nature portfolio

Peer Review File

Molecular heterogeneity of the non-human primate cochlea

Corresponding Author: Professor Renjie Chai

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)
In this manuscript, Chen et al. present a cross-species comparative study between mouse and macaque cochlear cell types. They profil more than 20643 nuclei from 1-year old and 11-year old crab-eating macaque cochlea, and 9470 nuclei from P21-25 murine cochlea using single-nucleus RNA sequencing on the 10x chromium platform. Nuclei were collected from cochlear epithelium, spiral ganglion, stria vascularis, spiral ligament, and other inner ear components including Reissner's membrane, macrophages and osteoblasts. Through a series of computational comparative analyses, the authors demonstrat conserved and divergent features between mouse and macaque cochlea and cellular diversity in spiral ganglion neurons. This cochlear snRNAseq dataset from non-human primates is a tremendous feat and ultimately a step closer to understanding the pathophysiology of the inner ear in mammals. However, unfortunately the manuscript in its current form has some major issues that do not make it acceptable for publication.

Major concerns:
1. Writing composition: Throughout the manuscript, there are innumerable grammatical and syntax errors that make reading it quite laborious. This reviewer initially began keeping track of these, but quickly became overwhelmed with this effort. I do suggest that the authors take a careful look at their writing.
2. Validation of results: There is sparce validation data for most of the manuscript. While there is one transcription factor that the authors validate as unique to the macaque (PBX3), there is none for the major cell types of the organ of Corti such as inner or out hair cells, or any of the supporting cell types. Without such validation, it is unclear if any of the presented data is biologically relevant.
3. A recent comprehensive snRNAseq dataset from Jean et al. (2023, PNAS) has 37 cell types described in mouse, with several of the cell types described (and validated) there not present in the dataset here. Any thoughts on the discrepancy here? Would using that now publicly available dataset provide a better comparison (at least the P20 data)?
4. Figures 1-5 lack some originality in presenting the data. Certain parts of the manuscript are a near complete recapitulation of Bakken et al., 2021 (Nature), including to the level of figure colors (e.g. Fig 3d here and Fig 2c in Bakken et al, among others).
5. I understand and appreciate that obtaining inner samples from macaques is difficult for a variety of reasons. However, there is a vast age difference between the two animals used here (1 year old and 11 year old). It would be much more convincing if there was a closer age match – for example two 1 year old or two 11 year old animals. Furthermore, in Fig 1b, there appears to be a couple of large groups of cells that are only in the 1yo macaque, and not the 11 year old. This is either batch effect or related to age differences. If related to age differences, then at least one additional specimen is needed per category. These cells groups are later labeled as Fibro2 and Fibro4_RMC.
6. Cell composition analysis (Fig 1e) cannot be inferred from percentages. This is not computationally sound. See (https://www.sc-best-practices.org/conditions/compositional.html) for details and explanation. This figure should either be removed or performed in a computationally rigorous manner.
7. How many hair cells of each sub-type are in the two datasets (mouse and macaque)? Did the authors make any attempt to harvest a larger number of hair cell nuclei? And why are the IHCs and OHCs so far apart on the UMAP? In nearly every other manuscript with scRNAseq from the inner ear, they're not that far apart in space. I understand that relation of cells cannot be fully inferred from UMAP, but I have not seen these cell types this far apart on a UMAP.
8. Re Fig 5d: Calb1 and Calb2 differences between mouse and macaque is not obvious here. Any attempts at quantification? That would be more convincing. It would be even more convincing if there was some in situ hybridization with quantification.
9. Did the authors pull out any tonotopic data? There's discussion of the hearing frequency differences between mouse and macaque, but is there any tonotopic evidence computationally in either the mouse and/or the macaque data?

10. The authors do not use a robust computational method for their chosen clustering resolution. It would behoove the authors to clearly perform a calculation to determine the optimal clustering for each of their datasets. For example, Bakken et al 2021 have a method to do this, and there are a few other methods available in the literature (e.g. Patterson-Cross et al., 2021, BMC Bioinformatics).
11. Methodology: there's a clear lack of details in the methods regarding how tissue was obtained in the macaque. Description of the dissection technique is lacking, what enzymes were used for dissociation and for how long, time from animal sacrifice to loading on the 10x Controller, etc…
12. Code availability: without proper and detailed code (for example shared on GitHub or similar platform), this reviewer cannot clearly see what was done from a computational perspective. While there are descriptions of all of the steps, and references to others who have done similar work, this is not a substitute for clear and well-annotated code for both peer-review and for the general scientific audience.
13. I have some concerns regarding the consistency of what is said and what is shown. For example, throughout the main manuscript, the authors refer to a 1 year old macaque, however, in the supplementals (Fig S1d), they write "Macaque_8M" a number of times. Is it 1 year old or 8 month old?
14. Where are the inner/outer hair cells in Figure S4a? I only see "HC" as a category without determination of IHC/OHCs. If there's claims of translational potential, it would be critical to rigorously show both inner and outer hair cell composition along with appropriate validation of these cell types. There's discussion of this work being important for pathological conditions such as age related hearing loss (ARHL). Approximately 80% of ARHL is secondary to cochlear hair cell loss.

Minor concerns:
1. Abstract: The last sentence mentions human stem cell-based models of disease. However, there's no data or any reference to stem cells anywhere else in the manuscript.
2. Introduction: line 92- reference is missing
3. Introduction: line 97-99- meaning is not clear
4. Introduction: line 106 and 109- the full forms of MET and AAV should be mentioned when the term is first introduced
5. Results: line 193- typo: 'uncvered'
6. Manuscript should say "glia" or "glial cells" not "glia cells"
7. Did the authors attempt another species comparison method?
8. Line 363-364: the human hearing frequency should be listed (20Hz to 20,000Hz).
9. Fig1a: Cartoon should not read "aged" macaque but "adult" macaque.

Reviewer #2

(Remarks to the Author)
Although it is of significance to reveal the cell composition and function diversity of non-human primate cochlea, and this study constructed a single-cell transcriptomic atlas of the macaque cochlea and discovered remarkable heterogeneity across and within cell types, this work is still rudimentary, requires a lot of follow-up work to improve.

1. The purpose of this study is to construct a single-cell atlas of the cochlea in non-human primates. However, the authors selected only one male individual aged 1 year and one female individual aged 11 years of the crab-eating macaque species for single-cell sequencing. Due to a great variation between individuals, the quantity of samples at the same gender and the same age in biological studies is generally more than 3. In order to avoid the effects of individual differences on the results, the authors should increase the samples for sequencing. It would be difficult to get the manuscript published in a decent journal like Nature Communications if the issue was not resolved.

2. Aging and sex itself must have a significant effect on the gene expression of the cochlea, how can the authors rule out the impact of age? In particular, there are obvious differences in the age of the individuals used in the manuscript.

3. In Figure 2B, it is evident that Fibro 2, Fibro4_RMC, and other cell types are predominantly found in the 1-year-old crab-eating monkey but not in 11-year-old monkey. Since the 1-year-old macaque is still at the developmental stage, this observation strongly suggests either a pronounced individual heterogeneity or potential developmental transition from the juvenile to the adult stage in the crab-eating monkey cochlea. Selecting sexually mature crab-eating macaque individual would be required. Similarly, the authors used the mice of the age of P21-25, more samples at different age and both genders are required.
4. The authors mentioned in the Ethical compliance section that there were 1-, 5-, and 11-year-old crab-eating monkey cochleae used for single-cell nuclear sequencing, but the data of 5-year-old sample was not found in the manuscript. The scientific rigor of the paper needs to be ensured.
5. The numbers of captured hair cells and SGNs are quite small. The authors should probe the diversity of hair cells, which is one of the most important cells of the cochlea. More analysis and related experimental validation are required.
6. More details about the steps for isolation of cochlear single cell nuclei should be provided.
7. This manuscript is very descriptive without a focused point or pathway. The authors are suggested to perform an in-depth analysis into the bioinformatic data, and more validation experiments are required. For example, the authors found that USH2A is highly expressed in the cochlear hair cells of monkeys. To make the results more convincing, IHC, IF, single cell-based qPCR should be performed. Further, to explore the role of USH2A, both loss of function and gain of function experiments in primary monkey cells are required.
8. The author employed the biomaRt package for the homology gene conversion between crab-eating macaques and mice.

However, using the human genome as an intermediate reference is not an optimal approach. Unlike the well-annotated reference genome in mice, the integrity of the macaque MF 5.0 reference genome seems relatively poor, with many genes lacking complete annotations. Consequently, biomaRt fails to perform homology annotation for these unannotated genes. Moreover, the author utilized the human genome as an intermediary and employed biomaRt (version: 2.42.1) with the Macaca_fascicularis_6.0 reference genome, which further amplifies errors in homology annotation. A more appropriate alternative would involve directly performing BLAST sequence alignment between crab-eating macaques and mouse. In addition, the authors are suggested to provide the list of homolog genes between crab-eating macaques and mouse.
9. In Fig. S18, in order to construct heatmaps of all subclasses of hearing loss genes, the authors subsampled 10 nuclei from each species. However, analyzing subclasses with a higher proportion in the population resulted in significant information loss. To reduce such noise, utilizing a sampling method with replacement might be a preferable choice.
10. It is a lack of details of the computational approaches and the code should be provided on the GitHub page.
11. A statistical approach to identify the appropriate resolution or find the number of clusters should be performed. There are a variety of packages can be used (i.e. see Patterson-Cross et al 2021 PMID: 33522897).
12. The number of cells for each cell group and each sample should be clearly annotated.

Minor points:
1. The authors need to explain "less than 15,000 genes" (lines 543 and 566) in the QC process.
2. The color annotations for cell types in Fig. 1b and 1d need to be aligned.
3. In Fig. S1 c, it seems that Chinese was used.
4. The log fold change in Line 595 should be changed to log2 fold change.

Reviewer #3

(Remarks to the Author)
'Xin Chen et al.' present in their paper a cellular map of the macaque cochlea at the single cell level and provide transcriptomic signatures for all the cell types they detected. To the best of my knowledge, it is the first time that a primate single cell transcriptome of the cochlea is proposed and has the potential to be highly valuable for the whole cochlear and auditory neuroscience field. The major therapy approach currently explored for genetic forms of deafness is gene therapy. Tens of preclinical studies in the mouse have or are currently investigating therapeutic approaches and strategies for many deafness genes. These therapeutic approaches to be safe need well designed promoters to ensure that the therapeutic cassettes target only the relevant cell types. This is one reason why there has now been a number of single cell transcriptomic studies in the mouse. In the view of translating these gene therapies from mice to humans, a single cell transcriptomic dataset in a non-human primate is essential to make sure the gene therapies designed in mice have a chance to be safe and to be rapidly translated to patients. It is in this context that 'Xin Chen et al.' provide a macaque transcriptomic single cell atlas of the cochlea based on the analysis of 20 600 nuclei. The authors have carried out their project using states of the art single cell sequencing techniques and analysis tools. Despite the quality of the data processing and graphical presentation, the manuscript presents several issues including two major ones, a) the low quality of English and b) the fact that the manuscript does not add much to the cochlear transcriptomic knowledge in the current state.

i) English. It is a shame that the authors did not use professional services from native scientific English speakers. The manuscript seems to be a direct translation of a foreign language using online tools. The consequences are the presence of many sentences with wrong grammar/syntax, the impossibility to understand some sentences (sometimes whole paragraphs), and probably many misunderstandings including in my assessment of the data. Here are just a few illustrations of the many issues:
-approximate wording: the title wording is unclear, what is a 'spiral ganglion hub' (line 149), to 'attach small portion of oligodendrocytes' should read 'to collect' (line157), what is a 'high specialized terminator?' (line 220),
-Coordinating conjunctions are often place at the start of sentences like 'And' (lines 154, 185,196).
-Whole paragraphs are hardly understandable (ex: lines 209-214)
-Major misunderstanding. The authors use the word 'diversity for cell types', which suggests that there are more cell types in the macaque than in the mouse like among glial cells. In fact, it seems that there are as many cell types. Rather, the macaque glial cells display more differentially expressed genes compared to mouse glial cells than in any other cochlear cell type (see abstract, paragraph from line 254).

ii) A general impression emerges that the conclusions are not drawn to the full potential of the data. There are of course many similarities between mouse and macaque. The authors try to pinpoint the differences. However, they seem to be of a limited impact and/or they are not confirmed with alternative techniques. Apart for PBX3, the other detected molecular claims have not been confirmed by another approach. For example, RNAscope, that is a robust mRNA detection technique could be used on a few precious macaque slices in some instances to confirm the hypothesis made by the authors. This concerns Transcription factor differences claims and glial cell expression differences between the mouse and macaque. In addition, the molecular calb1 marker difference could be of great importance in the auditory neuropathy field. In this field, it is of great debate whether the categorization of auditory nerve neurons found in rodents (low, middle and high threshold neurons) exist in primates and even more in humans. One would expect to see more explorations of this aspect using RNAscope or any other comparable method that is ethically compatible with the use of macaques.
One consequence of this lack of data confirmation is that the many form conclusions cannot be drawn. Here are a couple of examples:
-line 200: "these results suggest that a cohort fo TFs and effector genes can be used to classify cochlear cell types, but further verification is needed…"
-line288: "Future experiments should determine if these genes differed in species…"

-line 319: “ However, future experiments should determine…”.
-line 384, conclusion of the manuscript: “Different datasets, however, may require particular or combinatorial batch-corrected methods, since too strong correction might eliminate hidden variations in intro- or inter-species.”

iii) There are several generic conclusions that would deserve discussion. For example, I respectfully disagree with this affirmation: “By contrast, divergences across species provide strong evidence for adaptation or drift of non-human primate cochlea function, and of susceptibility to human-specific diseases”. There are of course difference in expression patterns of genes between different species. In several instances of the manuscript, the authors seem to want to push this idea that should be discussed in a more balanced way. The authors should give precise examples of human-specific diseases. To which ones do they refer? If one considers genetic forms of deafness for example, more than 150 genes have been identified. To the best of my knowledge, all of these genetic forms of deafness have been replicated either from human to mouse or from mouse to human. Moreover, there are more and more examples of particular audiological signatures associated with specific genetic forms of deafness that have been uncovered in mice and that were confirmed subsequently in patients. So there is clearly a very close resemblance in the genetic architecture and function of mouse and human cochleas. This does not necessarily mean of course that the transcriptomes are identical but it is important to address whether the differences observed in the manuscript may endanger or not the direct translation of therapies from mice to humans.

iv) Several methodological aspects were unclear:
-how many macaque cochleas were used in each sample? How many macaque replicates? It is likely that very few replicates were used for obvious reasons. However, this means that the differentially expressed genes between mouse have to be considered with care and require complemnetary methods (see point iii) above)
-the methods mention 1, 5, and 11 year old macaques. Are the macaques systematically mixed? Are there analysis between 1, 5, and 11 year old macaques.
-Only nuclei with more than 15000 genes were filtered out. This number seems very high and increases the risk of analyzing duplicates and or cells for which both the nuclear and cytosolic transcriptome were caught. A number like 5000 would be more reasonnable.
-Did the authors detect non circulating cells (lymphocites, macrophages etc…)? Were they filtered out during the procedure? How many out of the 20000 nuclei were finally exploited for the data presentation?

Minor issues.
i) Several sentences in the manuscript are not informative or lack relevance.
ii) Figures are generally well made. However, several problems need to be solves:
-many panels are unreadable due to too small characters (ex Fig. 1C, colour code about the nodes are unreadable in 2b,the colour codes for the cell types in the heat maps or clusters are generally unreadable (Fig. 6 for ex), the same colour code should be used between figures and species for the same cell type (6a, S18)
-there is a foreign text on S1C.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed my main concerns - thank you. The manuscripts is now written clearly and is much tighter & clearer throughout. While the number of animals used is low for the snRNAseq, nonetheless, it is high quality data that will be of tremendous use to the scientific community at large and constitutes a significant advancement worthy of publication in a high quality journal such as Nat Comm.

My one main concern that has not been addressed is that the code for generating all of their analyses has not been made available for peer-review. Not only does this ultimately need to be shared through github or a similar repository, it is this reviewer's opinion that it must also undergo the peer-review process as this is a critical part of the Methods section and must be presented in an acceptable manner. I understand that the raw data will be deposited in a publicly accessible repository. Finally, I do suggest that the authors consider depositing their data in gEAR as it will have maximum impact for the auditory neuroscience community and make the data accessible for scientists without much coding expertise (https://umgear.org/) (Orvis J, et al. Nat Methods. 2021 Jun 25. doi: 10.1038/s41592-021-01200-9, PMID: 34172972).

Minor comments:
153: type should be "types"
Fig3f legend: should say "left/right" as there's no top/bottom.
237: remove "to" between unlike & mouse SGNs
Fig4d: would be more clear and convincing to show the individual channels in gray-scale either in main figure or in supplement

Reviewer #2

(Remarks to the Author)
The latest revision has not significantly enhanced the manuscript. The authors have merely re-integrated the existing data, neglecting to expand the sample size of monkeys per group—a critical step to mitigate individual variability and validate the sequencing data's reliability. Without this resolution, publication in a reputable journal such as Nature Communications remains unlikely. Moreover, the manuscript requires further extensive validation to ensure its credibility.
The following concerns have been identified and require attention:
Major Issues:
1. The authors' responses have not adequately addressed the concerns raised. The current sample size is insufficient to overcome individual, gender, and age-related heterogeneity.
2. While the cross-species analysis revealed unique glial cell diversity, the authors have not clarified the distinctions between macaque and mouse glial cells or their functional implications. Experimental validation for macaque-specific markers such as GRIA2 and CLU is also needed.
3. In Figure 1c, the spatial distribution of two SGN subtypes in the macaque cochlea appears implausibly distant in the UMAP plot. Additional evidence and quantitative comparisons across samples are necessary.
4. In Figure 1d, the specificity of several marker genes, including ITGA8, CREB5, TMC1, CALB1, SLC17A8, PVALB, THSD4, BMP6, CLDN11, DCLK1, USH1C, GATA3, OTOA, and ENAH, is questionable. The authors must provide explanations.
5. The potential role of zinc finger transcription factors as cochlear epithelium markers in both species warrants further experimental validation.
6. In response to Review 2 of the P2P, the authors have not sufficiently addressed the reviewer's concerns, particularly the lack of experimental validation for U2H2A as a hair cell marker.
7. There is inconsistency in the ages of mice used across different figures (Figure S1b, Figure S2a and e, Figure S9b). Clarification of the data source for each sample is required.
Minor Issues:
1. Figure 2b requires annotation for the color bar.
2. The clarity of data representation in Figure 3e should be improved for enhanced readability.
3. Figure 3g requires clear annotation of its color bar for better understanding.
4. Additionally, several figure citations in the manuscript are improperly used, which could mislead the readers. Specifically, the text on line 272, “with many enriched in only one species (Figure 3d and e),” should actually refer to Figure 5d and e. Similarly, the statement on line 302, “with about half of the genes uniquely expressed or enriched in HCs (Figure 5; Figure S17),” should cite Figure 6.
5. The scale bar value is missing in the legend of Figure S1c.

Reviewer #3

(Remarks to the Author)
The major interest of the work by ‘Xin Chen et al.’ is that it provides a single cell transcriptomic dataset in a non-human primate essential to make sure the gene therapies designed in mice have a chance to be safe and to be rapidly translated to patients. The two major concerns that were raised have only been partially addressed:
i) The English writing was significantly improved making the manuscript more easily readable. However, there are still some language inaccuracies. More problematic is the fact that the authors did not increase the size of the text in the figures making many of them unreadable when printed on paper and requiring a zoom of 200-250% to be readable on the screen (see Fig.1d,2b,3b,3c,3d,3e,3f,4e,5b, 5c, 5g,6, supp fig1d,1e,1f, 1g,1h,2a,2b,2c,2d, 3a,3c,3d,3e,3g,3h, 4a, 4b, 12b,12c,12e,12g,13,14b,14c,14e,14g,15,17).
ii) Concerning the lack of additional cochlear transcriptomic knowledge, the improvements are limited probably owing to the filtering of the data obtained owing to the methodological set-up for several reasons: the limited number of macaques used, the use of aged tissue (compared to relatively young adult mice), and the use of single nucleus transcriptomics which provides more cells to the cost of sequencing depth. Despite these basic problems that are probably unsolvable, the authors could detect all key cochlear cell types and the data is potentially valuable as, to the best of my knowledge, no non-human primate cochlear transcriptome data is available yet. However, the authors should make clear the limitations of their data and not make overstatements. This point should be discussed extensively and in a balanced way.

Please find below more details:

1) The paragraph on cell diversity in the macaque cochlea is not very convincing owing to the limitations of the data mentioned above. The authors should be more careful in their conclusions as they are highly likely overstatements. Example: “glial cells in these two species differed in their transcriptional profiles”. The problem is the same with the section on the supporting cells : “this unexpected contrast between a small group of conserved genes and a large cohort of genes manifesting species-specific enrichment highlights the diversity of SC types across species”.

2) There is a similar problem in the “deafness associated genes across species section”.
The sentence “This indicates…that previously identified HL genes are mainly concentrated in HCs and are linked to HC functions” is a very likely overstatement. As mentioned in the review of the first submission, most if not all genetic forms of deafness have been replicated either from human to mouse or from mouse to human. Moreover, there are more and more

examples of particular audiological signatures associated with specific genetic forms of deafness that have been uncovered in mice and that were confirmed subsequently in patients. So there is clearly a very close resemblance in the genetic architecture and function of mouse and human cochleae. It is therefore highly unlikely that the macaque diverged in a major different direction from mice and humans. The probable reasons for the observations of the authors is that they used adult/aging macaques and most of the pathogenic genes are involved in cochlear development, many of them being in hair cells but many others also in other cell types (see Fig. 7 from JeanP et al. 2023, PNAS). For example, it is puzzling that GJB2, a major gene underlying around 50% of the deafness forms does not show much expression and differential expression within cochlear cell types (see supp Fig. 17 of the authors) whereas it is known to be involved in many cochlear cell types.

3) Concerning the “sensory neuron diversity in the macaque cochlea”, the authors pinpoint the key proteins lacking in macaque SGNs (versus mice) like pou4f1 and LypD1. Did the authors find potential new markers specific of type1c macaque neurons (and more generally other neurons)? I think the information is not mentioned and it could potentially be beneficial to try to demonstrate the existence of type1C neurons on human tissue.

4) The discussion should balance more the conclusions in the light of the limitations linked to the small number of macaque replicates, the use of adult macaques compared to young mice, and the fact that single nucleus transcriptomic sequencing has less depth of sequencing. The authors should indicate the average depth of sequencing (number of read per cells) and also give statistics on mitochondrial genes in the material and methods.

5) I do not understand why the authors wanted absolutely to test for the presence of electromotility in a macaque. The electromotile property of OHCs can be monitored with a non invasive technique, called distortion products acoustic emissions (DPOAEs). DPOAEs are well characterized in mice and humans. They probably are too in many non-human primates (eg: Valero et al Hearing Research 2008, PMC2567920). In addition, mutations in the Prestin gene have been found to underly hearing loss in patients (ex: Toht et al, PMID: 17786286). I therefore do not understand why the authors wanted to make the point that prestin is indeed expressed by macaque OHCs and underlies electromotility. The sole detection of prestin was a good enough indication that they had found OHCs.

6) Why reanalyzing the data by (Jean P. et al, PNAS 2023)? I could not find the reason in the manuscript. I guess the reason is the use of a UMAP analysis rather than t-sne. If this is the case, the precision should be made in the manuscript. In addition, the citation of the paper should be made systematically for each panel in supp Fig.2 with a citation like (Jean P. et al, PNAS 2023) and not just saying “by PNAS”. This is important to make sure there is no ambiguity between the authors’ data and other studies’ data. In supp Fig. 3, the authors should clearly state in the figure legends that they are back to their own data, too.

7) In supplementary Fig 1f, the scheme appears to be a copy-paste of the scheme found in Fig. 1b of Jean P. et al, 2023 PNAS. Moreover, Fig.1a also corresponds to Fig 1b of of Jean P. et al, 2023 PNAS with an additional layer. There is absolutely no source cited in the legends of these figures. Could the authors make the corrections and also confirm that there are no other figures copy-pasted from any other article/journal?

8) Other points:
-line94: ‘preferentially’ should be removed
-line 100: “extreme’ should be removed
-Line 112: “gene therapeutics” would better read “gene therapies”
-line 127: the adjective “non human primate” could be added to “constructing a cell atlas”.
-line 142: “in 1-year-old and 11-year old macaques” should read “in a 1-year-old and an 11-year old macaques”
-line 151: “These results strongly implicate” would better read as “These results shows”?
-line 156: “Oterferlin” should read otoferlin
- line 198: Maybe remind for the general reader what are Remak bundles.
-line 265: “to integrate the mouse” should read “to integrate our mouse” to avoid any ambiguity.
-Supp figures 6 and 7 are nice in that they can be put in parallel. However, the authors removed the GFAP from the mouse analysis. Why is it so whereas all other markers are exactly the same between macaque and mouse data?
-Please add the number of nuclei for each UMAP on the figures.
-Supplementary Fig. 17: lacking a scalebar
-Please verify on all the figures that a scale bar is present and readable

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed all of my concerns. This is a valuable dataset that will be highly useful to the auditory neuroscience community. I appreciate the authors' sharing their annotated code in GitHub to make this work reproducible. The URL for this should be mentioned in the methods section. Likewise, sharing the data through gEAR (for those that can't code), and depositing the raw data online will also be highly valuable to the auditory neuroscience community. Currently, I do not see where the raw data will be deposited - this should be added to the final manuscript

including the raw fastq files for all of the macaque snRNAseq data.

