nature portfolio

Peer Review File

Defects in hair cells disrupt the development of auditory peripheral circuitry

Corresponding Author: Professor Ulrich Mueller

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript from Bottom and colleagues expands on previous reports of the striking impact that mutation of key genes for hair cell transduction has on SGN gene expression and function. They show that not only is gene expression in the three SGN cell types (IA, AB, and IC) impacted, but pathfinding by type I and type II afferents is dramatically affected. The data are broadly consistent with a model whereby short- or medium-range pathfinding by the afferents requires signals from hair cells, presumably stimulated by transduction activity or its equivalent. One of the more clear and interesting results is that it is likely not glutamate transmission (the normal afferent signal), since mutations that abrogate glutamate signaling do not have the same impact.

While the manuscript's experiments each raise additional questions and call for more experiments, the manuscript is relatively complete as it stands. These additional questions (like definition of the critical window for Tmie expression) are well suited for a future manuscript and are not needed to complete this manuscript.

In particular, a critical translational question is whether the changes in SGN circuitry are reversible. Fortunately with AAV-Tmie injection at different ages, the authors will be able to define the critical window for SGN neurite pathfinding in future work.

There are a variety of issues that need to be resolved, but no significant major issues.

Comments

It seems less likely that a gene whose expression is directly regulated by Tmie or Pcdh15 expression is the factor affecting attraction of SGN neurites. It seems far more likely to me that it is hair cell depolarization, presumably controlled by mechanotransduction, is itself what controls the release of a factor; that would explain why Clrn1 loss causes only a partial phenotype compared to the other genes. As noted above, it would be a released factor other than glutamate that controls attraction.

Line 23. The word "maintains" is probably too much of a shorthand to get across what TMIE expression through AAV delivery does; be a little more explicit.

Line 58. "IHC" is also used later to abbreviate immunohistochemistry. Choose one abbreviation or the other (I highly recommend that IHC be used for inner hair cells.)

Lines 58-59. In both cases, "that" should be replaced by ", which"—the comma before "which" is important. There are a number of other places in the manuscript where "that" and "which" are used inappropriately. Not a big deal, but there is a precise way to use them.

Line 77. Add comma after "degeneration."

Line 131. I don't think you should abbreviate "differentially expressed." When the abbreviation comes up later, the nonaficionado doesn't know what it means.

Line 141. Add comma after “neurons25-28”

Lines 162-164. Are these differences statistically significant?

Line 176. The analysis Figure 2D needs to be explained much more thoroughly. What genes sets were subject to GO analysis?

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Line 195. Add comma after “endings.”

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Line 301. What does “severely increased” mean?

Lines 359. The apical end of the cochlea encodes low frequency sound, while the basal end encodes high frequency sound. You have this backwards.

Line 364. How do you know they are terminating and not turning and projecting elsewhere?

Line 400-401. Important: the data also say that hair cell activity, presumably electrical, is required for the signal to SGNs.

Line 402. Change “genes including cell adhesion molecules” to “genes, including those that encode cell adhesion molecules.”

Line 403. Add comma after “components.”

Line 411-412. It seems more likely that different genetic forms of hearing loss will affect the signals to SGNs simply by affecting activity to different degrees.

Line 418-419. You don’t need to be coy and say “It is tempting to speculate…” You are in fact speculating this—it’s reasonable—so just say that. That said, as noted above, I disagree with the speculation—I don’t think that the genes affected in DFNB6 mice, other than Tmie itself (and hence hair cell activity), underlie the phenotype.

Line 433. Change “mice” to “mouse hearing.”

Line 438-439. Restoring function of multiple cell types will be difficult; instead, it is important to define how plastic the SGN is so we know when to restore function in hair cells.

Lines 446-447. Give a little bit of information, e.g., gRNAs type of allele generated.

Line 460. What is “EBSS”?

Line 461. Fix “unitz.”

Line 464-465. This sentence makes no sense.

Line 470. Spell out “reverse transcriptase,” as “RT” is slang.

Line 472. Spell out what GEMs are.

Line 480. How much DNA?

Line 497. Give packaging information, e.g., where and how.

Line 510. Fix those superscripts (“or” doesn’t belong in the superscript; don’t try to shortcut).

Lines 508-530. You should be able to collapse these sections into a single section that doesn’t repeat information.

Lines 557-558. What is “drop-fixing”?

Lines 612 and 620. List exact lens, including numerical apertures.

Lines 666 and 676. Don’t use “IHC” as an abbreviation for “immunohistochemistry.” Also, what you are doing is more accurately called immunofluorescence.

Figure 1F. The text is not readable; you have plenty of space, make it bigger. A good goal is to have all text be 8 point at 100% view.

Figure 1G, left. These panels are far, far too dark. I recommend both adjusting the levels and using grayscale for the individual channels.

Figure 1G, right. These panels are hard to decipher because the text is so tiny.

Figure 3 and elsewhere. For the convenience of our colorblind colleagues, please use magenta/green. As above, I highly recommend grayscale for individual channels.

Figure 4 and elsewhere. Red/green/blue is a very challenging color combination. The blue is impossible to see when printed out, and is still bad in an electronic image.

Figures 5B-C and E-F. Why not have consistent units for IHCs and OHCs?

Figure 6 and elsewhere. Writing out numbers like “12.17 ± 0.2466” makes no sense. This implies greater precision in the error than the average, which makes no sense. I would use two (or even one) significant digits for the error, then use that the decide how many digits in the average. This should be “12.17 ± 0.25” or even “12.1 ± 0.2.”

Figure 8D. Why two different y-axis scales for IHCs and OHCs? And two different sized graphs?

Supplementary Figure 4. You defined “Tam” in the previous legend. Also, the red with black background is really hard to see.

Supplementary Figure 5. State here when the injections were done.

Reviewer #2

(Remarks to the Author)
One of the primary questions addressed by the authors is the extent to which hair cell defects during the early developmental stages impact the differentiation of spiral ganglion neurons (SGNs) and the formation of peripheral auditory circuitry.
To address this, Bottom et al investigated several mouse models representing genetic forms of hearing loss, including non-syndromic autosomal recessive forms of deafness (DFNB6, DFNB117) and Usher Syndrome type I (USH1F).
In their study, Bottom et al. initially focused on a mouse model for DFNB6, which lacks Tmie, a gene essential for hair bundle development and function. Using single-cell RNA sequencing, they compared the transcriptome of control and Tmie-/- mice at postnatal 21. Their analysis revealed that the inactivation of Tmie in auditory hair cells disrupts the expression of genes in the neurons they synapse with. These alterations involve a predominant up-regulation of genes. Many of these up-regulated genes are implicated in neuronal growth and axonal targeting, both of which are disrupted in the Tmie-/- mice. After injecting an AAV viral vector expressing GFP under the control of a type 1 neuron-specific promoter into the cochlea, they successfully labeled these neurons and demonstrated abnormal axonal targeting in Tmie-/- mice compared to the controls. Additionally, staining for NGFR revealed a significant increase in the number of NGFR+ fibers in the organ of Corti of Tmie-/- mice, accompanied by aberrant peripheral projections in genetically defined Type II SGNs.
They also report similar neuronal abnormalities in mouse models for DFNB117 and Usher Syndrome Type 1F, where primary hair cell defects arise from different mechanisms. Interestingly, gene therapy targeted at hair cells preserves hair cells and neurons in DFNB6 model mice. Their findings underscore the essential role of hair cell function in establishing normal peripheral auditory circuitry and highlight the importance of deafness treatment strategies focusing on restoring hair cell and neuronal function, even in cases where the primary defect originates in hair cells.

The study is very interesting and carefully presented with some weaknesses.

