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Phillip Steindel, Ph.D.
Journal of Visualized Experiments
1 Alewife Center Suite 200
Cambridge, MA 02140

RE: Revised manuscript JoVE60760

Dear Dr. Steindel,

We are pleased to submit a revised version of our manuscript, "Long range Channelrhodopsin-assisted circuit mapping of inferior colliculus neurons with blue and red-shifted channelrhodopsins" by Goyer and Roberts (JoVE60760). We thank the reviewers for their insightful and constructive evaluation of our manuscript. In response to reviewer comments, we made several revisions to the text, added data to Figures 2 and 3, revised the color scheme in Figure 2, and swapped the order of Figures 4 and 5. We have also highlighted protocol steps we think are important to include in filming. We hope the revised manuscript is suitable for publication in *JoVE*. Our responses to specific reviewer comments follow.

Sincerely,

Michael T. Roberts
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Department of Otolaryngology-Head & Neck Surgery
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Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have completed several additional read-throughs of the text and hope we have corrected all remaining spelling and grammar issues.

2. Please revise lines 315-326 to avoid textual overlap with previous publications (your eLife paper in this case).

Thank you for catching this. We have revised the text in this section, which can now be found at line numbers 352 – 365.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Drummond Nanoject

We have replaced all instances of commercial language with broader terminology (e.g., Drummond Nanoject is now nanoinjector).

Protocol:
1. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

We added an ethics statement before the first numbered step in the Surgery Preparations section of the protocol. See lines 122 – 125.

2. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted the essential steps of the protocol, adhering to the stated page limit.

3. Please include information (strain, sex, age) about mice used.

Information about the strain, sex, and age of mice used was added to the beginning of the Results section, lines 308 – 311.

4. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have split protocol steps into separate steps in instances where more than 2 – 3 actions were included, and we ensured that each protocol step addresses how the step is to be performed.

Figures and Tables:
1. Please remove the embedded figures and table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

We have removed the embedded figures and table from the manuscript and prepared them as separate files.

References:
1. Please do not abbreviate journal titles.

We replaced abbreviations of journal titles with full titles.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have double checked the Table of Materials to ensure that this information is included.

Reviewers' comments:
Reviewer #1:

Summary:
In the manuscript titled "Long-range channelrhodopsin-assisted circuit mapping of inferior colliculus neurons with blue and red-shifted channelrhodopsins". David Goyer & Michael T. Roberts described the channelrhodopsin assisted circuit mapping (CRACM) as a method for brain circuit mapping of long-range projections. The authors showed the ability of the neurons to traffic the red-shifted optogenetic probe to its terminals at the target area via long-range projections. The bilateral commissural connection between the two inferior colliculi and the contralateral connection between the right dorsal cochlear nucleus (DCN) and the left inferior colliculus were two examples of brain circuits studied here. In addition, the authors showed that CRACM method was able to selectively stimulate the projections expressing the optogenetic probe even if those projections were intermixed with other fibers, which was more efficient than electrical stimulation. However, there are some points the authors could consider to improve the manuscript

Major:

1. The authors did not mention any details about the subject used. The authors mentioned that it is a mouse with no information regarding the mouse's strain, its background if it is transgenic, and how it was obtained? from the context, we could know that it is a mouse whose VIP-neurons express a td-tomato. As such, did the VIP neurons of the mouse in figure 1 express td-tomato by through the crossing of two different mice under Cre-technology? The authors should explain.

We apologize for this oversight and thank the reviewer for pointing this out. We now include information about the mice used in the study (VIP-IRES-Cre x Ai14) at the beginning of the Results section, lines 308 – 311.

2. The authors also should mention the number of animals used.

Twenty-two mice were used in the study, and this is now stated at the beginning of the Results section, line 311.

3. Regarding the z-coordinate, the author should mention if the z-axis started from the surface of the skull or from the surface of the brain. Compared to the coordinates shown by Allen mouse brain atlas as a reference, the z-axis described here seem to be deeper. Considering the age of the mouse used here (21-30 days), the z-coordinates should be even shorter. The authors should comment on this.

Z-coordinates were measured from the elevation of the top of the skull at the lambda suture. This is now indicated in Protocol step 2.10, lines 189 – 190, and in the legend for Table 1. Our understanding is that the Allen Brain Atlas measures depth coordinates relative to the dorsal-most surface of the brain over the brain region to be injected. This presumably explains the difference between our Z-coordinates and those in the Allen Brain Atlas.

4. Why did Td-tomato give different colors in figure 1 "magenta" and figure 2 "red"? Is the td-tomato of different subtypes? or are they just different pseudo colors?

We believe the reviewer is referring to Figures 2 and 3 here. In these figures, the images are presented in pseudocolor. Originally, we represented tdTomato in magenta only in Figure 2 to make it easier for red-green colorblind individuals to differentiate the tdTomato and EGFP signals. We agree that it makes sense to retain this color scheme in Figure 3, and we have revised Figure 3 accordingly.

Minor:

1. Line 59: "Chlamydomonas reinhardtii" should be italic

This text is now italicized.

2. Since the work discussed an experimental method, the authors should write a statement showing that the animal's work was approved by IACUC under the institution's protocol of animal use as well as a safety concerns when it comes to using the laser for activation.