I have some minor comments:
Consider changing the title from "Elucidating the molecular diversity of non-human primate cochlea" to "Molecular heterogeneity of the non-human primate cochlea" or "Molecular diversity of the non-human primate cochlea"

Introduction - Number of inner and outer HCs in mice and in humans should have citations

Lines 204/205: "were strongly expressed in macaque HCs across species." Should this be in macaque HCs or just in HCs across species without "macaque" here?

Line 206: should be "conserved" instead of "conserve"

"RNASCOPE" is used multiple times or "RNAscope." Suggest keeping the format the same throughout. It should be "RNAscope™" as it's a trade-marked name from ACD Bio. Alternately, authors can just say "in situ hybridization" and just state once in the methods that the RNAscope™ platform was used.

Line 350: Should "HCs" here be "SCs"? I think it's supposed to be "This divergence in SCs..."

Figure 2a: the gray-scale UMAP has an arrow that brings out the IHC/OHC group. Is that arrow supposed to start where the black dots are? It looks like it's coming from an edge of a large cluster now. May need to be corrected.

(Remarks on code availability)
I have reviewed the code provided in github. I did not re-run the code, but in general, it appears well annotated and should give readers a chance to see exactly what was done. I am unable to re-run the code as the raw data or processed data has not been deposited online. Sharing the raw data is a critical part that must be deposited online prior to final publication of this important work.

Reviewer #3

(Remarks to the Author)
The authors addressed my concerns and it is appreciable that they increased the number of 5 year old macaques to make their data more significant. A few minor issues/typos were spotted concerning the readability of figures.

Minor issues

-line 98 : for clarity, the number of IHCs and OHCs should be mentionned for human cochlea for direct comparison with the mouse cochlea.

-line 206 : ‘conserve’ should read ‘conserved’

-line 273 : ‘were share’ should read ‘were shared’

-Fig. 1a: acronyms in the figure and in the legend do not always correspond :
Neu on the figure reads Type I and Type II in the legend
SS is on the figure and not in the legend
Osteocytes in the legend but not the diagram.
There are probably other inconsistencies.

Flg. 1 : ‘a’ and ‘b’ are not aligned. ‘c’ and ‘e’ are not aligned

Fig2: a) Some unreadable characters.
b) Missing ‘min’ and ‘max’ at the legend of expression level

Fig4: f), h) scale bars are unreadable

Fig5: f) legends too small

Fig6: c)b)d) all legends too small.

There are similar matters on several supplementary figures.

(Remarks on code availability)

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Dear Editor:

We express our sincere gratitude to you and the three reviewers for acknowledging the merit of our research and for providing insightful and constructive feedback, which has greatly enhanced the quality of our manuscript. We particularly note that while all reviewers appreciated our significant achievement in non-human primate cochlea research, they also identified areas for improvement in our writing style. We took this feedback earnestly and have carried out extensive revisions on our manuscript. Kindly refer to our general and point-by-point responses detailed below.

Reviewer #1 (Remarks to the Author):

In this manuscript, Chen et al. present a cross-species comparative study between mouse and macaque cochlear cell types. They profiled more than 20643 nuclei from 1-year old and 11-year old crab-eating macaque cochlea, and 9470 nuclei from P21-25 murine cochlea using single-nucleus RNA sequencing on the 10x chromium platform. Nuclei were collected from cochlear epithelium, spiral ganglion, stria vascularis, spiral ligament, and other inner ear components including Reissner's membrane, macrophages and osteoblasts. Through a series of computational comparative analyses, the authors demonstrated conserved and divergent features between mouse and macaque cochlea and cellular diversity in spiral ganglion neurons. This cochlear snRNAseq dataset from non-human primates is a tremendous feat and ultimately a step closer to understanding the pathophysiology of the inner ear in mammals. However, unfortunately the manuscript in its current form has some major issues that do not make it acceptable for publication.

We appreciate the reviewer's comments on our current work as a tremendous feat on non-human primate cochlea.

Major concerns:

1. Writing composition: Throughout the manuscript, there are innumerable grammatical and syntax errors that make reading it quite laborious. This reviewer initially began keeping track of these, but quickly became overwhelmed with this effort. I do suggest that the authors take a careful look at their writing.

We appreciate the reviewer's comments and take it seriously. We have corrected our grammatical and syntax errors and refined our manuscript thoroughly.

2. Validation of results: There is sparse validation data for most of the manuscript. While there is one transcription factor that the authors validate as unique to the macaque (PBX3), there is none for the major cell types of the organ of Corti such as inner or out hair cells, or any of the supporting cell types. Without such validation, it is unclear if any of the presented data is biologically relevant.

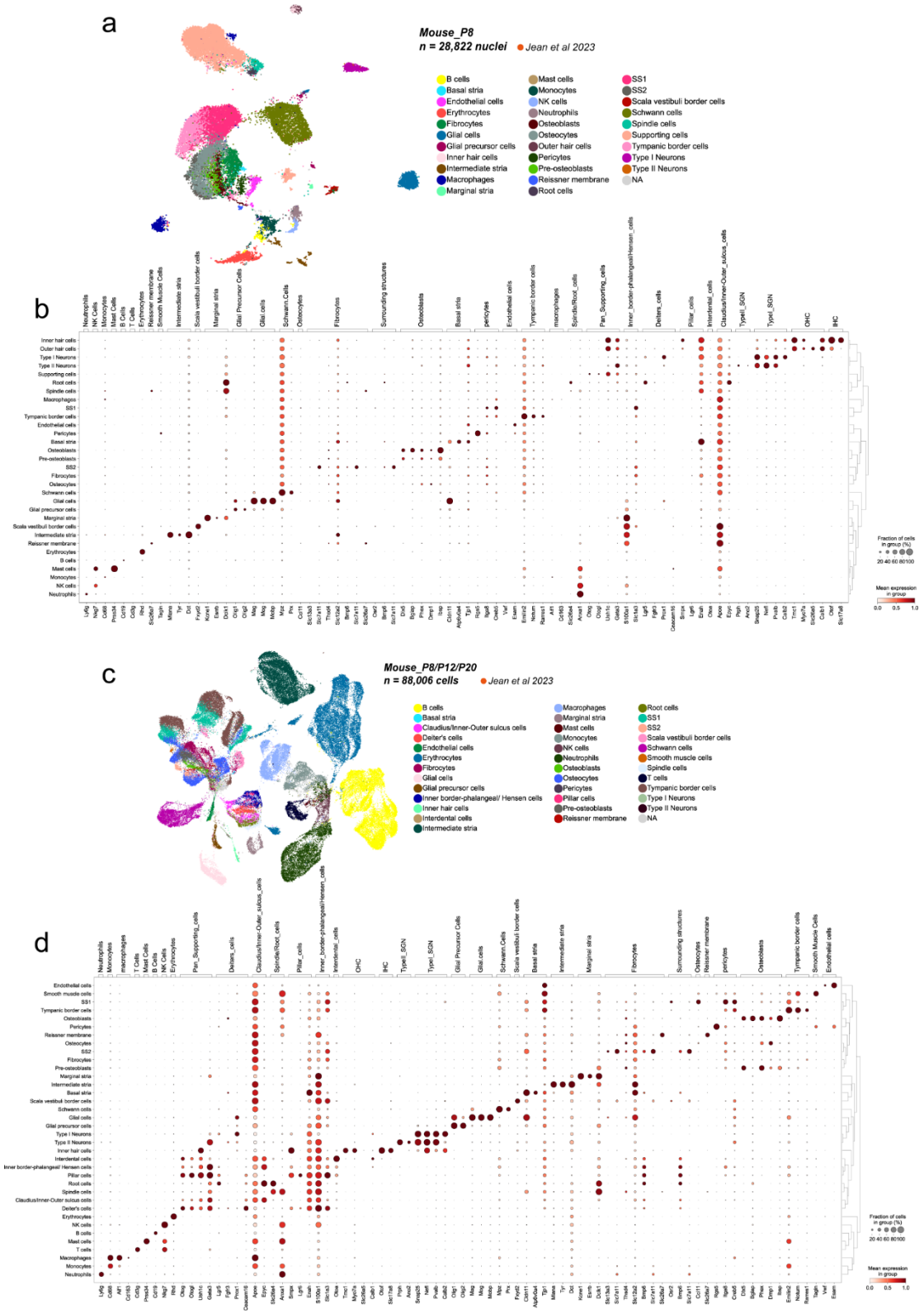
In this revised version, we've performed thorough validation of the primary cell types in the organ of Corti, including the inner and outer hair cells, which was a strong point of emphasis by the reviewer, as well as spiral ganglion neurons and their associated glial cells. After incorporating these validations into our updated manuscript, we're confident that this improved version will address your and the reviewers' concerns effectively.

3. A recent comprehensive snRNAseq dataset from Jean et al. (2023, PNAS) has 37 cell types described in mouse, with several of the cell types described (and validated) there not

present in the dataset here. Any thoughts on the discrepancy here? Would using that now publicly available dataset provide a better comparison (at least the P20 data)?

In our revised manuscript:

(i) As suggested by the reviewer, we have indeed reutilised the dataset from the 2023 PNAS paper.



Supplementary Figure 2

Single-cell or single-nucleus analysis of mouse cochlear cell types from PNAS, related to Figure 1.

a *UMAP (Uniform Manifold Approximation and Projection) plot representing cell types of P8 mouse cochleae annotated by PNAS.*

b *Dot plot depicting the expression levels of marker genes in each cell type from P8 mouse cochleae.*

c *UMAP plot, similar to (**a**), but for pooled P8, P12, and P20 mouse cochleae. The capture method employed single-cell dissection and resuspension, and cell-type annotation was performed by PNAS.*

d *Same as (**b**), but for P8, P12, and P20 single-cell mouse cochleae.*

(ii) Interestingly, we found that the well-validated markers from that paper are sufficient for our cell-type annotation, indicating that our in-house dataset is both comparable and comprehensible.

(iii) Additionally, besides immune cells and other structures such as surrounding structures and osteoblasts, we have managed to capture the entire cochlear cell types. These exhibit similarity to the major cell types found in Jean et al.'s study.

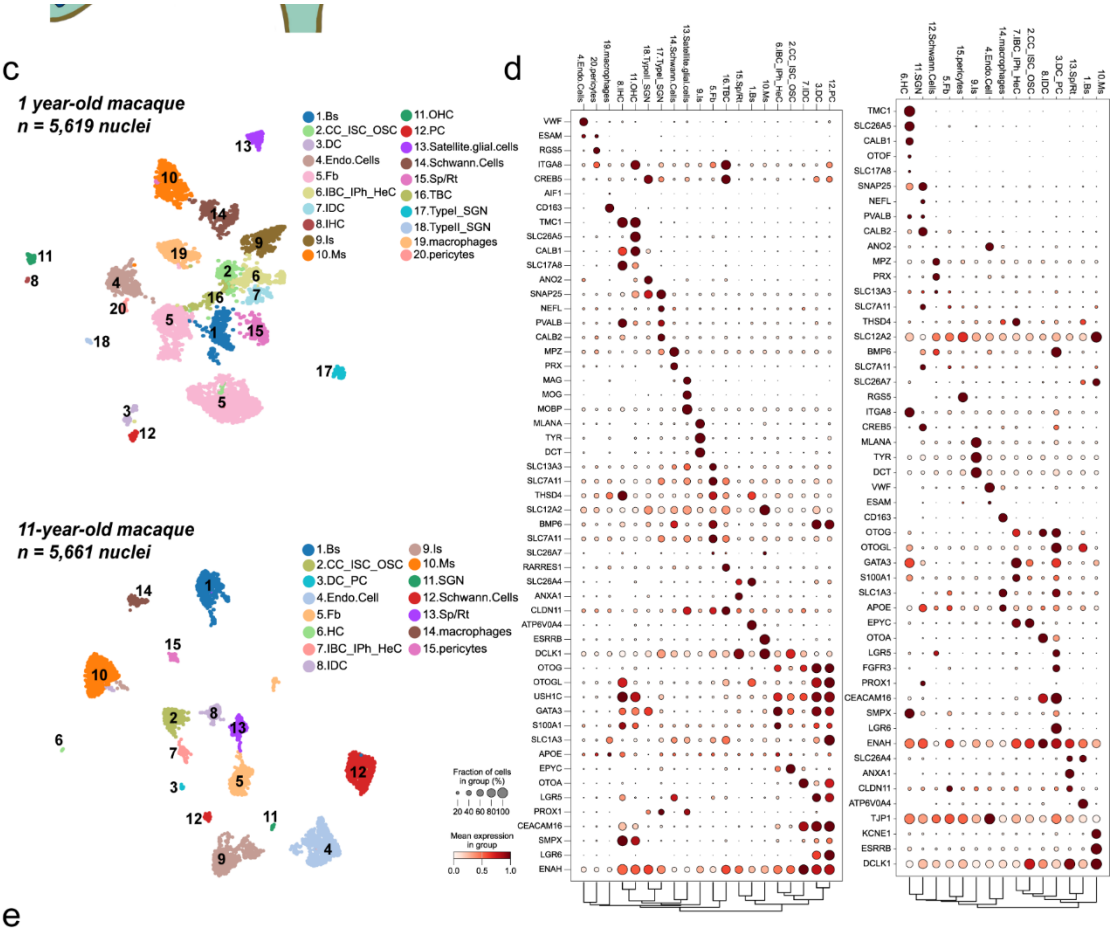


figure 1 A single-nucleus atlas of the macaque cochlea.

(c) *Uniform Manifold Approximation and Projection (UMAP) representation of whole-organ cochlear profiles (n = 5,619 nuclei for the 1-year-old macaque and 5,661 nuclei for the 11-year-old macaque) identified more than 20 distinct cochlear cell types.*

(d) *Dot plots displaying the mean expression level of well-characterized marker genes for each*

cell type (left: 1-year-old macaque; right: 11-year-old macaque).

4. Figures 1-5 lack some originality in presenting the data. Certain parts of the manuscript are a near complete recapitulation of Bakken et al., 2021 (Nature), including to the level of figure colors (e.g. Fig 3d here and Fig 2c in Bakken et al, among others).

We totally refined our figures and made it original.

5. I understand and appreciate that obtaining inner samples from macaques is difficult for a variety of reasons. However, there is a vast age difference between the two animals used here (1 year old and 11 year old). It would be much more convincing if there was a closer age match – for example two 1 year old or two11 year old animals. Furthermore, in Fig 1b, there appears to be a couple of large groups of cells that are only in the 1yo macaque, and not the 11 year old. This is either batch effect or related to age differences. If related to age differences, then at least one additional specimen is needed per category. These cells groups are later labeled as Fibro2 and Fibro4_RMC.

We sincerely thank the reviewer for acknowledging the challenges we faced in obtaining macaque cochlea samples. It's well recognized within the field of hearing neuroscience research that the dissection of juvenile and adult cochleae, let alone aged cochleae, in species ranging from mice and rats to pigs and larger animals such as macaques (as in our study), represents a formidable task. This undertaking is often fraught with numerous difficulties and failures, from the dissection process itself, to the task of loading onto the 10x Genomics platform, and even due to the scarcity of hair cells—the number of which can range from 3,000 in a mouse cochlea to 15,000 in a human cochlea. Given these inherent challenges, we have so far managed to profile one juvenile and one adult macaque cochlea for high-throughput sequencing, while being able to use one juvenile cochlea for electrophysiological recording and two adult macaque cochleae for validation purposes. We sincerely appreciate the reviewer's suggestion about maintaining consistency in sample collection, and our new results indicate that

(i) Preliminary findings suggest that the hair cells in a juvenile macaque have indeed reached functional maturity. This conclusion is supported by our introduction of electrical recording to measure the electromotility of the outer hair cell—a feature typically indicative of maturation in outer hair cells. Furthermore, our examination revealed that hair cells in the one-year-old macaque demonstrate full expression of mature hair cell markers, including those such as SLC17A8, SLC26A5, and ATP2A3, among others. This expression is managed in conjunction with fate determination factors including IKZF2 and TBX2, as shown in the data below:

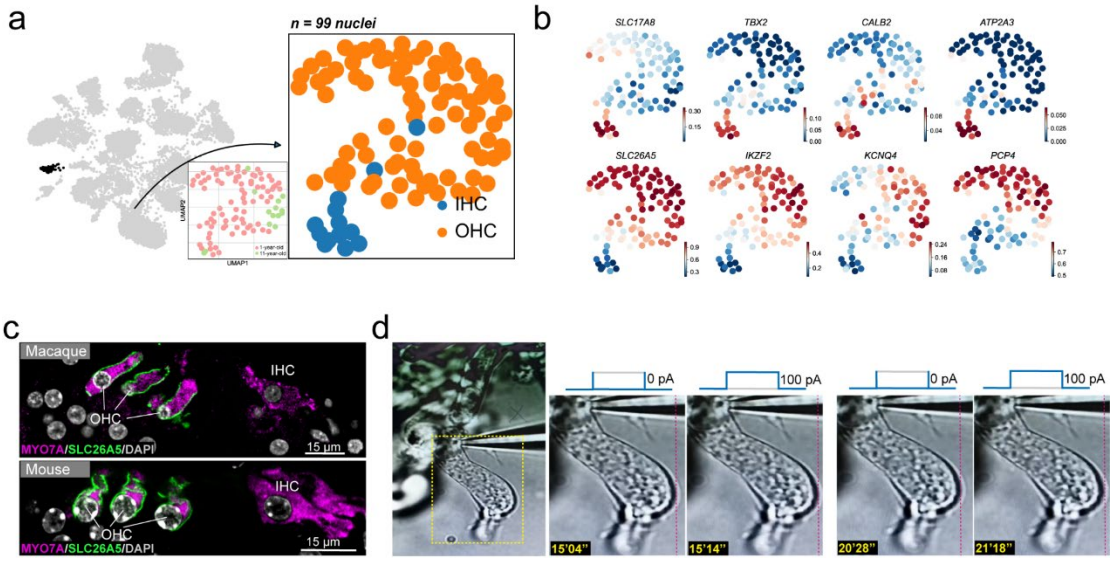


Fig. 2. Characterization of auditory receptors in the macaque cochlea.

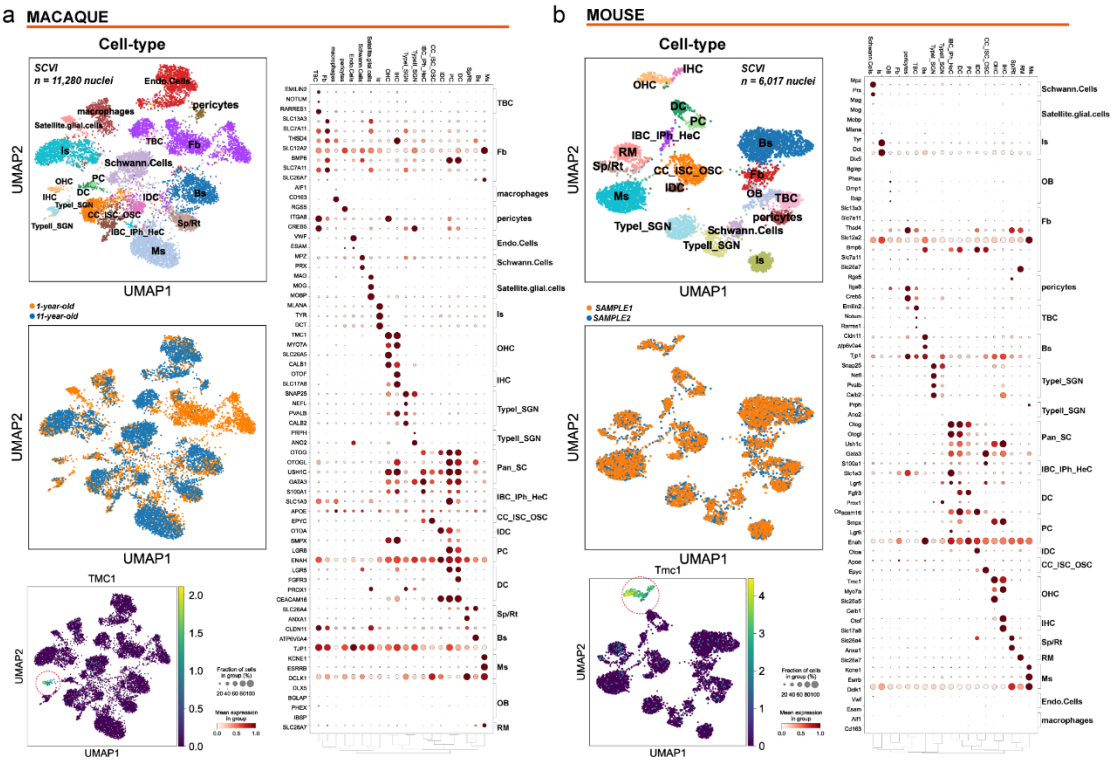
a *Uniform Manifold Approximation and Projection (UMAP) visualization of hair cells in the macaque cochlea, including inner hair cells (IHCs) and outer hair cells (OHCs).*

b *Top panel: Expression profile of canonical marker genes for IHCs, as previously identified in mouse models. Bottom panel: Expression profile of canonical marker genes for OHCs, as previously identified in mouse models.*

c *Confocal microscopy images demonstrating the localization of PRESTIN protein in macaque OHCs.*

d *Patch-clamp recordings revealing the electromotility of OHCs in response to current injection.*

(ii) Additionally, our data integration results indicate a successful combination and adequate mixing of these two samples. This further strengthens the reliability of our study and indicates a reliable overlap and correlation between the distinct sample sets.



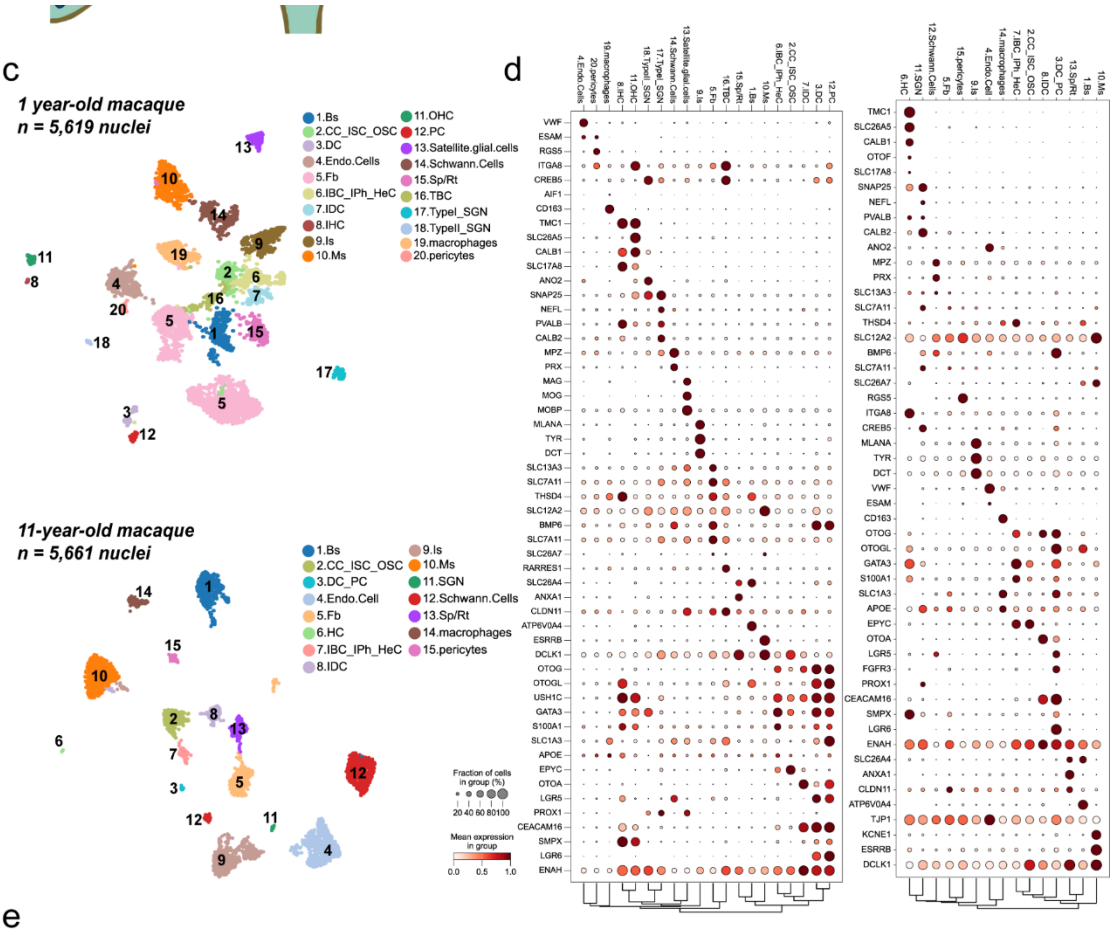
Supplementary Figure 4

Single-nuclei profiling of macaque and mouse cochlear cell types by scVI integration, related to Figure 2.

a *The left panel exhibits UMAP plots representation of cell types from the macaque cochleae while the right panel outlines the gene expression profiles pertinent to cell-type annotations.*

b *Similar to (**a**) but the analyses pertain to P21 mouse cochleae.*

(iii) Importantly, the markers used for cell-type annotation, typically associated with mature or terminal stages, align well within these two distinctive samples. This finding suggests a consistent cell-type identification across different developmental stages within our study.



