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TITLE:

Long range Channelrhodopsin-Assisted Circuit Mapping of Inferior Colliculus Neurons with Blue and Red-Shifted Channelrhodopsins

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SUMMARY:

Channelrhodopsin-assisted circuit mapping (CRACM) is a precision technique for functional mapping of long-range neuronal projections between anatomically and/or genetically identified groups of neurons. Here, we describe how to utilize CRACM to map auditory brainstem connections, including the use of a red-shifted opsin, ChrimsonR.

ABSTRACT:

When investigating neural circuits, a standard limitation of the in vitro patch clamp approach is that axons from multiple sources are often intermixed, making it difficult to isolate inputs from individual sources with electrical stimulation. However, by using channelrhodopsin assisted circuit mapping (CRACM), this limitation can now be overcome. Here, we report a method to use CRACM to map ascending inputs from lower auditory brainstem nuclei and commissural inputs to an identified class of neurons in the inferior colliculus (IC), the midbrain nucleus of the auditory system. In the IC, local, commissural, ascending, and descending axons are heavily intertwined and therefore indistinguishable with electrical stimulation. By injecting a viral construct to drive expression of a channelrhodopsin in a presynaptic nucleus, followed by patch clamp recording to characterize the presence and physiology of channelrhodopsin-expressing synaptic inputs, projections from a specific source to a specific population of IC neurons can be mapped with cell type-specific accuracy. We show that this approach works with both Chronos, a blue light-activated channelrhodopsin, and ChrimsonR, a red-shifted channelrhodopsin. In contrast to previous reports from the forebrain, we find that ChrimsonR is robustly trafficked down the axons of dorsal cochlear nucleus principal neurons, indicating that ChrimsonR may be a useful tool for CRACM experiments in the brainstem. The protocol presented here includes detailed descriptions of the intracranial virus injection surgery, including stereotaxic coordinates for

targeting injections to the dorsal cochlear nucleus and IC of mice, and how to combine whole cell patch clamp recording with channelrhodopsin activation to investigate long-range projections to IC neurons. Although this protocol is tailored to characterizing auditory inputs to the IC, it can be easily adapted to investigate other long-range projections in the auditory brainstem and beyond.

INTRODUCTION:

Synaptic connections are critical to neural circuit function, but the precise topology and physiology of synapses within neural circuits are often difficult to probe experimentally. This is because electrical stimulation, the traditional tool of cellular electrophysiology, indiscriminately activates axons near the stimulation site, and in most brain regions, axons from different sources (local, ascending, and/or descending) intertwine. However, by using channelrhodopsin assisted circuit mapping (CRACM)[1,2], this limitation can now be overcome[3]. Channelrhodopsin (ChR2) is a light activated, cation-selective ion channel originally found in the green alga *Chlamydomonas reinhardtii.* ChR2 can be activated by blue light of a wavelength around 450-490 nm, depolarizing the cell through cation influx. ChR2 was first described and expressed in *Xenopus* oocytes by Nagel and colleagues[4]. Shortly after that, Boyden and colleagues[5] expressed ChR2 in mammalian neurons and showed that they could use light pulses to reliably control spiking on a millisecond timescale, inducing action potentials ~10 ms after activation of ChR2 with blue light. Optogenetic channels with even faster kinetics have been found recently (e.g., Chronos[6]).

The basic approach to a CRACM experiment is to transfect a population of putative presynaptic neurons with a recombinant adeno-associated virus (rAAV) that carries the genetic information for a channelrhodopsin. Transfection of neurons with rAAV leads to the expression of the encoded channelrhodopsin. Typically, the channelrhodopsin is tagged with a fluorescent protein like GFP (Green Fluorescent Protein) or tdTomato (a red fluorescent protein), so that transfection of neurons in the target region can easily be confirmed with fluorescence imaging. Because rAAVs are non-pathogenic, have a low inflammatory potential and long-lasting gene expression[7,8], they have become a standard technique to deliver channelrhodopsins to neurons. If, after transfection of a putative presynaptic population of neurons, activation of a channelrhodopsin through light flashes elicits postsynaptic potentials or currents in the target neurons, this is evidence of an axonal connection from the transfected nucleus to the recorded cell. Because severed axons in brain slice experiments can be driven to release neurotransmitter through channelrhodopsin activation, nuclei that lie outside of the acute slice but send axons into the postsynaptic brain region can be identified with CRACM. The power of this technique is that the connectivity and physiology of identified long range synaptic inputs can be directly investigated.