Major comments:

1. My main concern revolves around the authors' omission of the role played by spontaneously released glutamate from inner hair cells (IHCs) and general synaptic activity during the pre-hearing period as influential factors in the development of synaptogenesis among SGNs and IHCs. The study lacks robust evidence to substantiate this exclusion for two main reasons: i) Their investigation using the VGLUT3 KO mouse model revealed that the lack of glutamate release by hair cells before hearing onset minimally affects the shaping of the peripheral auditory system, attributing little significance to synaptic activity during this period. However, the authors overlook the compensatory mechanisms. Notably, the heightened excitability observed in SGNs in these mice facilitates direct neuronal stimulation through potassium transients induced by supporting cells. My query concerns to how the authors reconcile these findings with the aforementioned observation.

ii) The widely accepted notion is that synaptic transmission plays a crucial role in the development of synaptogenesis among SGNs and IHCs. Dismissing such a hypothesis warrants a more comprehensive examination of the vGlut3 KO mouse model. Perhaps employing a method such as labeling neurons post-AAV injection, akin to the tmie model, could confirm this discovery, particularly considering that NGFR in type 2 neurons would typically remain unaffected if outer hair cells (OHCs) are intact, as observed in the VGLUT3 model.

2. To facilitate the reader's navigation through the various data, it would be beneficial to harmonize the ages at which the different studies were conducted.

3. Fig. 1: It would be advantageous to include gene expression kinetics at P30 rather than solely at P21, given the emphasis on neuronal development during this period.

Minor
1. Line 24, the statement "We conclude that hair cell function is crucial for the establishment of normal auditory circuits" may be overstated given that the study focuses solely on the peripheral auditory system.
2. Line 164: Is the difference in neuronal subtype distribution between KO and Htz significant?
3. To facilitate the reader's identification of OHCs in most figures, it is essential to include a marker for these cells, such as myosin 7A or myosin 6. This include Figures 4B,D, 6D,G and 8E.

Reviewer #3

(Remarks to the Author)
In this manuscript, Bottom et al have investigated the requirement of hair cell function for the establishment of normal auditory circuitry. To do this they have employed several mouse models of hearing loss, where the primary defect is within hair cells, and assessed these mice for neuronal defects. They conclude that hair cell function is critical for correct neuronal patterning and function, and as such they suggest that therapeutic intervention strategies for hair cell-based hearing loss must consider restoration of neuronal function as well as hair cell function.
The research question and techniques employed are sound. However, additional integration of these data with existing knowledge would help highlight the novelty and importance of the current work.

General comments:
Overall, I like the study, but the narrative does feel a little confused and disjointed. Furthermore, the discussion is too narrow and does not fully describe how these new data fit with existing knowledge, nor does it explore other possible explanations for these data.

Specific comments:
• Lines 162-164: The authors discuss the redistribution of SGN types in the Tmie KO compared with control. It is stated that type 1A and 1C are overrepresented. However, no statistical analysis is undertaken to confirm this.
• Line 264: Related to the previous comment, the authors state that the number of tdTomato labelled SGNs (labelling type 1C SGNs) did not differ between controls and Tmie-/- mice. Surely this is at odds with the previous statement ‘in the Tmie KO type 1A and 1C are overrepresented compared with control’. This discrepancy should be acknowledged and discussed.
• Throughout the manuscript, there is an unstated assumption that the genes for the knockout models are exclusively expressed only in hair cells, and are never expressed elsewhere in the cochlea e.g. spiral ganglion or surrounding supporting cells. The authors should provide evidence that these genes are never expressed (even transiently) in spiral ganglion or surrounding supporting cells at any point during development or the postnatal period, either through new data or by referencing previously published data. If hair cell-restricted expression of these specific genes cannot be confirmed, the impact of this on their findings and drawn conclusions should be acknowledged and discussed.
• Indeed, given the emphasis of the manuscript, please mention why hair cell-specific conditional knockout mice were not used in these experiments, rather than the standard conventional knockout mice that were used. Using hair cell-specific conditional knockout mice would help support the conclusion that correct hair cell function is critical for correct neuronal patterning.
• The authors present ribbon synapse data, in the form of anti-Glur2/3 and anti-Ribeye immunofluorescence labelling, imaging and counts, for all of their mutant mice except the Pcdh15av3J model. However, the authors then go on to compare the degree of severity of the synaptic phenotype observed in the Pcdh15av3J model. Given this comparison, I feel the synaptic phenotype data for the Pcdh15av3J model should be included in the manuscript.
• Line 313: The authors state they have produced a new Clrn2 knockout mouse model, which phenocopies a previously reported Clrn2 knockout. The authors then utilise this model for their studies. However, the authors provide no information or data regarding the generation or preliminary phenotyping of this new model. To allow critical assessment, information regarding the generation and phenotyping of this line needs to be included in this manuscript. ‘Data not shown’ is not acceptable.
• Line 334-335: The authors state there are no “obvious differences in NGFR+ SGN projections in Vglut3-/- mice”. However, these data are not quantified. The authors have quantified similar staining for their other mutants, therefore this should be

undertaken for the Vglut3-/- model.
• Line 359: Please correct the sentence. Apical is low-frequency and not high-frequency as stated. Similarly, basal is high-frequency and not low-frequency as stated.
• Figure 8: Are the improved low frequency ABR thresholds of the Tmie-/- mice injected with AAV.PHP.eB.TMIE::EGFP preserved over time? Or do they worsen with time, and become similar to the thresholds observed for the Tmie-/- mice injected with AAV.PHP.eB.EGFP?
• Figure 8: The authors suggest the recovery of MET function and synaptic defects are correlated with the efficiency of AAV delivery to the hair cells, rather than partial recovery of function in transduced hair cells. Given that not 100% of IHCs and OHCs are transduced in the Tmie-/- mice injected with AAV.PHP.eB.TMIE::EGFP, I feel the authors should ascertain whether there are significant synaptic differences between EGFP+ve and EGFP-ve hair cells within the same cochlear sample.
• For the AAV rescue study, why were injections undertaken using a 3-day window (P1-P3)? This could be a variable for the results achieved. Please determine the effect achieved by age of injection.
• Line 400: The conclusion that 'the findings suggest that the magnitude of SGN defects scales with the magnitude of functional impairment of hair cells' does not take into account anything to do with the temporal onset of expression of the genes that have been used to develop this statement. For the statement to be true, the authors need to compare the onset of MET dysfunction in the different models and show that they all onset at the same age point. If there is a difference in the age of onset, the differences in neuronal patterning could just be a reflection of the effects occurring at different stages of developmental maturation. For instance, perhaps expression of Tmie and Pcdh15 are required earlier than Clrn2. Again, by using a hair cell-specific conditional Tmie knockout mouse and inducing recombination at different late gestational/early postnatal ages, the authors would be able to investigate for a temporal effect.
• Please adhere to the ARRIVE guidelines, there is no mention of the genetic background of any of the mice utilised in these studies.
• Furthermore, please explain why only male mice were used for the sc-RNAseq study.
• Moreover, the statement that 'both sexes were used, and male and female numbers were used in approximately equal numbers' is too ambiguous. This could mean that all controls were female and all test mice were male, which would be a failure of experimental design. Please clarify the statement.
• In line with the journal's policy, t-values and degrees of freedom should be provided alongside p values for t-tests.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have carefully addressed all of my comments. I also believe that they responded well to the concerns raised by the other authors.

Reviewer #2

(Remarks to the Author)

Reviewer #3

(Remarks to the Author)
The authors have done a very good addressing the comments and concerns I raised in the initial round of review. The inclusion of new data, as well as improved statistical analyses of the existing data, help support the conclusions of their paper.

Reviewer comments- Bottom et al., Nat. Comm.

Reviewer #1 (Remarks to the Author):

This manuscript from Bottom and colleagues expands on previous reports of the striking impact that mutation of key genes for hair cell transduction has on SGN gene expression and function. They show that not only is gene expression in the three SGN cell types (IA, AB, and IC) impacted, but pathfinding by type I and type II afferents is dramatically affected. The data are broadly consistent with a model whereby short- or medium-range pathfinding by the afferents requires signals from hair cells, presumably stimulated by transduction activity or its equivalent. One of the more clear and interesting results is that it is likely not glutamate transmission (the normal afferent signal), since mutations that abrogate glutamate signaling do not have the same impact.

While the manuscript's experiments each raise additional questions and call for more experiments, the manuscript is relatively complete as it stands. These additional questions (like definition of the critical window for Tmie expression) are well suited for a future manuscript and are not needed to complete this manuscript.