(c) *Uniform Manifold Approximation and Projection (UMAP) representation of whole-organ cochlear profiles (n = 5,619 nuclei for the 1-year-old macaque and 5,661 nuclei for the 11-year-old macaque) identified more than 20 distinct cochlear cell types.*

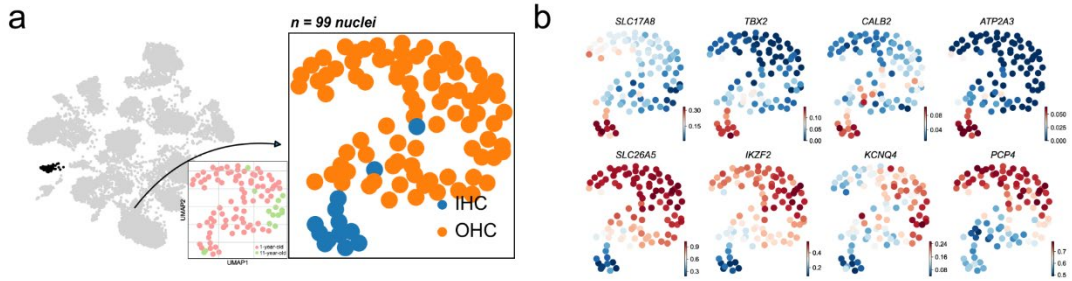
(d) *Dot plots displaying the mean expression level of well-characterized marker genes for each cell type (left: 1-year-old macaque; right: 11-year-old macaque).*

6. Cell composition analysis (Fig 1e) cannot be inferred from percentages. This is not computationally sound. See (https://www.sc-best-practices.org/conditions/compositional.html) for details and explanation.This figure should either be removed or performed in a computationally rigorous manner.

We accepted this suggestion and excluded this analysis.

7. How many hair cells of each sub-type are in the two datasets (mouse and macaque)? Did the authors make any attempt to harvest a larger number of hair cell nuclei? And why are the IHCs and OHCs so far apart on the UMAP? In nearly every other manuscript with scRNAseq from the inner ear, they're not that far apart in space. I understand that relation of cells cannot be fully inferred from UMAP, but I have not seen these cell types this far apart on a UMAP.

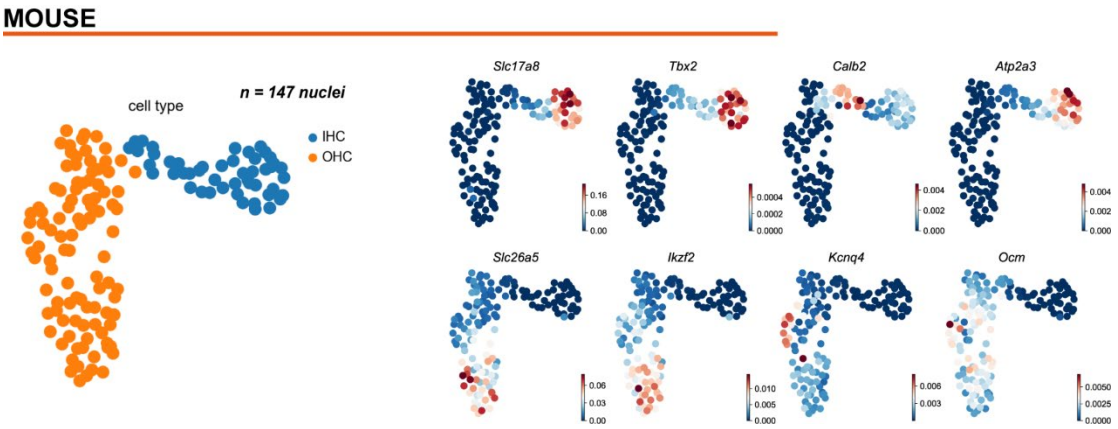
We are appreciative of the reviewer's insightful comments and have treated them with due seriousness. In our revised version, following a re-clustering of our dataset, we obtained (i) 99 hair cells in the macaque cochlea,



a *Uniform Manifold Approximation and Projection (UMAP) visualization of hair cells in the macaque cochlea, including inner hair cells (IHCs) and outer hair cells (OHCs).*

b *Top panel: Expression profile of canonical marker genes for IHCs, as previously identified in mouse models. Bottom panel: Expression profile of canonical marker genes for OHCs, as previously identified in mouse models.*

and (ii) 147 hair cells in the mouse cochlea.



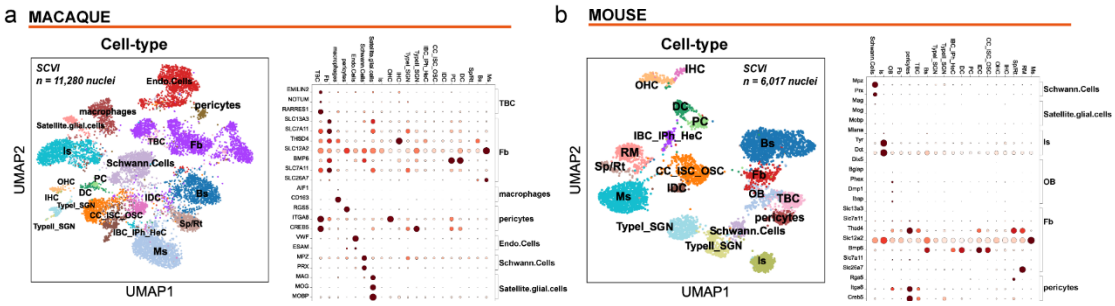
Supplementary Figure 5

Partitioning and re-clustering of mouse hair cell types, related to Figure 2.

Left: UMAP plot showing the integrated inner and outer hair cells of mouse hair cells. Right: the canonical inner hair cell markers (Slc17a8, Tbx2, Calb2, Atp2a3) and outer hair cell markers (Slc26a5, Ikzf2, Kcnq4, Ocm) were distinct expressed across the two populations.

Furthermore, (iii) by adopting the scVI integration method for dataset combination or conducting independent Leiden clustering on each sample, we've noticed that Inner Hair Cells (IHCs) and Outer Hair Cells (OHCs) now appear significantly closer in terms of their UMAP representation.

This revised approach has certainly improved the clarity and accuracy of our data representation.

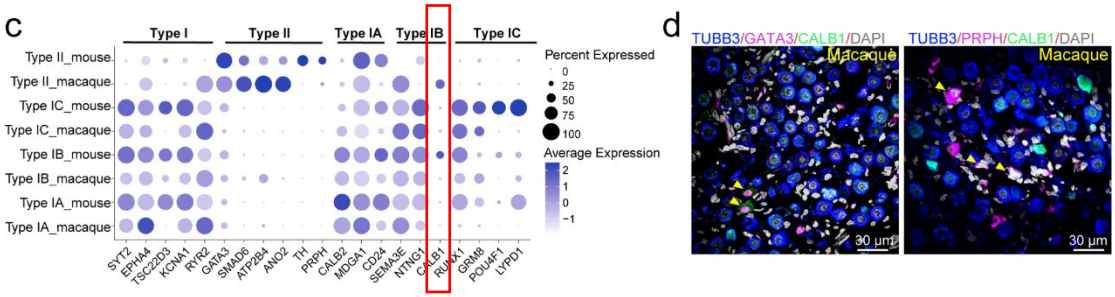


a *The left panel exhibits UMAP plots representation of cell types from the macaque cochleae while the right panel outlines the gene expression profiles pertinent to cell-type annotations.*

b *Similar to (**a**) but the analyses pertain to P21 mouse cochleae.*

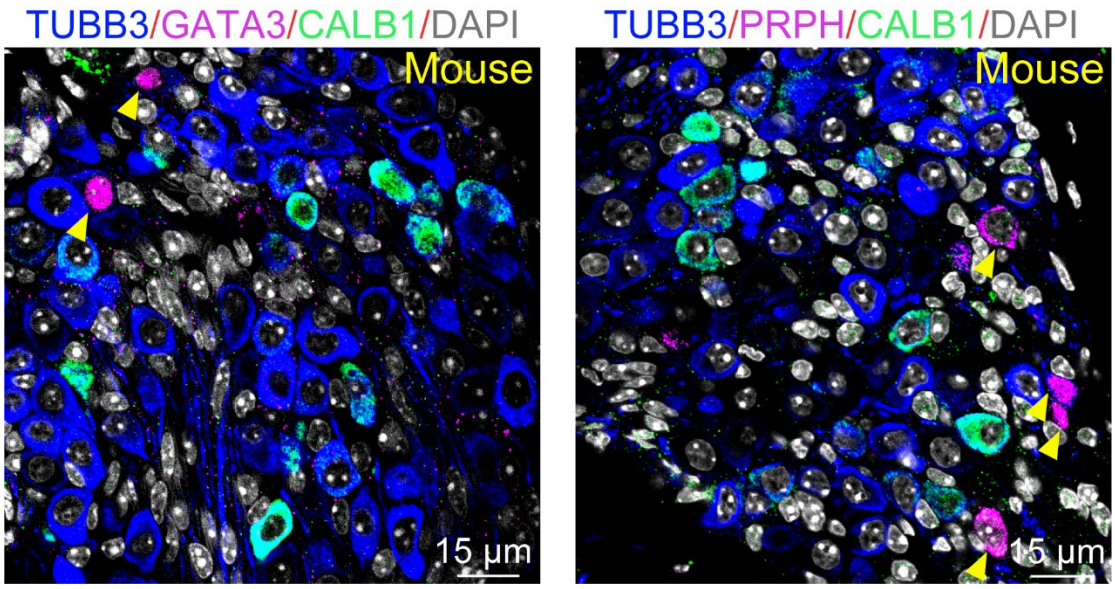
8. Re Fig 5d: Calb1 and Calb2 differences between mouse and macaque is not obvious here. Any attempts at quantification? That would be more convincing. It would be even more convincing if there was some in situ hybridization with quantification.

We deeply appreciate the reviewer's comments. In our revised manuscript, our findings reveal a distinctive trait in macaque cochlea: unlike in mice, Calb1 strongly co-localizes with Type II Spiral Ganglion Neurons (SGN) markers, including Gata3 and Prph. This conclusively confirms our sequencing data that Calb1 serves as a marker for Type II SGN in macaque cochlea. This unique pattern further sets apart the cochlear cellular architecture in macaques from that of mice, enhancing our understanding of species-specific differences.



c *A dot plot illustrating the gene expression levels of SGN subtype markers identified in mouse species, compared between macaque and mouse SGN subtype cells.*

d *Representative confocal images of immunofluorescence cryosection staining demonstrating CALB1 associated with GATA3 or PRPH localization in 5-year-old macaque (top) and 3-week-old mouse (bottom) SGNs.*



Supplementary Figure 11

Immunofluorescence staining of CALB1 with GATA3 or PRPH, related to Figure 4.

Representative confocal images of immunofluorescence cryosection staining demonstrating CALB1 associated with GATA3 or PRPH localization in P21 mouse SGNs.

9. Did the authors pull out any tonotopic data? There's discussion of the hearing frequency differences between mouse and macaque, but is there any tonotopic evidence computationally in either the mouse and/or the macaque data?

We are truly thankful for the reviewer's thoughtful comments. However, we believe that the issues raised extend beyond the scope of the current study. We hope to address these in a more comprehensive manner in future research endeavors.

10. The authors do not use a robust computational method for their chosen clustering resolution. It would behoove the authors to clearly perform a calculation to determine the optimal clustering for each of their datasets. For example, Bakken et al 2021 have a method to do this, and there are a few other methods available in the literature (e.g. Patterson-Cross et al., 2021, BMC Bioinformatics).

We are very appreciative of the reviewer's insightful comments. However, in this revised version of our manuscript, we have chosen to exclude the analysis involving the subdivision and sub-clustering of cell types. This decision was made to streamline our focus and maintain the conciseness of our study. Nonetheless, we acknowledge the potential importance of such an analysis for further understanding and we aim to incorporate it in subsequent research.

11. Methodology: there's a clear lack of details in the methods regarding how tissue was obtained in the macaque. Description of the dissection technique is lacking, what enzymes were used for dissociation and for how long, time from animal sacrifice to loading on the 10x Controller, etc…

We are sincerely grateful for the reviewer's comments and have taken them into careful consideration. To address the issues raised, we have endeavored to provide as much detail as possible in the method sections of our manuscript. Our aim is to enhance clarity and deepen understanding for our readers. We hope this improved elaboration satisfies the concerns expressed.

12. Code availability: without proper and detailed code (for example shared on GitHub or similar platform), this reviewer cannot clearly see what was done from a computational perspective. While there are descriptions of all of the steps, and references to others who have done similar work, this is not a substitute for clear and well-annotated code for both peer-review and for the general scientific audience.

We appreciate the emphasis on transparency and reproducibility in research and take these concerns very seriously. Accordingly, we will shortly be uploading the necessary codes to a public GitHub repository, along with the raw data associated with our research. This will enable interested parties to scrutinize, understand, and extend our work, thus fostering a more open scientific environment.

13. I have some concerns regarding the consistency of what is said and what is shown. For example,throughout the main manuscript, the authors refer to a 1 year old macaque, however, in the supplementals(Fig S1d), they write "Macaque_8M" a number of times. Is it 1 year old or 8 month old?

We sincerely regret the mistakes and oversights in our previous manuscript and want to express our apology for any confusion caused. For absolute clarity, here is the corrected information about the subjects used in our study:

For the sequencing portion, we utilized one 1-year-old and one 11-year-old macaque.

For the patch-clamp recording, we used a single 1-year-old macaque.

For validation purposes, both a 5-year-old and an 11-year-old macaque were used.

We hope this rectification clears up any misunderstandings, and we appreciate your understanding. We are dedicated to ensuring the accuracy and transparency of our research in future publications.

14. Where are the inner/outer hair cells in Figure S4a? I only see “HC” as a category without determination of IHC/OHCs. If there’s claims of translational potential, it would be critical to rigorously show both inner and outer hair cell composition along with appropriate validation of these cell types. There’s discussion of this work being important for pathological conditions such as age related hearing loss (ARHL). Approximately 80% of ARHL is secondary to cochlear hair cell loss.

We appreciated reviewer’s meticulous care for figuring out this detail and we take it seriously. In our new version, one can exactly observe both inner and outer hair cell not matter in separate dataset or combined dataset.

We truly appreciate the reviewer's careful and thorough examination of our work, which has helped us to improve the accuracy and clarity of our findings. We have taken these critiques seriously and revised our manuscript accordingly. In this new version, the details pertaining to both inner and outer hair cells are clearly depicted and can be readily observed in every dataset, whether analyzed separately or in a combined manner. This added precision should eliminate any potential ambiguity and enhance overall comprehension of the data presented. Once again, we thank the reviewer for their insightful comments and their commitment to upholding rigorous scientific standards.

Minor concerns:

1. Abstract: The last sentence mentions human stem cell-based models of disease. However, there's no data or any reference to stem cells anywhere else in the manuscript.

We appreciated reviewer's meticulous care. However, in this section, we attempt to provide a solid foundation for the development of stem cell-based models.

2. Introduction: line 92- reference is missing

We refined this.

3. Introduction: line 97-99- meaning is not clear

We refined this.

4. Introduction: line 106 and 109- the full forms of MET and AAV should be mentioned when the term is first introduced

We appreciated reviewer's reminder and refined this.

5. Results: line 193- typo: 'uncvered'

We refined this.

6. Manuscript should say "glia" or "glial cells" not "glia cells"

For uniform of context, we used 'glial cell' for rigorously understanding.

7. Did the authors attempt another species comparison method?

In our refined manuscript, we introduced both scVI and CCA algorithms for species comparison.

8. Line 363-364: the human hearing frequency should be listed (20Hz to 20,000Hz).

For reviewer's kind reminder, we have listed in the context.

9. Fig1a: Cartoon should not read "aged" macaque but "adult" macaque.

For reviewer's point-out, we refined this.

Reviewer #2 (Remarks to the Author):

Although it is of significance to reveal the cell composition and function diversity of non-human primate cochlea, and this study constructed a single-cell transcriptomic atlas of the macaque cochlea and discovered remarkable heterogeneity across and within cell types, this work is still rudimentary, requires a lot of follow-up work to improve.

We are grateful for the reviewer's insightful suggestions, which have been instrumental in enhancing our research. We have given their feedback careful consideration and have worked diligently to improve our manuscript. We've tried our best to address all the concerns and have further developed our scientific narrative, forming a more comprehensive and complete story.

We believe the modifications inspired by the reviewer's feedback not only rectify previous shortcomings but also enrich the overall substance and quality of our work. We look forward to hearing additional thoughts and comments on the revisions we've made.

1. The purpose of this study is to construct a single-cell atlas of the cochlea in non-human primates. However, the authors selected only one male individual aged 1 year and one female individual aged 11years of the crab-eating macaque species for single-cell

sequencing. Due to a great variation between individuals, the quantity of samples at the same gender and the same age in biological studies is generally more than 3. In order to avoid the effects of individual differences on the results, the authors should increase the samples for sequencing. It would be difficult to get the manuscript published in a decent journal like Nature Communications if the issue was not resolved.

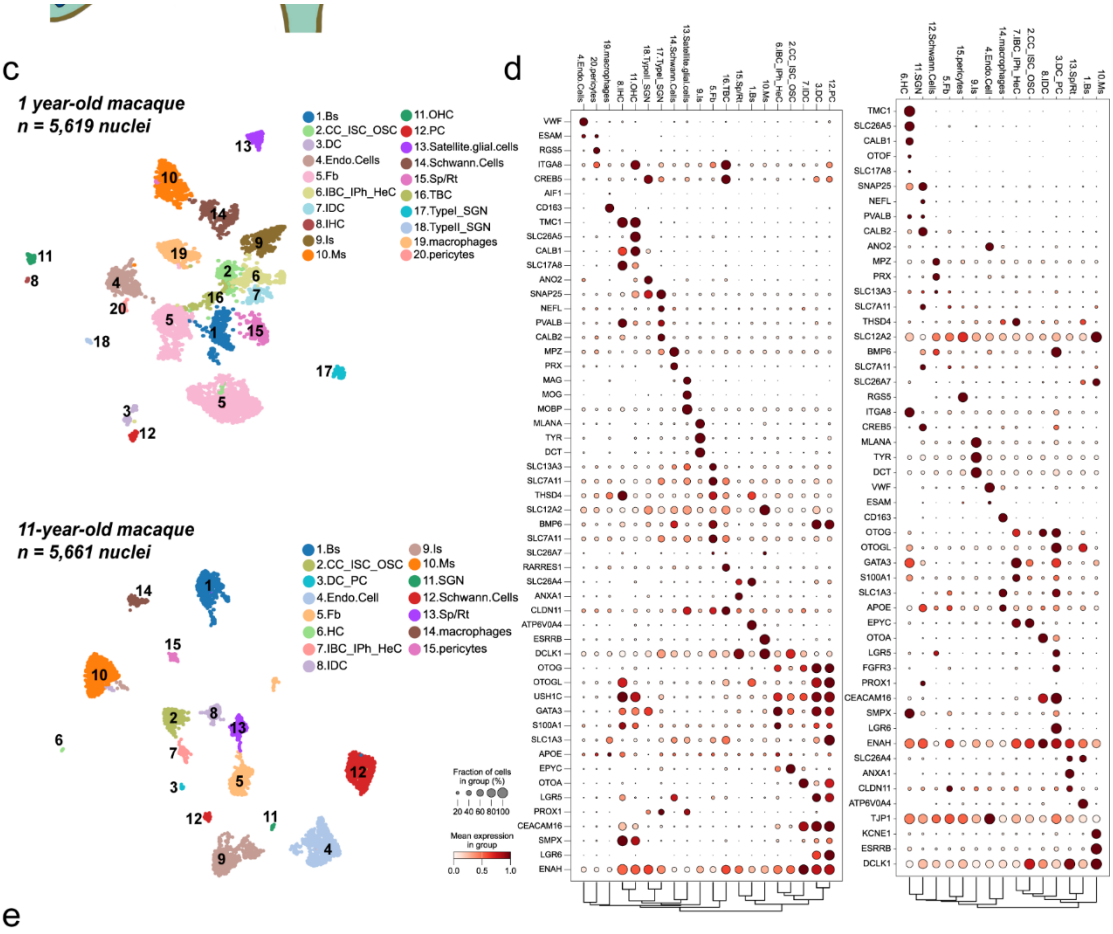
We are grateful for the reviewer's remarks and would like to address the concerns raised about the number of individuals sequenced.

(i) As the reviewer rightly acknowledged, the primary objective of our study is the generation of a single-cell atlas for the macaque cochlea. Therefore, utilizing samples from different life stages - juvenile and adult - appeared promising to give a holistic profile of the single-cell transcriptome. Thus, the two samples chosen for this purpose seemed logically sound and well within the scope of our study.

(ii) We anticipated potential variations between these two samples. Our primary presumption was that differences attributable to age and gender would not be substantial. Supporting this, our single-cell dataset convincingly demonstrated that well-validated markers for cochlear cell-type annotation were well-mapped into both our juvenile and adult macaques. The expression patterns between the two samples were not only similar but also comparable to those observed in mouse counterparts.

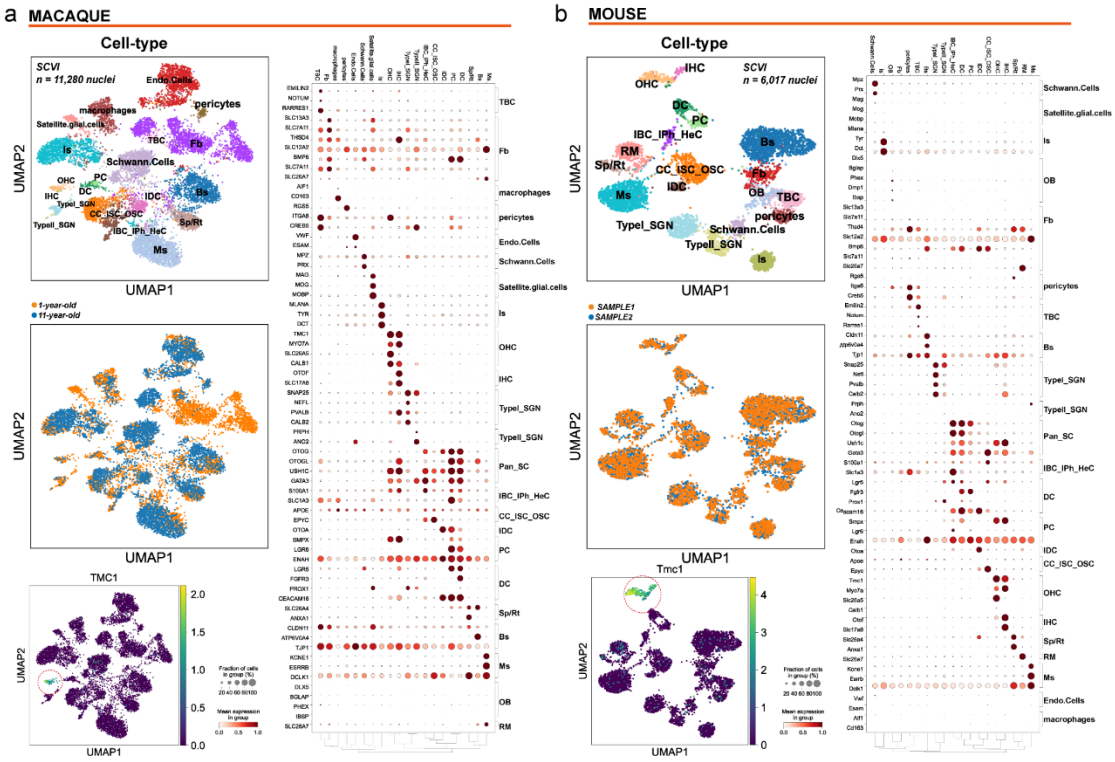
Moreover, we took advantage of the deep learning-based integration method, scVI, to mitigate any batch effects attributable to age- or gender-based variations. Our results clearly showed a successful correction of such batch effects.

Therefore, we are confident that the impact of these variations is minimal in our study. Our meticulous planning and methodological rigor ensure that our findings contribute meaningfully to our understanding of cochlear cell-type annotation.