In addition to channelrhodopsins that are excitable by blue light, investigators have recently identified several red-shifted channelrhodopsins[9,10], including Chrimson and its faster analog ChrimsonR, both of which are excited with red light of ~660 nm[6]. Red-shifted opsins are of interest because red light penetrates tissue better than blue light, and red light may have a lower cytotoxicity than blue light[10–12]. Red-shifted channelrhodopsins also open up the possibility of dual color CRACM experiments, where the convergence of axons from different nuclei on the same neuron can be tested in one experiment[6,13,14]. However, current red-shifted opsins often exhibit unwanted cross-activation with blue light[15–17], making two color experiments difficult. In

addition, some reports have indicated that ChrimsonR undergoes limited axonal trafficking, which can make it challenging to use ChrimsonR for CRACM experiments[16,17].

Nearly all ascending projections from the lower auditory brainstem nuclei converge in the inferior colliculus (IC), the midbrain hub of the central auditory pathway. This includes projections from the cochlear nucleus (CN)[18,19], most of the superior olivary complex (SOC)[20], and the dorsal (DNLL) and ventral (VNLL) nuclei of the lateral lemniscus[21]. Additionally, a large descending projection from the auditory cortex terminates in the IC[18–22], and IC neurons themselves synapse broadly within the local and contralateral lobes of the IC[23]. The intermingling of axons from many sources has made it difficult to probe IC circuits using electrical stimulation[24]. As a result, even though neurons in the IC perform computations important for sound localization and the identification of speech and other communication sounds[25,26], the organization of neural circuits in the IC is largely unknown. We recently identified VIP neurons as the first molecularly identifiable neuron class in the IC[27]. VIP neurons are glutamatergic stellate neurons that project to several long-range targets, including the auditory thalamus and superior colliculus. We are now able to determine the sources and function of local and long-range inputs to VIP neurons and to determine how these circuit connections contribute to sound processing.

The protocol presented here is tailored to investigating synaptic inputs to VIP neurons in the IC of mice, specifically from the contralateral IC and the DCN (**Figure 1**). The protocol can be easily adapted to different sources of input, a different neuron type or a different brain region altogether. We also show that ChrimsonR is an effective red-shifted channelrhodopsin for long range circuit mapping in the auditory brainstem. However, we demonstrate that ChrimsonR is strongly activated by blue light, even at low intensities, and thus, to combine ChrimsonR with Chronos in two-color CRACM experiments, careful controls must be used to prevent cross-activation of ChrimsonR.

PROTOCOL:

Obtain approval from the local Institutional Animal Care and Use Committee (IACUC) and adhere to NIH guidelines for the care and use of laboratory animals. All procedures in this protocol were approved by the University of Michigan IACUC and were in accordance with NIH guidelines for the care and use of laboratory animals.

1. Surgery preparations

1.1. Perform surgeries in aseptic conditions. Autoclave/sterilize all surgery tools and materials before surgery. Wear surgery gown and mask for the surgery.

1.2. Sanitize the surgery area (spray and wipe down with 70% ethanol), and place sterile towel drapes to cover the surgery area.

1.3. Prepare the recovery cage. Remove cage bedding to limit risk of asphyxiation. Put a heating pad under cage. Provide a food and water source.

1.4. Pull a glass capillary for the nanoinjector on a pipette puller. Cut or break off the tip to obtain an opening approximately 5 µm in diameter.

1.5. Bevel the capillary tip to an approximately 30° angle to improve tissue penetration and reduce clogging. Backfill the capillary with mineral oil and insert into a nanoliter injector.

1.6. Obtain an aliquot of the desired channelrhodopsin-encoding rAAV and dilute to the desired titer using sterile PBS.

NOTE: We have found that serotype 1 rAAVs work well for transfection of auditory brainstem nuclei. Specifically, rAAV1.Syn.Chronos-GFP.WPRE.bGH (blue light-activated channelrhodopsin) and rAAV1.Syn.ChrimsonR-tdTomato.WPRE.bGH (red-shifted channelrhodopsin), which are available from publically accessible repositories and vector cores, consistently yield the high expression levels and good long-range axonal trafficking of channelrhodopsins needed for CRACM experiments.

