. This is a solid, informative and clearly presented study.

Reviewer 2

Advance summary and potential significance to field

Itoh et al have demonstrated an important role for zebrafish Gsx2 in development of the Inferior Olivary neurons and explored the role of upstream signaling and Hox factors in IO specification from Ptf1a-_ve progenitors.

Comments for the author

The authors have put considerable effort into new experiments and analyses, which have improved the paper. They have also reworded some conclusions that were previously made too strongly to better fit with the data. I think the paper is essentially ready for publication, although some of the rewritten passages need careful proof editing for grammar errors and clarity. For example lines 364-5
'..might function at lowers in zebrafish'. " Lower levels" perhaps?
Lines 387/8 the word 'also' is repeated on both lines.
Line 68 in the introduction should be 'rhombomereS' (plural)

Reviewer 3

Advance summary and potential significance to field

my summary of the original version of the manuscript is unchanged. The main impact of the paper remains identifying a requirement for gsx2 in the specification of IO neurons.

Comments for the author

The authors have adequately addressed the concerns I raised in my review of the original version of the manuscript. In my opinion the paper is now appropriate for publication in Development. The

data support revising the title from stating that gsx2 "is involved in" to the stronger "is required for" IO specification.

Second revision

Author response to reviewers' comments

MS ID#: DEVELOP/2020/190603
MS TITLE: Gsx2 is involved in specification of neurons in the inferior olivary nuclei from Ptf1a-expressing neural progenitors in zebrafish
AUTHORS: Tsubasa Itoh, Miki Takeuchi, Marina Sakagami, Kazuhide Asakawa, Kenta Sumiyama, Koichi Kawakami, Takashi Shimizu, and Masahiko Hibi

> We thank all reviewers for their understanding of the significance of our study. We have carefully revised our manuscript at the reviewers' suggestions. Point-by-point detailed responses are provided below.

Reviewer 1 Advance Summary and Potential Significance to Field:
This manuscript provides new information about mechanisms of hindbrain patterning, inferior olivary neuron specification and hindbrain neural circuit formation as well as adding to our knowledge of Gsx2 function.

Reviewer 1 Comments for the Author:
The authors responded nicely to the first round of reviews and made numerous improvements to the manuscript. This is a solid, informative and clearly presented study.
> We are again grateful for the reviewer's high evaluation.

Reviewer 2 Advance Summary and Potential Significance to Field:
Itoh et al have demonstrated an important role for zebrafish Gsx2 in development of the Inferior Olivary neurons and explored the role of upstream signaling and Hox factors in IO specification from Ptf1a-_ve progenitors.

Reviewer 2 Comments for the Author:
The authors have put considerable effort into new experiments and analyses, which have improved the paper. They have also reworded some conclusions that were previously made too strongly to better fit with the data. I think the paper is essentially ready for publication, although some of the rewritten passages need careful proof editing for grammar errors and clarity.
> We thank the reviewer for taking a careful look at our typos and grammatical errors and pointing them out.

For example lines 364-5 '..might function at lowers in zebrafish'. " Lower levels" perhaps?
>We have changed "at lowers" to "at lower levels".

Lines 387/8 the word 'also' is repeated on both lines.
> We have changed this sentence to "We also found that mnx2b:GFF; UAS:GFP^{+} neurons located laterally to IOs were reduced in *gsx2* and *ptf1a* mutants (Fig. 2, 3)".

Line 68 in the introduction should be 'rhombomereS' (plural)
> We have changed it to 'rhombomeres".

In addition, we have carefully checked the rewritten passages. Furthermore, we had a native English proofread the manuscript. We have corrected as follows (the underline indicates corrections).
Line 27: "identity of neurons".
Line 39: "regulated independently of each other". Line 72: "that *Ptf1a* is required for the generation". Line 84: "control the differentiation".
Line 120: "we sought genes that are expressed". Line 150: "co-expressed in ~~the~~ neurons".

