**Point-by-point response to reviewers’ comments**

Thank you for your valuable suggestions on our manuscript (JoVE55054). We found your comments extremely helpful in improving and enriching our manuscript. We agree with all the points presented. However, we would like to address one issue differently than you suggested and explain this in a separate sheet at the end.

*Editorial comments:*

*1. Thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

A professional scientific editor has checked spelling and grammar of the manuscript.

*2. Define all abbreviations before use.*

We defined the following abbreviations:

[3.1.2] paraformaldehyde (PFA)

[3.1.3] ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-2Na).

3. 3.1.6: What is the series of ethanol dehydrations?

We now state this series as follows:

[3.1.6] (70%, two changes of 95%, three changes of 100%, each 1 hr).

*4. Formatting:*

*-Define all abbreviations at first occurrence (ie PFA).*

We have defined all abbreviations.

*-3.1.2 – PFA is toxic and requires a caution statement.*

We have added the following:

[3.1.2] Caution: PFA is toxic and should be handled carefully.

*-3.2.4 – Refer to Section 4 rather than “Step 4”.* Corrected.

*-Line 280 – Remove the stray underlined text.* Removed.

5. Grammar:

-Copyedit the manuscript for grammatical errors. Such editing is required prior to acceptance, and some errors are noted below:  
*-Correct the title and the short abstract so that appropriate articles are used (ie “to isolate the auditory bulla…”).*

We inserted the article “the” in the title and the short abstract:

[Title] Dissection of the Auditory Bulla...

[Short abstract] to isolate the auditory bulla

*-1.5 – “from the mouth”* Corrected to “into the mouse”.

*-3.1.2 – Delete “by sucking it out”* Deleted.

*-3.1.4 – “every other days”* Corrected to “every other day”

-4.1.1 –Clarify “place the tympanic membrane horizontally to be parallel to”.

We have changed this wording to:

[4.1.1] Adjust the orientation so that the neck and transversal lamina of the malleus are parallel to the horizontal bottom of the embedding dish (Figure 4A-C).

6. Additional detail is required:

-3.1.6 – Include a citation.

We cite An et al. (2003) Principles of Embedding and Common Protocols.

*-4.2.1 – Clarify the orientation in text rather than citing only a figure.*

We replaced “according to Figure 4F-G” with:

[4.2.1] so that the neck and transversal lamina of the malleus are perpendicular to the bottom of the embedding dish (Figure 4D-G).

*-4.5 – Include a citation.*

*Include a step at the end of the protocol for downstream analyses and include citations. This step should not be highlighted for filming.*

We have added information relevant to downstream analyses and cited the “Handbook of Histology Methods for Bone and Cartilage” (2003) for histological methods, “Reference 14” for bone labeling, and “Reference 3 for TRAP staining:

[4.5] For example, stain paraffin sections with hematoxylin and eosin (H&E), safranin O (for cartilage), or for tartrate-resistant acid phosphatase (TRAP) activity (for osteoclasts)3, or by immunohistochemistry. Undecalcified cryosections are suitable for bone labeling using fluorochromes 14, alizarin red staining for calcium, and immunofluorescence 42.

*7. Results:  
-Describe the data in Figure 5 in more detail. Are there any important features highlighted by the staining?*

We have added the following description:

[Representative results] The malleus attached to the tympanic membrane in the auditory bulla revealed ongoing endochondral ossification at P14 (Figure 5A). For bone labeling, calcein (30 µg/g bodyweight) was peritoneally injected into a P20 mouse, and bulla and capsule were isolated 24 hr later at P21. The sample without decalcification was embedded frozen and then cryosectioned at 6 μm using an adhesive film based on the method of Kawamoto 43. After nuclear staining with DAPI (4’,6-diamidino-2-phenylindole), the section was observed under a fluorescence microscope. Calcein signals (green) revealed new bone formation in the malleus (m), bulla and capsule (Figure 5B).

Horizontal sectioning of the malleal processus brevis (mPB) also shows the cochlea (Figure 5C).

*-Line 238 – A movie (Movie 1) has been cited; however, no movie has been provided for evaluation nor is there a corresponding figure legend for it. Please remove this citation.*

We now provide Movies 1-3, and inserted citations into protocols.