1.7. Follow injector instructions to front fill capillary with 1 – 3 µL of rAAV in sterile PBS.

2. Surgery

2.1. Put the animal into an induction chamber and induce anesthesia with 3% isoflurane in oxygen delivered via a calibrated isoflurane vaporizer. Observe the mouse until breathing becomes deep and slow and a toe pinch reflex is absent, about 3 – 5 minutes.

2.2. Transfer the animal to a stereotaxic frame. Secure the animal's head by putting its mouth on a palate bar with a gas anesthesia mask and by positioning non-perforating ear bars in both ear canals.

2.3. Insert a rectal temperature probe and switch on the homeostatic temperature controller.

2.4. Apply ophthalmic ointment to prevent eyes from drying out.

2.5. Administer preemptive analgesic (e.g., subcutaneous injection of 5 mg/kg carprofen).

2.6. Adjust isoflurane to 1 - 2.5%, according to the depth of the anesthetized state. Monitor temperature, breathing and color of mucous membranes at least every 15 minutes during the procedure.

2.7. Shave scalp with electric clippers. Disinfect scalp with three alternating swabs of povidone-iodine and 70% ethanol.

2.8. Make an incision in the scalp along the midline starting between the ears and continuing rostral to the eyes, exposing the lambda and bregma sutures. Push skin to the side and remove

periosteum from exposed bone if necessary.

2.9. Mark the lambda suture with a surgical marker, position the tip of the nanoinjector so that it is just touching lambda, and zero the micromanipulator coordinates. Use the nanoinjector tip and micromanipulator to measure the difference in elevation between the lambda and bregma sutures. Adjust palate bar height to bring lambda and bregma to within ±100 µm height difference.

2.10. Map the injection site using the nanoinjector tip and micromanipulator coordinate system and mark the site with a surgical marker. To inject the IC or DCN of P21 – P30 mice, use coordinates relative to the lambda suture, as shown in **Table 1**. Note that the Z depth in our coordinates is measured from the surface of the skull at lambda.

2.11. Use a micromotor drill with a 0.5 mm drill burr to perform a craniotomy over the injection site.

2.12. To ensure broad transfection of neurons in the target nucleus, make injections at various depths into the tissue (**Table 1**, Z coordinates), and, in the case of larger brain regions like the IC, make injections over the course of two or more penetrations at different X and Y coordinates (**Table 1**, Right IC penetration 1 and Right IC penetration 2).

2.13. Perform injections. For IC injections, deposit 20 nL of virus in intervals of 250 µm along the Z axis (injection depth) between 2250 µm and 1750 µm depth. For DCN injections, deposit 20 nL of virus at a depth of 4750 µm and 4550 µm, respectively.

2.14. After injection at each Z coordinate, wait 2 – 3 minutes before moving the injector to the next Z coordinate. This will allow time for the virus to diffuse away from the injection site, reducing the probability that virus will be sucked up the injection tract when the nanoinjector is repositioned.

2.15. After last injection in a penetration, wait 3 – 5 minutes before retracting nanoinjector from brain.

2.16. When the nanoinjector is removed from the brain between penetrations and between animals, eject a small volume of virus from the tip to check that the tip has not clogged.

2.17. After injections, use sterile PBS to wet the cut edges of the scalp and then gently move the skin back towards the midline. Close the wound with simple interrupted sutures using 6-0 (0.7 metric) nylon sutures.

2.18. Apply 0.5 – 1 mL of 2% lidocaine jelly to the wound.

2.19. Remove ear bars and temperature probe, turn off isoflurane, remove the mouse from the palate bar and transfer it to the recovery cage.

2.20. Monitor recovery closely. Once the animal is fully awake, moving around, and showing no signs of pain or distress, transfer it back into its cage and return the cage to the vivarium.

2.21. If surgeries will be performed on multiple animals in one day, use a hot bead sterilizer to sanitize surgery tools before next surgery.

3. Surgical follow up

3.1. Check animals daily for wound closure, infection, or signs of pain or distress over the next 10 days, adhering to the institution's animal care guidelines.

