RESEARCH ARTICLE

# REVISED Immunohistochemistry localises myosin-7a to cochlear efferent boutons [version 2; peer review: 2 approved]

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## Abstract

**Background:** Myosin 7a is an actin-binding motor protein involved in the formation of hair-cell stereocilia both in the cochlea and in the vestibular system. Mutations in myosin 7a are linked to congenital hearing loss and are present in 50% of Type-1 Usher syndrome patients who suffer from progressive hearing loss and vestibular system dysfunction.
**Methods:** Myosin 7a is often used to visualise sensory hair cells due to its well characterised and localised expression profile. We thus conducted myosin-7a immunostaining across all three turns of the adult rat organ of Corti to visualise hair cells.
**Results:** As expected, we observed myosin 7a staining in both inner and outer hair cells. Unexpectedly, we also observed strong myosin 7a staining in the medial olivocochlear efferent synaptic boutons contacting the outer hair cells. Efferent bouton myosin-7a staining was present across all three turns of the cochlea. We verified this localisation by co-staining with a known efferent bouton marker, the vesicular acetylcholine transporter.
**Conclusions:** In addition to its role in stereocilia formation and maintenance, myosin 7a or certain myosin-7a expression variants might play a role in efferent synaptic transmission in the cochlea and thus ultimately influence cochlear gain regulation. Our immunohistochemistry results should be validated with other methods to confirm these serendipitous findings.

## Keywords

Myosin 7a, medial olivocochlear fibres, hair cells, Usher syndrome

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Any reports and responses or comments on the article can be found at the end of the article.

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**REVISED** **Amendments from Version 1**

This version of the article has been updated to address the comments of the reviewers.

We have:

1. Provided an additional figure illustrating the organ of Corti, the location of hair cells, medial and lateral olivocochlear efferents, type1 and type 2 dendrites (Figure 1).

2. Added arrowheads to all figures showing images of the organ of Corti to indicate the position of the hair cells.

3. We have provided two additional figures (Figure 6 and Figure 7) showing orthogonal views of the imaged organ of Corti region which more clearly show the location of the round-shaped Myosin-7a positive structures in relation to the hair cells and VAChT in the efferent terminals.

4. We have updated the captions of figures to indicate the depth of the cholinergic efferent terminals below the apical surface of hair cells.

5. Added information about the age (in days) of the animals used to the methods section.

6. Added a short description of the role of the lateral olivocochlear fibers towards the end of the introduction.

7. In addition we have corrected several typos.

**Any further responses from the reviewers can be found at the end of the article**

## Introduction

Usher syndrome is an autosomal recessive disorder which affects hearing, vision and balance in approximately 4 to 17 per 100,000 people[1,2]. About 50% of hereditary hearing and vision loss cases have been linked to Usher syndrome[3]. Although the mechanisms underlying Usher syndrome are not entirely clear, many of the mutations that cause it affect proteins expressed in sensory hair cells. Studies on these proteins show that most of them are involved in the formation or maintenance of hair-cell stereocilia, which play a key role in the transmission of acoustic or vestibular stimuli[4].

One of such proteins is myosin-7a, an actin-binding motor protein. In hair cells myosin-7a has been linked to the transport of other proteins along the actin filaments inside stereocilia and the maintenance of stereocilia structure[5,6]. Thus, it plays an important role in stereocilia formation and maintenance. Mutations in myosin-7a have been linked to the most severe Usher syndrome, Type 1, and account for 50% of Usher Type 1 cases and 21% of all Usher syndrome cases[7–11]. Myosin-7a mutations have also been linked to non-syndromic deafness[12,13].

In addition to its presence in stereocilia, myosin-7a is present throughout the hair cell body and is not significantly expressed in the non-sensory cells of the organ of Corti. Given its localisation in hair cells and good availability of high-quality antibodies, myosin-7a is often used to stain hair cells selectively in cochlear immunohistochemistry investigations[14].

In the mammalian cochlea, two types of hair cells are present. Inner hair cells convert sound stimuli into electrical signals which can be transmitted to higher auditory processing regions. Whereas outer hair cells appear to be mostly involved in the enhancement of sound-induced vibrations in the cochlea, and thus increase the "gain" of the signal reaching the inner hair cells[15–17].

Studies indicate that the level of "gain" increase by the outer hair cells can be adjusted by cholinergic efferent fibres which originate in the brainstem and directly synapse onto the base of outer hair cells. Regulation of the "gain" by these medial olivocochlear fibres might be key to our ability to understand complex sounds such as speech in noisy environments and is thought to exert a protective effect when the ear is exposed to louder sounds[18–20].

A similar protective role is ascribed to the cholinergic lateral olivocochlear fibres which synapse onto the type 1 afferent neurons carrying sound information near the base of the inner hair cells[18] (Figure 1). During our investigation of the adult rat cochlea we not only observed myosin-7a staining in stereocilia and hair cell bodies, but also found strong myosin-7a staining in the medial olivocochlear boutons synapsing onto outer hair cells. This suggests that in addition to its role in hair cells, myosin-7a might play a role in cochlear gain regulation.

Our findings also suggest that myosin-7a mutations might contribute to hearing loss in Usher syndrome and nonsyndromic deafness patients by affecting efferent feedback function.

## Methods

The results described in this study were obtained as part of our research on the mechanisms of blast damage. All animal experiments were conducted under the Home Office project license P5B192285, were approved by the Imperial College AWERB Committee, and were in accordance with the UK Animals (Scientific Procedures) Act (1986). Some rats were subjected to a mild form of blast injury 3 months before cochlea isolation. The blast procedure was carried out using a compressed-gas driven shock tube of the Centre for Blast Injury Studies at Imperial College London. The configuration yielded a Friedlander pressure waveform with peak pressure of 230 kPa that simulates open-field detonations. We did not observe any immunostaining pattern differences between blasted and sham rats in the results described in this study. Hence we do not further distinguish between these two groups of rats in this article.