8. Discussion: Discuss the significance with respect to alternative methods, the limitations, and any troubleshooting/modifications that can be performed.

We now discuss the significance and troubleshooting as follows:

[Discussion] Dissecting the bulla from the head before sectioning has several advantages. First, postnatal cavitation and growth of the auditory bulla occurs most actively from P6 onwards and is complete by P14 50. The mesenchymal tissue between the tympanic membrane and cochlear wall is replaced by air through the cavitation process. Resultant air in the middle ear cavity can impede contact between tissues and liquids during fixation, decalcification and embedding. It is easier to remove air from the isolated auditory bulla by cutting off the anterior end (styliform process) rather than trying to do so in the unisolated bulla. Second, orientation of the malleus (and the tympanic membrane) is not vertical in the head. It is therefore easier to section the malleus in desired planes by embedding the isolated auditory bulla and capsule in a given orientation.

Cryo-sectioning conditions should be optimized according based on mouse age. For example, a less cool temperature inside the cryostat chamber is recommended for older mouse specimens to minimize damage to sections.

**Reviewers' comments:**

**Reviewer #1:**  
*Manuscript Summary:*  
*The authors deploy 6 figures to convey a dissection technique of the murine auditory bulla and the middle ear ossicles as well as tissue orientation guidelines for frozen and paraffin sectioning of the middle ear. A comparison of the mouse and human middle ears noting differences and similarities is also presented. The attention to anatomical detail is a particular strength of the manuscript.*  
  
*Major Concerns:  
Manuscript Title: The auditory bulla and ossicles are dissected but there is no dissection of the otic capsule as such. The title should be refined to state precisely the methods communicated. It would be ideal to include some hint about the histological approach offered as well. Something like: "Dissection of the auditory bulla in postnatal mice: isolation of the middle ear bones and histological analysis in two planes."*

Based on your suggestion, we changed the title to:

[Title] Dissection of the Auditory Bulla in Postnatal Mice: Isolation of the Middle Ear Bones and Histological Analysis

*1) Abbreviations in the legends: The authors list all of the abbreviations at the end of the legends but use abbreviations before formally introducing the term. The present format makes it difficult for the reader to follow the procedural step without shifting down to the abbreviations and then to the figure; the focus should be on assimilating the information in the figure, not tracking down abbreviations. Define the abbreviations on first use, do so dynamically, and then list only those abbreviations that were not referred to directly at the end of the legend.* Done.

*2) L111: IACUC issue: As written, it appears that the mouse is added to paper towels soaked in Isoflurane, which is not appropriate technique since anesthetic saturated paper towel could come into direct contact with the mouse and irritate mucous membranes causing pain. The mouse should be placed on a platform above the paper towels avoiding direct contact with the isoflurane soaked paper towels. The authors should clarify this critical issue.*

In the revision, we now state the following:

[1.1] Euthanize mice in a jar containing a platform above paper towels soaked in isoflurane or sevoflurane until respiratory ventilation ceases for more than a minute and then perform cervical dislocation. Be careful to avoid direct contact of mice with the soaked paper towels.

*3) L133. This sentence instructs us to dissect out the bulla and capsule together with surrounding tissues. But how? This is effectively the title of the manuscript but the precise dissection technique is not articulated. More descriptive input should be included here. Where do we put our forceps to perform this dissection?*

We described the procedure in the following passage, which is included in the revision:

[1.10] Under a binocular dissecting microscope, use forceps to pull apart the surrounding bones and scissors to cut the loosened boundary around the bulla and capsule (Figure 1F, Movie 1). The surrounding bones removed are the basioccipital (ventral border), exoccipital (ventro-posterior border), supraoccipital (posterior border), interparietal, parietal (dorsal border), squamosal (dorso-anterior border), alisphenoid (anterior border), and basisphenoid (antero-ventral border) bones.

*4) Section 1: the methodological steps need to be precisely linked to the figures and panels that help the reader understand the step articulated. A major weakness is that the reader needs to figure out for herself what the authors are trying to communicate instead of being led directly to the useful panel. The entire procedural section should carry a figure number and panel at each major instruction. The figures have this detail, but the prose does not lead us there effectively.*

[Protocol] We have inserted figure numbers into each major instructional section.