3.2. Wait 3 – 4 weeks before using animals in experiments to allow optimal expression of the channelrhodopsins.

4. Brain slice preparation and confirmation of injection target

4.1. For CRACM, use acutely prepared brain slices from transfected animals in standard in vitro electrophysiology experiments, described here only briefly (see Goyer et al. 2019 for a more detailed description[27]).

4.2. Prepare artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 12.5 D-glucose, 25 $NaHCO_3$, 3 KCl, 1.25 NaH_2PO_4, 1.5 $CaCl_2$, 1 $MgSO_4$. Bubble ACSF to a pH of 7.4 with 5% CO_2 in 95% O_2.

4.3. Perform all following steps, including in vitro electrophysiology, in near-darkness or red light to limit activation of channelrhodopsins.

4.4. Deeply anesthetize mouse with isoflurane and decapitate it quickly. Dissect the brain quickly in ~34 °C ACSF.

4.5. Cut coronal slices (200 – 250 µm) containing the IC in ~34 °C ACSF with a vibrating microtome and incubate the slices at 34 °C for 30 minutes in a holding chamber filled with ACSF bubbled with 5% CO_2 in 95% O_2. After incubation, store slices at room temperature until used for recordings.

4.6. If the injection target was not the IC, cut additional coronal slices of the injected brain region and check the transfection of the target nucleus under a fluorescence microscope. If there is no transfection in the target nucleus or additional transfection in different brain regions, do not continue with experiment.

5. In vitro recording & CRACM experiment

NOTE: To provide optical stimulation of Chronos and ChrimsonR, we use LEDs coupled to the

epifluorescence port of the microscope. However, lasers can be used instead of LEDs. If using lasers, obtain prior approval from institutional safety officials and follow appropriate guidelines for safe laser use.

5.1. Pull electrodes from borosilicate glass to a resistance of 3.5 – 4.5 MΩ. The electrode internal solution should contain (in mM): 115 K-gluconate, 7.73 KCl, 0.5 EGTA, 10 HEPES, 10 Na_2 phosphocreatine, 4 MgATP, 0.3 NaGTP, supplemented with 0.1% biocytin (w/v), pH adjusted to 7.3 with KOH and osmolality to 290 mOsm/kg with sucrose.

5.2. To make recordings, use standard patch clamp methods. Place the slice in a recording chamber under a fixed stage upright microscope and continuously perfuse with ACSF at ~2 mL/min. Conduct recordings near physiological temperature (~34 - 36 °C).

5.3. Patch neurons under visual control using a suitable patch clamp amplifier. Correct for series resistance, pipette capacitance and liquid junction potential.

5.4. During whole cell recordings, activate Chronos by delivering brief pulses (1 – 5 ms) of 470 nm light or ChrimsonR by brief pulses of 580 nm light through commercially available LEDs. Determine threshold of opsin activation and use a minimal stimulation protocol to elicit postsynaptic potentials. In general, use the shortest stimulus duration that elicits a PSP, and set the optical power to 120% of the threshold power required to elicit PSPs.

5.5. To confirm that the recorded changes in membrane potential are indeed synaptic inputs to the neuron, standard antagonists for excitatory/inhibitory postsynaptic receptors can be washed in during the experiment. To investigate different receptor contributions to a PSP (e.g. NMDA vs AMPA receptors), suitable receptor antagonists can be washed in. For each receptor antagonist, drug effects should reverse after washout.

5.6. Use the latency, jitter, and reliability of PSPs to confirm that light-activated synaptic inputs originate from direct, optical activation of synapses on the recorded neuron, as opposed to activation of channelrhodopsin-expressing synapses on an intervening neuron that synapses on the recorded neuron. In general, low latency (<2 ms), low jitter (<1 ms standard deviation in latency), and high reliability (>50%) indicate a direct synaptic connection from the channelrhodopsin expressing presynaptic neuron to the recorded neuron.

REPRESENTATIVE RESULTS:

We crossed VIP-IRES-Cre mice (*Vip*$^{tm1(cre)Zjh}$/J) and Ai14 Cre-reporter mice (B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J) to generate F1 offspring in which VIP neurons express the fluorescent protein tdTomato. F1 offspring of either sex were used, aged postnatal day (P) 21 to P70. A total of 22 animals were used in this study.