Cochleas were isolated from adult Sprague-Daley male rats (>400g) which corresponds to an age of 10 weeks and older. Rats were killed in accordance with UK Home Office Schedule 1 guidelines and decapitated. Intact cochleas were separated from the temporal bone, fixed and stored in 4% PFA at 4 °C for at least 24 hours. Data in this study were gathered from 11 cochleas obtained from 7 rats. Where possible we

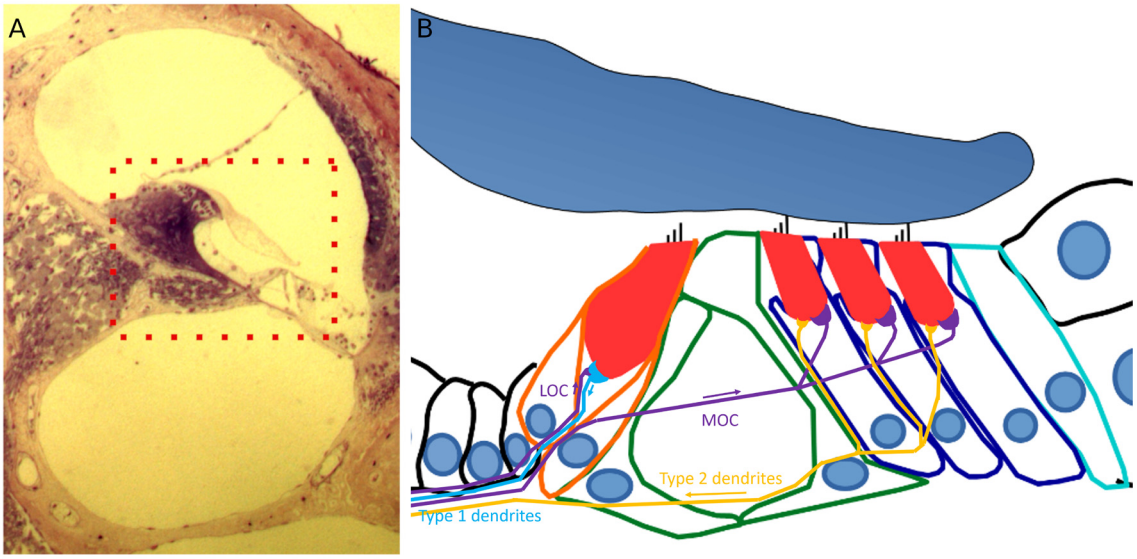


**Figure 1. Cross-section of the cochlea and the organ of Corti. A.** A section through the cochlea, rectangle depicts the location of the organ of Corti. **B.** A diagram representation showing a cross-section through the organ of Corti. Hair cells (red) can be subdivided into outer hair cells organised into three rows and inner hair cells. The medial and lateral efferent boutons and fibres (MOC and LOC) are depicted in purple. The afferent type 1 dendrites are depicted in blue and type 2 dendrites are shown in yellow. Cochlear section was a gift of Prof. Andrew Forge.

aimed to replicate data in 3 separately stained cochleas from 3 animals. We did not need to exclude any cochleas from our analysis.

Cochleas were washed 3 times for 5 minutes each in PBS to remove the PFA and transferred to a fresh batch of PBS. Excess tissue was removed and the bone covering the organ of Corti was carefully removed with tweezers. After exposing the organ of Corti, the tectorial membrane was peeled away with tweezers from the middle and base turns. To block nonspecific antigens, cochleas were incubated in blocking solution consisting of PBS, 0.1% Triton X-100 and 5% Normal Goat Serum for one hour at room temperature whilst placed on a laboratory rocker. After blocking, cochleas were moved to a fresh batch of blocking solution with primary antibodies and left to incubate and rock for 4 hours at room temperature. Subsequently, cochleas were washed 3 times for 5 minutes each using PBS and transferred to blocking solution with added secondary antibodies and phalloidin for 3 hours, again whilst rocking at room temperature. Finally, cochleas were washed 3 times for 5 minutes using PBS and stored in PBS until imaging.

Table 1 lists the primary antibodies used and their concentrations. Secondary anti-rabbit, anti-chicken and anti-guinea pig antibodies (Invitrogen) conjugated to Alexa fluorophores (488, 546, 594, 633) were used at a final concentration of 1:300 as summarised in Table 2. Phalloidin conjugated to Alexa 405 at a final concentration of 1:300 was added during the secondary antibody incubation step to visualise actin-rich stereocilia (A30104: Alexa Fluor™ Plus 405 Phalloidin Invitrogen). We used either anti-rabbit antibodies conjugated to Alexa 488 or 594 to visualise myosin-7a. We also used an anti-guinea pig antibody conjugated to Alexa 546 to visualise vesicular acetylcholine transporter (VAChT) as well as an anti-chicken antibody conjugated to Alexa 633 to visualise Neurofilament-Heavy (NF-H). Artificial look up table colors were applied to the captured images to better visualise and contrast the staining patterns. The chosen colours do not necessarily reflect the wavelength at which fluorescence was recorded.

A Leica SP5 upright confocal microscope with two-photon imaging capabilities was used to image the exposed organs of Corti. Cochleas were superglued to the lids of 55-mm diameter cell culture Petri dishes prior to imaging to ensure the organ of Corti would be in an appropriate orientation and immersed in PBS.