(c) *Uniform Manifold Approximation and Projection (UMAP) representation of whole-organ cochlear profiles (n = 5,619 nuclei for the 1-year-old macaque and 5,661 nuclei for the 11-year-old macaque) identified more than 20 distinct cochlear cell types.*

(d) *Dot plots displaying the mean expression level of well-characterized marker genes for each cell type (left: 1-year-old macaque; right: 11-year-old macaque).*



Supplementary Figure 4

Single-nuclei profiling of macaque and mouse cochlear cell types by scVI integration, related to Figure 2.

a *The left panel exhibits UMAP plots representation of cell types from the macaque cochleae while the right panel outlines the gene expression profiles pertinent to cell-type annotations.*

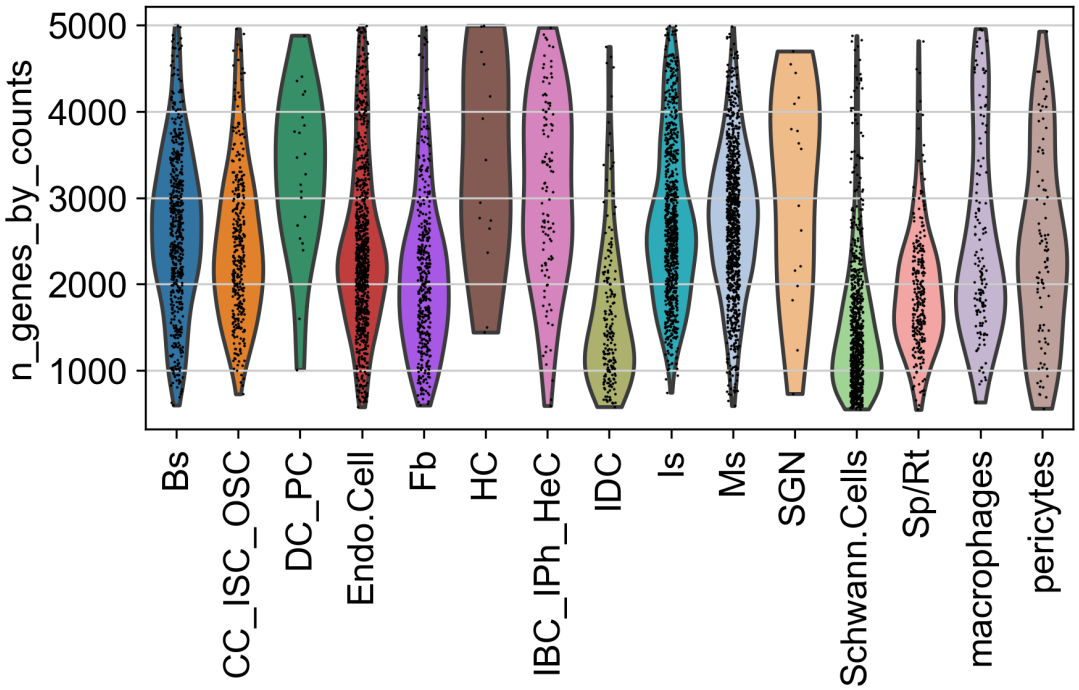
b *Similar to (**a**) but the analyses pertain to P21 mouse cochleae.*

(iii) We would like to additionally stress our commitment to ethical considerations in animal research. High standards of animal welfare were made a priority in our protocols, and to minimize any unnecessary distress, we kept the number of animals used to the absolute minimum necessary to achieve statistically significant results. Notwithstanding the small sample size, we managed to obtain a high-quality single-cell dataset that covers almost the entirety of cochlear cell types. Following up on the validation requested by the reviewers, our results confirm that increasing the number of replicates is not necessary in our case.

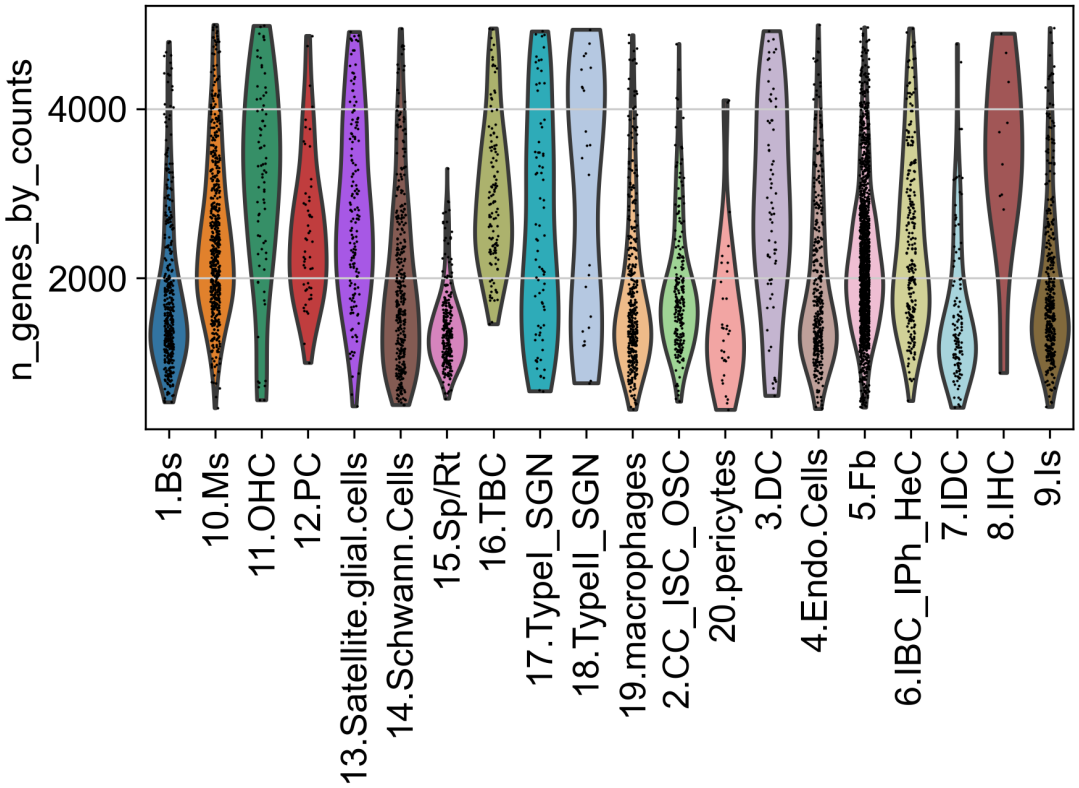
(iv) Moreover, we observed that the genes detected for each cell-type were remarkably similar across individuals, thereby substantiating our contention that individual differences likely have minimal impact on our results. This consistency in gene expression bolsters the validity of our findings and speaks to the reliability and reproducibility of our experimental strategy.

We believe that the steps and precautions undertaken in this study have given us robust, reliable results while respecting ethical guidelines for animal research. Once again, we appreciate the reviewer's insightful comments which have been instrumental in improving the quality and impact of our research.

11-year-old:



1-year-old:



2. Aging and sex itself must have a significant effect on the gene expression of the cochlea, how can the authors rule out the impact of age? In particular, there are obvious differences in the age of the individuals used in the manuscript.

We appreciate your careful reading and feedback. As we previously addressed the initial concern, we reiterate that the issues raised in your second concern are indeed similar and have been considered critically in our research process.

The emphasis of our study revolves around a careful balance between ethical practice in terms of animal usage and the scientific rigor required for generating valuable data. We have ensured that the samples chosen are appropriate and sufficient to produce reliable and comprehensive results. The carefully selected samples, spanning from juvenile to adult stage, offer a comprehensive perspective on the single-cell transcriptome of the macaque cochlea.

We have further validated that the potential influence of variations between the two samples, attributable to age and gender, is considerably mitigated by the use of the scVI method. This method successfully corrects for any batch effects, ensuring the reliability of our findings.

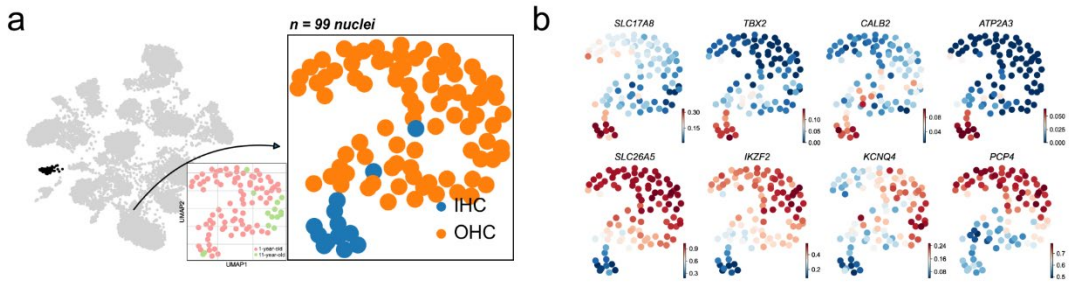
Furthermore, the congruence in the detection of genes across cell types corroborates our belief that individual differences have minimal impact on our results. This thereby upholds the validity and reliability of our findings.

We hope this clarification reinforces the integrity of our study, and we appreciate the insightful comments raised, which serves to strengthen our research.

3. In Figure 2B, it is evident that Fibro 2, Fibro4_RMC, and other cell types are predominantly found in the 1-year-old crab-eating monkey but not in 11-year-old monkey. Since the 1-year-old macaque is still at the developmental stage, this observation strongly suggests either a pronounced individual heterogeneity or potential developmental transition from the juvenile to the adult stage in the crab-eating monkey cochlea. Selecting sexually mature crab-eating macaque individual would be required. Similarly, the authors used the mice of the age of P21-25, more samples at different age and both genders are required.

We are thankful for the reviewer's further comments and suggestions. As with the previous points, the ideas raised in this query do hold similarities to prior issues, which principally revolved around diversifying our sample cohort.

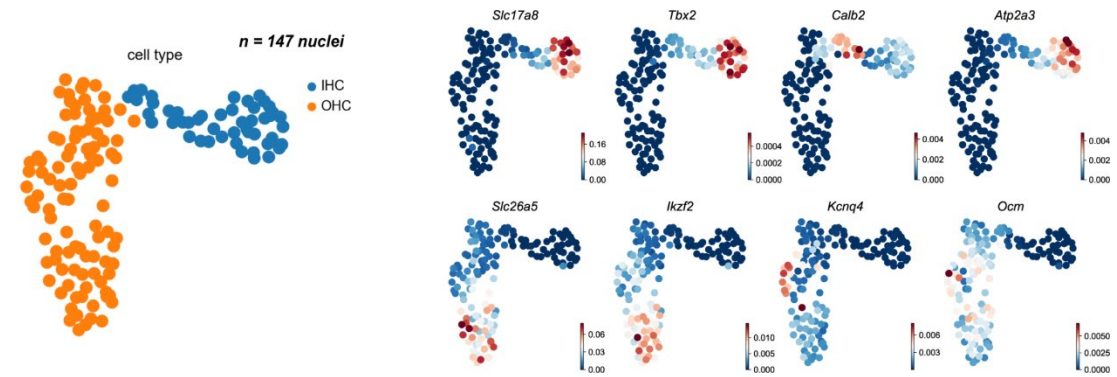
(i) In our current study, we've observed that the expression patterns of hair cells, sensory neurons, and glial cells in the macaque were indeed comparable with their counterparts in the mouse cochlea. This comparability was consistently observed across various life stages, attesting to a shared trend of gene expression in these cell types between the two specie



a *Uniform Manifold Approximation and Projection (UMAP) visualization of hair cells in the macaque*

cochlea, including inner hair cells (IHCs) and outer hair cells (OHCs).

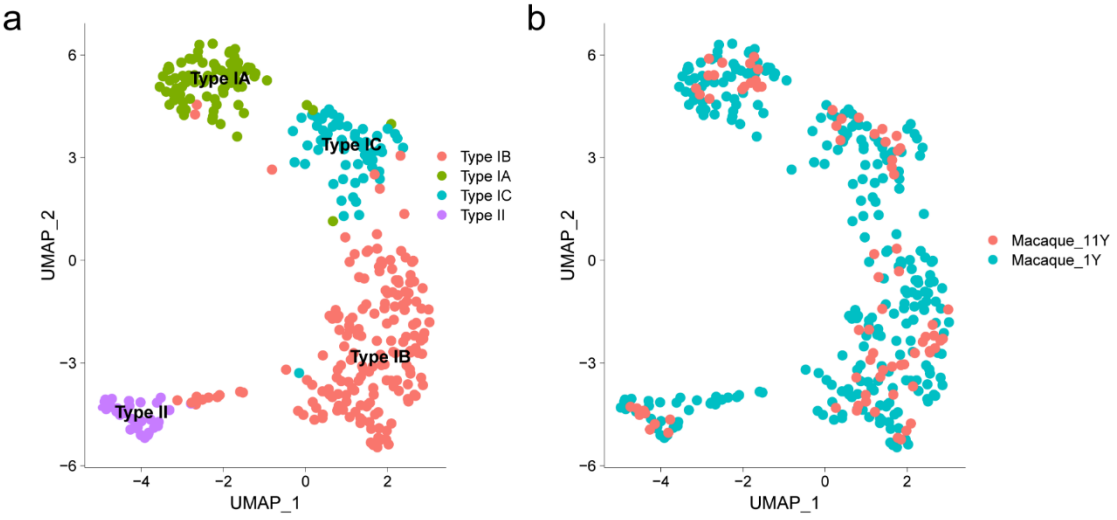
***b** Top panel: Expression profile of canonical marker genes for IHCs, as previously identified in mouse models. Bottom panel: Expression profile of canonical marker genes for OHCs, as previously identified in mouse models.*



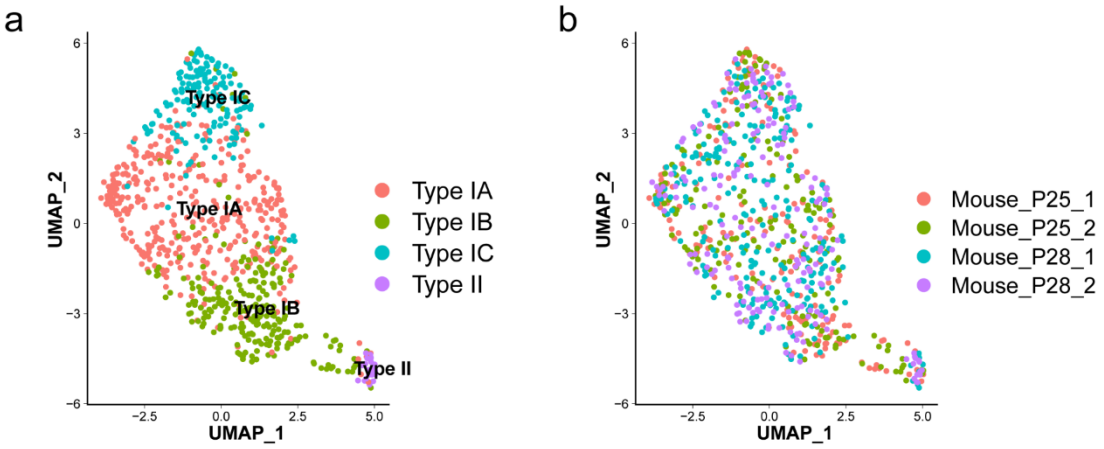
Supplementary Figure 5

Partitioning and re-clustering of mouse hair cell types, related to Figure 2.

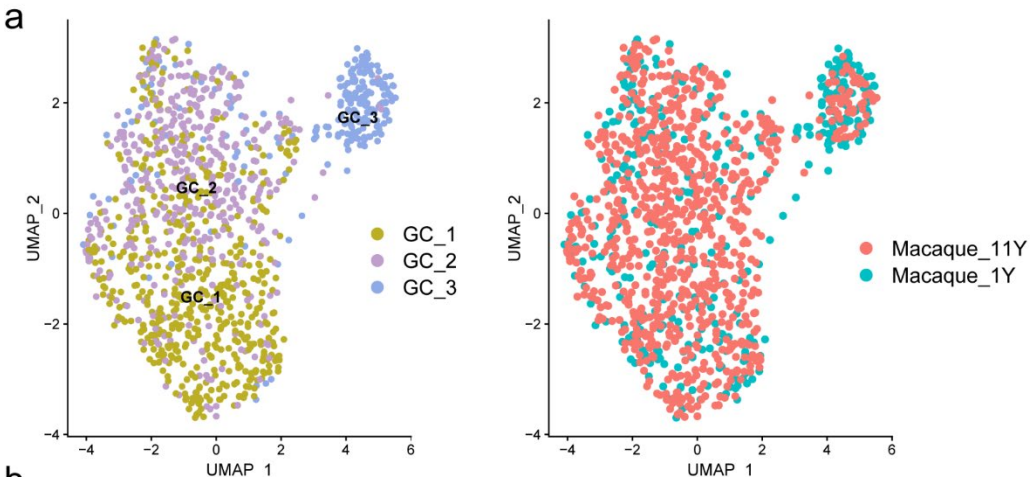
Left: UMAP plot showing the integrated inner and outer hair cells of mouse hair cells. Right: the canonical inner hair cell markers (Slc17a8, Tbx2, Calb2, Atp2a3) and outer hair cell markers (Slc26a5, Ikzf2, Kcnq4, Ocm) were distinct expressed across the two populations.



***a, b** UMAP representation of the re-clustering of macaque SGNs into 4 subtypes*



a, b *UMAP representation shows the re-clustering of mouse SGNs into 4 subtypes.*

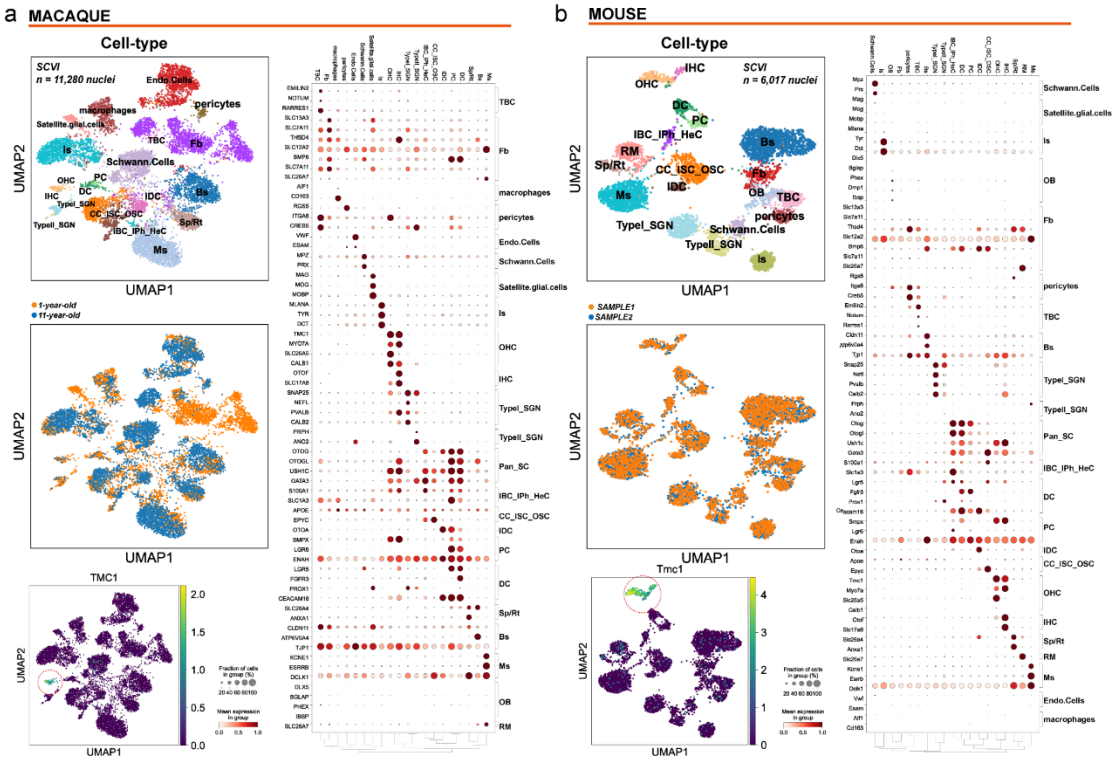


a *UMAP representation of the re-clustering of macaque glial cells, revealing three subtypes.*

(ii) Furthermore, when evaluating the mature markers used for cell-type identification, we also noticed that they exhibited similar expression patterns in both macaque and mouse models. Such similarities at a significant biological feature further substantiate our conviction that our chosen time point adequately captures the critical aspects of cochlear biology we intend to explore.

As such, considering the consistency observed across the considered cell types' expression patterns and mature markers between the two species, we argue that additional sequencing data at multiple ages may not be necessary for our current study's purpose. While we acknowledge that including diverse age groups could potentially deepen our understanding of cochlear biology, such investigations could be independently pursued in future studies without detracting from the validity of the insights gleaned from the current work.

We look forward to continuing this scientific discourse and cooperating with reviewers to address any remaining concerns to ensure our study's robustness and credibility.



Supplementary Figure 4

Single-nuclei profiling of macaque and mouse cochlear cell types by scVI integration, related to Figure 2.

a *The left panel exhibits UMAP plots representation of cell types from the macaque cochleae while the right panel outlines the gene expression profiles pertinent to cell-type annotations.*

b *Similar to (**a**) but the analyses pertain to P21 mouse cochleae.*

4. The authors mentioned in the Ethical compliance section that there were 1-, 5-, and 11-year-old crab-eating monkey cochleae used for single-cell nuclear sequencing, but the data of 5-year-old sample was not found in the manuscript. The scientific rigor of the paper needs to be ensured.

We greatly appreciate the reviewer's attentive feedback and apologize for any oversights in our previous versions. In our revised manuscript, we have clearly specified the use of macaque cochleae from different age groups and sexes for various experiments to ensure higher transparency and comprehension of our methodologies.

To rewrite the specific section: "For the purpose of single-nucleus RNA sequencing (snRNA-seq), cochleae from a 1-year-old male and an 11-year-old female macaque were utilized. Immunostaining experiments were performed using cochleae from a 5-year-old male and an 11-year-old male macaque. Electromotility recordings were obtained from a 1-year-old male macaque."

This revision aims to better distribute the use of various individual subjects for different research aspects, allowing us to capture age and gender differences more accurately. Once again, we thank the reviewer for their careful consideration and efforts in improving our study.

5. The numbers of captured hair cells and SGNs are quite small. The authors should probe the diversity of hair cells, which is one of the most important cells of the cochlea. More analysis and related experimental validation are required.

We highly appreciate the reviewer's suggestion. In our revised manuscript, we have placed notable emphasis on hair cells. Upon revisiting our single-cell datasets, we found that the number of captured hair cells constituted approximately 0.9% of the overall count in macaque cochleae (99/11,280) and 2.5% of the total in mouse cochleae (147/6,017). In parallel, captured spiral ganglion neurons (SGNs) were around 3% in the macaque cochleae (351/11,280) and 13% of the total in mouse cochleae (792/6,017). We would also like to note that around 7-8 mouse cochleae were pooled per replicate in this study.

To put these figures into perspective, the proportions adhere to the trend observed in recently published studies:

(i) Jean et al. (2023, PNAS): At postnatal day 8 (P8), the snRNA-seq method captured 1% of hair cells (318/28,822) and 2% of SGNs (577/28,822). Employing scRNA-seq at P8, P12, and P20, 0.3% of hair cells (247/88,006) and 0.2% of SGNs (151/88,006) were captured.

(ii) Kolla et al. (2020, Nature Communications): At P7, scRNA-seq of the cochlear epithelium resulted in the capture of 7.8% of hair cells (265/3,377).

(iii) Xu et al. (2022, Frontiers in Cellular Neuroscience): At P14, 16.7% of hair cells (419/2,513) were captured using scRNA-seq. The same technique, at P28, accounted for 4.2% of captured hair cells (1269/30,144).

Thus, we maintain that the percentages of captured hair cells and SGNs in our study remain within the ranges reported in the literature and sufficiently inform our analyses.

REFERENCES:

Jean, P., Wong Jun Tai, F., Singh-Estivalet, A., Lelli, A., Scandola, C., Megharba, S., Schmutz, S., Roux, S., Mechaussier, S., Sudres, M., et al. (2023). Single-cell transcriptomic profiling of the mouse cochlea: An atlas for targeted therapies. Proc Natl Acad Sci U S A 120, e2221744120.

Kolla, L., Kelly, M.C., Mann, Z.F., Anaya-Rocha, A., Ellis, K., Lemons, A., Palermo, A.T., So, K.S., Mays, J.C., Orvis, J., et al. (2020). Characterization of the development of the mouse cochlear epithelium at the single cell level. Nat Commun 11, 2389.

Xu, Z., Tu, S., Pass, C., Zhang, Y., Liu, H., Diers, J., Fu, Y., He, D.Z.Z., and Zuo, J. (2022). Profiling mouse cochlear cell maturation using 10x Genomics single-cell transcriptomics. Front Cell Neurosci 16, 962106.

6. More details about the steps for isolation of cochlear single cell nuclei should be provided.

We deeply appreciate the constructive feedback from the reviewers. We understand the need for clear and comprehensive methodological descriptions to ensure reproducibility and trust in our results. With this in mind, we have taken the appropriate measures to refine our METHODs

section further.

The process of single-nuclei isolation, amongst other critical experimental procedures, is now elucidated in finer detail. We have carefully outlined the individual step-by-step procedures, including the materials used, time points, temperatures, handling methods, and all other necessary considerations that might influence the results of this step.

We hope these additions will improve the clarity and understanding of our methods and address any concerns or ambiguities. We remain open to further suggestions and corrections to improve the quality of our work. Thank you once again for your keen attention and invaluable input.

7. This manuscript is very descriptive without a focused point or pathway. The authors are suggested to perform an in-depth analysis into the bioinformatic data, and more validation experiments are required. For example, the authors found that USH2A is highly expressed in the cochlear hair cells of monkeys. To make the results more convincing, IHC, IF, single cell-based qPCR should be performed. Further, to explore the role of USH2A, both loss of function and gain of function experiments in primary monkey cells are required.

We greatly value the reviewer's suggestion regarding a comprehensive analysis of PBX3 function. However, we must respectfully communicate that an extensive investigation into PBX3's role strays considerably from the primary intent of our present research.

The current study primarily focuses on constructing a comprehensive cell atlas of macaque cochlea. While we acknowledge that PBX3 potentially plays an interesting and relevant role in the cochleae, detailed explorations of its function would necessitate an independent, dedicated study, given the complexity and depth of such work.

We certainly agree that your suggestion could pave the way for future research directions and potentially contribute significantly to the body of knowledge in this area. We hope to, or indeed encourage others to, further explore this line of investigation in subsequent studies.

Thank you again for your insightful comments and suggestions. They highly contribute to shaping and refining our future research inquiries.