Stereotaxic injection of AAV1.Syn.Chronos-GFP.WPRE.bGH into the right IC of VIP-IRES-Cre x Ai14 mice using coordinates shown in **Table 1** resulted in strong Chronos-EGFP expression in the right IC (**Figure 2A**). Visual inspection of Chronos-EGFP fluorescence indicated that most of the somata

labeled in the right IC were located in the central nucleus of the IC (ICc), but labeled somata were sometimes also present in the dorsal cortex of the IC (ICd) and occasionally in the lateral cortex of the IC (IClc). The targeting and extent of transfection should be checked for every animal used in an experiment, as expression of channelrhodopsins in non-targeted regions can lead to false positives. To achieve broader or more restricted expression of Chronos, the amount of deposited virus as well as the stereotaxic coordinates can be easily adjusted to achieve the desired outcome.

Stereotaxic injection of AAV1.Syn.Chronos-GFP.WPRE.bGH into the DCN (see coordinates in **Table 1**) of VIP-IRES-Cre x Ai14 mice resulted in strong transfection of DCN neurons (**Figure 2B**). To confirm selective transfection of the DCN, the brainstem of every animal should be sliced to verify that EGFP expression was present and limited to the DCN. If there is no transfection or if there is considerable expression of EGFP in the auditory nerve or VCN, recordings should not be performed. Using the coordinates shown in **Table 1** with a total injection volume of 40 nL, Chronos-EGFP expression will be limited to the DCN in most cases. EGFP-labeled axons were present in the left (contralateral) ICc 3 weeks after injection for both IC and DCN injection sites (**Figure 2A right, 2B right**).

To test the long-range trafficking of ChrimsonR, AAV1.Syn.ChrimsonR-tdTomato.WPRE.bGH was injected into the right DCN of VIP-IRES-Cre x Ai14 mice, using the same coordinates as with Chronos injections. ChrimsonR injection led to strong expression in the DCN, with tdTomato fluorescence visible in cells and fibers (**Figure 3A**). In the contralateral ICc, fibers strongly labeled with tdTomato were clearly visible after 3 weeks, demonstrating the long-range trafficking capability of the ChrimsonR-tdTomato construct when injected into auditory brainstem nuclei (**Figure 3B**). Optical activation of ChrimsonR elicited EPSPs in IC VIP neurons (**Figure 3C**), indicating that ChrimsonR is a useful tool for long-range CRACM experiments when the experimental parameters demand the use of red light instead of blue light. However, we found that ChrimsonR was readily activated with blue light, showing the same threshold for blue light activation as Chronos (**Figure 4**). The sensitivity of ChrimsonR to blue light means that special care must be taken to distinguish between inputs transfected with ChrimsonR or Chronos in the same animal[13].

When targeting recordings to VIP neurons in the contralateral (left) ICc after injections into the right IC, blue light flashes elicited excitatory postsynaptic potentials (EPSPs) or inhibitory postsynaptic potentials (IPSPs). This confirms commissural projections to VIP neurons. To analyze commissural EPSPs and IPSPs separately, we used receptor antagonists to block IPSPs during EPSP recordings, and vice versa. Representative EPSPs and IPSPs recorded during CRACM experiments are shown in **Figure 5**. IPSPs were observed in 6 out of 12 tested ICc VIP neurons. IPSPs were small (1.53 mV ± 0.96 mV) and had moderate kinetics. The 10 – 90% rise times of IPSPs were 7.8 ms ± 2.1 ms, halfwidths were 15.1 ms ± 6.8 ms, and decay time constants were 32.4 ms ± 17.0 ms (**Figure 5A**, left). IPSPs were mediated by $GABA_A$ receptors, as they were blocked by 5 µM gabazine, a $GABA_A$ receptor antagonist (**Figure 5A**, right; n = 6). EPSPs were observed in 11 out of 27 ICc VIP neurons tested. EPSPs were also small (1.52 mV ± 1.08 mV) and had moderate kinetics. The 10 – 90% rise times of EPSPs were 8.3 ms ± 4.3 ms, halfwidths were 19.6 ms ± 7.6

ms, and decay time constants were 43.5 ms ± 16.8 ms (**Figure 5B** "Control"). EPSPs were mediated by AMPA and NMDA receptors. Application of 50 µM D-AP5, an NMDA receptor antagonist, reduced EPSP halfwidth (14.3 ms ± 4.7 ms, p = 0.006) and trended toward reducing the rise time (6.3 ms ± 1.6 ms, p = 0.09), decay time constant (30.6 ms ± 7.3 ms, p = 0.06), and EPSP amplitude (1.38 ± 0.33 mV, p =0.105; ANOVA for repeated measurements with Tukey post-hoc test). Application of 10 µM NBQX, an AMPA receptor antagonist, blocked the remainder of the EPSP (**Figure 5B** "+ AP5 & NBQX").