*Minor Concerns:*  
*Abstract: last sentence: what does "various aspects of auditory ossicles" mean here? Be precise in the value of this work.*

[Long abstract] We replaced “various aspects” with “pathological, developmental and evolutionary aspects”.

*L64: fix "opto"* Corrected.

*L65-68: This OM discussion is not well linked to the preceding ideas. Work middle ear infection into this paragraph better by perhaps noting how frequently OM brings kids into the ENT office! Make the role of the middle ear vital to the reader; OM is an enormous clinical problem that uses critical clinical resources to address. So your method could be useful for interrogation of animal models of OM.*

Based on the reviewer’s comment, we revised the following paragraph of the introduction:

[Introduction] Animal models of ear conditions are needed, given the importance of hearing and ear health to the well-being of patients of all ages. For example, otitis media is an extremely common ear infection seen in human infants and children, and severe, acute otitis media and its complications can occur if the condition is not treated with appropriate antimicrobials 9. Mouse models of otitis media could prove useful in understanding the pathogenesis and in developing treatments 10,11.

*92-96: Do not use "it" serially here. Also, this summary paragraph is precisely the rationale for the new title: isolation of ossicles and histological sections.*

[Introduction] We replaced “Secondly, it demonstrates” with “Secondly, this protocol demonstrates....”

*L115: neck muscle tissue*

[1.2] We inserted the word “neck”, as requested.

*L117:decapitate not "cut off the head". How large are the scissors? Are they sharp? Give us relevant details.*

We now provide relevant details as follows:

[1.3] Decapitate mice at the cervical region using 14-cm sharp surgical scissors.

*L166: how do we remove the stapes? Where do we grab it safely? Which direction do we pull in?*

We now provide the following information:

[2.2.2] Insert a sewing needle (or a marking pin) into the obturator foramen of the stapes and lift up the stapes.

*L181: every other day* Corrected.

*L183: Store in 70% ethanol but did we get from aqueous solution to 70% ethanol by graded ethanols to 70% ethanol? Is the 70% ethanol made in PBS?*

We now provide the following information:

[3.1.5] Optional: Transfer to 70% ethanol through graded alcohol series (30%, 50%, 70% in water).

*L280: identify the posterior cranial fossa in Fig. 1*

[Figure 1] We have now labeled the posterior cranial fossa (pcf).

*L280: there are small black arrows that are unidentified.*

We are sorry but we do not see black arrows in this figure; possibly the reviewer is mistaken about them or could clarify the question.

*L289: Fig. 2 is called "Isolation of the malleus" but it is never truly shown isolated from the middle ear until Fig. 6. Make the figure title accurate. Dissection of the malleus?*

We have now changed the figure title to:

[Figure 2 title] Dissection of the malleus.

*L304: Dissection of the incus and stapes?*

We have now changed the figure title to:

[Figure 3 title] Dissection of the incus and stapes.

*L307: the scissor icon is hard to see; outline in black.*

[Figure 3B] We have replaced the icon with a “needle tip” icon.

*L313: the dashed lines referred to are not present. The arrowhead is not defined? Also, why are the bullae in A,D,F green? The green middle ear bones n B,C,E,G need to be labeled in some way so we can use this anatomical information to understand the required orientation. Also, the microCT image should be labeled so we have some common anatomical landmarks for appreciating the orientation required. We need to be able to repeat this method and get the results shown in the representative data section and this figure is not properly rendered to achieve this goal.*

We have extensively revised the figure legend and improved Figure 4. We now also state that the bullae are green because the photo was taken with a color filter:

[Figure 4 legend] **Orienting the auditory bulla and capsule during embedding for longitudinal (parasagittal, A-C) and horizontal sectioning (D-E) of the malleus. (A-C)** The neck and transversal lamina of the malleus are placed parallel to the bottom of embedding dish. **(A)** Side view: micro-CT image to show embedding of the right malleus in the bulla (pseudocolored blue). The malleus and incus are pseudocolored green. Dashed line, the desired cutting plane. Solid line, bottom of embedding dish. m, malleus; arrowheads, dorsal crest. M, medial; L, lateral; D, dorsal; V, ventral. **(B)** Top view: Micro-CT image. Note that the anterior end of the bulla (styliform process) was removed.i, incus. **(C)** Top view: micrograph (taken with a color filter). AC, anterior (superior) semicircular canal; Sf, subarcuate fossa; Sp, styliform process. A, anterior; P, posterior; D, dorsal; V, ventral. **(D-F)** The processus brevis of the malleus is placed perpendicular to the bottom of embedding dish. **(D)** Side view: Micro-CT image to show embedding of the right malleus. Dashed line, the desired cutting plane. Solid line, bottom of embedding dish. **(E)** Top view: Micro-CT image. mM, malleal manubrium. **(F)** Top view: micrograph (taken with a color filter). Scale bars, 1 mm. Micro-CT images were obtained at a voxel resolution of 5 m, as previously described 7