The Argon 488, Diode 543, Diode 594 and Diode 633 were used for single-photon excitation of the Alexa dyes conjugated to the secondary antibodies and the Mai Tai eHP DeepSee 5332 laser set to a wavelength of 800 nm was used for two-photon excitation of the phalloidin-conjugated Alexa 405. A 25x/0.95NA water immersion objective (HCX IRAPO L25x/0.95 W) was used. Images were recorded in 12 bits at a resolution of 1024 x 1024 and further processed in ImageJ (https://imagej.nih.gov/ij/)

## Results

Cochleas were stained using the myosin-7a antibody and phalloidin to visualise hair cells and stereocilia, and the NF-H antibody was used to visualise afferent and efferent fibers. Myosin-7a

antibodies in conjunction with secondary anti-rabbit Alexa 488 conjugated antibodies visualised inner and outer hair cells across all 3 cochlear turns in the rat, consistent with a number of previous investigations[21–23] (Figure 2 and Figure 3). In addition, in the outer hair cell region, high-intensity myosin-7a staining was visible manifesting as oval structures which appeared to be just below the outer hair cells. These oval structures were visible in all 3 turns of the rat organ of Corti with typically 1 to 3 of them apparent below each outer hair cell. As we did not find any equivalent finding published in the literature we investigated if this staining was due to nonspecific binding of the secondary antibodies. To try to exclude this possibility we used a different secondary anti-rabbit antibody conjugated to Alexa 594 instead of Alexa 488. The oval structures were still visible when using the Alexa-594 conjugated anti-rabbit antibody (Figure 4). As an additional control, we also omitted the primary myosin-7a antibody. With no primary antibody present no oval structures were visible.

As the observed oval structures resembled medial olivocochlear boutons that synapse onto the outer hair cells, we co-stained

**Table 1. Primary antibodies used in the study.**

| Antibody | Antigen | Host | Supplier | Dilution |
|---|---|---|---|---|
| PA1-936 | Mouse myosin-7a | Rabbit polyclonal | Invitrogen | 1:300 |
| 139 105 | Rat VAChT | Guinea pig polyclonal | Synaptic Systems | 1:300 |
| AB5539 | Bovine NF-H | Chicken polyclonal | Sigma-Aldrich | 1:600 |

**Table 2. Secondary antibodies used in the study.**

| Antibody | Antigen | Host | Supplier | Dye | Dilution |
|---|---|---|---|---|---|
| A11008 | Rabbit IgG | Goat polyclonal | Invitrogen | Alexa Fluor 488 | 1:300 |
| A11012 | Rabbit IgG | Goat polyclonal | Invitrogen | Alexa Fluor 594 | 1:300 |
| A21103 | Chicken IgY | Goat polyclonal | Invitrogen | Alexa Fluor 633 | 1:300 |
| A11074 | Guinea pig IgG | Goat polyclonal | Invitrogen | Alexa Fluor 546 | 1:300 |

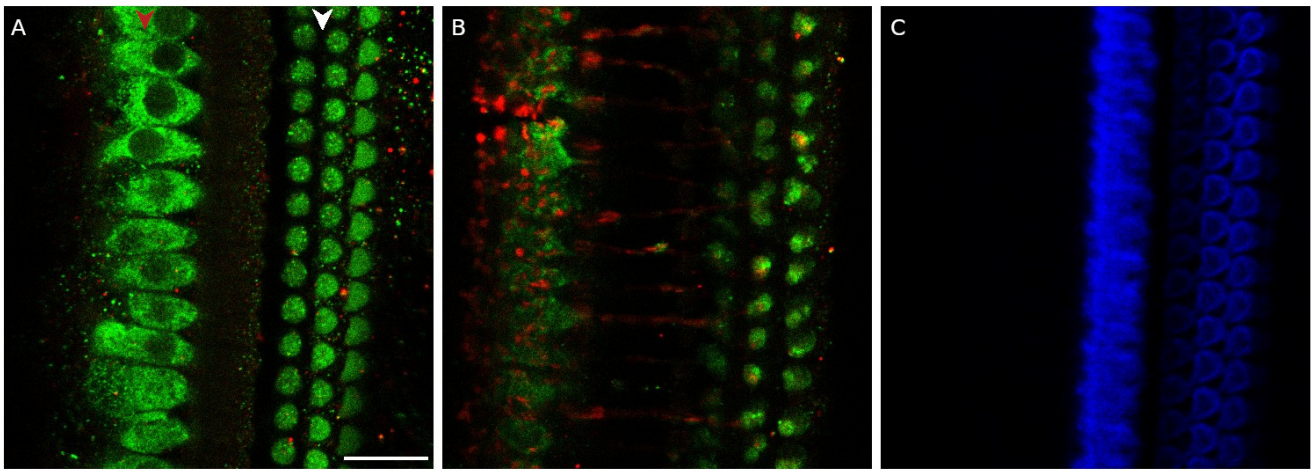


**Figure 2. Myosin-7a staining of medial olivocochlear boutons in the basal turn of the rat organ of Corti. A.** Composite picture showing myosin-7a (green) and NF-H (red) staining at the level of the top of outer hair cells and **B.** below the outer hair cells at the level of the medial olivocochlear boutons, which were located approximately 12 µm below the apical surface of the outer hair cells. **C.** Phalloidin (blue) staining of the same organ of Corti fragment showing the tops of the outer hair cells. Red arrowhead indicates the row of the inner hair cells, white arrowhead indicates the three rows of outer hair cells. Scale bar is 20 µm. Data was replicated in 3 cochleas from 3 rats.