Recordings of VIP neurons in the left ICc after DCN injections revealed EPSPs evoked by blue light flashes, confirming synaptic inputs from the DCN to VIP neurons in the IC. DCN CRACM experiments were conducted with GABAergic and glycinergic blockers in the bath to block spontaneous IPSPs. We found that 2 – 5 ms pulses of blue light elicited EPSPs in 19 of 25 neurons tested. Light-evoked EPSPs had moderate amplitudes (2.85 mV ± 2.98 mV) and relatively slow rise times (4.2 ms ± 1.3 ms), halfwidths (20.6 ms ± 14.4 ms) and decay time constants (22.0 ms ± 6.7 ms) (n = 6 cells, data not shown).

All PSPs evoked by Chronos or ChrimsonR activation were elicited in an all-or-none fashion and did not scale their amplitude with increasing optical power (data not shown). However, this result depends on the number and physiology of the transfected synapses providing input to a particular neuron and is therefore likely to vary depending on the particular combination of axonal projection and neuron type being investigated.

FIGURE AND TABLE LEGENDS:

Figure 1: rAAV injection sites and experimental setup. (**A**) Injection site and experimental setup to investigate commissural projections. Left: An rAAV construct (e.g., rAAV1.Syn.Chronos-GFP.WPRE.bGH) is injected into the right IC and targeted recordings are performed in the contralateral IC. Right: During patch clamp recordings, Chronos-transfected fibers are excited with blue light to elicit postsynaptic potentials. (**B**) Injection site and experimental setup to investigate long-range projections from DCN to IC. Left: The ChrimsonR construct is injected into the right DCN and targeted recordings are performed in the contralateral IC. Right: During patch clamp recordings, ChrimsonR-transfected fibers are excited with red light to record ChrimsonR evoked postsynaptic potentials.

Figure 2: Chronos-EGFP expression after injections into IC and DCN. (**A**) Left: Image of coronal IC slice showing tdTomato-labeled VIP neurons throughout the IC (magenta) and Chronos-EGFP expression in somata localized to the injection sites in the right IC (green). Right: Higher magnification image showing a recorded VIP neuron (white-green) in the IC contralateral to the injection site. Green puncta are Chronos-EGFP expressing projections from the contralateral IC. (**B**) Image of coronal brainstem slice showing Chronos-EGFP expression in the DCN after rAAV-Chronos-EGFP injection. Left: Image of the DCN, showing transfected DCN and EGFP-positive fibers entering the dorsal acoustic stria. Middle: Higher magnification image showing Chronos-transfected cell bodies in the DCN. Right: Chronos-EGFP expression in fibers and terminals in the contralateral IC from long range DCN – IC projections.

Figure 3: ChrimsonR-tdTomato expression in the DCN and DCN projections to the IC. (**A**) Image of a coronal brainstem slice from a mouse in which the right DCN was injected with rAAV1.Syn.ChrimsonR-tdTomato.WPRE.bGH. Left: Image of ChrimsonR expression in the right DCN. Right: Higher magnification image of right DCN showing strong transfection of neurons with ChrimsonR. (**B**) High magnification image of ChrimsonR-transfected DCN axons in the IC. (**C**) Optogenetically evoked EPSPs recorded from a VIP neuron in the IC contralateral to the rAAV-ChrimsonR injected DCN. Original traces are shown in light grey and average EPSP is in red. Orange box indicates timing of 590 nm light pulse. Scale bars show 20 ms/0.5 mV.