Line 156-157: “These axons are potentially from the nucleus commissure of Wallenberg, which send mossy fibers that project to GCs, as reported for other teleost species (Xue et al., 2004)”.
Line 172: “co-expressed in ~~the~~ neurons”
Line 182: “In addition, ~~we also found that~~ mnx2b:GFF;UAS:GFP^{+} neurons”. Line 197-198: “as in *gsx2* mutants”.
Line 198-199: “Currently, the identity of these neurons is not known, but they are likely derived from *gsx2*- and *ptf1a*-expressing neuronal progenitors”.
Line 207: ‘roles’.
Line 212: “regulated independently of each other in the caudal hindbrain”. Line 227: “These data”.
Line 234: “The inhibition of”.
Line 242-246: “Our findings suggest that the Fgf signal suppressed rostral expansion of *gsx2* expression, thereby limiting the *gsx2*-expressing IO progenitor domain to r7. The expansion of *gsx2* expression by inhibition of the Fgf signal likely led to the expansion of IO progenitors and a subsequent increase in IO neurons”.
Line 268-270: “The reduction in *gsx2* expression by inhibition of the RA signal likely led to the reduction of IO progenitors and the subsequent reduction of IO neurons”.
Line 296: “Consistent with this, single-cell RNA sequencing analysis revealed that *gsx2* and *ptf1a* were co-expressed in dorsomedial progenitor cells of the hindbrain (Tambalo et al., 2020)”.
Line 307: “Role of Ptf1a in the development of hindbrain neurons”.
Line 316: “also contribute to the development of DCN neurons in mice and of crest cells in zebrafish”
Line 322: “mutants showed a reduction”.
Line 334: “there is a similarity between DCN and MON: they both receive input from hair cells”.
Line 359: “in the spinal cord of zebrafish”.
Line 361-364: “A previous study showed the presence of IOs in the hindbrain of *Gsx2* mutant mice although it is not clear whether IO neurons were reduced (Szucsik et al., 1997), suggesting that the IO phenotype of *Gsx2* mouse mutants are milder than in the phenotype of the *gsx2* zebrafish mutant”.
Line 365: “reported for the development of mouse LGE”.
Line 390: “In addition, ~~we also found~~ that mnx2b:GFF;UAS:GFP^{+} neurons”. Line 411: “an expansion of”.
Line 417: “limits the IO progenitor domain”.
Line 425: “IO neuronal identity”.
Line 448: “in the *aldh1a2* morphant or in DEAB-treated larvae”.
Line 457-460: “The zebrafish *gsx2* gene has an about 800-bp element on the 3′ side of the open reading frame that displays partial sequence homology with the mouse *Gsx2* enhancer that can drive transgene expression in the mouse ventral telencephalon and caudal hindbrain”.
Line 462: “further analysis is required to understand”.
Line 612-614: “Since 28C;UAS:GFP^{+} cells that were located in this region extended their axons rostrally, they were considered as IO neurons and the number of ~~the~~ GFP^{+} cells were counted manually”.
Line 949: “the *gsx2* mutant”.
Line 993: “expression extends to”.
Line 1006: “in the hindbrain of the control”. Line 1015: “DMSO-treated”.

Reviewer 3 Advance Summary and Potential Significance to Field:
My summary of the original version of the manuscript is unchanged. The main impact of the paper remains identifying a requirement for gsx2 in the specification of IO neurons.

Reviewer 3 Comments for the Author:
The authors have adequately addressed the concerns I raised in my review of the original version of the manuscript. In my opinion the paper is now appropriate for publication in Development. The data support revising the title from stating that gsx2 "is involved in" to the stronger "is required for" IO specification.
> We thank the reviewer for understanding that our conclusions have been strengthened by the revision in the manuscript. At the reviewer’s suggestion, we have changed the title to “Gsx2 is required for specification of neurons in the inferior olivary nuclei from Ptf1a- expressing neural progenitors in zebrafish”.

Third decision letter

MS ID#: DEVELOP/2020/190603

MS TITLE: Gsx2 is required for specification of neurons in the inferior olivary nuclei from Ptf1a-expressing neural progenitors in zebrafish

AUTHORS: Tsubasa Itoh, Miki Takeuchi, Marina Sakagami, Kazuhide Asakawa, Kenta Sumiyama, Koichi Kawakami, Takashi Shimizu, and Masahiko Hibi
ARTICLE TYPE: Research Article

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