8. The author employed the biomaRt package for the homology gene conversion between crab-eating macaques and mice. However, using the human genome as an intermediate reference is not an optimal approach. Unlike the well-annotated reference genome in mice, the integrity of the macaque MF 5.0reference genome seems relatively poor, with many genes lacking complete annotations. Consequently, biomaRt fails to perform homology annotation for these unannotated genes. Moreover, the author utilized the human genome as an intermediary and employed biomaRt (version: 2.42.1) with the Macaca_fascicularis_6.0 reference genome, which further amplifies errors in homology annotation. A more appropriate alternative would involve directly performing BLAST sequence alignment between crab-eating macaques and mouse. In addition, the authors are suggested to provide the list of homolog genes between crab-eating macaques and mouse.

We deeply value your suggestion and would like to extend our appreciation. In current high-throughput single-cell/nuclei RNA sequencing, a characteristic limitation is the low number of

detected genes, which is typically around 1-2k median genes per cell. These genes detected tend to be the highly expressed ones in the individual cell under investigation.

Due to this, the choice of reference genome for alignment doesn't have as much weighting as it might otherwise. Therefore, we assert that using the Macaca_fascicularis_5.0 genome serves adequately for our high-throughput sequencing purposes.

When performing a cross-species comparative analysis, the specifies-specific gene ids or gene symbols need to be mapped to their respective one-to-one orthologues. The R package, biomaRt, is frequently used in this transformation process as it enables efficient retrieval of large amounts of data from various species.

The references cited below have successfully utilized biomaRt or an online Biomart multi-species comparison filter in their work:

Garcia-Alonso, L., Lorenzi, V., Mazzeo, C.I., Alves-Lopes, J.P., Roberts, K., Sancho-Serra, C., Engelbert, J., Marečková, M., Gruhn, W.H., Botting, R.A., et al. (2022). Single-cell roadmap of human gonadal development. Nature 607, 540-547.

Cross-species comparison. We compared the transcriptional signatures of the cell types identified in our human scRNA-seq to their mouse counterparts, considering all developmental stages combined. Mouse gene identifiers were converted to human genes using ENSEMBL Biomart multi-species comparison filter. Genes with several mappings were discarded. Furthermore, genes associated with the cell cycle were removed to avoid biases. Before training the model, human cell types

Hain, D., Gallego-Flores, T., Klinkmann, M., Macias, A., Ciirdaeva, E., Arends, A., Thum, C., Tushev, G., Kretschmer, F., Tosches, M.A., et al. (2022). Molecular diversity and evolution of neuron types in the amniote brain. Science 377, eabp8202.

Lizard and mouse genes were subsetted to one-to-one orthologs that are expressed in both species, according to the orthology annotations provided by Ensembl (*Pogona* assembly pv1.1 and mouse assembly GRCm38.p6, one-to-one orthologues downloaded from Ensembl on 1 May 2019).

Lust, K., Maynard, A., Gomes, T., Fleck, J.S., Camp, J.G., Tanaka, E.M., and Treutlein, B. (2022). Single-cell analyses of axolotl telencephalon organization, neurogenesis, and regeneration. Science 377, eabp9262.

Cross-species cell type correlations

Axolotl brain cell types were compared with cells sampled from mouse (*3*, *25*, *26*) and turtle (*2*) brain. Pairwise ortholog gene correspondences were directly obtained from Ensembl (*61*, *62*). Spearman correlation was used for pairwise cross-species comparisons, using the corr.test function

Yu, B., Zhang, Q., Lin, L., Zhou, X., Ma, W., Wen, S., Li, C., Wang, W., Wu, Q., Wang, X., et al. (2023). Molecular and cellular evolution of the amygdala across species analyzed by single-nucleus transcriptome profiling. Cell Discov 9, 19.

Integrating snRNA-seq datasets across species

To identify homologous cell types across species, we performed three rounds of dataset integration: Mammal Datasets (all nuclei from mammals), Inhibitory Neuronal Datasets (inhibitory neurons from mammals), and BLA Excitatory Neuronal Datasets (nuclei from BLA cell group in mammals). We employed the Seurat (v4.0.5)[79] workflow to integrate datasets across diverse species. The vignette of SeuratDisk (https://github.com/mojaveazure/seurat-disk) was used to convert AnnData objects to Seurat objects. The BiomaRt[84] package was applied to reduce raw matrices to only orthologous genes (13,478 genes) with one-to-one orthologues defined in humans, macaques, and mice. The *SelectIntegrationFeatures* func-

Bakken, T.E., Jorstad, N.L., Hu, Q., Lake, B.B., Tian, W., Kalmbach, B.E., Crow, M., Hodge, R.D., Krienen, F.M., Sorensen, S.A., et al. (2021). Comparative cellular analysis of motor cortex in human, marmoset and mouse. Nature 598, 111-119.

Integrating Cv3 snRNA-seq datasets across species. To identify homologous cell types across species, we used Seurat's SCTransform workflow to perform a separate supervised integration on each cell class across species. Raw expression matrices were reduced to include only those genes with one-to-one orthologues defined in the three species (14,870 genes; downloaded from NCBI Homologene (https://www.ncbi.nlm.nih.gov/homologene) in November 2019; RRID SCR_002924) and placed into Seurat objects with accompanying metadata. To avoid

We trust that this adequately clarifies our choice of methodology and illuminates the reasoning behind our decisions. We remain grateful for the opportunity to improve and tighten our research through insightful recommendations such as yours.

9. In Fig. S18, in order to construct heatmaps of all subclasses of hearing loss genes, the authors subsampled 10 nuclei from each species. However, analyzing subclasses with a higher proportion in the population resulted in significant information loss. To reduce such noise, utilizing a sampling method with replacement might be a preferable choice.

We thank the reviewer's comments. In our revised version, we first normalized the expression matrix of each cell-types then visualized with heatmap.

We are genuinely appreciative of the reviewer's constructive comments. Based on your feedback, we revised our approach in the subsequent version of our manuscript.

Specifically, we implemented normalization of the expression matrix for each cell type prior to

visualization. The normalization process essentially takes into account the variances in sequencing depths across different cells, which provides consistency and comparability.

Subsequent to the normalization, we prepared a heatmap. The heatmap is a graphical representation of the data where individual values contained in the matrix are expressed as colors, providing an intuitive, visual representation of the intricate dataset.

The normalized expression matrix visualized within the heatmap allows for better discernment and interpretation of crucial information, such as the variability and behavior of gene expression across various cell types.

Thank you once again for your valuable feedback that has significantly contributed to improving the robustness and interpretability of our work.

10. It is a lack of details of the computational approaches and the code should be provided on the GitHub page.

We express our gratitude for the reviewer's reminder. Ensuring full transparency and reproducibility is indeed a cornerstone of high-quality, credible research.

In response to your suggestion, we will promptly upload both our codes and raw sequencing data. The code will provide others with the ability to replicate our methods and analyses, while the raw data will support an open and comprehensive understanding of our findings.

We aim to uphold best practices in data integrity, allowing for peer validation and promoting collaboration within our scientific community. We will make sure that all of our data and coding work associated with this study is accessible and appropriately documented for clear understanding.

Thank you again for your valuable input. We appreciate your assistance in strengthening the integrity of our research.

11. A statistical approach to identify the appropriate resolution or find the number of clusters should beperformed. There are a variety of packages can be used (i.e. see Patterson-Cross et al 2021 PMID:33522897).

We extend our gratitude to the reviewer for the thoughtful suggestions. In alignment with your feedback, we have revised our approach to no longer subdivide the cell-types into subclusters.

Following the cellular taxonomy published in PNAS in 2023, we have expanded our scope to include all cochlear cell-types. This comprehensive approach encapsulates cell-types from the cochlear epithelium, the spiral ganglion, the lateral wall, and immune cells.

We agree this holistic perspective provides a more accurate reflection of the cellular diversity and complexity within the cochlea. By not over-segmenting the cell-types into subclusters, we ensure a representation that adheres more closely to the biological reality and reduces the risk of forced clustering or overinterpretation.

Thank you once again for your invaluable guidance. We trust these adjustments enhance the rigor and relevance of our analyses.

12. The number of cells for each cell group and each sample should be clearly annotated.

In our new version, we have listed the number of cells for each cell group/sample in a table form.

We genuinely appreciate the reviewer's insight. In our revised manuscript, we have indeed incorporated a new table listing the number of cells corresponding to each cell group/sample.

This table provides a clear, structured breakdown of our sample set, enhancing the readability and understanding of our data. It will allow readers to easily comprehend the distribution and the diversity of cells across different groups or samples, thus providing essential context for interpreting our findings.

We believe that this adjustment enhances the transparency of our study and boosts the interpretability of our results. Thank you once again for your astute guidance, which has significantly contributed to improving our manuscript.

Minor points:

1. The authors need to explain "less than 15,000 genes" (lines 543 and 566) in the QC process.

In our new version, we have filtered out more cells and obtain high quality insuring data accuracy and reliability. the QC is that '*Cells expressing more than 5,000 genes and less than 500 counts were excluded from further analysis*'.

2. The color annotations for cell types in Fig. 1b and 1d need to be aligned.

We have refined this in our new version.

3. In Fig. S1 c, it seems that Chinese was used.

We have corrected this in our new version.

4. The log fold change in Line 595 should be changed to log2 fold change.

We have corrected this in our new version.

Reviewer #3 (Remarks to the Author):

'Xin Chen et al.' present in their paper a cellular map of the macaque cochlea at the single cell level and provide transcriptomic signatures for all the cell types they detected. To the best of my knowledge, it is the first time that a primate single cell transcriptome of the cochlea is proposed and has the potential to be highly valuable for the whole cochlear and auditory neuroscience field. The major therapy approach currently explored for genetic forms of deafness is gene therapy. Tens of preclinical studies in the mouse have or are currently investigating therapeutic approaches and strategies for many deafness genes. These therapeutic approaches to be safe need well designed promoters to ensure that the therapeutic cassettes target only the relevant cell types. This is one reason why there has now been a number of single cell transcriptomic studies in the mouse. In the view of translating these gene therapies from mice to humans, a single cell transcriptomic dataset in a non-human primate is essential to make sure the gene therapies designed in mice have a chance to be safe and to

be rapidly translated to patients. It is in this context that ‘Xin Chen et al.’ provide a macaque transcriptomic single cell atlas of the cochlea based on the analysis of 20 600 nuclei. The authors have carried out their project using states of the art single cell sequencing techniques and analysis tools. Despite the quality of the data processing and graphical presentation, the manuscript presents several issues including two major ones, a) the low quality of English and b) the fact that the manuscript does not add much to the cochlear transcriptomic knowledge in the current state.

We are grateful for the reviewer's expert guidance and professional advice. As stated above, we believe our research holds significant value for the field of auditory neuroscience and offers a foundational base for preclinical therapy. Nevertheless, we also acknowledge the constructive criticisms raised and recognize the need for improvements in our manuscript.

In response to your recommendations and identified imperfections, our modified manuscript now includes the following changes:

(i) We have entrusted the editing of our manuscript to native English speakers skilled in the scientific field. This has substantially improved language clarity, coherence, and overall presentation.

(ii) We have considerably expanded on cochlear transcriptomic knowledge in our manuscript. This expansion is reflected across the introduction, results, and discussion sections, providing readers with a deeper and more rounded understanding of our research context.

We believe these actions address the issues raised and significantly enhance both the scientific and the communicative value of our manuscript. We kindly invite you to refer to our revised document for a detailed reflection of these changes.

Once again, we express our sincere gratitude for your insightful input that has helped us improve our work substantially.

i) English. It is a shame that the authors did not use professional services from native scientific English speakers. The manuscript seems to be a direct translation of a foreign language using online tools. The consequences are the presence of many sentences with wrong grammar/syntax, the impossibility to understand some sentences (sometimes whole paragraphs), and probably many misunderstandings including in my assessment of the data. Here are just a few illustrations of the many issues:

-approximate wording: the title wording is unclear, what is a ‘spiral ganglion hub’ (line 149), to ‘attach small portion of oligodendrocytes’ should read ‘to collect’ (line157), what is a ‘high specialized terminator?’ (line220),

-Coordinating conjunctions are often place at the start of sentences like ‘And’ (lines 154, 185,196).

-Whole paragraphs are hardly understandable (ex: lines 209-214)

-Major misunderstanding. The authors use the word ‘diversity for cell types’, which suggests that there are more cell types in the macaque than in the mouse like among glial cells. In fact, it seems that there are as many cell types. Rather, the macaque glial cells display more

differentially expressed genes compared to mouse glial cells than in any other cochlear cell type (see abstract, paragraph from line 254).

We'd like to express our sincere apologies for any inconvenience our previous manuscript may have caused the reviewers and the editor. We recognize the need for clearer and more precise scientific communication. Therefore, we've taken your feedback seriously and have enlisted the assistance of native English speakers with a strong grounding in scientific research to revise, refine, and polish our manuscript to improve the clarity, precision, and overall quality of the writing.

We truly appreciate your patience and the diligent attention you've given to our work. We hope that our renewed efforts to enhance our manuscript meet with your approval and help convey our research findings in a more understandable and professional manner. Thank you for your continued guidance and support.

ii) A general impression emerges that the conclusions are not drawn to the full potential of the data. There are of course many similarities between mouse and macaque. The authors try to pinpoint the differences. However, they seem to be of a limited impact and/or they are not confirmed with alternative techniques. Apart for PBX3, the other detected molecular claims have not been confirmed by another approach. For example, RNAscope, that is a robust mRNA detection technique could be used on a few precious macaque slices in some instances to confirm the hypothesis made by the authors. This concerns Transcription factor differences claims and glial cell expression differences between the mouse and macaque. In addition, the molecular calb1 marker difference could be of great importance in the auditory neuropathy field. In this field, it is of great debate whether the categorization of auditory nerve neurons found in rodents (low, middle and high threshold neurons) exist in primates and even more in humans. One would expect to see more explorations of this aspect using RNAscope or any other comparable method that is ethically compatible with the use of macaques.

One consequence of this lack of data confirmation is that the many form conclusions cannot be drawn. Here are a couple of examples:

-line 200: “these results suggest that a cohort of TFs and effector genes can be used to classify cochlear celltypes, but further verification is needed…”

-line288: “Future experiments should determine if these genes differed in species…”

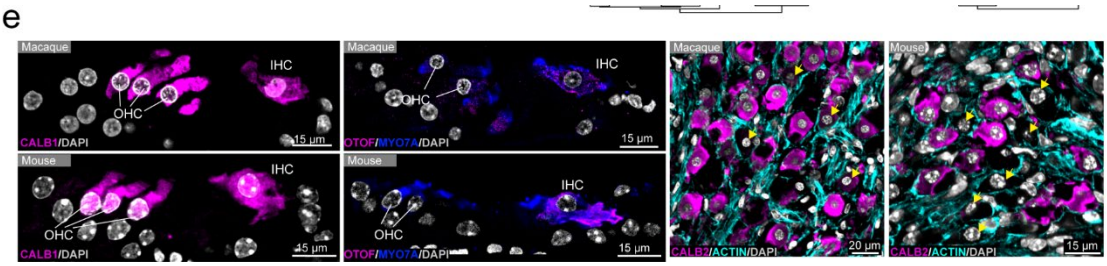
-line 319: “However, future experiments should determine…”.

-line 384, conclusion of the manuscript: “Different datasets, however, may require particular or combinatorial batch-corrected methods, since too strong correction might eliminate hidden variations in intro- or inter-species.”

We continue to express our gratitude for the insightful reminders and suggestions from the reviewer. Your suggestions underscore the necessity of employing an independent validation method to substantiate the conclusions drawn in our study.

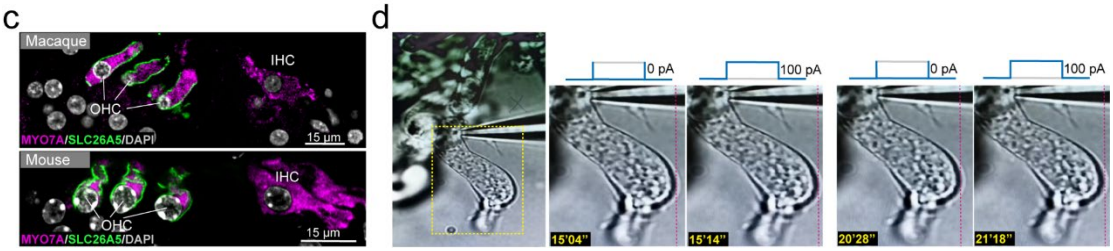
In accordance with your advice, we have introduced additional validation results in our updated manuscript to reinforce our conclusions. Specifically:

(i) Validation of sequenced data:



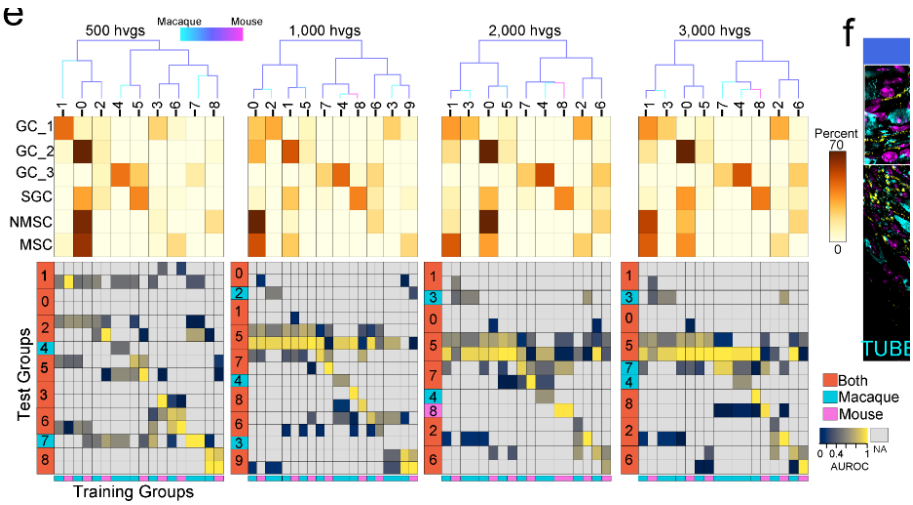
e *Immunofluorescence results confirming the expression of hair cell markers, including CALB1 and OTOF, and the neuronal marker CALB2 in the macaque cochlea.*

(ii) validation of OHC maturation and electromotile activity:

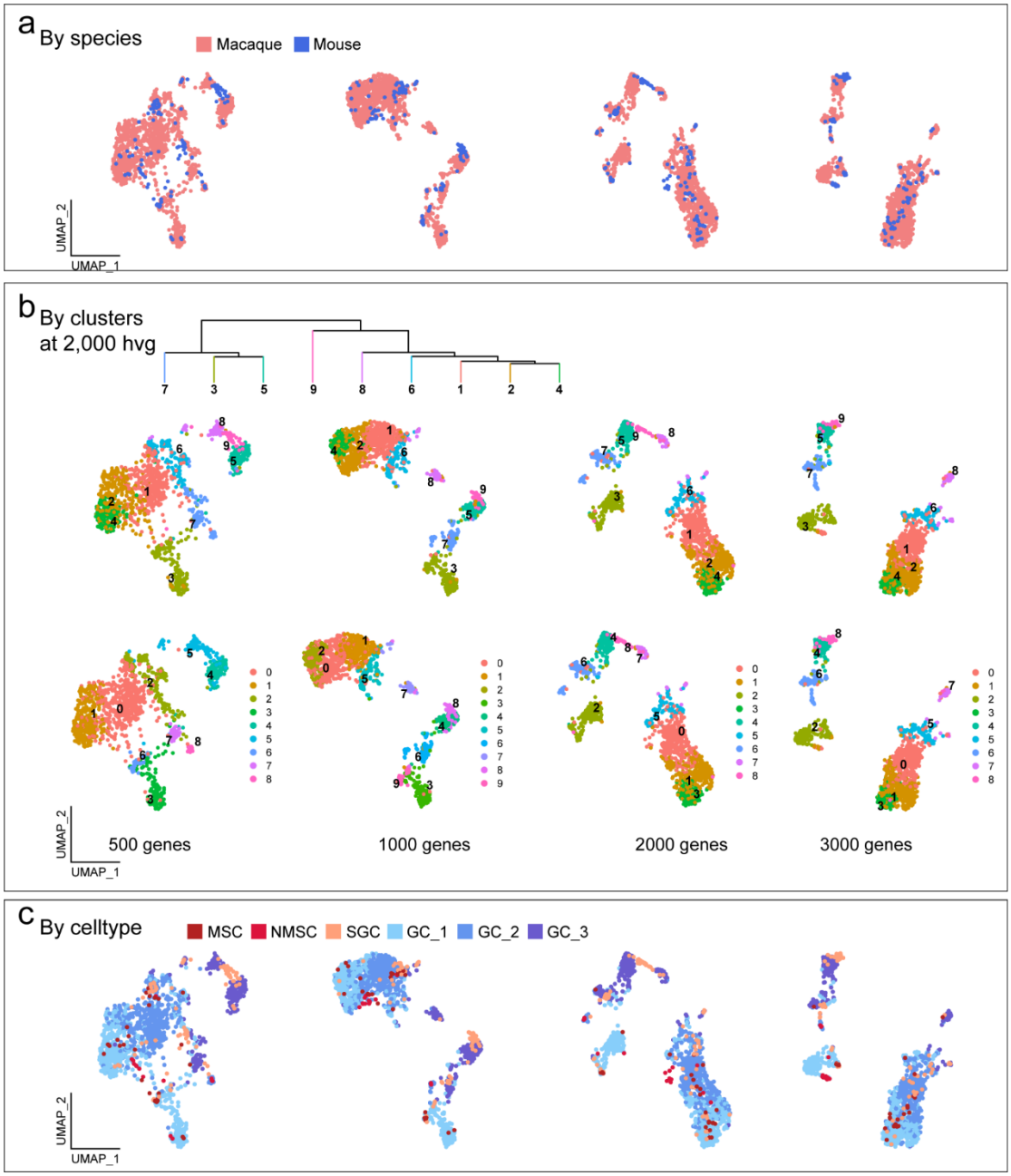


c *Confocal microscopy images demonstrating the localization of PRESTIN protein in macaque OHCs.*
d *Patch-clamp recordings revealing the electromotility of OHCs in response to current injection.*

(iii)apply bioinformatics analysis to confirm the heterogeneity of glial cell in macaque that differ from mouse species.



e *Top panel: Hierarchical clustering of the average expression profiles of integrated glial cell clusters; branches are colored by species composition. Middle panel: Matrices displaying the percentage of cells in each integrated glial cluster (columns), with original subtypes grouped by species (rows). Bottom panel: Heatmaps illustrating the transcriptomic similarity of these integrated clusters within the original glial subtypes, determined by applying MetaNeighbor analysis.*



Supplementary Figure 8

Integration of macaque and mouse glial cell data via the Seurat CCA-based pipeline by applying different numbers of highly variable genes (hvgs), related to Figure 3.

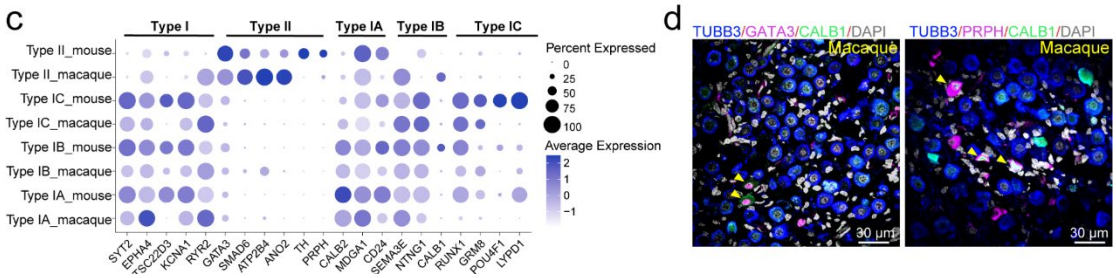
a *UMAP representations of single nuclei colored by species after CCA-based integration, sequentially executed with differing numbers of hvgs, indicate unchanged species mixing.*

b *Top: hierarchical clustering of the average expression profiles was computed for the clusters identified in the integrated space (2000 hvgs), and colors in the terminal branches indicate the cluster number in the integrated data. Middle: UMAP representation of the integrations with colors computed with 2000 hvgs, which correspond to the color in the dendrogram. Bottom: UMAP plots of Seurat integrations with their original cluster number. Cells in the other UMAPs (500, 1000, and 3000 hvgs) are colored with the same colors used in the referenced integration (2000 hvgs), which indicates that the high-level structure of the data manifold is relatively stable when applying different numbers of hvgs.*

c *UMAP representation of single nuclei colored by glial cell subtypes after CCA-based integration running*

with 500, 1000, 2000, and 3000 hvgs.

(iv)validation of CALB1 that is a type II sensory neuron marker.



c *A dot plot illustrating the gene expression levels of SGN subtype markers identified in mouse species, compared between macaque and mouse SGN subtype cells.*

d *Representative confocal images of immunofluorescence cryosection staining demonstrating CALB1 associated with GATA3 or PRPH localization in 5-year-old macaque (top) and 3-week-old mouse (bottom) SGNs.*

These complimentary methods and results work collaboratively to further strengthen the validity of our findings and more definitively support the conclusions we've drawn. We trust this comprehensive validation will address your concerns.