Figure 4: Activation of ChrimsonR by low levels of blue light. (**A**) Activation of Chronos in commissural projections by pulses of 470 nm blue light. Top: Original traces (light grey) and average (cyan) of Chronos-driven IPSPs, evoked at an optical power slightly above the threshold for Chronos activation (scale bars show 20 ms/0.5 mV). Bottom: Relationship between optical power at 470 nm and the probability of observing a Chronos-evoked PSP. (**B**) Activation of ChrimsonR with 470 nm blue light. Top: Original traces (light grey) and average (red) of ChrimsonR-driven EPSPs, evoked with 470 nm blue light at the same optical power used in **A**, **top** (scale bars show 20 ms/0.5 mV). Bottom: Relationship between optical power at 470 nm and the probability of observing a ChrimsonR-driven PSP. Note that the threshold for blue light activation of ChrimsonR PSPs was identical to the threshold for eliciting Chronos PSPs.

Figure 5: Characterization of light-evoked PSPs from commissural synapses onto VIP neurons. The right IC was injected with rAAV1.Syn.Chronos-GFP.WPRE.bGH and recordings were made from VIP neurons in the contralateral ICc. (**A**) Optogenetically-evoked IPSPs were evoked by 2 – 5 ms blue light flashes (left) while EPSPs were blocked by 10 µM NBQX and 50 µM D-AP5. IPSPs were abolished by gabazine (right). (**B**) Optogenetically-evoked EPSPs were evoked by 2 – 5 ms blue light flashes (left) while IPSPs were blocked with 1 µM strychnine and 5 µM gabazine. Wash-in of 50 µM D-AP5 significantly reduced the halfwidth and decay time constant of light-evoked EPSPs (middle). Wash-in of 10 µM NBQX abolished the remaining EPSP (right). Original traces in **A** and **B** are shown in light grey, and averages from up to 50 individual traces are shown in black. From Goyer et al., 2019.

Table 1: Stereotaxic coordinates for rAAV injections into IC and DCN. All coordinates are relative to lambda in µm. Z coordinates are measured from the dorsal surface of the skull at lambda.

DISCUSSION:

We have found that CRACM is a powerful technique for identifying and characterizing long range synaptic inputs to neurons in the mouse IC. Following the protocol detailed here, we achieved robust transfection of neurons in the DCN and IC as well as reliable axonal trafficking of Chronos and ChrimsonR to synaptic terminals in the IC. Additionally, we demonstrated that this technique enables the measurement and analysis of postsynaptic events, including PSP amplitude, halfwidth, decay time, and receptor pharmacology. Our experience suggests that this approach can be readily adapted to perform functional circuit mapping experiments throughout the auditory brainstem and beyond.

Overall, the specificity of optogenetic circuit mapping provides a distinct advantage over electrical stimulation of axons. Viral transfections provide the ability to spatially and molecularly restrict the expression of channelrhodopsins to a targeted population of presynaptic neurons. In contrast, electrical stimulation cannot differentiate between axons originating from different presynaptic nuclei when those axons are intermingled, as is the case in most brain regions. Electrical stimulation can also initiate both orthodromic and antidromic spikes, further complicating matters when a distal stimulation site contains axons originating from neurons located near the recording site.

To ensure stable expression and good axonal trafficking of a channelrhodopsin, choosing the right viral vector and serotype is paramount. We found that stable expression of Chronos in IC neurons was achieved with a serotype 1 rAAV including a Chronos or ChrimsonR construct combined with a woodchuck hepatitis posttranscriptional regulatory element (WPRE) and bovine growth hormone (BGH) polyadenylation signal. rAAV serotype 5 failed to produce functional opsins in the IC, but was functional in the DCN (data not shown). The rigorous validation of injection coordinates for every experiment and ensuring that opsin expression is restricted to the targeted brain region is similarly important. A meaningful identification of inputs is only possible if expression of the opsin is carefully checked and documented for every experiment.

The rAAV1.ChrimsonR construct used in the above protocol yielded stable expression and good axonal trafficking of the protein, even in long range projections from the DCN to the IC (Figure 3). This makes ChrimsonR a suitable opsin for (long-range) circuit mapping. A red-shifted opsin like ChrimsonR can be useful if the experimental parameters require light penetration deep into the tissue, but the experimenter must be aware that all currently available red-shifted opsins show some cross-activation with blue light. Although some studies have argued that ChrimsonR and Chronos may be separately activated[6,14,16], our data suggest that great care must be taken with this approach. A recent report details additional methods that should be used if attempting to separate red-shifted opsins from Chronos[13]. Therefore, when using ChrimsonR and Chronos in two color CRACM experiments, carefully designed control experiments need to be executed to ensure clear separation of blue and red-shifted opsin activation.