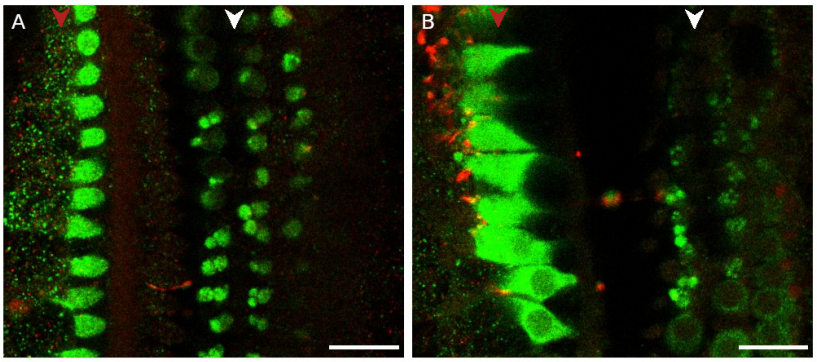


**Figure 3. Myosin-7a stains medial olivocochlear boutons in the middle and apical turn of the rat organ of Corti. A.** Medial olivocochlear boutons are visible below the outer hair cells in the mid and in **B.** the apical turn. (green – myosin-7a, red – NF-H). Red arrowhead indicates the row of the inner hair cells, white arrowhead indicates the three rows of outer hair cells. The medial olivocochlear boutons were located approximately 15 to 25 µm below the apical surface of the outer hair cells. Scale bars are 20 µm. Mid-turn data were replicated in 3 cochleas from 3 rats. Apical region data were replicated in 3 cochleas from 2 rats.

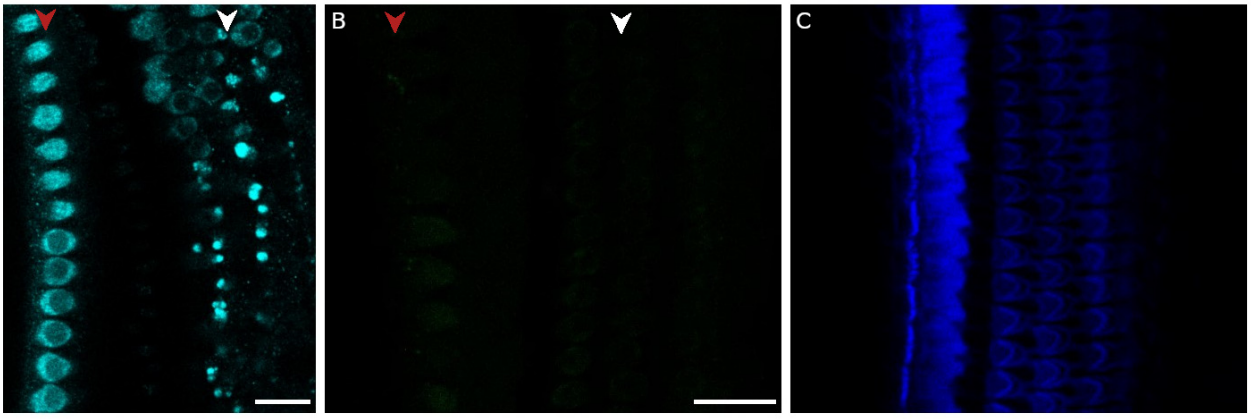


**Figure 4. Changing the secondary antibody did not change the myosin-7a staining pattern. A.** Myosin-7a staining (cyan) in the apical cochlear turn using an Alexa-594 anti-rabbit secondary antibody instead of Alexa-488 antirabbit (Note that the cyan colour does not correspond to the wavelength of the fluorescent signal). **B.** Negative control with only secondary Alexa 488 antibody (left) and phalloidin (right). Red arrowhead indicates the row of the inner hair cells, white arrowhead indicates the three rows of outer hair cells. Scale bars are 20 µm. Each control was conducted on a single cochlea, each from a different rat.

with an antibody against the VAChT, which is used to visualise medial and lateral olivocochlear boutons. The oval structures visualised using the myosin-7a antibody co-stained with the VAChT. In addition, the VAChT antibody visualised the lateral olivocochlear boutons in the inner hair cell region, which were not visualised by myosin-7a staining (Figure 5).

To further confirm the location of the myosin-7a staining in the outer hair cell region relative to hair cells and the VAChT we created orthogonal views showing a cross-section of the organ of Corti at locations in which the oval myosin-7a staining structures were observed (Figure 6 and Figure 7). These show that the myosin-7a-rich oval structures are located towards the bottom of the outer hair cells, that the shape of their cross-section is not inconsistent with the shape of synaptic boutons and that their localisation corresponds with that of the VAChT. In addition, the myosin-7a-rich oval structures can be distinguished from the outer hair cells thanks to the higher fluorescent signal level.

## Discussion

Our results suggest that in addition to being present in hair cells, myosin-7a may also be present in the medial olivocochlear boutons contacting outer hair cells.

