Convergence of type 1 spiral ganglion neuron subtypes onto principal neurons of the anteroventral cochlear nucleus

Nicole Wong, Sydney Brongo, Evan Forero, Connor Cook, Shuohao Sun, Amanda Lauer, Ulrich Mueller, and Matthew Xu-Friedman

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Dear Dr. Xu-Friedman:

Thank you for submitting your manuscript to The Journal of Neuroscience.

We have received the reviews of your paper, "Convergence of type 1 spiral ganglion neuron subtypes onto principal neurons of the anteroventral cochlear nucleus" (JN-RM-1507-24), which are appended to this email. Based on the reviewers' comments and our editorial assessment, we would like to reconsider your manuscript at The Journal of Neuroscience following major revisions. We hope that you will be able to address the reviewers' concerns in full and resubmit the manuscript, along with a point-by-point reply to the reviews that indicates your response to each concern. Before we make a decision about publication, we will have your revision reviewed by the reviewers and editorial team.

Your revision must include the manuscript with new text highlighted, as well as a clean copy of the manuscript. Please carefully review your paper at this time for any corrections in style or substance. Please consult our Revised Submission Checklist for details on preparing your revision: https://www.jneurosci.org/sites/default/files/files/JN_Revised_Submissions_Checklist.pdf.

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Your submission must also include publication-quality figures, each in a separate EPS or TIFF (300 dpi) file. Please make sure your figures adhere to style requirements to avoid delays in manuscript processing. Detailed guidelines for figures are available here: https://www.jneurosci.org/content/information-authors#figures

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Thank you for giving us the opportunity to consider your paper for publication in The Journal of Neuroscience. Please let us know if you have any questions or concerns.

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Yours sincerely,

Gavan McNally
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Editor-in-Chief
The Journal of Neuroscience

Manuscript Instructions

Reviewing Editor :

The authors use a combination of optogenetics, in vitro intracellular recordings, in vivo extracellular recordings, and immunohistochemistry to examine the anatomical and physiological specificity of 2 types of SGN onto the cochlear nucleus. Unexpectedly, they find no differences in these two neuron types. This is a nice example of a null result that advances our understanding of the brain. The authors discuss the implications for this convergence of processing streams on auditory function. The reviewers have made useful suggestions for improving the manuscript, including condensing some of the figures (e.g. Figures 1-3). I would encourage the authors to keep the relevant information as main, rather than supplementary, figures, but to consider how they may be more efficiently presented.

Reviewer #1 (Rationale for Significance Rating for Authors):

In the manuscript by Wong et al, the authors use optogenetic targeting of subtypes of spiral ganglion afferent neurons (SGN) to probe whether acoustic processing in the cochlear nucleus is performed in segregated streams of information corresponding to the SGN classes. Contrary to expectations, the authors found in immunohistochemistry, in vitro electrophysiology, and in vivo electrophysiology experiments that there is a convergence of SGN subtypes onto bushy cells in the AVCN, which helps to inform the information processing of this class of neurons that are critical for hearing.

Reviewer #1 :

In the manuscript by Wong et al (JN RM-1507-24) the authors use genetic mouse models to stimulate sub-populations of auditory nerve afferents to test whether these neurons evoke different responses in their target neurons in the cochlear nucleus. Prior work from other labs has defined populations of primary afferent (spiral ganglion neuron; SGN) subtypes that have different spontaneous rates and thresholds to sound-evoked excitation (along with other cellular and synaptic specializations) and can be identified by genetic markers. These subtypes of neurons are thought to encode different features of the sound, and would therefore be expected to evoke different responses in ascending neurons in the cochlear nucleus to preserve the diversity of single neuron responses in parallel paths of encoding. The authors combine cell-type specific targeting for labeling and optogenetics in two of the three different neuronal subtypes to 1. Demonstrate that optogenetic activation of genetically-identified subpopulations of SGN pre-synaptic axons in vitro while recording from their post-synaptic bushy cell targets in the AVCN is a viable way of assaying the synaptic functions of these neurons 2. Determine the complement of SGN subtypes onto bushy cells, 3. test whether subtype specific SGN projections have different synaptic effects on cochlear nucleus bushy cells in vitro, and 4. Determine whether bushy cells that receive SGN subtype-specific projections have different activity patterns that match the expected activity patterns of the SGN subtypes (spontaneous and sound-driven activity rates). They found that, contrary to expectations, SGN subtype-specific synapses onto bushy cells were remarkably similar in in vitro experiments. More critically, the different subtypes of SGNs did not evoke different response types in bushy cells - instead, SGNs with different responses converged onto bushy cells. This unexpected finding is therefore important for our understanding of processing of acoustic information in the cochlear nucleus.