In particular, a critical translational question is whether the changes in SGN circuitry are reversible. Fortunately with AAV-Tmie injection at different ages, the authors will be able to define the critical window for SGN neurite pathfinding in future work.

There are a variety of issues that need to be resolved, but no significant major issues.

Comments

It seems less likely that a gene whose expression is directly regulated by Tmie or Pcdh15 expression is the factor affecting attraction of SGN neurites. It seems far more likely to me that it is hair cell depolarization, presumably controlled by mechanotransduction, is itself what controls the release of a factor; that would explain why Clrn1 loss causes only a partial phenotype compared to the other genes. As noted above, it would be a released factor other than glutamate that controls attraction.
We entirely agree with the reviewer. We now state that changes in electrical activity and membrane potential of hair cells could play an important role in regulating SGN differentiation.

Line 23. The word "maintains" is probably too much of a shorthand to get across what TMIE expression through AAV delivery does; be a little more explicit.
The text has been corrected to explicitly state that expression of TMIE via AAV restores connectivity and hearing.

Line 58. "IHC" is also used later to abbreviate immunohistochemistry. Choose one abbreviation or the other (I highly recommend that IHC be used for inner hair cells.)
The text has been adjusted to use the abbreviation IHC exclusively for inner hair cells. As per comment below, we now use the term "immunofluorescence" instead of "immunohistochemistry".

Lines 58-59. In both cases, “that” should be replaced by “, which”—the comma before “which” is important. There are a number of other places in the manuscript where “that” and “which” are used inappropriately. Not a big deal, but there is a precise way to use them.
Erroneous uses of “that” have been changed to “which” throughout the text.

Line 77. Add comma after “degeneration.”
Corrected in text.

Line 131. I don’t think you should abbreviate “differentially expressed.” When the abbreviation comes up later, the nonaficionado doesn’t know what it means.
The abbreviation DE has been removed from text.

Line 141. Add comma after “neurons25-28”
Corrected in text.

Lines 162-164. Are these differences statistically significant?
We ran one-tailed t-tests on subtype composition percentages after normalizing subtype numbers to total SGN numbers for each individual sample. Results of these tests revealed only a significant decrease in IB percentages between control and *Tmie*$^{-/-}$ mice (p = 0.0290). We have added these data to the text.

Line 176. The analysis Figure 2D needs to be explained much more thoroughly. What genes sets were subject to GO analysis?

For Figure 2D, the following was done:

1. We did a differential expression analysis for each subtype-genotype combination. (for example, IA-control vs other cells, IB-KO vs others).
2. For each set of differentially expressed genes, those with q-value < 0.00001, were used for GO analysis using the GO database (including GO_Molecular Function, GO_Cellular Component, and GO_Biological Process)
3. The histology-related terms in the plot were manually selected from the GO terms with Adjusted p-value < 0.1.

We have modified the text in the “Bioinformatic analysis” section of the Methods to explicitly explain the work flow.

Line 194. Change to “projections.”
Corrected in text.

Line 195. Add comma after “endings.”
Corrected in text.

Lines 197-198. Does this sentence mean that the type II SGN projections express GFP-synapsin or are the type II SGNs visualized by a different channel? The sentence is unclear.

The sentence has been fixed to convey that high titer AAV-synapsin-GFP injections often labels type II SGNs in addition to type I SGNs.

Line 265. The word “vast” seems like an exaggeration.
The text has been corrected to remove use of “vast”.

Line 269. Since this is fixed tissue, how do you know that there are no “stable” connections?
The text has been corrected to remove mention of “stable” connections.

Line 280. Add comma after “affected.”
Corrected in text.

Line 298. Change “(USH1F) that” to “(USH1F), which.”
Corrected in text.

Line 301. What does “severely increased” mean?
The text has been corrected to state the statistically significant increase in $NGFR^{+}$ terminals near IHCs.

Lines 359. The apical end of the cochlea encodes low frequency sound, while the basal end encodes high frequency sound. You have this backwards.
We apologize for this unnecessary mistake. The text has been corrected.

Line 364. How do you know they are terminating and not turning and projecting elsewhere?
The use of “terminal” or “nerve terminal” throughout the text was meant to refer to the morphological ending of SGNs imaged in fixed tissues. We have carefully corrected the text to make clear that we are not referring to an active process but a morphological feature. To achieve this, we repeatedly refer to “the morphology of nerve fibers”.

Line 400-401. Important: the data also say that hair cell activity, presumably electrical, is required for the signal to SGNs.
We have changed the text to indicate that perturbations in the electrical activity of hair cells presumably contributes to the phenotypic changes.

Line 402. Change “genes including cell adhesion molecules” to “genes, including those that encode cell adhesion molecules.”
Corrected in text.

Line 403. Add comma after “components.”
Corrected in text.

Line 411-412. It seems more likely that different genetic forms of hearing loss will affect the signals to SGNs simply by affecting activity to different degrees.
We have included the statement that the signals might include electrical activity of hair cells, which might be affected to different degrees in different genetic forms of hearing loss.

Line 418-419. You don’t need to be coy and say “It is tempting to speculate…” You are in fact speculating this—it’s reasonable—so just say that. That said, as noted above, I

disagree with the speculation—I don't think that the genes affected in DFNB6 mice, other than Tmie itself (and hence hair cell activity), underlie the phenotype.
Corrected in text.

Line 433. Change "mice" to "mouse hearing."
Corrected in text.

Line 438-439. Restoring function of multiple cell types will be difficult; instead, it is important to define how plastic the SGN is so we know when to restore function in hair cells.
We agree and have adjusted the text to indicate that defining the time window for neuronal plasticity will be useful to determine when restoration of gene function in hair cells can rescue neuronal defects.

Lines 446-447. Give a little bit of information, e.g., gRNAs type of allele generated.
Clrn2$^{-/-}$ mice were generated using CRISPR/Cas9 technology to delete a 48bp region of the *Clrn2* allele spanning portions of the 5'UTR and exon 1, including the start codon. Detailed information regarding the generation of the mice (including target-specific crRNA sequence) is now included in the Methods section. Additionally, confirmation of the MET current reduction phenotype in *Clrn2*$^{-/-}$ hair cells previously reported (Dunbar, *et al*. 2021. *EMBO Mol Med*) is now included in new Supplementary Data 6.

Line 460. What is "EBSS"?
EBSS is Earle's Balanced Salt Solution. We now explain this in the text.

Line 461. Fix "unitz."
Corrected in text.

Line 464-465. This sentence makes no sense.
The sentence was incomplete due to the missing chemical component that was added at 8 ul/ml concentration (RNAse inhibitor). This has been corrected in text.

Line 470. Spell out "reverse transcriptase," as "RT" is slang.
Corrected in text.

Line 472. Spell out what GEMs are.
Corrected in text.

Line 480. How much DNA?
A range of 172-222 total ng cDNA was used for library prep. This has now been explicitly stated in the text.

Line 497. Give packaging information, e.g., where and how.
Packaging information has been added to text.

Line 510. Fix those superscripts ("or" doesn't belong in the superscript; don't try to shortcut).
Corrected in text.

Lines 508-530. You should be able to collapse these sections into a single section that doesn't repeat information.
Lypd1-CreERT2 and *TH-CreER* mouse line methodology have now been combined into a single section.

Lines 557-558. What is "drop-fixing"?
Drop-fixing refers to fixation of freshly dissected tissues via immersion in fixative as opposed to tissue dissected following fixation via perfusion. Usage of fresh tissue in P11 timepoints has been clarified in text.

Lines 612 and 620. List exact lens, including numerical apertures.
Exact lens information with NAs now included in "Image acquisition" section of Methods.

Lines 666 and 676. Don't use "IHC" as an abbreviation for "immunohistochemistry." Also, what you are doing is more accurately called immunofluorescence.
As mentioned above, text mentions of immunohistochemistry have been changed to "immunofluorescence".

Figure 1F. The text is not readable; you have plenty of space, make it bigger. A good goal is to have all text be 8 point at 100% view.
Text size in Fig. 1F and throughout have been corrected to be at least 8 point at 100% view.