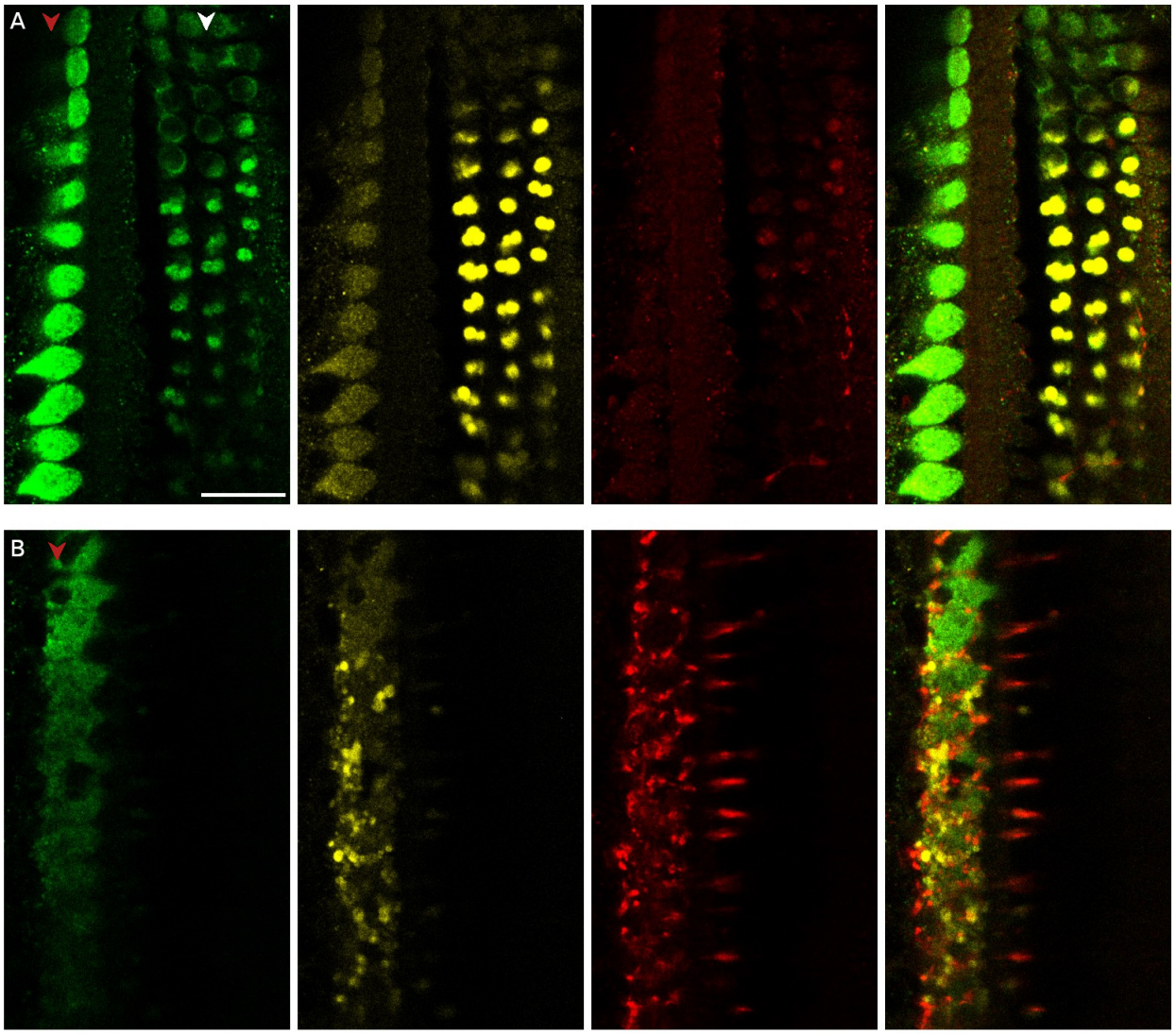


**Figure 5. Myosin-7a staining visualises medial olivocochlear boutons but not lateral olivocochlear boutons. A.** Myosin-7a (green), VAChT (yellow), NF-H (red) and composite at the plane of the medial olivocochlear boutons. **B.** Myosin-7a (green), VAChT (yellow), NF-H (red) and composite at the plane of the lateral olivocochlear boutons. Red arrowhead indicates the row of the inner hair cells, white arrowhead indicates the three rows of outer hair cells. The medial olivocochlear boutons were located approximately 12 µm below the apical surface of the outer hair cells. The lateral olivocochlear boutons were located approximately 20 µm below the apical surface of the inner hair cells. Scale bar is 20 µm. Data were replicated in 2 cochleas from 2 rats.

We have observed myosin-7a staining in the medial olivocochlear boutons across all three turns of the cochlea and verified that the staining pattern we observe is not due to unspecific staining related to the secondary antibody. We have also independently confirmed localisation to the medial olivocochlear boutons by using a known cochlear efferent bouton marker VAChT. In contrast to myosin-7a staining, VAChT staining also visualised the lateral olivocochlear boutons in the inner hair cell region, which is a further positive control validating that the myosin-7a staining in the medial olivocochlear boutons is specific. Future work should validate myosin-7a localisation to medial olivocochlear boutons in other species and using other experimental methods.

Several studies investigating myosin-7a expression in the cochlea have been conducted in the past and it is not clear why it has not been observed in medial olivocochlear boutons previously. One possibility is that the polyclonal antibody we have used to visualise myosin-7a recognises an epitope, which is present in myosin-7a isoforms specific to the medial