Overall the work is clear and well written, statistics are appropriate, and figures are carefully prepared and detailed. Multiple techniques are combined in this comprehensive study that examines the SGN subtypes at the genetic, protein, in vitro synaptic, and in vivo sound response levels. Some unanswered questions are regarding whether the convergence of SGN subtypes would occur in other species, but current genetic tools limit these experiments to mice. The discussion appropriately considers potential species-specific differences. A weakness is that only two of the three genetically identified subpopulations SGN are manipulated in the optogenetic experiments. Some sections of the methods require additional information, detail below. There are also a few areas of the text that would benefit from additional detail / clarity, below.

- The mouse lines used target two of the three SGN classes. It could be stated more directly earlier in the text why there is not a good line for targeting the third (1b) subtype. Is it that one does not exist, is not specific enough, or is it that the two extremes of spontaneous rates / thresholds were selected for these difficult experiments?

- For figure 2, it may help to point out that these mouse lines are CreERT2, and therefore may have 'leaky' Cre expression even in the absence of tamoxifen (a good example is Koundakjian et al 2007 from the Goodrich lab). Therefore, any expression in neurons in mice without tamoxifen (the TI- mice) is not non-specific, it is also likely specific but simply sparse.

- Additional methods / n / detail required:
o What is the LJP for the internal solutions and was it compensated for?
o AVCN immunohistochemistry section requires more detail: What was analyzed in the custom protocol? Intensity? Volume? Co-localization? How many sections / mice? Were serial section analyzed for the volume measurements? Complete endbulbs?
o In the in vivo electrophysiology section, please give more detail about the 15 ms noise burst (or provide a reference) and how the resulting responses were identified as a positive response (eg what time window used for response detection, how much of

an increase in spiking, other relevant details).
o In vivo recordings: how long between tones used for RLF? How long between optogenetic pulses?
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- Results section for figure 1: what area of the SGN was covered for the imaging and analysis? How many images per cochlea?

- Results text figure 4A: It is stated that optical and electrical stimulation activated different synapses, but it is unclear why this would occur - I would expect that the same axons could be activated by either stimulation, especially because the electrical stimulation will be non-specific. Please clarify this, possibly by describing what steps were used to intentionally activate different populations (electrode or LED placement?)?

- Results text for figure 4E - what was the paired pulses inter-stimulus interval?

- The strontium experiment in figure 5 is very nice!

- In the in vivo experiments, the most power can come from the 'non-expressing' experiments, as those will have the most clearly defined populations of SGN inputs. The 'expressing' experiments can have any mix of 1a, 1b, or 1c inputs. However, in both mouse lines, it is unknown whether there are unidentified 1b inputs. This does not change the overall conclusions, but should be acknowledged in the discussion.

Reviewer #2 :

Reviewer 2 has declined to share their comments.

We are very grateful to the reviewers and editors for their time and their thoughtful comments. We have addressed all the points raised by the reviewers, as detailed below.

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> Targeting the 1b SGN subtype requires a unique marker, and a mouse line that tags it. Calbindin (Calb1) is a candidate marker for 1b SGNs, showing little overlap with SGN labelling for Pou4f1 or Calb2 in our hands (data not shown here). The experiments in this study require a tamoxifen-inducible calbindin-Cre line. Jackson has a constitutive line, which would likely express ChR2-eYFP in all SGNs, because calbindin is widely expressed at young ages. We are not aware of such a tamoxifen-inducible line. We have added a brief remark along these lines (l. 119, 310).

- For figure 2, it may help to point out that these mouse lines are CreERT2, and therefore may have 'leaky' Cre expression even in the absence of tamoxifen (a good example is Koundakjian et al 2007 from the Goodrich lab). Therefore, any expression in neurons in mice without tamoxifen (the TI- mice) is not non-specific, it is also likely specific but simply sparse.