Figure 1G, left. These panels are far, far too dark. I recommend both adjusting the levels and using grayscale for the individual channels.
Levels have been uniformly increased across all Fig. 1G panels and all single channel images have been changed to grayscale here and throughout the manuscript.

Figure 1G, right. These panels are hard to decipher because the text is so tiny.
Text size in figures have been corrected to be at least 8 point at 100% view.

Figure 3 and elsewhere. For the convenience of our colorblind colleagues, please use magenta/green. As above, I highly recommend grayscale for individual channels.
All single channel images have been changed to grayscale throughout the manuscript. All two-color images have been changed to magenta/green and all three-color images have been changed to cyan/yellow/red for colorblind viewer compatibility.

Figure 4 and elsewhere. Red/green/blue is a very challenging color combination. The blue is impossible to see when printed out, and is still bad in an electronic image.
All three-color images have been changed to cyan/yellow/red for easier viewing and colorblind viewer compatibility.

Figures 5B-C and E-F. Why not have consistent units for IHCs and OHCs?
To make consistent with CTBP2 puncta measures in IHCs (# puncta/10 IHCs), CTBP2 puncta in OHCs measures were re-calculated and are now displayed as # puncta/10 OHCs. This has also been made consistent in CTBP2 quantification in *Vglut3*-KO mice (Fig. 7E).

Figure 6 and elsewhere. Writing out numbers like "12.17 ± 0.2466" makes no sense. This implies greater precision in the error than the average, which makes no sense. I would use

two (or even one) significant digits for the error, then use that the decide how many digits in the average. This should be “12.17 ± 0.25” or even “12.1 ± 0.2.”
All figure legends have been corrected to two significant digits for all means and SEMs.

Figure 8D. Why two different y-axis scales for IHCs and OHCs? And two different sized graphs?
Graphs have been combined for consistent size and scale.

Supplementary Figure 4. You defined “Tam” in the previous legend. Also, the red with black background is really hard to see.
Repeat “Tam” definition removed. Single color red image changed to grayscale as per above comment.

Supplementary Figure 5. State here when the injections were done.
Time of injections (P1) added into text.

Reviewer #2 (Remarks to the Author):

One of the primary questions addressed by the authors is the extent to which hair cell defects during the early developmental stages impact the differentiation of spiral ganglion neurons (SGNs) and the formation of peripheral auditory circuitry.
To address this, Bottom et al investigated several mouse models representing genetic forms of hearing loss, including non-syndromic autosomal recessive forms of deafness (DFNB6, DFNB117) and Usher Syndrome type I (USH1F).
In their study, Bottom et al. initially focused on a mouse model for DFNB6, which lacks Tmie, a gene essential for hair bundle development and function. Using single-cell RNA sequencing, they compared the transcriptome of control and Tmie-/- mice at postnatal 21. Their analysis revealed that the inactivation of Tmie in auditory hair cells disrupts the expression of genes in the neurons they synapse with. These alterations involve a predominant up-regulation of genes. Many of these up-regulated genes are implicated in neuronal growth and axonal targeting, both of which are disrupted in the Tmie-/- mice. After injecting an AAV viral vector expressing GFP under the control of a type 1 neuron-specific promoter into the cochlea, they successfully labeled these neurons and demonstrated abnormal axonal targeting in Tmie-/- mice compared to the controls. Additionally, staining for NGFR revealed a significant increase in the number of NGFR+ fibers in the organ of Corti of Tmie-/- mice, accompanied by aberrant peripheral projections in genetically defined Type II SGNs.
They also report similar neuronal abnormalities in mouse models for DFNB117 and Usher Syndrome Type 1F, where primary hair cell defects arise from different mechanisms. Interestingly, gene therapy targeted at hair cells preserves hair cells and neurons in DFNB6 model mice. Their findings underscore the essential role of hair cell function in establishing normal peripheral auditory circuitry and highlight the importance of deafness treatment strategies focusing on restoring hair cell and neuronal function, even in cases where the primary defect originates in hair cells.

The study is very interesting and carefully presented with some weaknesses.

Major comments:

1. My main concern revolves around the authors' omission of the role played by spontaneously released glutamate from inner hair cells (IHCs) and general synaptic activity during the pre-hearing period as influential factors in the development of synaptogenesis among SGNs and IHCs. The study lacks robust evidence to substantiate this exclusion for two main reasons: i) Their investigation using the VGLUT3 KO mouse model revealed that the lack of glutamate release by hair cells before hearing onset minimally affects the shaping of the peripheral auditory system, attributing little significance to synaptic activity during this period. However, the authors overlook the compensatory mechanisms. Notably, the heightened excitability observed in SGNs in these mice facilitates direct neuronal stimulation through potassium transients induced by supporting cells. My query concerns to how the authors reconcile these findings with the aforementioned observation.

We have revised the discussion of the manuscript to better integrate our findings with previous studies in the field. Previous studies from the laboratory of our colleague Dwight Bergles have shown that glutamate release from hair cells onto SGNs is important to trigger spontaneous action potentials, and that NMDA receptors are involved in this process. The previous studies have also shown that in NMDA receptor knock-outs, survival of SGNs is affected, and that perturbing spontaneous activity patterns in cochlear supporting cells affects auditory circuit function as analyzed by in vivo imaging of the inferior colliculus. However, no study has analyzed effects of glutamate release on SGN morphology. Our study shows that in MET deficient mice, ribbon synapses and SGN morphology are far more drastically affected than in *Vglut3*$^{-/-}$ mice. This does not question the importance of glutamate signaling for auditory circuit development but provides strong evidence that the phenotype of MET mutant mice cannot be explained alone by defects in glutamate signaling. We do not find this particularly surprising because inactivation of MET in hair cells likely affects them profoundly. In fact, it is already known that MET inactivation leads to defects in hair cell morphology and the expression of genes such as whirlin. Inactivation of MET might affect the release of signaling molecules (via changes in electrical activity?) and expression of synaptic genes that contribute to the observed phenotypic changes. In response to the reviewer’s comments, we have substantially extended the discussion to address these points.

ii) The widely accepted notion is that synaptic transmission plays a crucial role in the development of synaptogenesis among SGNs and IHCs. Dismissing such a hypothesis warrants a more comprehensive examination of the vGlut3 KO mouse model. Perhaps employing a method such as labeling neurons post-AAV injection, akin to the tmie model, could confirm this discovery, particularly considering that NGFR in type 2 neurons would typically remain unaffected if outer hair cells (OHCs) are intact, as observed in the VGLUT3 model.

To further address our initial findings in *Vglut3* KO mice, we used sparse labelling of Type I SGNs via AAV-hSyn-mCherry injections at P3 and analysis at P21, mirroring the timepoint used in *Tmie* KO mice (Fig. 7F). We found that nearly all labeled SGNs analyzed displayed normal IHC contact in KOs at levels nearly identical to control littermates (Fig. 7G, H). These results further support the notion that, besides glutamate release, other factors contribute to regulating connectivity patterns between IHCs and type I SGNs . However, our results must be interpreted with caution as they do not rule out more subtle effects of glutamate release on other SGN developmental aspects such as refinement, basolateral targeting accuracy or physiological characteristics. We have adjusted the text to explicitly discuss this point.

2. To facilitate the reader's navigation through the various data, it would be beneficial to harmonize the ages at which the different studies were conducted.
We have made every effort to standardize ages. Fig. 1,2,3,6,7,8,9,S1,S5 and S7 use P21 animals. Figs. 4 and 5 uses P20-P23 animals, which in our opinion is sufficiently close to P21.

In Fig. 3, 4, and 5, we use additional earlier time points (P11 and P14) to capture developmental processes within the appropriate time window.

In Fig. S3 and S4, we analyze at P28 because tamoxifen injection is conducted at P21.