*L326: Add to each panel the animal age and method of preparation. P6 Longitudinal Paraffin added to panel A for example.*

[Figure 6] Information relevant to age, method, orientation, and staining (labeling) has been added to each panel.

*L333: Remarkably, the mouse ossicles in panel B are not mentioned and should be. Presumably they were imaged at the same magnification as the human ossicles.*

We have now added the following information:

[Figure 6B legend] Ossicles of P31 mouse are imaged at the same magnification as that used for human ossicles.

*L276: Panels E and F should be oriented so that their angles match precisely. Cochlea might be abbreviated CO and CC could be used for common crus.*

[Figure 1] We have added an orientation symbol to Panel F (rather than rotating the image). We now abbreviate cochlea as Co.

*L289: Adjust the brightness, contrast, and midtones of Panel H so there is better delineation of the 3 ossicles.*

[Figure 2] We have replaced Panel H with a new photo to better delineate the ossicles.

**Reviewer #2:**  
*Manuscript Summary:*  
*This is a well-written description of how to isolate the bulla and ossicles. The information about embedding the bulla for sectioning could be very useful but needs some clarification.*  
  
*Major Concerns:*  
*-The cryostat images (Fig 5B,C) are not very nice. Figure 5B was stained with alizarin complexone but this looks like H & E. A better example should be shown or Alizarin red should be used. The difference in angle between 5A and B is also quite minor (we can see the orbicular apothesis and part of the manubrium of the malleus in both indicating a similar angle). Are the authors sure they have orientated the samples correctly?*

[Figure 5B] Based on your comments, we replaced Alizarin red staining with calcein bone labeling. We also now provide information relevant to orientation in each panel of Figure 5.

*-What are the planes of section referring to? It would be helpful if they were the positions of the bulla in the head, which they don't appear to be.*

In the revision we have improved explanation of the planes in both Figures 4 and 5.

*What is the benefit of dissecting the bulla from the head before sectioning? This should be mentioned.*

We now discuss the benefit of dissecting the bulla before sectioning in the following passage:

[Discussion] Dissecting the bulla from the head before sectioning has several advantages. First, postnatal cavitation and growth of the auditory bulla occurs most actively from P6 onwards and is complete by P14 50. The mesenchymal tissue between the tympanic membrane and cochlear wall is replaced by air through the cavitation process. Resultant air in the middle ear cavity can impede contact between tissues and liquids during fixation, decalcification and embedding. It is easier to remove air from the isolated auditory bulla by cutting off the anterior end (styliform process) rather than trying to do so in the unisolated bulla. Second, orientation of the malleus (and the tympanic membrane) is not vertical in the head. It is therefore easier to section the malleus in desired planes by embedding the isolated auditory bulla and capsule in a given orientation.

*-The orbicular apothesis is not the processus brevis, this is a different structure (see papers by Mason).*

Please see separate response sheet at the end.

*Minor Concerns:*  
*-Method wise, I would suggest they don't jump from PBS to 70% Ethanol but use a graded series instead.*

We now provide the following information:

[3.1.5] Optional: Transfer to 70% ethanol through graded alcohol series (30%, 50%, 70% in water).

*-Histoclear should be given as an alternative to xylene, as xylene (due to its carcinogenic nature) is not allowed in many labs in Europe.*

We now provide the following information:

[3.1.6] Optional: Substitute xylene with Histo-Clear, which is a non-toxic and non-flammable histological clearing agent.