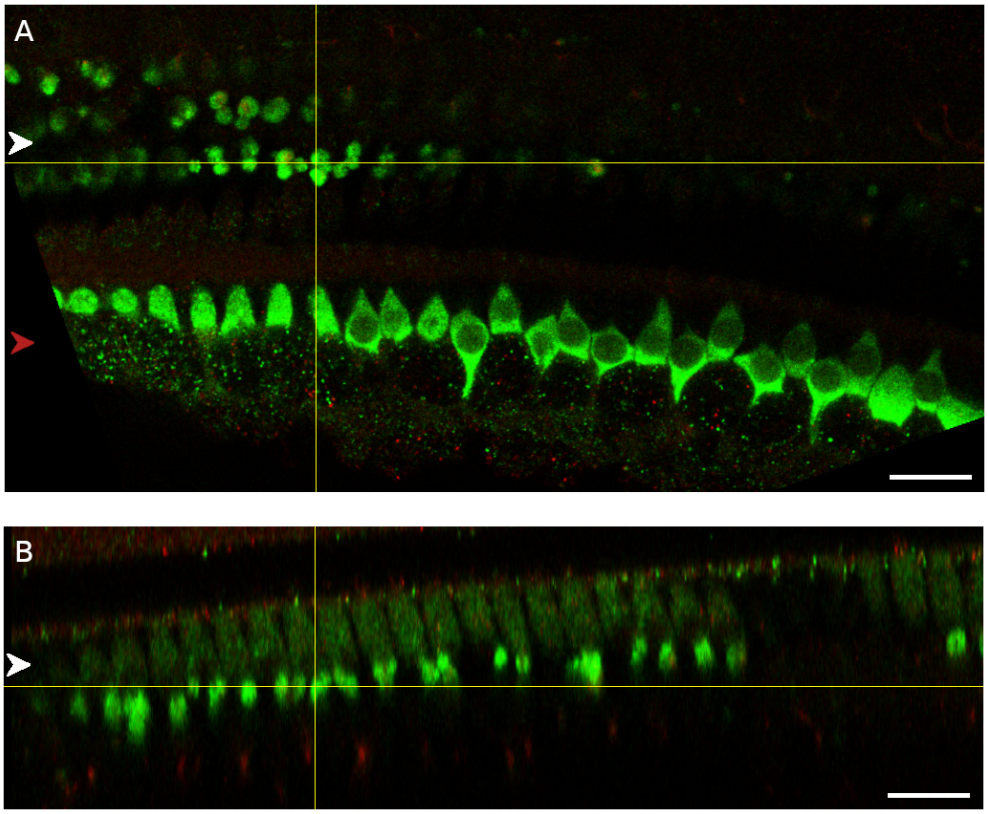


**Figure 6. Strong myosin-7a staining is visible below the outer hair cells in the mid cochlear turn. A.** Top down view showing myosin-7a (green) and NF-H (red) staining. The yellow x-axis represents the plane along which the orthogonal view shown in **B** was created. **B.** Orthogonal view showing a cross-section of the organ of Corti along the yellow x-axis shown in **A**. Red arrowhead indicates the row of the inner hair cells, white arrowhead indicates the three rows of outer hair cells or a single row in the orthogonal view. The medial olivocochlear boutons were located approximately 15 µm below the apical surface of the outer hair cells. Scale bar is 20 µm.

olivocochlear boutons. We have determined that the antibody we have used was raised using an N-terminal antigenic region of myosin-7a, in contrast to other popular myosin-7a antibodies raised using antigenic regions in the C-terminal region of myosin-7a used in many other publications[6,14,24]. It is not clear if all myosin-7a isoforms would have this same C-terminal region. The C-terminal region of myosins is documented to be variable, and differences might affect the subcellular distribution and function of different myosin isoforms[25,26].

According to the website of the antibody's manufacturer, this particular myosin-7a antibody has been used in the past to visualise myosin-7a as part of other published studies. These however have not involved staining of the adult rat cochlea and were mostly done on mice before the onset of hearing[27–29]. Therefore, if myosin-7a presence in medial olivocohlear boutons is related to maturation state or this particular epitope is present only in the rat, then no evidence of staining would have been present in these studies.

Although we have attempted to exclude nonspecific staining, it is worth noting that there is a possibility the myosin-7a antibody we used stains a different protein present in the medial olivocochlear boutons. However, as many of the antibodies which can be used to visualise the medial olivocochlear boutons, such as VAChT, also label the lateral olivocochlear boutons, it is interesting to note that the myosin-7a antibody stains for a protein that is only found in medial olivocochlear boutons. Therefore, even if a different protein than myosin-7a is responsible for the staining pattern we observed, it would be still advantageous to use it as a highly specific marker of medial olivocochlear boutons, as well as potentially important to determine its function.

If myosin-7a is indeed present in the medial olivocochlear boutons, it could hint at another important role this protein plays within the cochlea and possibly have consequences for our understanding of the mechanisms underlying Usher syndrome and congenital hearing loss.