3. Fig. 1: It would be advantageous to include gene expression kinetics at P30 rather than solely at P21, given the emphasis on neuronal development during this period.
Single cell RNA sequencing experiments are extremely expensive (~$50,000 for the samples in this manuscript). We therefore had to limit our analysis to the most essential time point. We focused on P21 for several reasons. First, SGN survival is not affected in *Tmie* mutant mice at P21, but cell death is observed at P30. Thus, a P30 time point might confound true expression changes with loss of RNA due to cell degeneration. Second, major steps in the development of SGN synapses with IHCs have taken place by P21 followed by synaptic refinement up to P28. Thus, we cover an essential developmental time frame. Third, we had previously shown by immunofluorescence that gene expression programs in SGNs are already perturbed at P21 (Sun et al., 2018. *Cell*), which is consistent with the RNAseq data shown here. Fourth, our histological analysis shown in this manuscript demonstrates that SGN defects are already severe at P21 and thus likely a consequence of perturbations in earlier developmental processes. We therefore would not expect that a P30 time point would provide significant new insights that would change our conclusions. We therefore find that a P21 time point is adequate, but we agree that the study of other time points, presumably before P21, would be informative and we are planning to conduct them.

Minor
1. Line 24, the statement "We conclude that hair cell function is crucial for the establishment of normal auditory circuits" may be overstated given that the study focuses solely on the peripheral auditory system.
Text has been corrected to explicitly state “peripheral auditory circuitry”.

2. Line 164: Is the difference in neuronal subtype distribution between KO and Htz significant?
We ran one-tailed t-tests on subtype composition percentages after normalizing subtype numbers to total SGN numbers for each individual sample. Results of these tests revealed only a significant decrease in IB percentages between control and *Tmie*$^{-/-}$ mice (p = 0.0290). We have revised the text to clarify this point.

3. To facilitate the reader's identification of OHCs in most figures, it is essential to include a marker for these cells, such as myosin 7A or myosin 6. This include Figures 4B,D, 6D,G and 8E.
We were not able to co-stain with OHC markers for these figure panels because available antibodies to identify these cells such as those recognizing MYO7A and MYO6 are raised in

rabbits, just as the NGFR antibodies. However, rows of OHCs can clearly be identified by our DAPI staining. We also have stained parallel samples with MYO7A antibodies and could include those images next to the NGFR images. However, it is difficult to fit these panels into the figures without significantly shrinking the size of all images, which would make it more difficult to view nerve fibers. Since visualization of nerve fibers is crucial, we have not included labels and instead highlight the position of OHCs with labels on the figures. If the reviewer insists that labeling of OHCs is essential to show, we could provide revised figures or even move data to the supplement, but we find that the results are sufficiently well documented to support our conclusions.

Reviewer #3 (Remarks to the Author):

In this manuscript, Bottom et al have investigated the requirement of hair cell function for the establishment of normal auditory circuitry. To do this they have employed several mouse models of hearing loss, where the primary defect is within hair cells, and assessed these mice for neuronal defects. They conclude that hair cell function is critical for correct neuronal patterning and function, and as such they suggest that therapeutic intervention strategies for hair cell-based hearing loss must consider restoration of neuronal function as well as hair cell function.
The research question and techniques employed are sound. However, additional integration of these data with existing knowledge would help highlight the novelty and importance of the current work.

General comments:
Overall, I like the study, but the narrative does feel a little confused and disjointed. Furthermore, the discussion is too narrow and does not fully describe how these new data fit with existing knowledge, nor does it explore other possible explanations for these data.

Specific comments:
• Lines 162-164: The authors discuss the redistribution of SGN types in the Tmie KO compared with control. It is stated that type 1A and 1C are overrepresented. However, no statistical analysis is undertaken to confirm this.
We thank the reviewer for this comment because we had not clearly addressed this point. We ran one-tailed t-tests on subtype composition percentages after normalizing subtype numbers to total SGN numbers for each individual sample. Results of these tests revealed only a significant decrease in IB percentages between control and *Tmie*$^{-/-}$ mice (p = 0.0290). We have revised the text to clarify this point.

• Line 264: Related to the previous comment, the authors state that the number of tdTomato labelled SGNs (labelling type 1C SGNs) did not differ between controls and Tmie-/- mice. Surely this is at odds with the previous statement ‘in the Tmie KO type 1A and 1C are overrepresented compared with control’. This discrepancy should be acknowledged and discussed.
Statistical analysis (from above) ultimately revealed that IC composition was not significantly different between controls and *Tmie*$^{-/-}$ mice, and thus is not at odds with the results seen in Supp. Fig. 3C,D. However, subtype composition based on *Lypd1-CreERT2* genetic labeling and the designated IC composition from single cell RNAseq may not be identical due to the bioinformatic approach of subtype designation. In brief, the bioinformatic

approach first utilized high confidence seed cells from control samples to generate predictions. For IC neurons, these high confidence cells express a multitude of genes amongst *Lypd1* that will also factor into the assignment of cells in the rest of the analysis. This may result in some cells that have lower levels of *Lypd1* but high levels of other IC genes being classified as IC.

• Throughout the manuscript, there is an unstated assumption that the genes for the knockout models are exclusively expressed only in hair cells, and are never expressed elsewhere in the cochlea e.g. spiral ganglion or surrounding supporting cells. The authors should provide evidence that these genes are never expressed (even transiently) in spiral ganglion or surrounding supporting cells at any point during development or the postnatal period, either through new data or by referencing previously published data. If hair cell-restricted expression of these specific genes cannot be confirmed, the impact of this on their findings and drawn conclusions should be acknowledged and discussed.

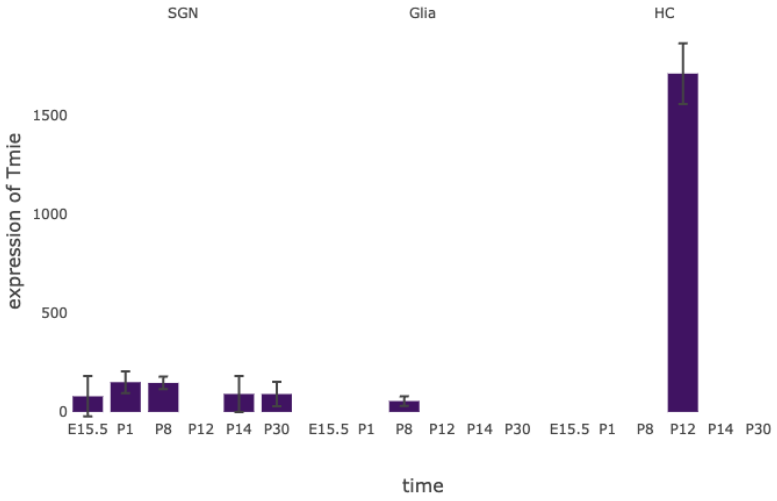
Based on data in previous publications, it appears likely that the majority of genes for the knockout models used within this study are largely limited in their cochlear expression to hair cells:

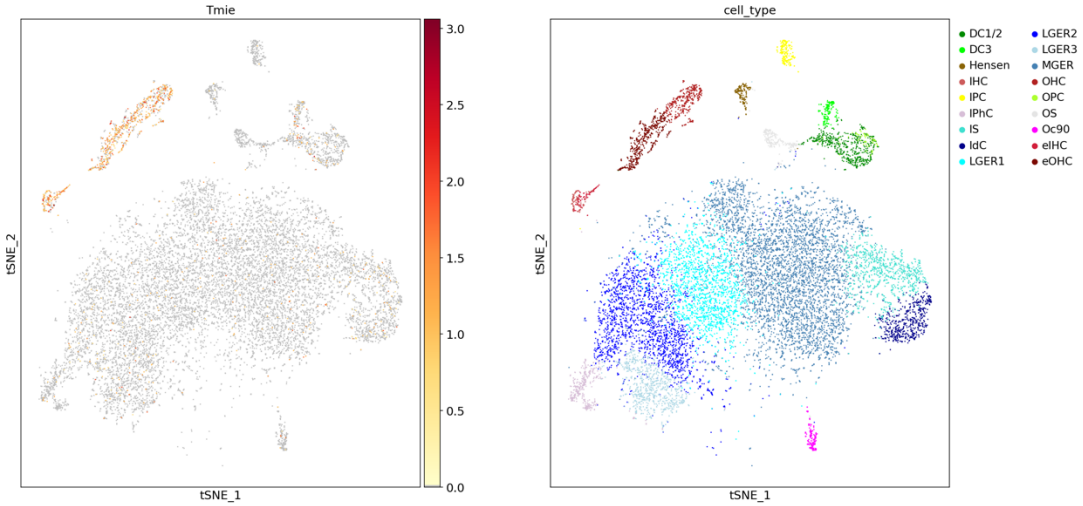
1. **Tmie**- *Tmie* LacZ knock-in mice used for localization show *Tmie* expression only in hair cells as early as P1 (Zhao et al., 2014. *Neuron*). Similarly, *Tmie*-HA C terminal tagged mice show TMIE-HA protein expression exclusively in stereocilia and cell bodies of IHCs and OHCs. (Cunningham et al., 2020. *Neuron*).
2. **Pdch15**- *Pdch15* gene expression (via ISH) was detected in developing sensory epithelium in the mouse embryonic cochlear duct (Murcia and Woychik, 2001. *Mechanisms of Development*). Pdch15 protein expression (via immunofluorescence) was found to be localized to the stereocilia of IHCs and OHCs between E16-P21, with some expression found in the cuticular plates and cell bodies (Ahmed et al., 2003. *Human Molecular Genetics*). Pdch15 protein expression (via immunofluorescence) was shown to localize specifically to the lower tip link of hair cell stereocilia (Kazmierczak et al., 2007. *Nature*)
3. **Vglut3 *(Slc17a8)***- Vglut3 protein expression (via immunofluorescence) was not detected anywhere in the mouse organ of Corti except in IHCs between E15-P21 (Seal et al., 2008. *Neuron*). *Vglut3-P21-iCreER* mice crossed with *Ai14* reporter show recombination in IHCs and no recombination in SGNs at any age tested. However, there is recombination in cochlear glia at early ages which decreases with age (Li et al., 2018. *Hearing Research*).
4. **Clrn2**- *Clrn2* is a more recently discovered gene involved in hearing and data for endogenous localization in the mouse cochlea has not yet been produced. Using an injectoporation approach to drive overexpression of GFP-tagged Clrn2 in cochlear explant cultures, Clrn2-GFP was found to be trafficked to the stereocilia of hair cells (Dunbar et al., 2019. *EMBO Molecular Medicine*), however this result may not mimic endogenous conditions.

In addition to previous publications using *in situ* cochlear detection of the aforementioned genes and their protein products in mice, we used the UMgEAR database (umgear.org) for exploring RNA sequencing datasets of the mouse inner ear. For UMgEAR, two main datasets were used for expression analysis- Kolla et al., 2020. *Nature Communications* (single cell RNAseq of P1 mouse cochlear epithelium) and Li et al., 2020. *eLife* (bulk RNAseq of mouse SGNs at E15.5, P1, P8, P12, P14, P30, plus cochlear glia at P8 and hair

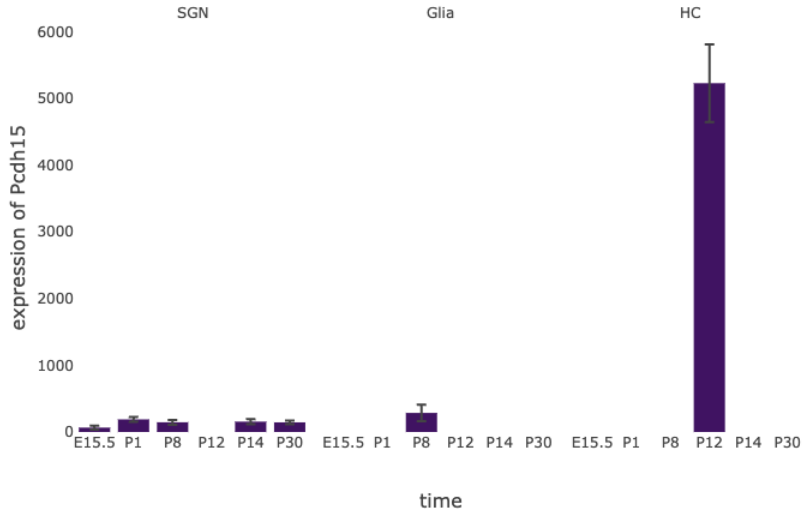
cells at P12). Below are attached images of plots derived from these data sets for each of the 4 genes listed.

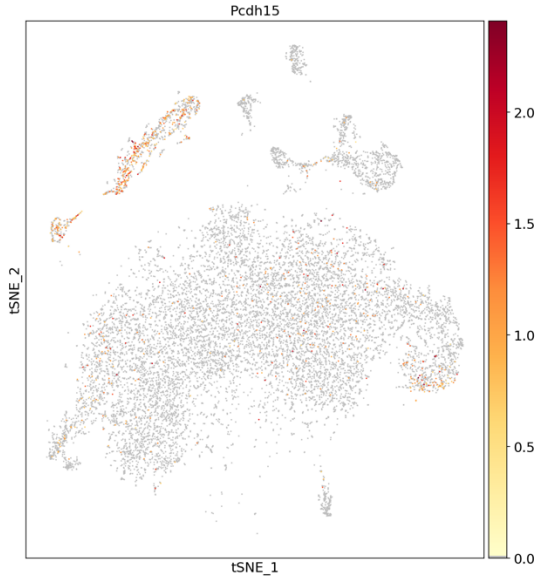
Tmie:

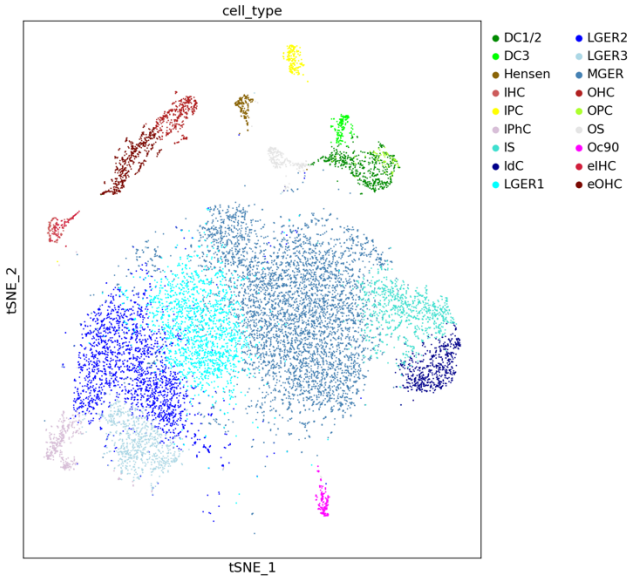




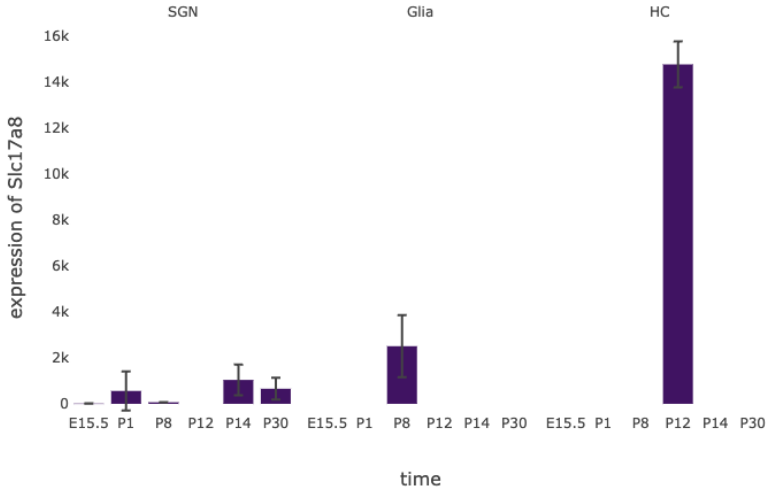
Pdch15:

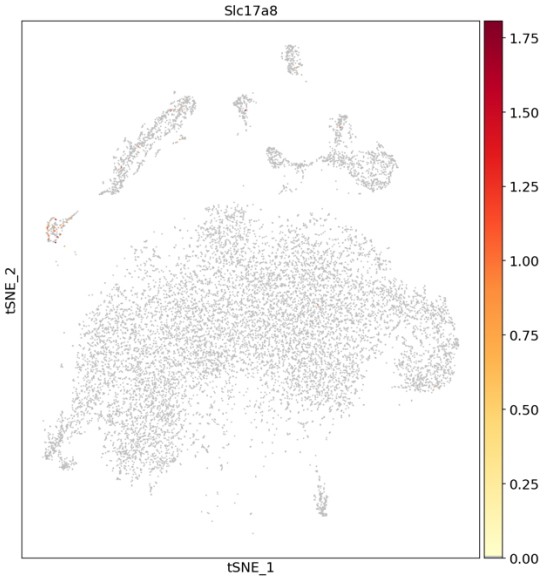

SGN
Glia
HC
6000
5000
4000
3000
2000
1000
0
expression of Pcdh15
E15.5 P1 P8 P12 P14 P30 E15.5 P1 P8 P12 P14 P30 E15.5 P1 P8 P12 P14 P30
time

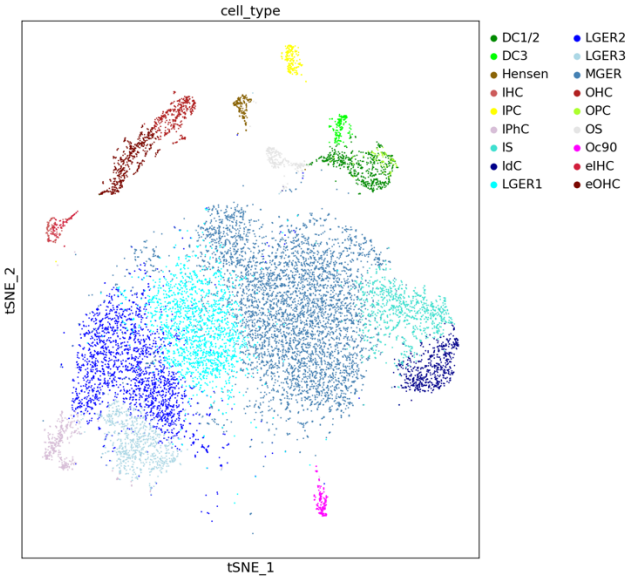




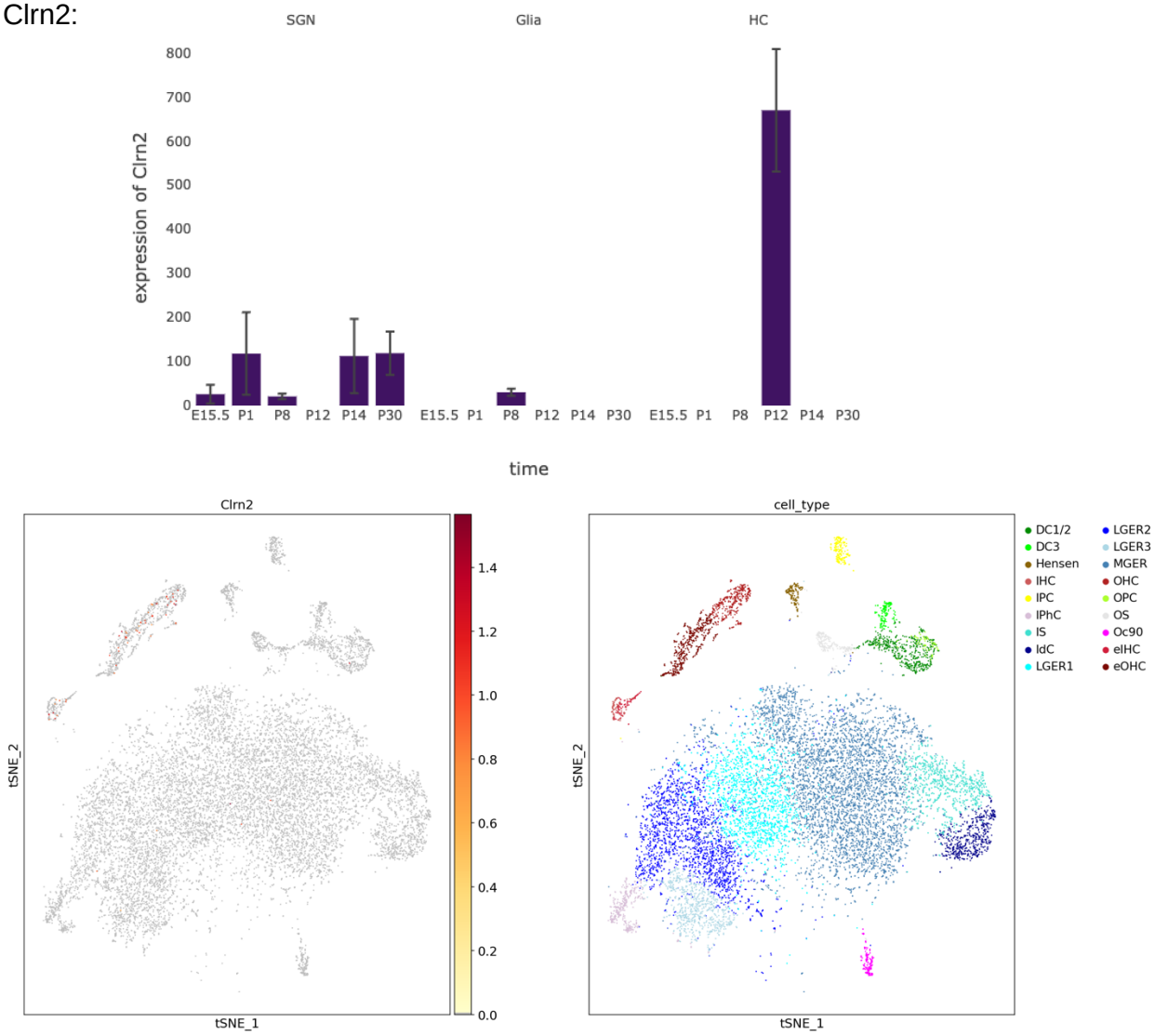
Vglut3 (Slc17a8):







Clrn2:



These plots exemplify that high specificity of transcripts are detected for each of these genes to both IHCs and OHCs or only IHCs (for *Vglut3/Slc178a*). Although we cannot unequivocally rule out mRNA or protein expression in other cochlear cell types, the *in situ* data collated above and gene expression data shown here strongly support the conclusion that loss of these 4 genes in hair cells largely drive the phenotypes described within this manuscript.

However, to further support our findings, we also have generated hair cell specific conditional knock-outs for *Tmie* as described below.

• Indeed, given the emphasis of the manuscript, please mention why hair cell-specific conditional knockout mice were not used in these experiments, rather than the standard conventional knockout mice that were used. Using hair cell-specific conditional knockout mice would help support the conclusion that correct hair cell function is critical for correct neuronal patterning.

While the expression of MET components used for genetic knock out mice used in this study are largely limited to hair cells (see above comment), we agree that hair-cell specific deletion of these genes would provide stronger evidence that loss of gene expression in hair cells causes the observed phenotype. We therefore generated hair-cell specific knock-outs for *Tmie*. To accomplish this, we crossed two previously described mouse lines: 1. *Gfi1*-GCE mice; a tamoxifen-inducible driver line with *CreERT2* restricted to hair cells in the cochlea (Tang et al., 2019. *Genesis)* and 2. *Tmie*^{FL} mice; a line carrying a floxed allele at exon 5 of the *Tmie* gene (Zhao et al., 2014. *Neuron*). This allowed for time-specific deletion of *Tmie* exclusively in cochlear hair cells. Using these mice, we found that neonatal deletion of *Tmie* only in hair cells phenocopied defects in SGN projections (using NGFR staining) and synaptic development (using CTBP2 and GLUR2 staining) seen in *Tmie*$^{-/-}$ mice. Overall, these new data (now shown in revised Fig. 6) support the conclusion that loss of Tmie expression in hair cells causes the defects in hair cell innervation by SGNs.