*-The dotted lines outlining the different components of the EAM are based on what? Are the authors sure they have the delineations correct?*

We replaced the photo in question and have improved the Figure 2A legend to read:

[Figure 2A legend] The sulcus tympanicus (ST, dashed arrow) is the attachment site of the tympanic membrane. The bone lateral to the ST is part of the external ear, and the bone medial to the ST forms the floor of the middle ear cavity.

*-The authors mention that it is difficult to isolate the bulla before P12. This should be correlated with known descriptions of bulla development (Richter et al etc). The authors should also refer to the timing of cavitation.*

We now discuss this point as follows:

[Discussion] First, postnatal cavitation and growth of the auditory bulla occurs most actively from P6 onwards and is complete by P14 50. The mesenchymal tissue between the tympanic membrane and cochlear wall is replaced by air through the cavitation process. Resultant air in the middle ear cavity can impede contact between tissues and liquids during fixation, decalcification and embedding. It is easier to remove air from the isolated auditory bulla by cutting off the anterior end (styliform process) rather than trying to do so in the unisolated bulla.

*-There are a few typos in the Table of materials  
For example Eosin, containers, stainless, Haematoxylin.*  
[Table of materials] These have now been carefully checked.  
  
**Reviewer #3:**  
*Manuscript Summary:*  
This was a well-written and generally clear paper which explains how to access and view a small but important part of the anatomy of mice. I can see it being useful to auditory researchers who are starting in this field. The good-quality photographs were useful aids which illustrate the techniques.  
  
*Major Concerns:*  
My three main comments for improvement of this paper are all relatively minor and easy to implement:  
*1) In the methodology section, the step-by-step instructions should be explicitly linked with the figure panels showing those stages, where applicable. This would make it easier for the reader to see where he/she is in the process.*

Done.

*2) Although the developmental biology literature has used the term "processus brevis" to mean "orbicular apophysis", this is a mistake which has simply been repeated in several papers. As explained in more detail below, the term "processus brevis" should be removed from this paper (as the true processus brevis is not referred to or illustrated) and the text and figures should refer consistently to the orbicular apophysis.*

Please see separate response sheet below.

*3) Micro-CT imaging is mentioned in this paper and CT-derived images are presented in Figure 4. However, the paper does not describe how these scans were made and processed.*

We now provide micro-CT method:

[Figure 4 legend] Micro-CT images were obtained at a voxel resolution of 5 µm as previously described (Kanzaki et al, 2011).

*Minor Concerns:*  
*Line 42: The auditory ossicles are usually the smallest bones in the body, but this is not true in all mammals. See, for example, the relatively enormous ossicles of golden moles, as discussed in Mason, 2013 (already on the reference list).*

We have now changed the Abstract and the Discussion to state:

[Abstract] In most mammals, … the smallest bones

[Representative Results] It is worth noting that the malleus head relative to body size is massively enlarged in species such as the golden mole, demonstrating significant variability in allometric relationships of “the smallest” bones (Mason, 2013).

*Line 64: "optoacoustic" should read "otoacoustic".* Corrected.

*Line 70: Although it is true to say that most of the auditory ossicles are formed by endochondral ossification, this is not true of the goniale, as the authors acknowledge on line 271. The goniale is considered a part of the malleus. Here's a reference:  
Rodríguez Vázquez, J.F., Mérida Velasco, J.R. & Jiménez Collado, J. (1991) A study of the os goniale in man. Acta Anatomica 142: 188-192.*

We have now inserted the following information:

[Introduction] except for the goniale part of the malleus (Rodriguez Vazquez, 1991; Tucker, 2004)”.

*Line 109: As well as putting a whole figure reference here, particular numbered steps should be explicitly linked to the appropriate parts of Figure 1. For example, step 1.7 seems to equate to panel 1A, and it should say so. This applies also to Figures 2 to 5. See major comments.* Corrected.

*Line 112: "respiration" should be replaced with e.g. "respiratory ventilation".*

Corrected.

*Line 131: "further underneath (lateral to)". "Underneath" would normally be taken to mean "ventral to", so how does this equate to "lateral to"? This needs rephrasing.*

[1.8] We have now replaced “underneath” with “lateral to”.