Again, we appreciate your invaluable input and hope that our efforts in revising the manuscript align with your recommendations.

iii) There are several generic conclusions that would deserve discussion. For example, I respectfully disagree with this affirmation: “By contrast, divergences across species provide strong evidence for adaptation or drift of non-human primate cochlea function, and of susceptibility to human-specific diseases”. There are of course difference in expression patterns of genes between different species. In several instances of the manuscript, the authors seem to want to push this idea that should be discussed in a more balanced way. The authors should give precise examples of human-specific diseases. To which ones do they refer? If one considers genetic forms of deafness for example, more than 150 genes have been identified. To the best of my knowledge, all of these genetic forms of deafness have been replicated either from human to mouse or from mouse to human. Moreover, there are more and more examples of particular audiological signatures associated with specific genetic forms of deafness that have been uncovered in mice and that were confirmed subsequently in patients. So there is clearly a very close resemblance in the genetic architecture and function of mouse and human cochleae. This does not necessarily mean of course that the transcriptomes are identical but it is important to address whether the differences observed in the manuscript may endanger or not the direct translation of therapies from mice to humans.

We appreciate and are highly indebted to the reviewer for the professional advice and insightful recommendations aimed at refining our manuscript, particularly the discussion section. Your expertise and guidance have played a crucial role in further enhancing our manuscript by bringing a deeper level and clarity of analysis, thereby making our research more robust and its implications clearer.

We have applied the comments to the fullest extent in the revised manuscript and hope the updated version meets your expectations. Your role in this process is immensely valued. Once again, thank you for your invaluable contributions.

iv) Several methodological aspects were unclear:

-how many macaque cochleas were used in each sample? How many macaque replicates? It is likely that very few replicates were used for obvious reasons. However, this means that the differentially expressed genes between mouse have to be considered with care and require complemnetary methods (see point iii) above)

We are grateful for the reviewer's suggestion regarding the clarification of the sample used in our study. Upon your recommendation, we have taken steps to fully detail and explain our choice of sample within the revised manuscript.

The sample selection and its specifics are now comprehensively outlined, which we believe not only provides clear context but also aids in the understanding of our research approach and results.

Once again, we appreciate your valuable insights and hope the revisions made in our manuscript satisfactorily address your concerns. We trust the latest version of our manuscript is now more precise and informative. Thank you for your guidance.

-the methods mention 1, 5, and 11 year old macaques. Are the macaques systematically mixed? Are there analysis between 1, 5, and 11 year old macaques.

We greatly value the reviewer’s suggestion. Following your advice, we have provided a clear, in-depth demonstration of the sample used in our study within the updated manuscript. This includes additional details such as the specifics of the sample selection, the relevance to our study, and how the samples were processed and analyzed.

By integrating these changes into the revised version, we've attempted to increase the manuscript's clarity and provide a more robust base for our findings. Your contribution has been instrumental in enhancing the quality of our work.

Once again, we express our gratitude for your professional guidance. We hope our modifications meet your expectations and reinforce the reliability and integrity of our study.

-Only nuclei with more than 15000 genes were filtered out. This number seems very high and increases the risk of analyzing duplicates and or cells for which both the nuclear and cytosolic transcriptome were caught.A number like 5000 would be more reasonnable.

We wholeheartedly concur with your comments about enhancing the quality control (QC) process. We have incorporated stringent QC measures into our study as based on your suggestions. Specifically, we have now set conditions wherein cells that express more than 5,000 genes and less than 500 counts are excluded from subsequent analysis.

This additional QC step ensures a higher level of accuracy and reliability in our data by eliminating potential outliers or incorrect readings that could distort our results, thereby lending

a higher degree of rigor and credibility to our research.

We trust this adjustment positions our manuscript favorably in terms of precision, trustworthiness, and methodological robustness. We appreciate your expert insights to enhance the quality of our work. Again, thank you for your constructive feedback.

-Did the authors detect non circulating cells (lymphocites, macrophages etc…)? Were they filtered out during the procedure? How many out of the 20000 nuclei were finally exploited for the data presentation?
In our single-nuclei isolation protocol, we only captured macrophages with a low proportion and was not filtered out for further analysis. Finally after QC, we used 11,280 nuclei from macaque cochleae and 6,017 nuclei from mouse cochleae for downstream analysis including Leiden clustering, DEGs calculation and data integration et al.

Minor issues.
i) Several sentences in the manuscript are not informative or lack relevance.
Thanks for the reviewer's reminder, we totally corrected
ii) Figures are generally well made. However, several problems need to be solves:
-many panels are unreadable due to too small characters (ex Fig. 1C, colour code about the nodes are unreadable in 2b,the colour codes for the cell types in the heat maps or clusters are generally unreadable(Fig. 6 for ex), the same colour code should be used between figures and species for the same cell type (6a,S18)
-there is a foreign text on S1C.
Thanks for the reviewer's reminder, we have refined and corrected.

Dear Editor,

We sincerely appreciate your letter dated July 9, 2024, regarding our manuscript entitled "Elucidating the Molecular Diversity of Non-Human Primate Cochlea." We are deeply grateful for the opportunity to resubmit our work and for your continued support of our research. We would also like to extend our sincere thanks to the esteemed reviewers for their comprehensive and insightful feedback, which has been invaluable in enhancing the scientific quality of this study.

The reviewers' primary concern centered on sample size limitations. In response to this critical issue, we have significantly strengthened our study by incorporating three additional non-human primate cochlear samples in the revised version. Through rigorous discussion and additional experiments, we have meticulously addressed each of the reviewers' comments. Our team has implemented substantial modifications to improve the manuscript's scientific rigor, clarity of presentation, and overall coherence. Below, we provide point-by-point responses detailing the most important revisions made in response to the reviewers' recommendations.

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed my main concerns - thank you. The manuscripts is now written clearly and is much tighter & clearer throughout. While the number of animals used is low for the snRNAseq, nonetheless, it is high quality data that will be of tremendous use to the scientific community at large and constitutes a significant advancement worthy of publication in a high quality journal such as Nat Comm.

We are profoundly grateful for the reviewers' high praise that " it is high quality data that will be of tremendous use to the scientific community at large " This acknowledgment further reinforces our commitment to advancing scientific research through rigorous and impactful datasets.

My one main concern that has not been addressed is that the code for generating all of their analyses has not been made available for peer-review. Not only does this ultimately need to be shared through github or a similar repository, it is this reviewer's opinion that it must also undergo the peer-review process as this is a critical part of the Methods section and must be presented in an acceptable manner. I understand that the raw data will be deposited in a publicly accessible repository. Finally, I do suggest that the authors consider depositing their data in gEAR as it will have maximum impact for the auditory neuroscience community and make the data accessible for scientists without much coding expertise(https://umgear.org/) (Orvis J, et al. Nat Methods. 2021 Jun 25. doi: 10.1038/s41592-021-01200-9, PMID: 34172972).

We express our gratitude for the reviewer's reminder. We appreciate the emphasis on transparency and reproducibility in research and take these concerns very seriously.

In response to your suggestion, we have uploaded our codes to a public GitHub repository, please find in *https://github.com/Shinnyee/Cell-atlas-of-non-human-primate-cochlea*. and will be sooner in gEAR portal as well as raw sequencing data. The code will provide others with the ability to replicate our methods and analyses, while the raw data will support an open and comprehensive understanding of our findings.

We aim to uphold best practices in data integrity, allowing for peer validation and promoting collaboration within our scientific community. We will make sure that all of our data and coding

work associated with this study is accessible and appropriately documented for clear understanding.

Thank you again for your valuable input. We appreciate your assistance in strengthening the integrity of our research.

Minor comments:

153: type should be "types"

We have refined this in our new version.

Fig3f legend: should say "left/right" as there's no top/bottom.

We have refined this in our new version.

237: remove "to" between unlike & mouse SGNs

We have refined this in our new version.

Fig4d: would be more clear and convincing to show the individual channels in gray-scale either in main figure or in supplement.

We have refined this in our new version.

Reviewer #2 (Remarks to the Author):

The latest revision has not significantly enhanced the manuscript. The authors have merely re-integrated the existing data, neglecting to expand the sample size of monkeys per group—a critical step to mitigate individual variability and validate the sequencing data's reliability. Without this resolution, publication in a reputable journal such as Nature Communications remains unlikely. Moreover, the manuscript requires further extensive validation to ensure its credibility. The following concerns have been identified and require attention:

We are grateful for the reviewer's insightful suggestions, which have been instrumental in enhancing our research. We have given careful considerations and have worked diligently to improve our manuscript. We've tried our best to address all the concerns, including expanding the sample sizes and providing more validation. We have further developed our scientific narrative, forming a more comprehensive and complete story.

Major Issues:

1. The authors' responses have not adequately addressed the concerns raised. The current sample sizes insufficient to overcome individual, gender, and age-related heterogeneity.

We are grateful for the reviewer's remarks and would like to address the concerns raised about the number of sample sizes sequenced.

The primary objective of our study is the generation of a single-cell atlas for the macaque cochlea. Therefore, utilizing samples from different life stages - juvenile and adult - appeared promising to give a holistic profile of the single-cell transcriptome. To address potential heterogeneity across individuals, genders, and ages, we have significantly increased the sample size compared with the initial version.

For absolute clarity, here is the information about the subjects used in our study:

For the sequencing portion, we utilized one 1-year-old, one 11-year-old macaque and two 5-year-old macaques (one provided two cochleae).

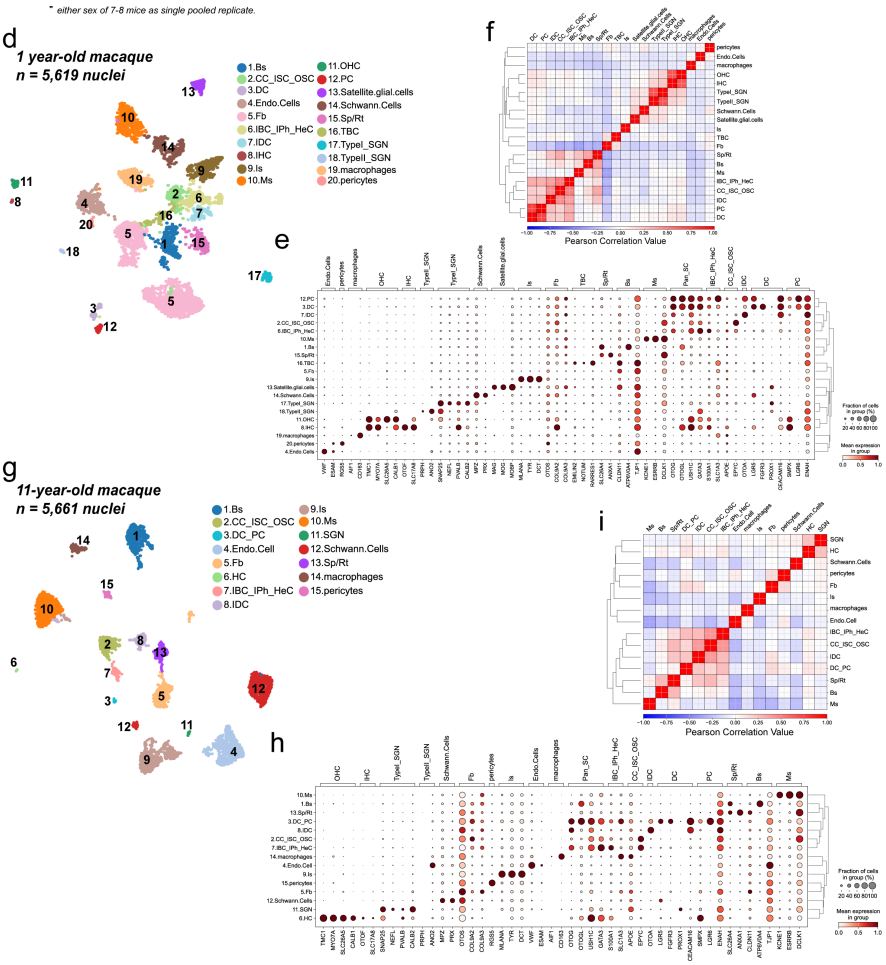
For the patch-clamp recording, we used a single 1-year-old macaque.
For validation purposes, both three 5-year-old and an 11-year-old macaque were used.



c A timeline for displaying macaque cochlea sampling across ages for snRNA-seq and cell-type validation. A total of 4 macaque cochleae were used for sequencing and of 4 macaque cochleae were used for validation and one for electrophysiological recording.

(ii) Our single-cell dataset demonstrated that well-validated markers for cochlear cell-type annotation were well-mapped into both our 1-,5-,11-macaques. The expression patterns between the five samples were not only similar but also comparable to those observed in mouse counterparts.

(iii) Additionally, our data integration results indicate a successful combination and adequate mixing of these five samples. This further strengthens the reliability of our study and indicates a reliable overlap and correlation between the distinct sample sets.



d Uniform Manifold Approximation and Projection (UMAP) plot of the 1-year-old macaque cochlea

identified more than 20 distinct cell types. A total of 5, 619 high-quality nuclei were captured prior to subsequential integration. These cell types include basal stria cells (Bs), root cells (Rt), intermediate stria cells (Is), spindle cells (Sp), marginal stria cells (Ms), endothelial cells (Endo.cells), pericytes, smooth muscle cells, pillar cells (PC), Deiters's cells (DC), inner border cells/inner phalangeal cells/Hensen's cells (IBC, IPh, HeC), interdental cells (IDC), Claudius cells/inner sulcus cells/outer sulcus cells (CC, ISC, OSC), inner hair cells (IHC), outer hair cells (OHC), type I spiral ganglion neurons (TypeI SGN), type II spiral ganglion neurons (TypeII SGN), satellite glial cells, Schwann cells, osteocytes (OB), Reissner's membrane (RM), tympanic border cells (TBC), and fibrocytes (Fb).

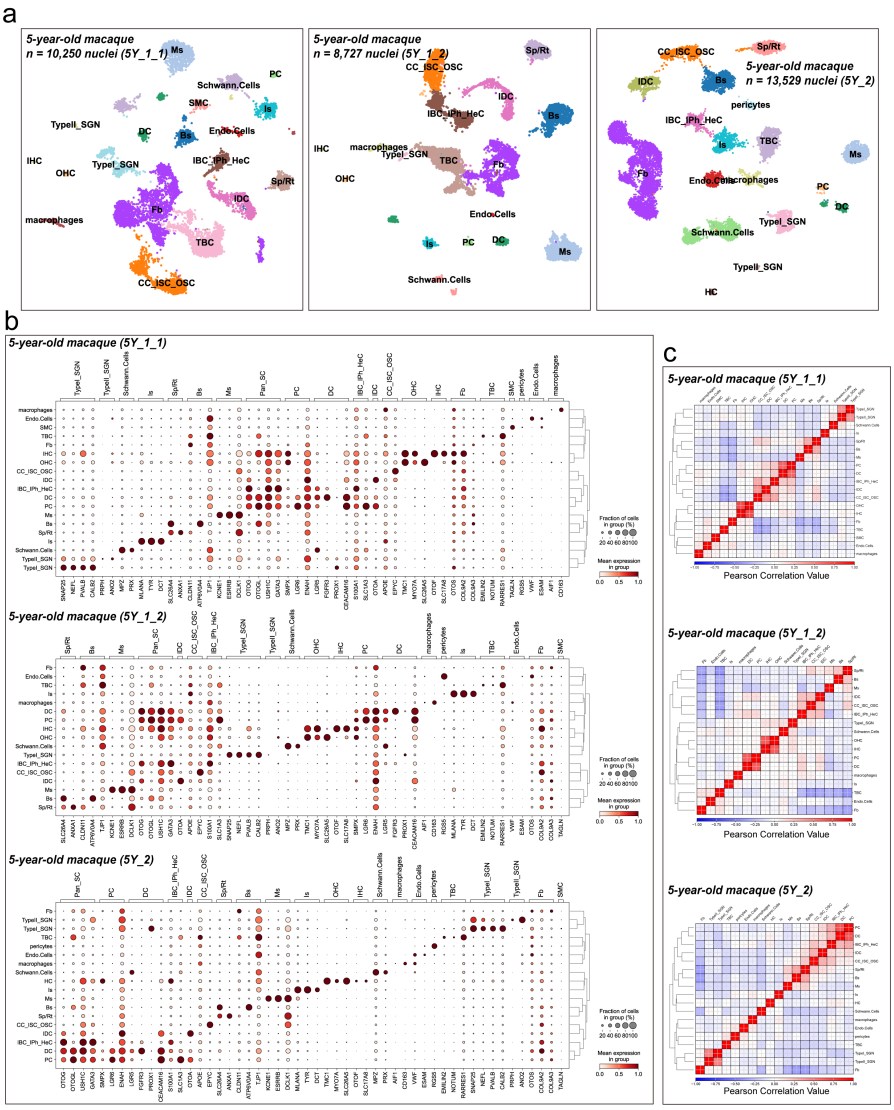
e A dot plot displaying the variance-scaled, log-transformed expression of marker genes (x-axis) identified in mouse species as a reference for mapping 1-year-old macaque cochlear cell types (y-axis). The main markers for manual annotation are from Jean et al. PNAS, 2023.

f Pearson correlation heatmap showing the similarity of transcriptomic expression patterns within cell types in the macaque cochlea (1-year-old).

g Same as (d), but for the 11-year-old dataset.

h Same as (e), but for the 11-year-old dataset.

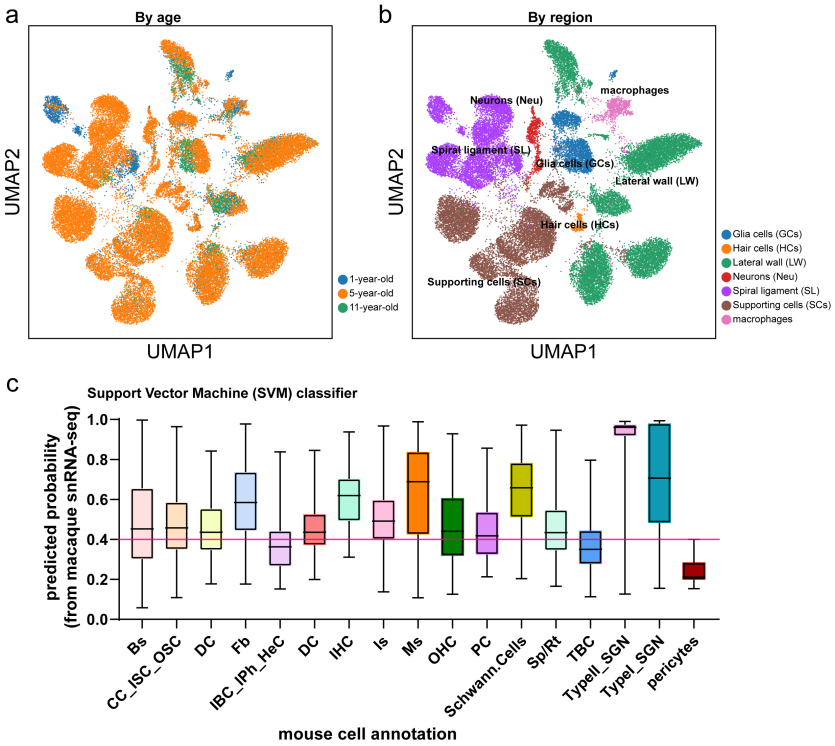
i Same as (f), but for the 11-year-old dataset.



a Uniform Manifold Approximation and Projection (UMAP) plot of the 5-year-old macaque cochlear single-cell transcriptomes, which was identified more than 20 distinct cell types. A total of 32,506 high-quality nuclei from three individual cochleae were captured prior to subsequential integration.

b A dot plot displaying the variance-scaled, log-transformed expression of marker genes (x-axis) identified in mouse species used for manual cell-type annotation for the 5-year-old macaque cochlea (y-axis). The marker gene list is mainly from Jean et al. PNAS, 2023.

c Pearson correlation heatmap showing the similarity of transcriptomic expression patterns within cell types in the macaque cochlea (5-year-old).



a Uniform Manifold Approximation and Projection (UMAP) plot of the integrative macaque cochlear single-nucleus transcriptomes, which are from 1-, 5-, and 11-year-old macaque cochleae. We used scVI (single-cell variation inference) for data integration and batch correction.

b UMAP plot displaying the main cochlear regions, including the cochlear epithelium (hair cells and supporting cells), spiral ganglion (neurons and glial cells), lateral walls, spiral ligament and macrophages.

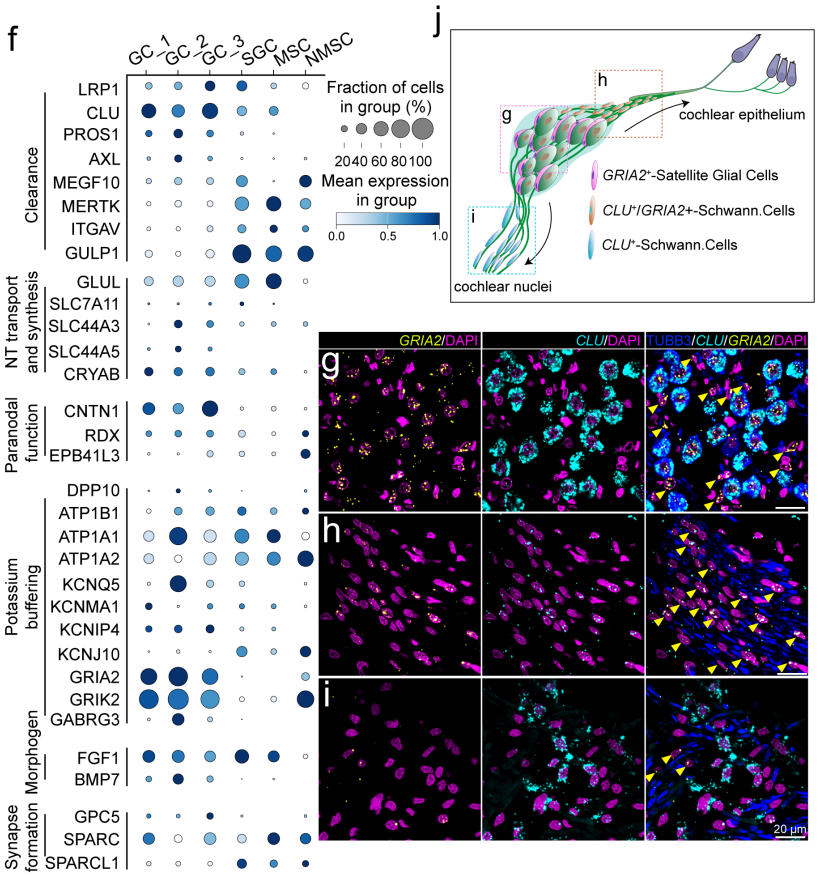
c Boxplot showing the predicted probabilities (y-axis) of macaque cell types that were label-transferred onto manually postnatal days (P)25 mouse cell types (x-axis) with a Support Vector Machine (SVM) model as a classifier trained on the macaque cells. The box extends from the lower to upper quartile values of the data, with a line indicating a median value of 0.4.

Once again, we appreciate your valuable insights and hope the revisions made in our manuscript satisfactorily address your concerns. We trust the latest version of our manuscript is now more precise and informative. Thank you for your guidance.

2. While the cross-species analysis revealed unique glial cell diversity, the authors have not clarified the distinctions between macaque and mouse glial cells or their functional implications. Experimental validation for macaque-specific markers such as GRIA2 and CLU is also needed.

We highly appreciate the reviewer's suggestion. In our revised manuscript, we have added the experimental validation for macaque-specific markers, GRIA2 and CLU. Our RNAscope results further confirmed that peripheral glial cells in macaque can be roughly divided into two types (Fig. 3j), namely GRIA2-positive satellite glial cells that are mainly enriched in modiolus region (Fig. 3g) and CLU-/GRIA2-positive or sole CLU-positive Schwann cells that are preferentially abundant along the outside region of modiolus as neuronal fiber projects into both cochlear epithelium (Fig. 3h) and cochlear nuclei (Fig. 3i), respectively. Our in situ hybridization results of GRIA2 and CLU are corresponding to that of sequencing data illustrating the wide expression of GRIA2 in all glial cells in macaque and selectively enriched expression of CLU in clusters of

GC_1/GC_3 in macaque glial cells (Fig. 3f).



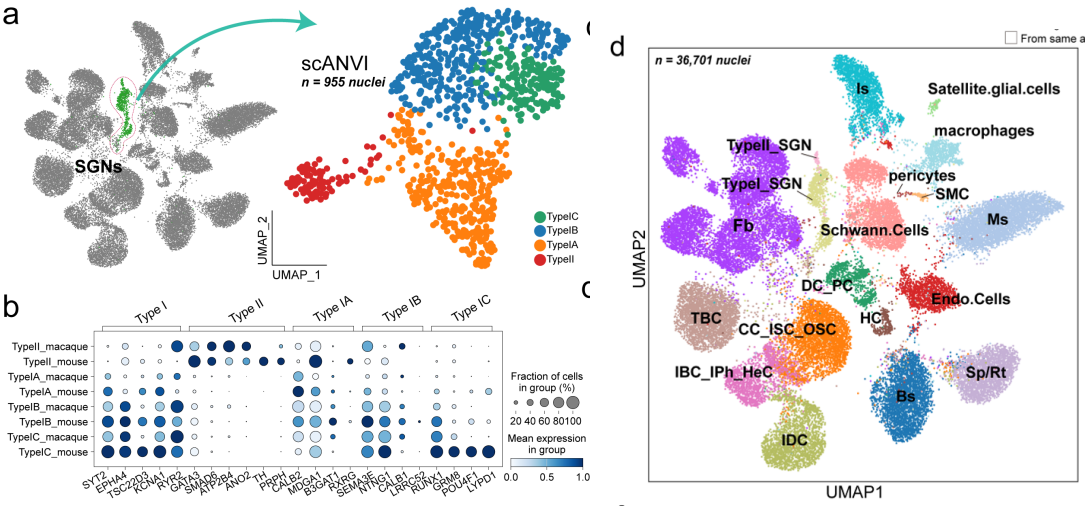
f A dot plot displaying the variance-scaled, log-transformed expression of selected genes (y-axis) associated with astrocyte functions in macaque and mouse glial subtypes (x-axis).

g-i, RNAscope assay confirmed the localization of macaque-specific genes: GRIA2 (glutamate receptor 2) and CLU (clusterin) mRNA in glial cell types. The regions selected for illustration are corresponding to region in modiolus (g), region that project to cochlear epithelium (h) and region that project to cochlear nuclei (i).

j Schematic diagram displaying GRIA2+ and/or CLU+ glial cell type in macaque cochlea.