> The reviewer raises an important clarification. The concern we had related to the timing of Cre expression. Lypd1 and Calb2 are more broadly expressed among SGNs until P14–21, so tamoxifen-independent Cre activation at these early stages could lead to expression of eYFP in SGNs that only temporarily express Calb2 or Lypd1. However, we observed only very sparse labelling, if any, in non-tamoxifen-injected animals, which is unlikely to include enough off-target labelling to influence our results. We have clarified this in the text (l 327ff).

- Additional methods / n / detail required:
o What is the LJP for the internal solutions and was it compensated for?

> We calculated the liquid junction potential as 6.6 mV for voltage-clamp and 8.8 mV for current-clamp internal solutions with respect to normal ACSF. The liquid junction potential was not compensated for. We have added remarks along these lines to the methods (l 173).

o AVCN immunohistochemistry section requires more detail: What was analyzed in the custom protocol? Intensity? Volume? Co-localization? How many sections / mice? Were serial section analyzed for the volume measurements? Complete endbulbs?

VGluT1-immunoreactive puncta were traced in optical sections, and subtype identity was determined by co-expression with calretinin-immunoreactivity (i.e. subtype 1a-like), eYFP (1c-like), or neither (1b-like). There were 3-5 confocal series collected in 3 mice. We reconstructed puncta across adjacent optical sections, to determine the VGluT1-immunoreactive puncta volumes. Cells were reconstructed if they appeared to be fully enclosed in the series, so we believe endbulbs to be complete. We have expanded the explanation in the Methods (l 220ff).

o In the in vivo electrophysiology section, please give more detail about the 15 ms noise burst (or provide a reference) and how the resulting responses were identified as a positive response (eg what time window used for response detection, how much of an increase in spiking, other relevant details).

The noise burst was generated by passing random numbers to the DAQ at 10 kHz, at an intensity of ~92 dB SPL. If any spikes were observed during a 50 ms window during and after the noise burst, it was taken as evidence of a nearby unit. We added this information about the search stimulus to the Methods (l 271ff).

o In vivo recordings: how long between tones used for RLF? How long between optogenetic pulses?

The interval between tone presentations or optogenetic pulses was ~0.2 s. We added this information to the Methods section (l 279).

o In in vivo recordings, was the "neuroptic" an artifact of the LED electronics or was it neural activity?

We have heard others use the term “neuroptic” to mean a local field potential triggered by optogenetic stimulation, similar to the neurophonic commonly observed during sound presentation. However, we made no direct study of its origin, so we have reworded this section to be more cautious (l 280ff).

- Results section for figure 1: what area of the SGN was covered for the imaging and analysis? How many images per cochlea?

Data were taken from a single confocal series in each sample (3 mice, 1 or 2 cochleas per mouse). We did not preserve information about the specific frequency location in the cochlea. We added this information to the Methods (l 206ff). Our goal here was to evaluate if YFP expression was in the cell type we expected. It is unlikely that promoter specificity or Cre leakage would vary by tonotopy.

- Results text figure 4A: It is stated that optical and electrical stimulation activated different synapses, but it is unclear why this would occur - I would expect that the same axons could be activated by either stimulation, especially because the electrical stimulation will be non-specific. Please clarify this, possibly by describing what steps were used to intentionally activate different populations (electrode or LED placement?)?

Care was taken with both electrical and optogenetic stimulation *in vitro* to activate only a single input, by minimizing intensity of light or electrical stimulation. For (renumbered) Fig. 3ABE, we tested if electrical and optogenetic stimuli activated the same input by stimulating in pairs, first electrical then optogenetic and vice versa. If we saw depression after the second stimulus, then the input was shared. We show data only from distinct electrical and optogenetic inputs here. We modified the wording at l 362 to try to clarify.

- Results text for figure 4E - what was the paired pulses inter-stimulus interval?

We have added clarification of the interval (15 ms) in the methods (l 182) and in the figure legend (now Fig. 3) to make this information easier to find.

- The strontium experiment in figure 5 is very nice!

Thanks!

- In the in vivo experiments, the most power can come from the 'non-expressing' experiments, as those will have the most clearly defined populations of SGN inputs. The 'expressing' experiments can have any mix of 1a, 1b, or 1c inputs. However, in both mouse lines, it is unknown whether there are unidentified 1b inputs. This does not change the overall conclusions, but should be acknowledged in the discussion.

We have added some remarks about this complex issue in the discussion (l 590ff).

Reviewer #2 :

Reviewer 2 has declined to share their comments.