*Lines 146, and 254-5. The term "processus brevis" is NOT synonymous with "orbicular apophysis": the two are different processes (see major comments above).  
To expand on this, the term "processus brevis" has been widely used - wrongly - in the developmental biology literature to refer to a prominent process of the mouse malleus, the one labelled in the current paper (e.g. in Fig. 6A). However, this term seems to have been borrowed, incorrectly, from the human anatomical literature. The "processus brevis" in humans is also known as the "lateral process", a projection at the base of the manubrium. Mice have this too. However, the authors are describing and labelling in their figures the "orbicular apophysis", a different process which has no equivalent in humans. See Mason (2013) for a discussion and diagrams. All uses of "processus brevis" in this paper should be changed to "orbicular apophysis", in text and figures. Mason (2013) can be cited as a reference, to avoid confusion.*

We understand this point. However, we would like to use both “processus brevis” and “orbicular apophysis”. Please see the separate sheet relevant to this issue below.

*Line 173, 196, 235: "Styliform process" could perhaps be mistaken by those familiar with the human ear as the "styloid process", but the authors are actually referring to the processus styliformis, a little process of bone contributing to the Eustachian tube. This should be made clearer. N.B. "styliform" is an adjective so always needs to be paired with a noun (i.e. always write "the styliform process", not just "the styliform").*

[1.10] We have added, “Note that the styliform process (Sp), which supports the tympanic opening of the Eustachian tube 41, is distinct from the styloid process of the temporal bone.”

[3.1.2],[ 3.2.2] “the styliform” was replaced by “the styliform process”.

*Line 181: change "every other days" to "every other day".* Corrected.

*Lines 215-6: The method discussed here suggests that the tympanic membrane should be parallel to the bottom of the embedding dish, in order to get sagittal sections (N.B. this should be "parasagittal", i.e. parallel to the sagittal plane down the midline). This seems to imply that the tympanic membrane in mice is oriented in the parasagittal plane. However, it is in fact inclined at an angle of maybe 20-30 degrees to the vertical, according to the diagram in van Kampen (1905: p.553). Has this orientation been taken into consideration?  
van Kampen, P.N. (1905) Die Tympanalgegend des Säugetierschädels. Gegenbaurs Morphologisches Jahrbuch 34: 321-722.*

[4.1] Corrected to “Longitudinal (parasagittal) sectioning of the malleus”

We also added, “Note that the tympanic membrane and malleus are inclined at an angle at approximately 30 degrees to the vertical in the head (Figure 4A, Fig. 59 in Kampen, 1905).”

*Lines 222-4: Along similar lines to the above, is the manubrium really exactly horizontal? I suggest that it should be made clear that the planes referred to (parasagittal and horizontal) are only approximate. "Vertical to the bottom" should presumably read "perpendicular to the bottom".*

We explained that the planes are relative to the malleus in Figure 4.

[4.1.1] Note that the tympanic membrane is inclined at an angle at 20-30 degrees to the vertical in the mouse head (Figure 4A, Fig. 59 in Kampen, 1905).

[4.2.1] [4.3.1] Corrected to "perpendicular to the bottom".

Line 238: Movie 1 is not included within the material provided to me as a Reviewer.

We now provide Movies 1-3, and provide references to them.

*Lines 253, 262: Birds have wings of many different shapes and aspect ratios, so this is not a very useful description! The sword analogy is better.*

[Representative results] The “bird wing-like” was replaced by “gliding-seagull-wing-like”.

*Lines 257, 271: "...where the gonium is located". The goniale (which is the more usual term for this ossification) forms part of the anterior process, but in my experience it is impossible to determine where the goniale fuses with the endochondral parts of the malleus. Perhaps these two passages should be rephrased, or alternatively the authors could describe and show more clearly in the diagrams where they believe the union to be.*

We clarified this point in the Figure 2H legend:

[Figure 2H legend] “Go, goniale (fused to the malleus and the tympanic ring)”

*Line 263: The anatomical axis is the line through the anterior process of the malleus and the short process of the incus, as described here. However, many vibrometric studies have shown that this rarely coincides with the true axis of rotation, especially in human ossicles. Some of the references referred to in the text discuss this; see below for another.*

*Willi, U.B., Ferrazzini, M.A. & Huber, A.M. (2002) The incudo-malleolar joint and sound transmission losses. Hearing Research 174: 32-44.*

We now provide the following information:

[Representative results] In human ossicles, vibrometric studies reveal that the incudo-malleolar joint is mobile rather than functionally fixed (Willi, 2002).