We have added a statement about IACUC approval at the beginning of the Surgery Preparations section of the protocol, lines 122 – 125. In addition, we mentioned on lines 286 – 287 that our optical stimulation of Chronos and ChrimsonR was performed with LEDs, and therefore laser safety issues do not apply. However, we realize that some may prefer to use lasers. We therefore added a statement to the beginning of the "In vitro recording and CRACM experiment" section directing readers to obtain approval before using lasers and to follow appropriate safety guidelines, lines 268 – 271.

3. Lines 230, 231, and 233, did the authors dissect the brain at 34oC or 4oC?

Many labs, including ours, have found that nuclei in the auditory brainstem and midbrain remain healthier when sliced at near physiological temperature. Accordingly, our protocol, which is directed toward work in the brainstem, indicates that brains were/should be dissected at 34 degrees C.

Reviewer #2:

Manuscript Summary.
IC neurons receive various inputs including ascending, descending, commissural, and local inputs from the CN, the SOC, the DNLL, the VNLL, and the AC. The intermingling of axons from several different sources has made it difficult to characterize physiological properties of IC neuron in specific IC circuits. The channelrhodopsin-assisted circuit mapping (CRACM) is very useful and powerful approach for overcoming this limitation. Authors established CRACM combined with electrophysiological recordings to identify two different input sources (ascending and commissural inputs) to the IC using Chronos and

ChrimsonR. However, ChrimsonR has exhibited unwanted cross-activation with blue light. In this manuscript, authors also report that ChrimsonR is activated with blue light showing the same threshold for blue light activation as Chronos. Although there are still some issues to perform dual color CRACM experiments using Chromos and ChrimsonR, it would be worthy to address if ChrimsonR is an effective channelrhodopsin for long range circuit mapping in the auditory brainstem.

Major Concerns:

In this manuscript, authors described that "During patch clamp recordings, ChrimsonR-transfected fibers are excited with red light to record ChrimsonR evoked postsynaptic potential" in Figure 1B. However, the authors only show the recording data of EPSPs in IC VIP neurons which was elicited ONLY blue light activation of ChrimsonR (Figure 5B), and not show the data obtained red light activation of ChrimsonR (580 or ~660 nm). Figure 5B, which shows the limitation of ChrimsonR for using the dual color CRACM in the IC, is important information for investigators to design the experiment carefully. However, I recommend to include the additional data, which authors described in Figure 1B, with the same format with Figure 5B: ChrimsonR-driven EPSPs evoked with 660 nm (or 580 nm) red light, and relationship between optical power at 660 nm and the probability of observing a ChrimsonR-driven PSP. This information is very useful for investigators who want to use ChrimsonR in the auditory brainstem.

We thank the reviewer for this helpful suggestion. We have added an example of EPSPs evoked by activating ChrimsonR with 590 nm light to Figure 3C. Unfortunately, during our previous experiments we did not collect a full input-output curve for EPSP probability versus 590 nm light intensity, so we have not added such a plot here. However, such an input-output function is well-documented in the original study describing ChrimsonR: see Figure 2D of Klapoetke et al., 2014, doi: 10.1038/nmeth.2836.

Minor Concerns:

1. In Figure 2A, the inclusion of the higher magnification image of the left IC (showing the projection, likely Figure 3B) is recommended.

We have updated Figure 2A to show a higher magnification image of the Chronos-EGFP-labeled commissural projection to the contralateral IC.

2. In Protocol, please address animal (mouse) strain, age, and sex

We now include this information at the beginning of the Results section, lines 308 – 311.

3. Please address the viral information in detail for other investigators to easily follow your protocol. Are rAA1.Syn.Chronos-GFP. WPRE.bGH (blue ligh-activated channelrhodopsin) and rAAV1.Syn.ChrimsonR-tdTomato.WPRE.bGH (red137 shifted channelrhodopsin) commercially available? Or how to prepare these viruses?

We obtained these viruses from Addgene and have indicated the source and catalog number in the Table of Materials. Our understanding of JoVE policy is that we are not permitted to mention Addgene in the main text of the manuscript. However, we added text to protocol step 1.7 (lines 148 – 149) to indicate that these viruses are available from publically accessible repositories and vector cores.

4. Page 8, line 257, how about the relationship between the optical power and the amplitude of PSP? Linear relationship? Or exhibiting the saturation? Or on-and-off?

This is an important point. In our experiments, we did not observe any change in PSP amplitude with increasing light intensity, and therefore our responses were essentially on-and-off. However, this result is likely to depend heavily on the particular neuron and synapses under study. We have added sentences addressing this issue to the end of the Results section, lines 375 – 379.

5. Page 8, line 322, what is the amplitude of EPSPs in the presence of AP5?

The EPSP amplitude trended toward a decrease ($p = 0.105$) in the presence of D-AP5. We have added this information to the Results text, lines 362 – 363.

6. Are Figure 4A and B the original traces (light grey) and average (black) from the representative VIP neuron?

Yes, thank you for catching that we neglected to indicate this in the figure legend. Note that we have swapped the order of Figures 4 and 5 so that the numbering matches the order in which these figures are encountered in the text. The Figure 5 legend (previously Figure 4 legend) now includes a sentence to address the meaning of the grey and black traces, lines 431 - 432.

7. Figure 4 legend, 50 μm to be corrected to 50μM (Capital)

Thank you for catching this typo. The Figure 5 legend (previously Figure 4 legend) has been corrected, lines 424 – 433.