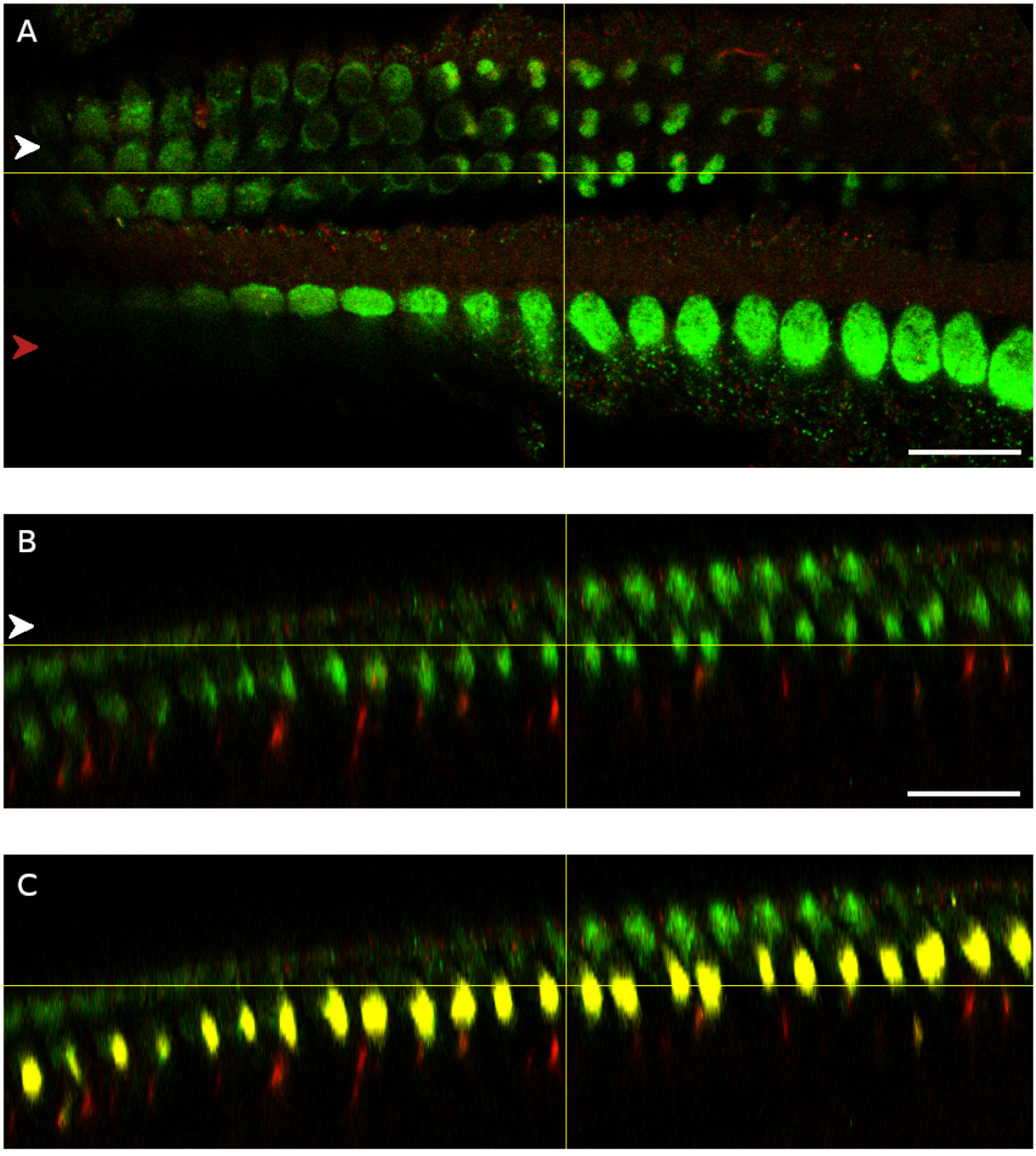


**Figure 7. Strong myosin-7a and VAChT staining is visible below the outer hair cells in the basal cochlear turn. A.** Top down view showing myosin-7a (green) and NF-H (red) staining. The yellow x-axis represents the plane along which the orthogonal views shown in **B** and **C** were created. **B.** Orthogonal view showing a cross-section of the organ of Corti along the yellow x-axis shown in **A. C.** Orthogonal view showing a cross-section of the organ of Corti along the yellow x-axis shown in **A** and VAChT staining (yellow). Red arrowhead indicates the row of the inner hair cells, white arrowhead indicates the three rows of outer hair cells or a single row in the orthogonal view. The medial olivocochlear boutons were located approximately 12 µm below the apical surface of the outer hair cells. Scale bars are 20 µm.

## Data availability

### Underlying data

Dryad: Immunohistochemistry localises myosin-7a to cochlear efferent boutons, https://doi.org/10.5061/dryad.9s4mw6mhz[30]

This project contains the following underlying data:

- A zipped file containing tiff files organised in folders based on which figure in this publication the data are associated with.

## Reporting guidelines

Zenodo: ARRIVE checklist for 'Immunohistochemistry localises myosin-7a to cochlear efferent boutons', https://doi.org/10.5281/zenodo.5763739[31]



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# Open Peer Review

## Current Peer Review Status: ✔ ✔

**Version 2**

Reviewer Report 23 February 2022

https://doi.org/10.21956/wellcomeopenres.19617.r48849



✔ **Walter Marcotti**
School of Biosciences, University of Sheffield, Sheffield, South Yorkshire, S10 2TN, UK

The authors have fully addressed my points.

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Auditory Neuroscience

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

**Version 1**

Reviewer Report 24 January 2022

https://doi.org/10.21956/wellcomeopenres.19271.r47885



✔ **Hiroshi Hibino**
Osaka University Graduate School of Medicine, Osaka, Japan

This study examined the localization of myosin-7a in the cochlea of adult rat by immunohistochemistry. This actin-binding protein is involved in the formation of hair-cell's stereocilia. The authors detected the strong myosin-7a immunoreactivity not only in the hair-cell's elements as reported previously but also in the medial olivocochlear efferent synaptic boutons

contacting the outer hair cells. The signal was clearly observed even when the secondary antibody was changed to the different one. Moreover, minimum fluorescence was visible in the experiment without the primary myosin-7a antibody. These observations sufficiently support their idea that myosin-7a is also present in the efferent synaptic boutons.

The quality of the data is convincing and the text is well written. To improve the manuscript, I described a few minor comments in the followings.

Minor issues

1. For a broad array of readers including non-experts, I request the authors to describe an illustration of an organ of Corti in the manuscript. In addition, they should point to the positions of outer hair cells and inner hair cells by arrows or arrowheads in all the figures.

2. I suggest the authors to show the depth information in all the figures. For instance, information of the distance between the apical or upper surface of hair cells and the immunolabeling in the button will be helpful.

3. Describe the age of the animals examined in this study.

4. In page 6, left column, 2$^{nd}$ paragraph; 'Nterminal' is typo?

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Hearing

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 12 Feb 2022

**Andrei Kozlov**, Imperial College London, London, UK

We thank our reviewer Professor Hiroshi Hibino for the time and effort he has spent to carefully evaluate our manuscript and for his helpful recommendations for improvement. We provide the reviewers' comments (in *italics*) and our responses to each point below.