*Line 265: should read "and the two are almost parallel".* Corrected.

*Line 265: Maier & Ruf (2016) did not examine mouse and human ossicles, so it is not clear how this paper is relevant here. The authors should go to the original sources for their references.*

We have now replaced Maier & Ruf (2016) with Fleischer (1978).

*Lines 265-6: Noting my earlier comments about this, this statement is incorrect - it is the orbicular apophysis (not the processus brevis) which "is a prominent semi-spherical protrusion in mice, while in humans it is not apparent". The true processus brevis is present in both, representing the projecting root of the manubrium at the point where it inserts into the tympanic membrane at the opposite end to the umbo.*

We agree with this statement. Please see the separate sheet relevant to this issue.

*Line 270: There is no need to put quotation marks around "lamina" here: this is a well-established descriptive term for mouse malleus structure. It is usually called the transversal lamina.*

We now removed quotation marks around “transversal lamina”

*Lines 271-2: It seems odd to say that the tympanic bone anchors the malleus to the skull, when the tympanic bone is normally taken to be part of the skull!*

We have now deleted “both of which anchor the malleus to the skull”.

*Line 277: "Medial surface of the right skull" should read e.g. "medial surface of the right half of the bisected, skinned head".*

Corrected.

*Line 279: There is only a single arrowhead pointing to the "dorsal crest" in panels 1D and 1E. Please include second arrowheads at the other end of the crest, because at present there is more than one crest-like structure visible in panels 1D and 1E which the arrow might be indicating.*

[Figure 1DEF and Figure 4A-F] We have now added the requested second arrowhead.

*Line 285: a period is erroneously underlined here.* Corrected.

Line 290: Specify whether this is a left or right bulla.

The word “right” has been inserted into the legend:

[Figure 2A legend] Ventrolateral view of a right auditory bulla and capsule.

*Line 300: Be consistent in the use of "gonium" or "gonial" throughout the paper ("gonial" or "goniale" is the commoner term which I personally would prefer). Also - can this actually be distinguished in panel 2H? I don't think it can.*

We use the term “goniale” throughout the revised manuscript. The Figure 2H legend was changed to, “Go, goniale (fused to the malleus and the tympanic bone)”.

*Line 301: Make it clear that the air-bubble is within the middle ear cavity, seen through the tympanic membrane.*

We revised the legend to read:

[Figure 2C legend] Arrow, air bubble in the middle ear cavity seen through the tympanic membrane.

*Line 306: We are asked to note that "the short crus of the incus is fixed by the posterior ligament", but this ligament is not visible in any of the photomicrographs here.*

We have now clarified this point in the Figure 3A legend:

[Figure 3A legend] Note that the short crus (iCB, Crus breve) of the incus (i) is fixed by the posterior ligament (not shown).

Line 313: Make it clear whether we are looking at a left or a right bulla here.

[Figure 4AD legend] The word “right” is now inserted.

*Line 314: There is no explanation anywhere in this article about how CT images were obtained, reconstructed or coloured (see major comments). A short paragraph at least is required!*

We have added the following sentence to the Figure 4 legend and cited a reference:.

[Figure 4 legend] Micro-CT images were obtained at a voxel resolution of 5 µm as previously described (Kanzaki, 2011).

*Line 335: Make it clear that Fig. 6B also shows the mouse ossicles, to scale. It should also explain in the caption what the dotted lines and the curved arrow represent.*

[Representative results] We have now added “at the same magnification.”

[Figure 6B legend] Ossicles of P31 mouse are imaged at the same magnification as that used for human ossicles. Curved arrows indicate the angle between the anatomical axis and the manubrium (dotted lines).

*Lines 338, 342: I don't see the muscular process (mp) labelled in the figure.*

[Figure 6 legend] We now indicate “black asterisk (muscular process of the malleus)” and later “white asterisk (muscular process of the stapes)”.

*Line 339: As referred to previously, the label "mPB" actually points to the orbicular apophysis, which is NOT synonymous with the processus brevis. I have never heard the term "tuber mallei", which I suggest is deleted.*

We have now deleted “Tuber mallei”. Please see the separate sheet relevant to mPB versus orbicular apophysis issue.