3. In Figure 1c, the spatial distribution of two SGN subtypes in the macaque cochlea appears implausibly distant in the UMAP plot. Additional evidence and quantitative comparisons across samples are necessary.

We are appreciative of the reviewer's insightful comments and have treated them with due seriousness. In our revised version, following a re-clustering of our dataset, we obtained 955 SGNs in the macaque cochlea. And now the two SGN subtypes in the macaque cochlea appear to be closer embedding.



a Left panel: Highlighted SGN subclass from the whole macaque cellular map in the main Uniform Manifold Approximation and Projection (UMAP) plot. Right panel: UMAP plot depicting 4 subtypes after joint-embedding and re-clustering of the macaque SGN subclasses (type I and type II), which was re-clustered using scANVI (single-cell ANnotation using Variational Inference)-based integration.
b A dot plot illustrating the variance-scaled, log-transformed expression of SGN subtype markers (x-axis) identified in mouse species for comparison between macaque and mouse SGN subtype cells (y-axis).
d Uniform Manifold Approximation and Projection (UMAP) representation of whole-organ cochlear profiles identified more than 20 distinct cochlear cell types.

4. In Figure 1d, the specificity of several marker genes, including ITGA8, CREB5, TMC1, CALB1, SLC17A8, PVALB, THSD4, BMP6, CLDN11, DCLK1, USH1C, GATA3, OTOA, and ENAH, is questionable. The authors must provide explanations.

Thank for the thorough review.

(i)Upon systematically re-examining the listed genes, we have verified that ***ITGA8***, ***CREB5***, and ***THSD4*** lack prior evidence as cell-type-specific markers, and they have now been excluded from the marker gene *Dot plot* in the updated manuscript.

(ii) The genes ***SLC17A8***, ***OTOA***, ***USH1C***, ***CLDN11***and ***BMP6*** were referenced from ***Jean P. et al, 2023 PNAS***.

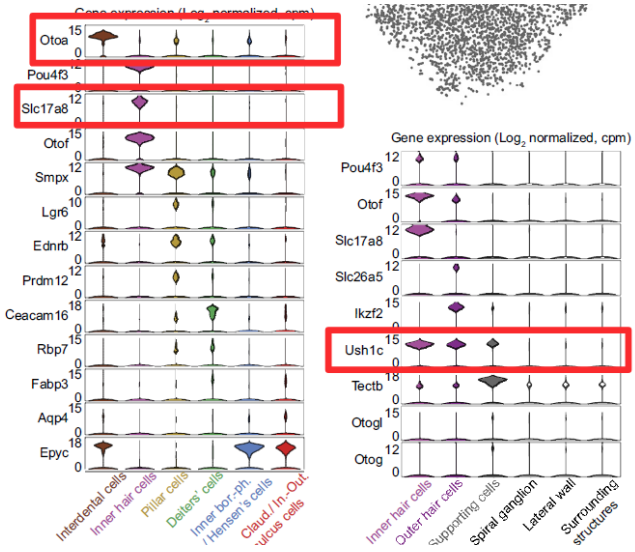
Our analysis aligns with the literature as follows: *SLC17A8* serves as a validated marker gene for inner hair cells (IHCs), *OTOA* is predominantly expressed in interdental cells (IDC), and *USH1C* shows co-expression in both hair cells (HCs) and supporting cells.

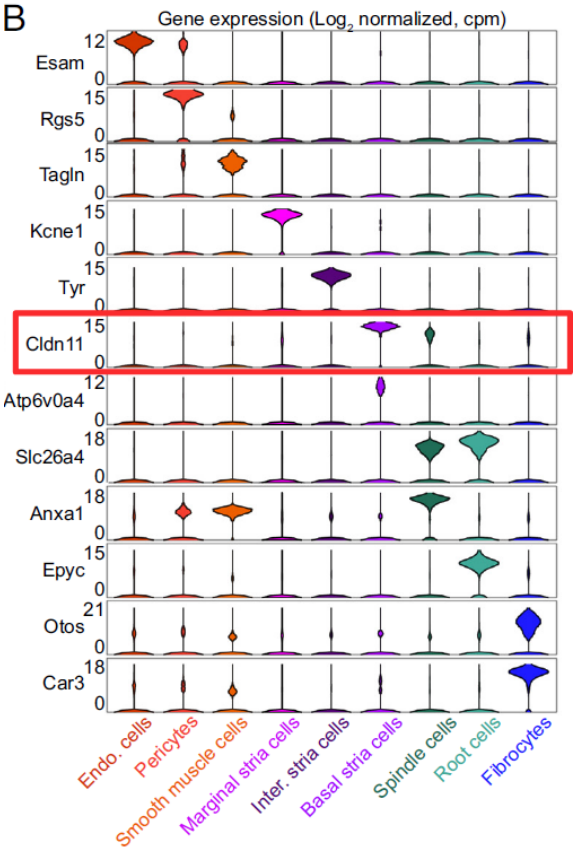
Discrepancies were noted for *CLDN11*: while it is reported to localize primarily to basal stria cells (Bs) in mice, our data revealed its expression in macaque spindal/root cells (Sp/Rt), satellite glial cells, as well as tympanic border cells (TBCs) and fibrocytes (Fb). We attribute this divergence to the current reliance on murine marker genes for macaque cell-type annotation, as no single-cell sequencing data specific to macaques are yet available. Such cross-species differences in gene expression patterns are an inherent limitation of this approach.

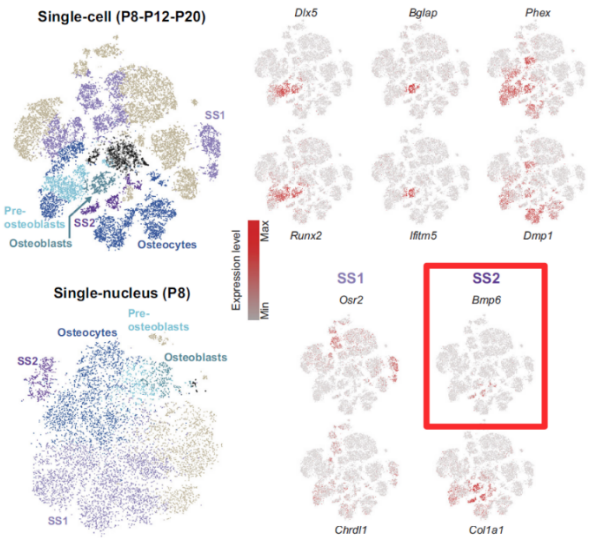
Regarding *BMP6*, the cited study (*Jean P. et al, 2023 PNAS*) focuses exclusively on osteocytic populations in its "Transcriptomic characterization of the osseous cell types" section, reporting *BMP6* expression in surrounding structure 2(SS2) but does not report its

expression profiles in other cell types. In contrast, our macaque data demonstrate that *BMP6* is predominantly highly expressed in the DC_PC cluster, with additional expression observed in Schwann cells, type Ⅱ SGN, and fibroblasts (Fb).

Jean, Philippe et al. "Single-cell transcriptomic profiling of the mouse cochlea: An atlas for targeted therapies." Proceedings of the National Academy of Sciences of the United States of America vol. 120,26 (2023): e2221744120. doi:10.1073/pnas.2221744120





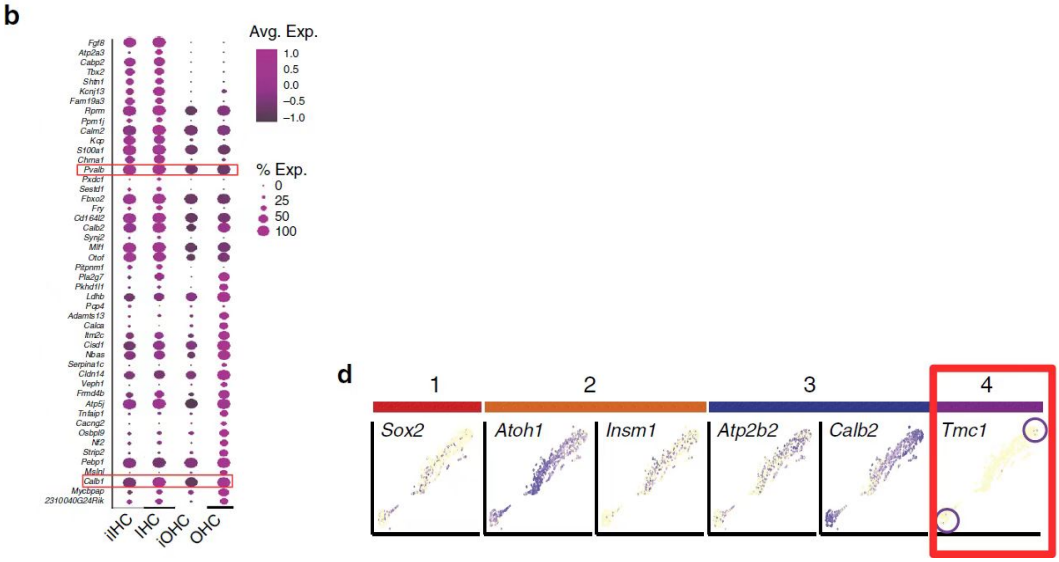


(iii) The references for **PVALB**, **CALB1**, and **TMC1** are derived from the Kolla, Likhitha et al,2020 Nature Communication. The literature reports that PVALB and CALB1 are expressed in hair cells, with TMC1 serving as a marker for mature hair cells[1]. Notably, *CALB1* has been widely documented in mice as a marker gene for type Ⅰ B spiral ganglion neurons (SGNs) [2]. However, in our macaque data, *CALB1* expression was predominantly observed in type Ⅱ SGNs, a divergence that is comprehensively discussed in the revised manuscript (Fig. 4). Additionally, *PVALB*—a calcium-binding protein primarily localized to the spiral ganglion (SG)—is ubiquitously expressed across all SGN subtypes, consistent with its established role in neuronal calcium homeostasis[3].

[1] Kolla, Likhitha et al. "Characterization of the development of the mouse cochlear epithelium at the single cell level." Nature communications vol. 11,1 2389. 13 May. 2020, doi:10.1038/s41467-020-16113-y

[2] Sun, Shuohao et al. "Hair Cell Mechanotransduction Regulates Spontaneous Activity and Spiral Ganglion Subtype Specification in the Auditory System." Cell vol. 174,5 (2018): 1247-1263.e15. doi:10.1016/j.cell.2018.07.008

[3] Kaur, C et al. "Comparison of unbiased stereological estimation of total number of cresyl violet stained neurons and parvalbumin positive neurons in the adult human spiral ganglion." Journal of chemical neuroanatomy vol. 93 (2018): 30-37. doi:10.1016/j.jchemneu.2017.06.004



(iiii)The gene **GATA3** was referenced from Xu, Zhenhang et al, 2022 Frontiers in cellular neuroscience.

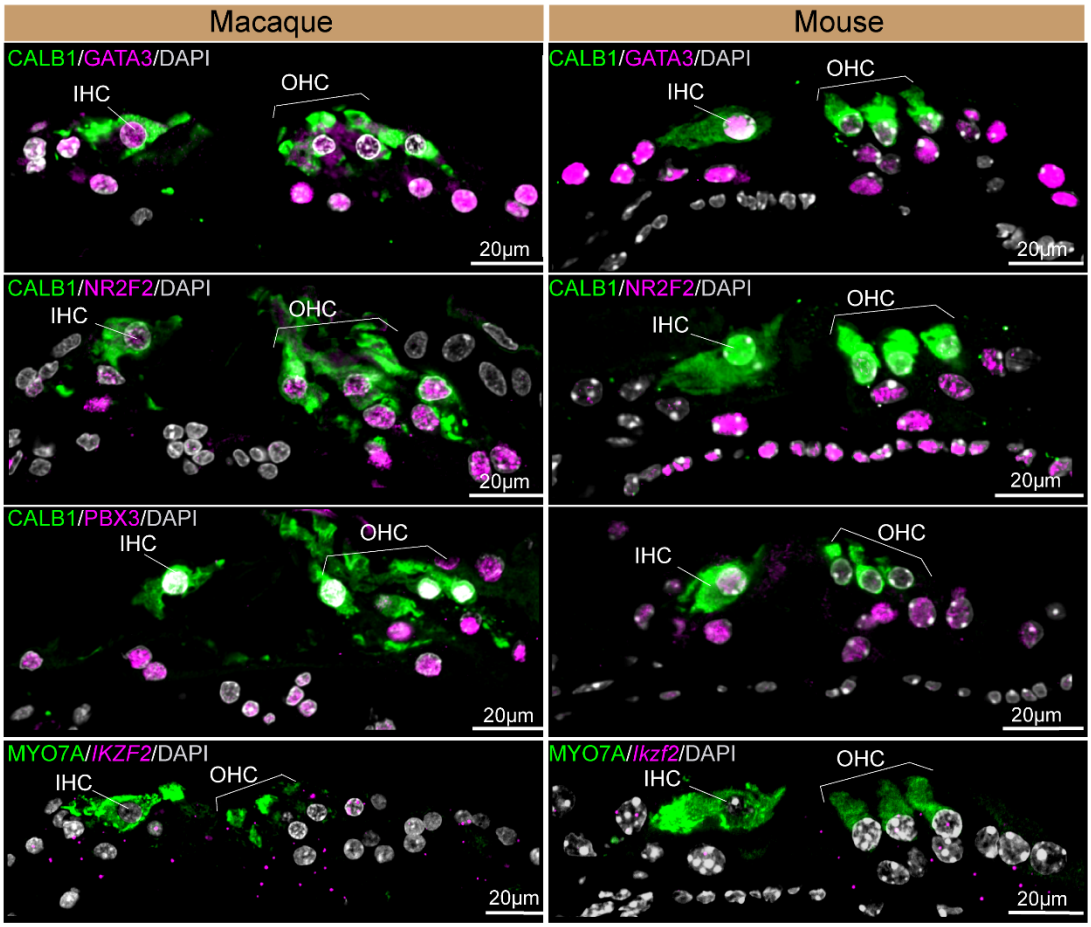
Xu, Zhenhang et al. "Profiling mouse cochlear cell maturation using 10× Genomics single-cell transcriptomics." Frontiers in cellular neuroscience vol. 16 962106. 18 Aug. 2022, doi:10.3389/fncel.2022.962106

Cell type	Marker genes
OHC	*Myo6*, *Slc26a5*, Ocm (Self et al., 1999; Yu and Tang, 2008; Simmons et al., 2010)
IHC	*Myo6*, *Slc17a8*, *Otof* (Roux et al., 2006, 2009; Seal et al., 2008)
Spiral ganglion neuron	*Tubb3*, *Nefh* (Barclay et al., 2011; Locher et al., 2013)
Microglia	*Cx3cr1* (Jurga et al., 2020)
IPhC/IB/DC/PC/HeC	*Sox2* (Hume et al., 2007)
IPh/IB/Spiral ligament (SLg)	*Slc1a3* (Jin et al., 2003; Glowatzki et al., 2006; Udagawa et al., 2021)
Schwann cell	*Mpz*, *Mbp* (Kim et al., 2017)
Schwann cell (SC)/Satellite glial cell (SGC)	*Plp1* (Wan and Corfas, 2017)
Outer sulcus cell (OSC)/Inner sulcus cell (ISC)/DC/PC	*Gjb2* (Crispino et al., 2011)
Inner sulcus cell (ISC)/Hensen cell (HeC)/IPhc/IB	*Gata3* (Nishimura et al., 2017; Walters et al., 2017)
Reissner's membrane (RM)	*Vmo1* (Peters et al., 2007)
Tympanic border cell (TBC)	*Emilin2* (Amma et al., 2003)
Spiral limbus (SLb) /Spiral ligament (SLg)	*Coch* (Robertson et al., 2001)
Marginal cell (MC)	*Kcnq1*, *Kcne1* (Tian and Johnson, 2020; Milon et al., 2021)
Intermediate cell (IC)	*Dct*, *Met* (Tian and Johnson, 2020; Milon et al., 2021)
Spindle cell/Root cell (SpC/RC)	*Slc26a4* (Milon et al., 2021)
Basal cell (BaC)	*Cldn11* (Milon et al., 2021)
Macrophage	*Cx3cr1* (Kaur et al., 2015; Rai et al., 2020)
B cell	*Cd79a* (Browne et al., 2003; Rai et al., 2020)
T cell	*Cd3g*, *Cd4* (Hong et al., 2011)
Granulocyte	*Itgam* (Fernekorn et al., 2007)

(iiiii) Intriguingly, **DCLK1** and **ENAH** show broad expression across nearly all macaque cell types, a phenomenon potentially attributable to species-specific regulatory mechanisms.

5. The potential role of zinc finger transcription factors as cochlear epithelium markers in both species warrants further experimental validation.

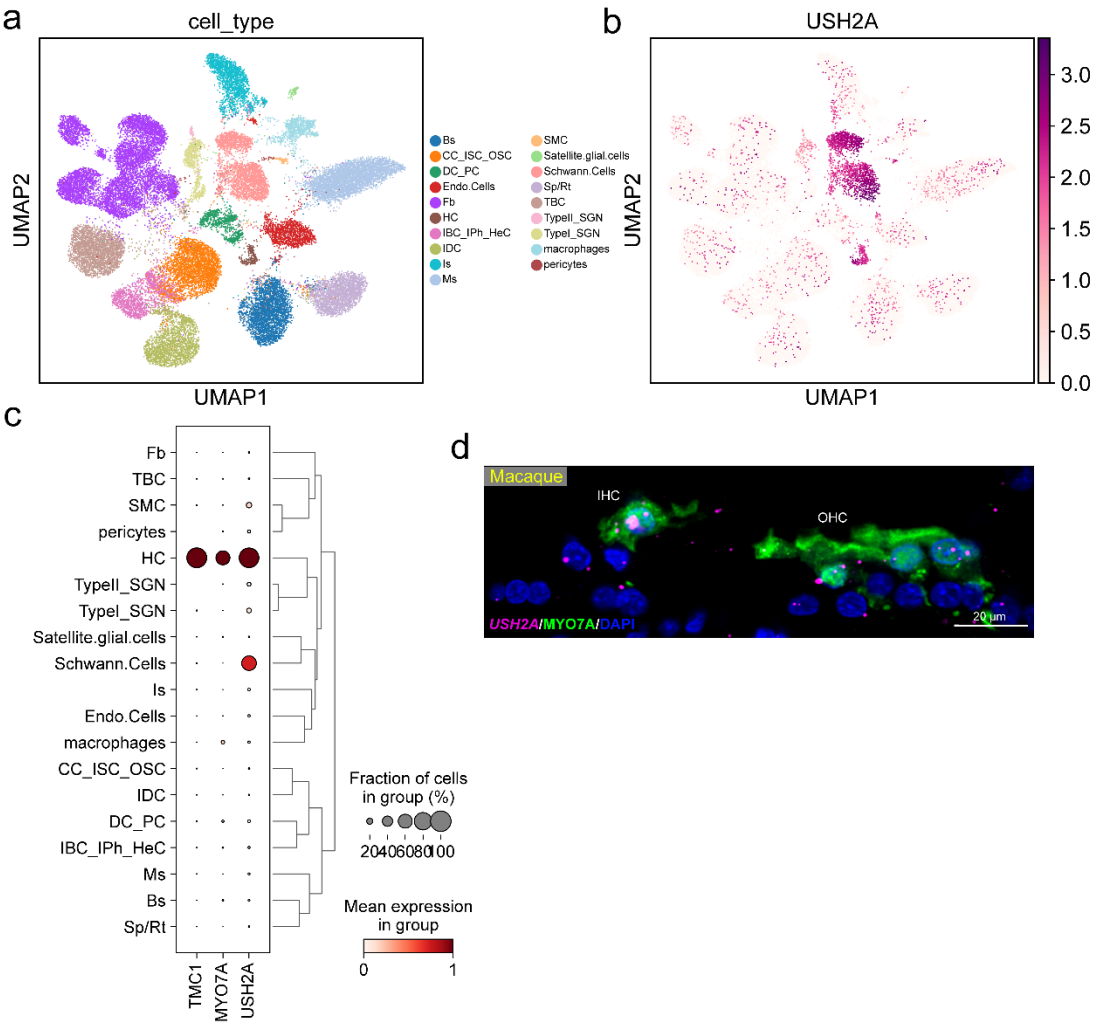
We are thankful for the reviewer's further comments and suggestions. In our revised version, we have added the experimental validation for zinc finger transcription factors, *Ikzf2*.



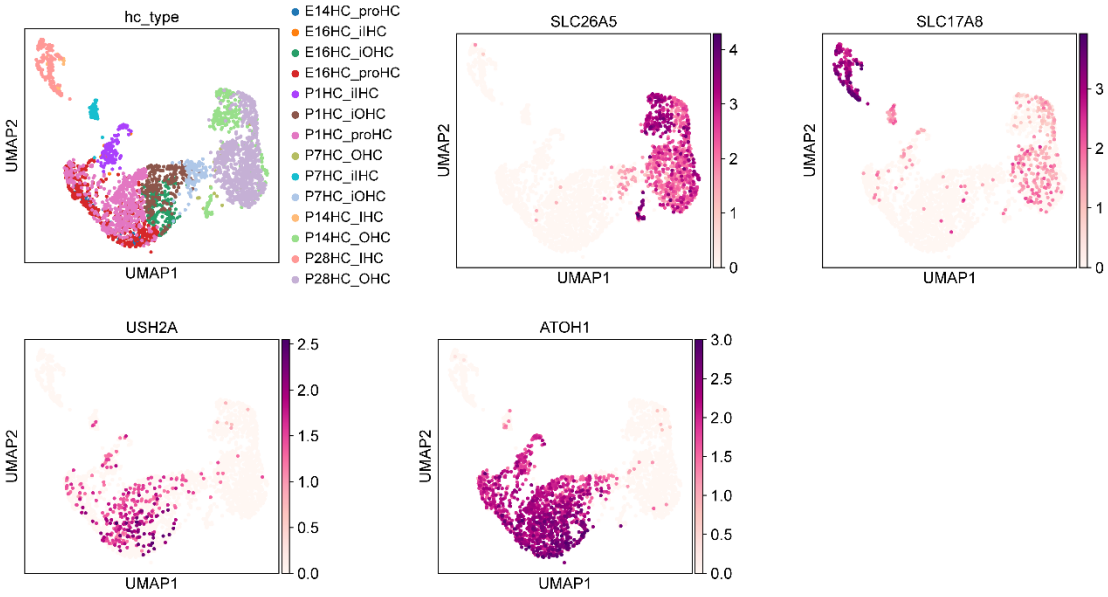
i Representative confocal images of immunofluorescence cryosection staining demonstrating IKZF2 (Ikaros family zinc finger protein 2), GATA3 (trans-acting T-cell-specific transcription factor GATA-3), NR2F2 (COUP transcription factor 2) and PBX3 localization in 5-year-old macaque cochlea.

6. In response to Review 2 of the P2P, the authors have not sufficiently addressed the reviewer's concerns, particularly the lack of experimental validation for U2H2A as a hair cell marker.

In our latest manuscript, we have performed RNAscope validation of USH2A expression in hair cells, which we believe should address the reviewer’s concerns.



And also USH2A transient expression in prenatal mouse hair cells as both confirmed by our scRNA-seq data and paper from *PNAS,2007 (Usherin is required for maintenance of retinal photoreceptors and normal development of cochlear hair cells).*



7. There is inconsistency in the ages of mice used across different figures (Figure S1b, Figure

S2a and e, Figure S9b). Clarification of the data source for each sample is required.