• The authors present ribbon synapse data, in the form of anti-Glur2/3 and anti-Ribeye immunofluorescence labelling, imaging and counts, for all of their mutant mice except the Pcdh15av3J model. However, the authors then go on to compare the degree of severity of the synaptic phenotype observed in the Pcdh15av3J model. Given this comparison, I feel the synaptic phenotype data for the Pcdh15av3J model should be included in the manuscript.
Synaptic data (stains for GLUR2 and CTBP2) were acquired and quantified in P21 *Pdch15 av3J* mice (revised Fig.7D, E). These new data reveal similar effects due to *Pdch15* loss as seen in *Tmie*$^{-/-}$ mice.

• Line 313: The authors state they have produced a new Clrn2 knockout mouse model, which phenocopies a previously reported Clrn2 knockout. The authors then utilise this model for their studies. However, the authors provide no information or data regarding the generation or preliminary phenotyping of this new model. To allow critical assessment, information regarding the generation and phenotyping of this line needs to be included in this manuscript. 'Data not shown' is not acceptable.
Clrn2$^{-/-}$ mice were generated using CRISPR/Cas9 technology to delete a 48bp region of the *Clrn2* allele spanning portions of the 5'UTR and exon 1, including the start codon. Detailed information regarding the generation of the mice (including target-specific crRNA sequence) is now included in the Methods section. Additionally, confirmation of the MET current reduction phenotype in *Clrn2*$^{-/-}$ hair cells previously reported (Dunbar, *et al*. 2021. *EMBO Mol Med*) is now included in new Supplementary Data 6.

• Line 334-335: The authors state there are no "obvious differences in NGFR+ SGN projections in Vglut3-/- mice". However, these data are not quantified. The authors have quantified similar staining for their other mutants, therefore this should be undertaken for the Vglut3-/- model.
We have now quantified NGFR staining data in *Vglut3*$^{-/-}$ mice. The results are now shown in revised Fig. 8E.

• Line 359: Please correct the sentence. Apical is low-frequency and not high-frequency as stated. Similarly, basal is high-frequency and not low-frequency as stated.
We apologize for this avoidable mistake. Corrected.

• Figure 8: Are the improved low frequency ABR thresholds of the Tmie-/- mice injected with AAV.PHP.eB.TMIE::EGFP preserved over time? Or do they worsen with time, and become similar to the thresholds observed for the Tmie-/- mice injected with AAV.PHP.eB.EGFP?
To address this, we injected a new set of *Tmie*$^{-/-}$ mice at P1 with AAV.PHP.eB.EGFP, tested ABRs at P21 (original timepoint), then re-tested the same mice for ABRs at P42 (3 weeks later). We found that the improved ABR thresholds in response to click, 8 kHz, and 12 kHz stimulation seen at P21 were preserved at P42, with paired t-tests showing no significant differences between subjects at the two ages. The results of these analyses, as well as the thresholds across all frequencies tested are now described in the text and plotted in Supplementary Data 7 D, E.

Of note, we are still keeping these mice and will conduct further recordings at later ages but did not want to hold up the manuscript any further. We can add later time points during the time when the manuscript is undergoing review of the revisions and would add a later time point if the manuscript is accepted for publication.

• Figure 8: The authors suggest the recovery of MET function and synaptic defects are correlated with the efficiency of AAV delivery to the hair cells, rather than partial recovery of function in transduced hair cells. Given that not 100% of IHCs and OHCs are transduced in the Tmie-/- mice injected with AAV.PHP.eB.TMIE::EGFP, I feel the authors should ascertain whether there are significant synaptic differences between EGFP+ve and EGFP-ve hair cells within the same cochlear sample.
In response to the reviewer's suggestion, we have now carried out the experiments. We quantified CTBP2 and GLUR2 staining in both AAV.TMIE.GFP^{+} and AAV.TMIE.GFP^{-} IHCs of *Tmie*$^{-/-}$ mice (injected at P1) at P21. We found significant differences between GFP^{+} and GFP^{-} IHCs in colocalized CTBP2/GLUR2 synaptic puncta number and percentage (revised Supp Fig. 7J-L) based on whether individual cells were positive for AAV.TMIE transduction or not. These results suggest that recovery of individual cell synapse properties using AAV.TMIE acts in a cell-autonomous manner.

• For the AAV rescue study, why were injections undertaken using a 3-day window (P1-P3)? This could be a variable for the results achieved. Please determine the effect achieved by age of injection.
For the initial experiment, injections were undertaken using a 3 day window due to availability of animals and research materials. The majority (5/7) of *Tmie*$^{-/-}$ mice were injected with AAV.TMIE at P1 and the low numbers of mice with AAV.TMIE injections at P2 (1/7) and P3 (1/7) did not allow for statistical comparison of age of injection on effects. However, the addition of new mice for longitudinal study of ABR rescue (above) were all injected at P1, adding statistical power to this age of injection. Thus, we decided to remove all included data points (in ABR, transduction efficiency, synaptic quantification, NGFR quantification) from the two mice injected at P2 and P3 (and their control littermates) to maintain consistency of age of injection (only P1).

• Line 400: The conclusion that 'the findings suggest that the magnitude of SGN defects scales with the magnitude of functional impairment of hair cells' does not take into account anything to do with the temporal onset of expression of the genes that have been used to develop this statement. For the statement to be true, the authors need to compare the onset of MET dysfunction in the different models and show that they all onset at the same age point. If there is a difference in the age of onset, the differences in neuronal patterning could just be a reflection of the effects occurring at different stages of developmental maturation.

For instance, perhaps expression of Tmie and Pcdh15 are required earlier than Clrn2. Again, by using a hair cell-specific conditional Tmie knockout mouse and inducing recombination at different late gestational/early postnatal ages, the authors would be able to investigate for a temporal effect.
We agree that it would be interesting to investigate temporal effects of MET inactivation using conditional knock-out mice. However, we find that this is beyond the scope of the current manuscript, which aims at demonstrating that defects in hair cell function can affect their innervation by SGNs. Of note, all mouse models that we are studying here have been extensively characterized for their defects in MET (by us and others) and they lack transduction (Tmie, Pcdh15) or have reduced transduction (Clrn2) already in the first few postnatal days when MET channels start to mature (Algramam et al. 2001., *Nature Genetics*; Zhao et al., 2014. *Neuron*; Dunbar et al., 2019. *EMBO Molecular Medicine*). Thus, there are not significant differences in the time of MET deficiency in the mouse lines under study. However, we agree that it is difficult to conclude from three mouse models that there is a scaling effect. We therefore have revised the text to note this as a possibility but that further studies will be necessary to clarify this point.

• Please adhere to the ARRIVE guidelines, there is no mention of the genetic background of any of the mice utilised in these studies.
Genetic backgrounds of all mice used in the study is now stated in the “Animal models” section of the Methods.

• Furthermore, please explain why only male mice were used for the sc-RNAseq study.
Single cell RNA sequencing studies are commonly carried out in one sex to avoid potential differences in gene expression between samples being confounded by sex-specific gene expression differences. For example, in principle component analysis, sex-specific differences can drive clustering of cells. We learned this ourselves the hard way. In an unrelated experiment, we have sequenced RNA from SGNs from wild-type and mutant mice at P0 and initially had not paid attention to sex. We subsequently noticed that by chance all wild-type animals were male, while all mutants were female. Sex-specific difference severely skewed our analysis and large expression differences in gender specific genes obscured our principle component analysis and top differentially expressed genes. We had to redo the entire experiment by focusing on one sex only.
One might argue that we should conduct our analysis separately in males and females but opted to focus for cost reasons to focus on one sex but then analyze expression changes and phenotype in both males and females.

• Moreover, the statement that ‘both sexes were used, and male and female numbers were used in approximately equal numbers’ is too ambiguous. This could mean that all controls were female and all test mice were male, which would be a failure of experimental design. Please clarify the statement.
Our text was ambiguous. We routinely track sex of animals and ensure that we study animals of both genders as controls and experimental animals. We have revised the text accordingly.

• In line with the journal’s policy, t-values and degrees of freedom should be provided alongside p values for t-tests.
T-values and degrees of freedom have now been provided for all t tests and are stated within figure legends.