Lines 351-355: It is not clear enough here whether the middle ear cavity of mice of this age is filled with fluid normally, and "cavitation" refers to the natural event whereby the fluid is replaced with air, or whether the fluid is appearing as a post-mortem artefact and "cavitation" is simply the process of bubbles forming therein. Please be clearer about when the fluid in the middle ear is removed, in ontogeny.

We now clarify the point in the following:

[Discussion] Dissecting the bulla from the head before sectioning has several advantages. First, postnatal cavitation and growth of the auditory bulla occurs most actively from P6 onwards and is complete by P14 50. The mesenchymal tissue between the tympanic membrane and cochlear wall is replaced by air through the cavitation process. Resultant air in the middle ear cavity can impede contact between tissues and liquids during fixation, decalcification and embedding. It is easier to remove air from the isolated auditory bulla by cutting off the anterior end (styliform process) rather than trying to do so in the unisolated bulla.

*Fig. 1F: It is not clear what the styliform process label is actually indicating here, since no process is visible.*

We have replaced the Figure 1F photo and clearly labeled the styliform process (Sp).

*Figures 2, 5, 6: All references to the "processus brevis" in captions and figures should be changed to "orbicular apophysis" (see above).*

Please see the separate sheet about this issue.

*Fig. 2D: The arrow labelling the malleus head appears to be pointing to the base of the anterior process. The head is nearer to the articulation with the incus.*

Corrected.

*Fig. 3: The arrow in panel B and the cross in panel C are not explained in the caption.*

Both the arrow and the cross are now explained:

Asterisk, muscular process of the stapes.

X indicates the cut end of the stapedial artery (SA).

Fig. 6A: As per my previous comment, the label attached to the head of the mouse malleus seems to be too far towards the anterior process. I would regard the swollen region next to the articulation with the incus as the true head, i.e. somewhere to the right of the current label.

*Materials spreadsheet: "tangsten" should presumably read "tungsten", "steinless conteiners" should read "stainless containers", "rotater" should be "rotator", "haemotoxyrin" should be "haemotoxylin", "eodin" should be "eosin", "vender" should be "vendor", "silan" should be "silane".*

All of these errors have been corrected in the revision.

**“Processus brevis” versus “orbicular apophysis”**

Both Reviewers #2 and #3 asked that the term “processus brevis” be replaced by “orbicular apophysis”. We agree that the orbicular apophysis is a correct term. However, we would like to 1) use the term “processus brevis” in the paper for the following reason, and 2) explain to the readers in the Discussion why we prefer the term.

**The term is established:**

“Processus brevis” has been used to indicate the orbicular apophysis for more than two decades particularly in the field of mouse developmental biology. For example, there were 2,640 citations of papers published during the 90's that used this terminology.

Rijli et al (1993) Cell [439 times cited]

Satokata et al (1994) Nature Genetics [840 times cited]

Martin et al (1995) Genes & Development [212 times cited]

Rivera-Pérez et al (1995) Development [210 times cited]

Yamada et al (1995) Development [206 times cited]

Houzelstein (1997) Mechanisms of Development [113 times cited]

Depew et al (1999) Development [230 times cited]

Xu et al (1999) Nature Genetics [394 times cited]

We believe that the current manuscript we submit to JoVE should not use terminology different from these publications.

"Therefore, we would like to use the term processus brevis in the paper and then explain why we prefer that term at the end of the “Representative results” section as follows:

[Discussion] In mouse, the correct term for the prominent semi-spherical protrusion of the malleus is “orbicular apophysis”. Nevertheless, the term “processus brevis” has been widely used to indicate the orbicular apophysis for more than two decades, particularly among mouse developmental biologists 16,20,22-25. “Processus brevis” originally referred to the lateral process (processus lateralis), which differs from the orbicular apophysis. In humans, a lateral process resembling a slight conical projection forms the general line of attachment to the tympanic membrane, extending from the manubrium (not seen in Fig. 6B, medial view). In mice, the lateral process is also a projection of the manubrium at the opposite end to the umbo 48. The pars flaccida of the tympanic membrane is above the lateral process of the malleus. Orbicular apophysis is not apparent in the human malleus.