*For a broad array of readers including non-experts, I request the authors to describe an illustration of an organ of Corti in the manuscript. In addition, they should point to the positions of outer hair cells and inner hair cells by arrows or arrowheads in all the figures.*

We have added an illustration of the organ of Corti and a cross-section of the cochlea showing its localization in Figure 1 in the updated version of the manuscript. We have also used arrowheads to indicate the position of inner and outer hair cells in all figures.

*I suggest the authors to show the depth information in all the figures. For instance, information of the distance between the apical or upper surface of hair cells and the immunolabeling in the button will be helpful.*

We have added information about the depth of the bouton below the apical surface of the hair cells to the figure captions.

*Describe the age of the animals examined in this study.*
We have added information about the age of the animals to the 2nd paragraph of the methods section.

*In page 6, left column, 2nd paragraph; 'Nterminal' is typo?*
Yes, this was a typo. It has been corrected in the updated version of the manuscript.

***Competing Interests:*** No competing interests were disclosed.

Reviewer Report 18 January 2022

https://doi.org/10.21956/wellcomeopenres.19271.r48008



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**Walter Marcotti**
School of Biosciences, University of Sheffield, Sheffield, South Yorkshire, S10 2TN, UK

The focus of this short manuscript is to describe the distribution of Myo7a in the rat cochlea. The experimental work is performed using a combination of immunostaining and confocal imaging. The authors claim that Myo7a is not only expressed in the sensory hair cells, as previously

demonstrated, but also in the efferent terminals of the medial olivocochlear fibres contacting the OHCs. This is, in principle, an interesting finding with several potential functional implications, as stated by the authors. Although this work provides a very nice descriptive investigation, there are several aspects that need some additional consideration. This will require either additional experiments or some clear statements indicating the several limitations of this brief study.

One of the most unclear aspects of the work is the discrepancy between some of the data provided. Figures 1 and 2 show very little, or no overlap between Myo7a and neurofilament, which should label the efferent terminals. However, using the same antibodies, the authors claim the opposite in Figure 4 (complete overlap; see also the last point below). Considering that the images should be representative of the results, I am unsure which piece of evidence I should consider. Maybe the use of an additional anti-Myo7a antibody should be sought.

Also, have the authors considered the possibility that these round-shaped Myo7a positive structures could be part of unhealthy OHCs or something else? This could be easily addressed by double labelling the OHCs with another hair cell marker (e.g. Myo6).

From the data provided, it is also very difficult to appreciate the possible overlap highlighted in Figure 4. How are the authors able to distinguish between overlap (Myo7a in both in the OHCs and efferent terminals) or its juxtaposition (Myo7a in OHCs and NF or VAChT in the efferent terminals) from the images provided?

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Not applicable

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Partly

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Auditory Neuroscience

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 12 Feb 2022

**Andrei Kozlov**, Imperial College London, London, UK

We would like to thank our reviewer Professor Walter Marcotti for the time and effort he has spent to carefully evaluate our manuscript and for the helpful recommendations for improvement. We provide the reviewers' comments (in *italics*) and our responses to each point below.

*One of the most unclear aspects of the work is the discrepancy between some of the data provided. Figures 1 and 2 show very little, or no overlap between Myo7a and neurofilament, which should label the efferent terminals. However, using the same antibodies, the authors claim the opposite in Figure 4 (complete overlap; see also the last point below). Considering that the images should be representative of the results, I am unsure which piece of evidence I should consider. Maybe the use of an additional anti-Myo7a antibody should be sought.*

The results in several publications we reviewed whilst writing the manuscript were consistent with the data we obtained. Similarly to our results, neurafilament staining did not visualise well the efferent boutons; instead antibodies against other markers such as VAChT, or anti-choline acetyltransferase (ChAT) were used for this purpose. Examples can be seen in Figure1 (Froud *et. al.*, 2015: https://www.nature.com/articles/ncomms8115#MOESM736), Figure 4 (Lang *et. al.*, 2011: https://pubmed.ncbi.nlm.nih.gov/21061038/), Figure 4 (Seist et. al., 2020: https://www.frontiersin.org/articles/10.3389/fnmol.2020.00087/full) and Figure 5 (Kujawa and Liberman 2005: https://www.jneurosci.org/content/29/45/14077/tab-figures-data).

NF-H staining visible in Figure 5 at the location of the medial olivocochlear boutons is comparable to the NF-H staining of the organ of Corti fragment shown in Figure 2. This is not clear in Figure 2 as it depicts a composite image showing both myosin 7a and NF-H staining but can be verified by viewing the freely available Dryad dataset linked to the publication.

*Also, have the authors considered the possibility that these round-shaped Myo7a positive structures could be part of unhealthy OHCs or something else? This could be easily addressed by double labelling the OHCs with another hair cell marker (e.g. Myo6).*

We have not conducted separate double labelling experiments, however the localization of the Myo7a positive structures clearly overlaps with the localization of VAChT as shown in the orthogonal views found in Figures 6 and 7 in version 2 of the manuscript.

*From the data provided, it is also very difficult to appreciate the possible overlap highlighted in Figure 4. How are the authors able to distinguish between overlap (Myo7a in both in the OHCs and efferent terminals) or its juxtaposition (Myo7a in OHCs and NF or VAChT in the efferent terminals) from the images provided?*

To better show the position of the round-shaped Myo7a positive structures we have created orthogonal views of the image stacks we captured (Figures 6 and 7 in the updated version of the manuscript). Views were captured along the X-axis shown on the top-down views in

those figures.

The orthogonal views appear to confirm that the round-shaped Myo7a positive structures are separate from OHCs and are located below them. Furthermore, the orthogonal views confirm the colocalisation of these structures with the efferent bouton marker VAChT.

***Competing Interests:*** No competing interests were disclosed.