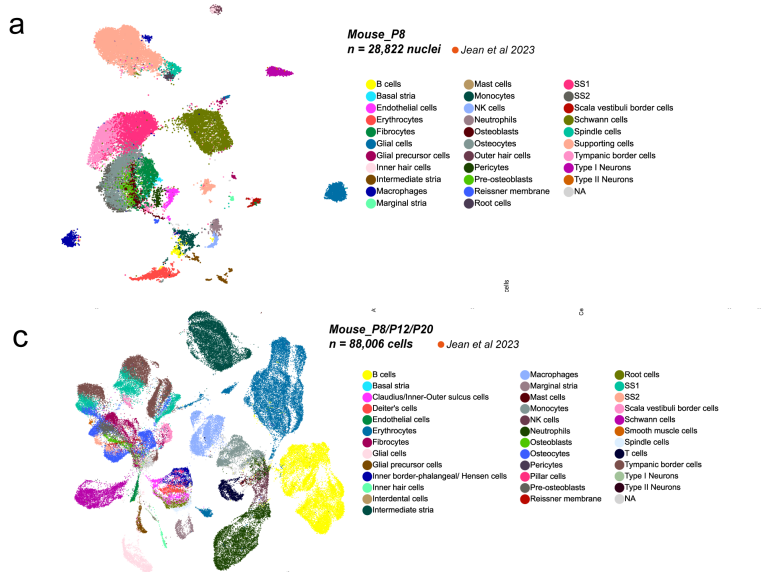
We greatly appreciate the reviewer's attentive feedback and apologize for any oversights in our previous versions. In our revised manuscript, we have clarified the data source for each mouse in both the figures and figure legends.

b

Species	RNA-seq (Cv3)	Details
Crab-eating Macaque	5，619	1-year-old, male
Crab-eating Macaque	32,506	5-year-old, male [*from 2 animals, 10,250 (5Y_1_1); 8,727 (5Y_1_2); 13,529 (5Y_2)*]
Crab-eating Macaque	5，661	11-year-old, female
C57BL/6J Mouse	6,775 (2*)	3，419 (P25_1) 3，356 (P25_2)

* *either sex of 7-8 mice as single pooled replicate.*

b A table summarizing sample information, including species, collected nuclei after quality control, biological replicates, and sex.

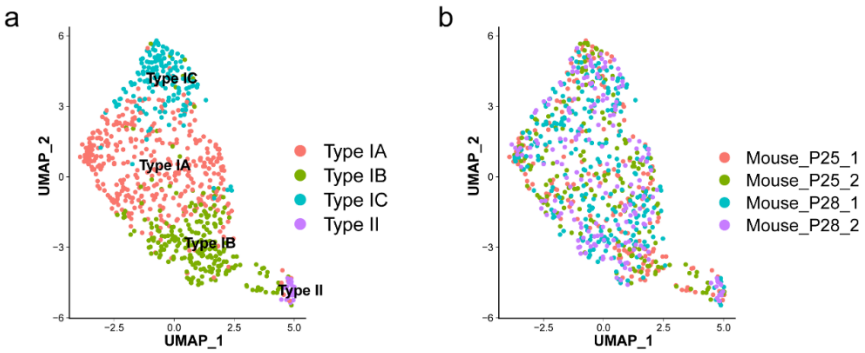


*a UMAP (Uniform Manifold Approximation and Projection) plot representing cell types of postnatal days **(P)8** mouse cochleae from Jean et al. PNAS, 2023.*

b Dot plot depicting the variation-scaled, log-transformed expression levels of marker genes (x-axis) in each cell type (y-axis) from P8 mouse cochleae.

*c UMAP plot, similar to (a), but for pooled **P8, P12, and P20** mouse cochleae. The capture method employed single-cell dissection and resuspension, and cell-type annotation was performed by Jean et al. PNAS, 2023.*

*d Same as (b), but for **P8, P12, and P20** single-cell mouse cochleae.*



*a, b UMAP (Uniform Manifold Approximation and Projection) representation shows the re-clustering of mouse SGNs from the postnatal days **(P)25/28** cochlear snRNA datasets. 4 subtypes including three type I subtypes (IA, IB, IC) and one type II subtype are embedding and visualized. We used our own sequenced dataset of postnatal days**(P) 25** mouse cochleae and published P28 mouse cochleae (Zhenhang Xu et al. 2022, Front Cell Neurosci.) as a whole for SGN subtype analysis.*

Minor Issues:

1. Figure 2b requires annotation for the color bar.

We have refined this in our new version.

2. The clarity of data representation in Figure 3e should be improved for enhanced readability.

We have moved this diagram into the attached diagram S10d to make the figures more logical and clear.

3. Figure 3g requires clear annotation of its color bar for better understanding.

We have refined this in our new version.

4. Additionally, several figure citations in the manuscript are improperly used, which could mislead the readers. Specifically, the text on line 272, "with many enriched in only one species (Figure 3d and e)," should actually refer to Figure 5d and e. Similarly, the statement on line 302, "with about half of the genes uniquely expressed or enriched in HCs (Figure 5; Figure S17)," should cite Figure 6.

We have refined this in our new version.

5. The scale bar value is missing in the legend of Figure S1c.

We have refined this in our new version.

Reviewer #3 (Remarks to the Author):

The major interest of the work by 'Xin Chen et al.' is that it provides a single cell transcriptomic dataset in a non-human primate essential to make sure the gene therapies designed in mice have a chance to be safe and to be rapidly translated to patients. The two major concerns that were raised have only been partially addressed:

We sincerely thank the reviewer for acknowledging a single cell transcriptomic dataset in a non-human primate is essential to make sure the gene therapies to be safe to patients.

i)The English writing was significantly improved making the manuscript more easily readable. However, there are still some language inaccuracies. More problematic is the fact that the authors did not increase the size of the text in the figures making many of them unreadable when printed on paper and requiring a zoom of 200-250% to be readable on the screen (see Fig.1d,2b,3b,3c,3d,3e,3f,4e,5b, 5c,5g,6, supp fig1d,1e,1f, 1g,1h,2a,2b,2c,2d,

3a,3c,3d,3e,3g,3h, 4a, 4b,12b,12c,12e,12g,13,14b,14c,14e,14g,15,17).

We'd like to express our sincere apologies for any inconvenience our previous manuscript may have caused the reviewers and the editor. We've taken your feedback seriously and continued to revise, refine, and polish our manuscript constantly to improve the clarity, precision, and overall quality of the writing. In our new version, we have refined the size of the text in figures to make the manuscript more easily readable.

We truly appreciate your patience and the diligent attention you've given to our work. We hope that our renewed efforts to enhance our manuscript meet with your approval and help convey our research findings in a more understandable and professional manner. Thank you for your continued guidance and support.

ii) Concerning the lack of additional cochlear transcriptomic knowledge, the improvements are limited probably owing to the filtering of the data obtained owing to the methodological set-up for several reasons: the limited number of macaques used, the use of aged tissue (compared to relatively young adult mice), and the use of single nucleus transcriptomics which provides more cells to the cost of sequencing depth. Despite these basic problems that are probably unsolvable, the authors could detect all key cochlear cell types and the data is potentially valuable as, to the best of my knowledge, no non-human primate cochlear transcriptome data is available yet. However, the authors should make clear the limitations of their data and not make overstatements. This point should be discussed extensively and in a balanced way.

We sincerely thank the reviewer's recognition for the value of our data. In our new vision, we had added two 5-year-old macaques (one provided two cochleas) for snRNA seq and two 5-year-old macaques for validation compared with the initial version. The present samples have compensated the heterogeneity stemming from individual variability, gender distinctions, and age-related factors.

We appreciate the valuable suggestions and have revised our statements to ensure more rigorous and impartial analysis and presentation of the data. We express our sincere gratitude for your insightful input that has helped us improve our work substantially.

Please find below more details:

1) The paragraph on cell diversity in the macaque cochlea is not very convincing owing to the limitations of the data mentioned above. The authors should be more careful in their conclusions as they are highly likely overstatements. Example: "glial cells in these two species differed in their transcriptional profiles". The problem is the same with the section on the supporting cells: "this unexpected contrast between a small group of conserved genes and a large cohort of genes manifesting species-specific enrichment highlights the diversity of SC types across species".

We appreciate the reviewer's comments and take it seriously. We have corrected our grammatical and syntax errors and refined our manuscript thoroughly. We have revised the conclusions of the manuscript in new vision with a more rigorous and accurate description.

We rewrite these sentences in '***Glial cell diversity in the macaque cochlea***' section and '***The similarity and specificity of expression profiles in the cochlear epithelium***' section, please these changes in our revised manuscript.

2) There is a similar problem in the "deafness associated genes across species section". The

sentence "This indicates…that previously identified HL genes are mainly concentrated in HCs and are linked to HC functions" is a very likely overstatement. As mentioned in the review of the first submission, most if not all genetic forms of deafness have been replicated either from human to mouse or from mouse to human. Moreover, there are more and more examples of particular audiological signatures associated with specific genetic forms of deafness that have been uncovered in mice and that were confirmed subsequently in patients. So there is clearly a very close resemblance in the genetic architecture and function of mouse and human cochleae. It is therefore highly unlikely that the macaque diverged in a major different direction from mice and humans. The probable reasons for the observations of the authors is that they used adult/aging macaques and most of the pathogenic genes are involved in cochlear development, many of them being in hair cells but many others also in other cell types (see Fig. 7 from Jean P et al. 2023, PNAS). For example, it is puzzling that GJB2, a major gene underlying around 50% of the deafness forms does not show much expression and differential expression within cochlear cell types (see supp Fig. 17 of the authors) whereas it is known to be involved in many cochlear cell types.

We sincerely appreciate your valuable feedback and have thoroughly revised the manuscript accordingly. We trust that the updated figures and accompanying descriptions in the latest version will adequately address the concerns you raised.

Following the expansion of our sample size and subsequent re-construction of the disease-associated molecular atlas, we observed that the majority of these genes exhibit cell-type-specific expression patterns. For instance, POU4F3—a well-established deafness gene—is predominantly expressed in hair cells, with its mutations known to cause DFNA15, a common autosomal dominant hearing loss disorder [1]. The gene Gjb2, which encodes connexin 26 for gap junctions, is broadly expressed in supporting cells and cells of the lateral wall [2]. Mutations in Gjb2 are associated with both congenital hearing loss and later-onset deafness [3]. Notably, our data demonstrate highly conserved expression patterns across 1-, 5-, and 11-year-old macaques, further validating the robustness of our dataset. Cross-species comparative analysis reveals that deafness-associated genes are evolutionarily conserved between macaques and mice, sharing analogous expression profiles.

[1] Zhang, Chi et al. "A Novel Nonsense Mutation of POU4F3 Gene Causes Autosomal Dominant Hearing Loss." Neural plasticity vol. 2016 (2016): 1512831. doi:10.1155/2016/1512831

[2] Chen, Sen et al. "The spatial distribution pattern of Connexin26 expression in supporting cells and its role in outer hair cell survival." Cell death & disease vol. 9,12 1180. 5 Dec. 2018, doi:10.1038/s41419-018-1238-x

[3] Oguchi, Tomohiro et al. "Clinical features of patients with GJB2 (connexin 26) mutations: severity of hearing loss is correlated with genotypes and protein expression patterns." Journal of human genetics vol. 50,2 (2005): 76-83. doi:10.1007/s10038-004-0223-7

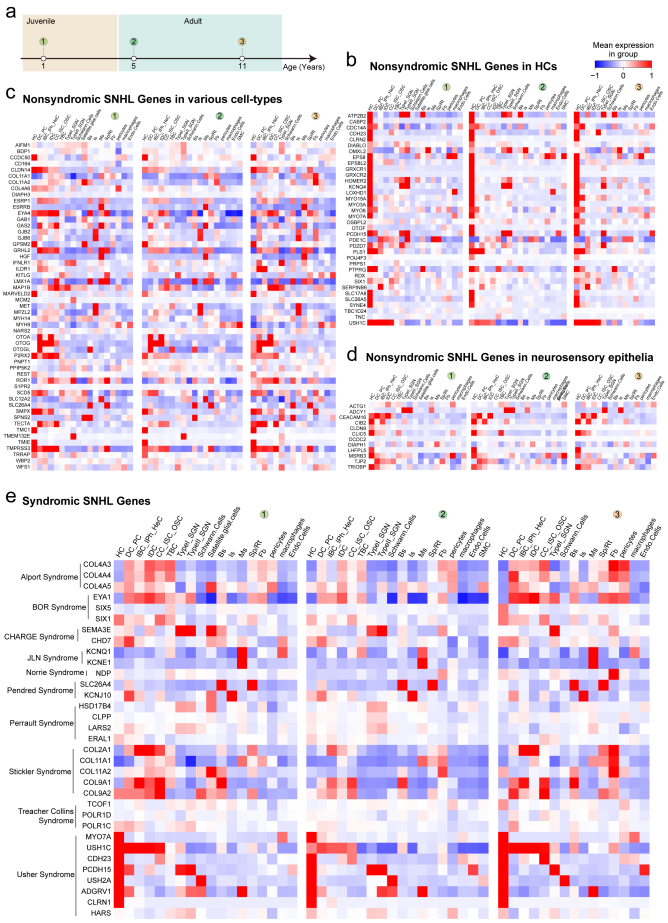


Fig. 6 Mapping deafness-related genes in macaque cochlea.

a Temporal ordering of snRNA-seq data from juvenile (one-year-old) macaque to adult macaques (5-year-old and 11-year-old) for constructing the disease map of sensorineural hearing loss (SNHL).

b Heat map showing the variance-scaled, log-transformed expression pattern of nonsyndromic SNHL genes (y-axis) that are greatly localized in hair cells across the whole cochlear cell types (x-axis).

c Heat map showing the variance-scaled, log-transformed expression of nonsyndromic SNHL genes (y-axis) in multiple inner ear cell types across the whole cellular map(x-axis).

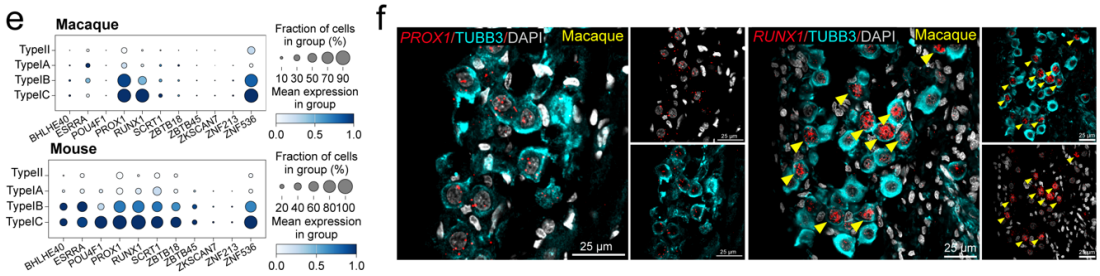
d Heat map showing the variance-scaled, log-transformed expression of nonsyndromic SNHL genes (y-axis) in neurosensory epithelia across the whole cochlear cell types (x-axis).

e Heat map showing the variance-scaled, log-transformed expression pattern of selective syndromic SNHL genes (y-axis) localized in the whole cochlear cell types (x-axis). BOR: Branchio-Oto-Renal syndrome; JLN: Jervell & Lange-Nielsen syndrome; CHARGE: coloboma, heart defect, atresia choanae, restricted growth and development, genital abnormality, and ear abnormality syndrome.

3) Concerning the "sensory neuron diversity in the macaque cochlea", the authors pinpoint the key proteins lacking in macaque SGNs (versus mice) like pou4f1 and LypD1. Did the authors find potential new markers specific of type1c macaque neurons (and more generally other neurons)? I think the information is not mentioned and it could potentially be beneficial to try to demonstrate the existence of type1C neurons on human tissue.

We appreciate and are highly indebted to the reviewer for the professional advice and insightful recommendations aimed at refining our manuscript. In our new version, we thought

RUNX1 is essential for type IC SGN specification. Immunolabeling and RNASCOPE assay confirmed its selectively expressed in SGN subtypes.



e Dot plots showing the variance-scaled, log-transformed expression of transcription factors (TFs, x-axis) with mutually exclusive expression in type I SGNs in macaque and mouse snRNA-seq data (y-axis).
f Representative confocal images of immunofluorescence cryosection staining demonstrating TUBB3 associated with PROX1 (prospero homeobox protein 1) or with RUNX1 (Runt-related transcription factor 1) localization in 5-year-old macaque SGNs.

4) The discussion should balance more the conclusions in the light of the limitations linked to the small number of macaque replicates, the use of adult macaques compared to young mice, and the fact that single nucleus transcriptomic sequencing has less depth of sequencing. The authors should indicate the average depth of sequencing (number of read per cells) and also give statistics on mitochondrial genes in the material and methods.

In accordance with your advice, we have introduced additional samples in our updated manuscript to reinforce our conclusions.

In our new vision, we had added two 5-year-old macaques (one provided two cochleas) for sn-RNA seq and one 5-year-old macaques for validation compared with the initial version. These enhancements were implemented to address potential variations associated with ages.

As suggested by the reviewer, we have indicated the average depth of sequencing (number of read per cells) and given statistics on mitochondrial genes in the material and methods.

We look forward to continuing this scientific discourse and cooperating with reviewers to address any remaining concerns to ensure our study's robustness and credibility.

5) I do not understand why the authors wanted absolutely to test for the presence of electromotility in a macaque. The electro motile property of OHCs can be monitored with a non invasive technique, called distortion products acoustic emissions (DPOAEs). DPOAEs are well characterized in mice and humans. They probably are too in many non-human primates (eg: Valero et al Hearing Research 2008, PMC2567920). In addition, mutations in the Prestin gene have been found to underly hearing loss inpatients (ex: Toht et al, PMID: 17786286). I therefore do not understand why the authors wanted to make the point that prestin is indeed expressed by macaque OHCs and underlies electromotility. The sole detection of prestin was a good enough indication that they had found OHCs.

Prestin is the motor protein of cochlear OHCs [1]. The electromotility of outer hair cells (OHCs) can be electrophysiologically characterized via patch-clamp recording techniques. However, to date, no patch-clamp recordings of cochlear hair cell electromotility have been reported in non-human primates. The electromotility of the outer hair cell is also a feature typically indicative of maturation in outer hair cells [2]. The presence of electromotility in cochlear hair cells of 1-year-

old macaques confirms their functional maturation at this age.

[1] Liberman, M Charles et al. "Prestin is required for electromotility of the outer hair cell and for the cochlear amplifier." Nature vol. 419,6904 (2002): 300-4. doi:10.1038/nature01059

[2] Chessum, Lauren et al. "Helios is a key transcriptional regulator of outer hair cell maturation." Nature vol. 563,7733 (2018): 696-700. doi:10.1038/s41586-018-0728-4

6) Why reanalyzing the data by (Jean P. et al, PNAS 2023)? I could not find the reason in the manuscript. I guess the reason is the use of a UMAP analysis rather than tsne. If this is the case, the precision should be made in the manuscript. In addition, the citation of the paper should be made systematically for each panel in supp Fig.2 with a citation like (Jean P. et al, PNAS 2023) and not just saying "by PNAS". This is important to make sure there is no ambiguity between the authors' data and other studies' data. In supp Fig. 3, the authors should clearly state in the figure legends that they are back to their own data, too.

As the reviewer says, the use of a UMAP analysis rather than tSNE is exactly why we reanalyzed the data by (Jean P. et al, PNAS 2023). Their comprehensive work has characterized the transcriptomic signatures covering nearly all cochlear cell types and developed cell type–specific markers.

We have indeed reutilized the dataset. We are grateful to the reviewers for their feedback concerning the unclear citation of data in our work. We have treated them with due seriousness. In our revised version, we have explicitly indicated the data sources in both figures and figure captions to prevent any unnecessary misunderstandings or ambiguities.

7) In supplementary Fig 1f, the scheme appears to be a copy-paste of the scheme found in Fig. 1b of Jean P. et al, 2023 PNAS. Moreover, Fig.1a also corresponds to Fig 1b of Jean P. et al, 2023 PNAS with an additional layer. There is absolutely no source cited in the legends of these figures. Could the authors make the corrections and also confirm that there are no other figures copy-pasted from any other article/journal?

We sincerely apologize for the insufficient attribution of image sources. Figures 1a and Supplementary Figure 1f are reconstructed schematic diagrams based on *Fig. 1b* from Jean P. et al. (*PNAS, 2023*). We have now explicitly acknowledged this reference in the revised figure legends and conducted a thorough review of the entire manuscript to ensure all citations are clearly labeled. Upholding academic integrity remains our fundamental commitment and obligation. We deeply appreciate the reviewers' attention to this matter and their constructive feedback.

8) Other points:

-line94: 'preferentially' should be removed-line 100: "extreme' should be removed

-Line 112: "gene therapeutics" would better read "gene therapies"

We have refined this in our new version.

-line 127: the adjective "non human primate" could be added to "constructing a cell atlas".

We have refined this in our new version.

-line 142: "in 1-year-old and 11-year old macaques" should read "in a 1-year-old and an 11-year old macaques"

We have refined this in our new version.

-line 151: “These results strongly implicate” would better read as “These results shows”?

We have refined this in our new version.

-line 156: “Oterferlin” should read otoferlin

We have refined this in our new version.

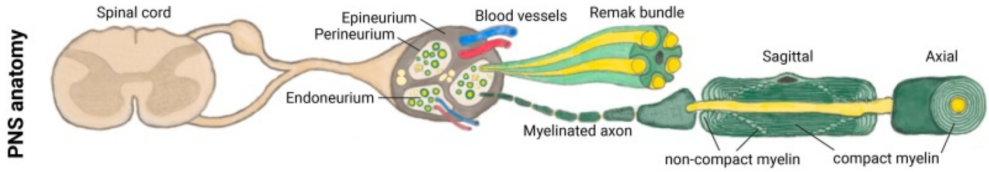
- line 198: Maybe remind for the general reader what are Remark bundles.

We gratefully acknowledge the reviewers' thoughtful consideration and will incorporate a concise definition of the *Remak bundle* within the revised manuscript to ensure conceptual clarity for general readers.

Derived from the embryonic neural crest, Schwann cells differentiate into myelinating cells or bundle multiple unmyelinated axons into Remak fibers [1]. Perinatally, in rodents, SCs radially sort and subsequently myelinate individual axons larger than 1 µm in diameter, while small calibre axons become organised in non-myelinating Remak bundles [2].

[1] Kidd, Grahame J et al. “Biology of Schwann cells.” Handbook of clinical neurology vol. 115 (2013): 55-79. doi:10.1016/B978-0-444-52902-2.00005-9

[2] Bosch-Queralt, Mar et al. “Schwann cell functions in peripheral nerve development and repair.” Neurobiology of disease vol. 176 (2023): 105952. doi:10.1016/j.nbd.2022.105952

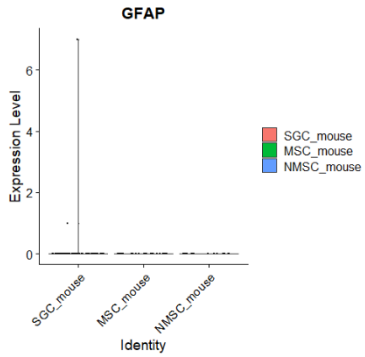


-line 265: “to integrate the mouse” should read “to integrate our mouse” to avoid any ambiguity.

We have refined this in our new version.

-Supp figures 6 and 7 are nice in that they can be put in parallel. However, the authors removed the GFAP from the mouse analysis. Why is it so whereas all other markers are exactly the same between macaque and mouse data?

We appreciate this remark. Due to low count detection of GFAP, we directly remove the GFAP in the mouse glial cell analysis. Thanks again to point this out.



-Please add the number of nuclei for each UMAP on the figures.

We have refined this in our new version.

-Supplementary Fig. 17: lacking a scalebar-Please verify on all the figures that a scale bar is present and readable

We have refined this in our new version.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed all of my concerns. This is a valuable dataset that will be highly useful to the auditory neuroscience community. I appreciate the authors' sharing their annotated code in GitHub to make this work reproducible. The URL for this should be mentioned in the methods section. Likewise, sharing the data through gEAR (for those that can't code), and depositing the raw data online will also be highly valuable to the auditory neuroscience community. Currently, I do not see where the raw data will be deposited - this should be added to the final manuscript including the raw fastq files for all of the macaque snRNAseq data.
Response: Thank you for your positive assessment of our research. We have now included the code URL in the Code Availability section of the manuscript. The single-cell RNA sequencing data generated in this study have been deposited in the Genome Sequence Archive (GSA) under accession number CRA032615. We hope that our data will prove valuable and beneficial to researchers in the field of auditory neuroscience.

I have some minor comments:
Consider changing the title from "Elucidating the molecular diversity of non-human primate cochlea" to "Molecular heterogeneity of the non-human primate cochlea" or "Molecular diversity of the non-human primate cochlea"
Response: Thank you for your suggestion. We have adopted it and changed the article title to "Molecular heterogeneity of the non-human primate cochlea".

Introduction - Number of inner and outer HCs in mice and in humans should have citations
Response: We have added it.

Lines 204/205: "were strongly expressed in macaque HCs across species." Should this be in macaque HCs or just in HCs across species without "macaque" here?
Response: We have refined it.

Line 206: should be "conserved" instead of "conserve"
Response: We have refined it.

"RNASCOPE" is used multiple times or "RNAscope." Suggest keeping the format the same throughout. It should be "RNAscope™" as it's a trade-marked name from ACD Bio. Alternately, authors can just say "in situ hybridization" and just state once in the methods that the RNAscope™ platform was used.
Response: Thank you for your suggestion. We have standardized the term to "RNAscope™" throughout the manuscript.

Line 350: Should "HCs" here be "SCs"? I think it's supposed to be "This divergence in SCs..."
Response: We have refined it.

Figure 2a: the gray-scale UMAP has an arrow that brings out the IHC/OHC group. Is that arrow supposed to start where the black dots are? It looks like it's coming from an edge of a large cluster now. May need to be corrected.
Response: We have refined it.

Reviewer #1 (Remarks on code availability):

I have reviewed the code provided in github. I did not re-run the code, but in general, it appears well annotated and should give readers a chance to see exactly what was done. I am unable to re-run the code as the raw data or processed data has not been deposited online. Sharing the raw data is a critical part that must be deposited online prior to final publication of this important work.
Response: We agree with the importance of data sharing. The single-cell RNA sequencing data generated in this study have been deposited in the Genome Sequence Archive (GSA) under accession number CRA032615.