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DISCLOSURES:

The authors have nothing to disclose.

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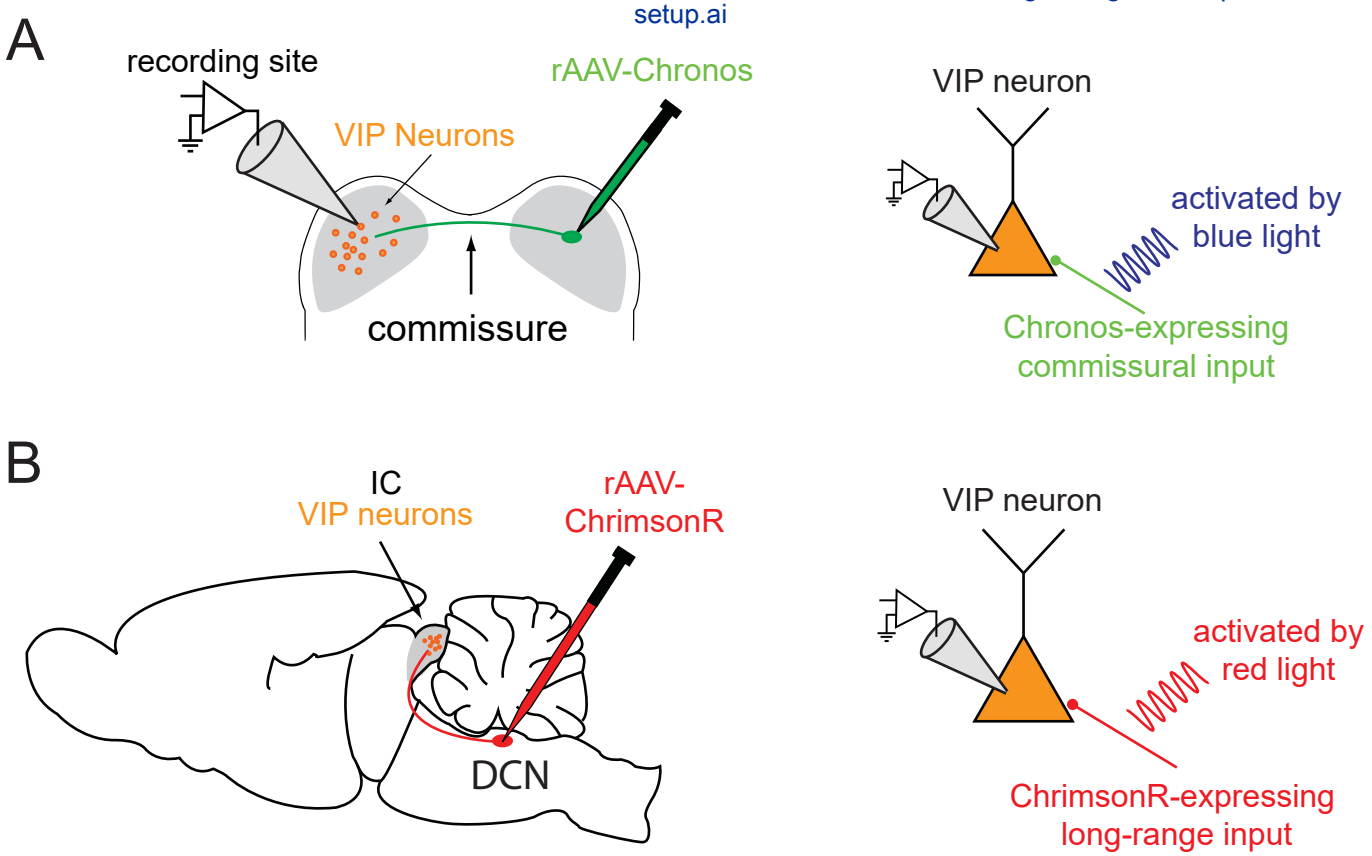
539

Figure 1

Click here to access/download;Figure;Figure 1 Experimental setup.ai



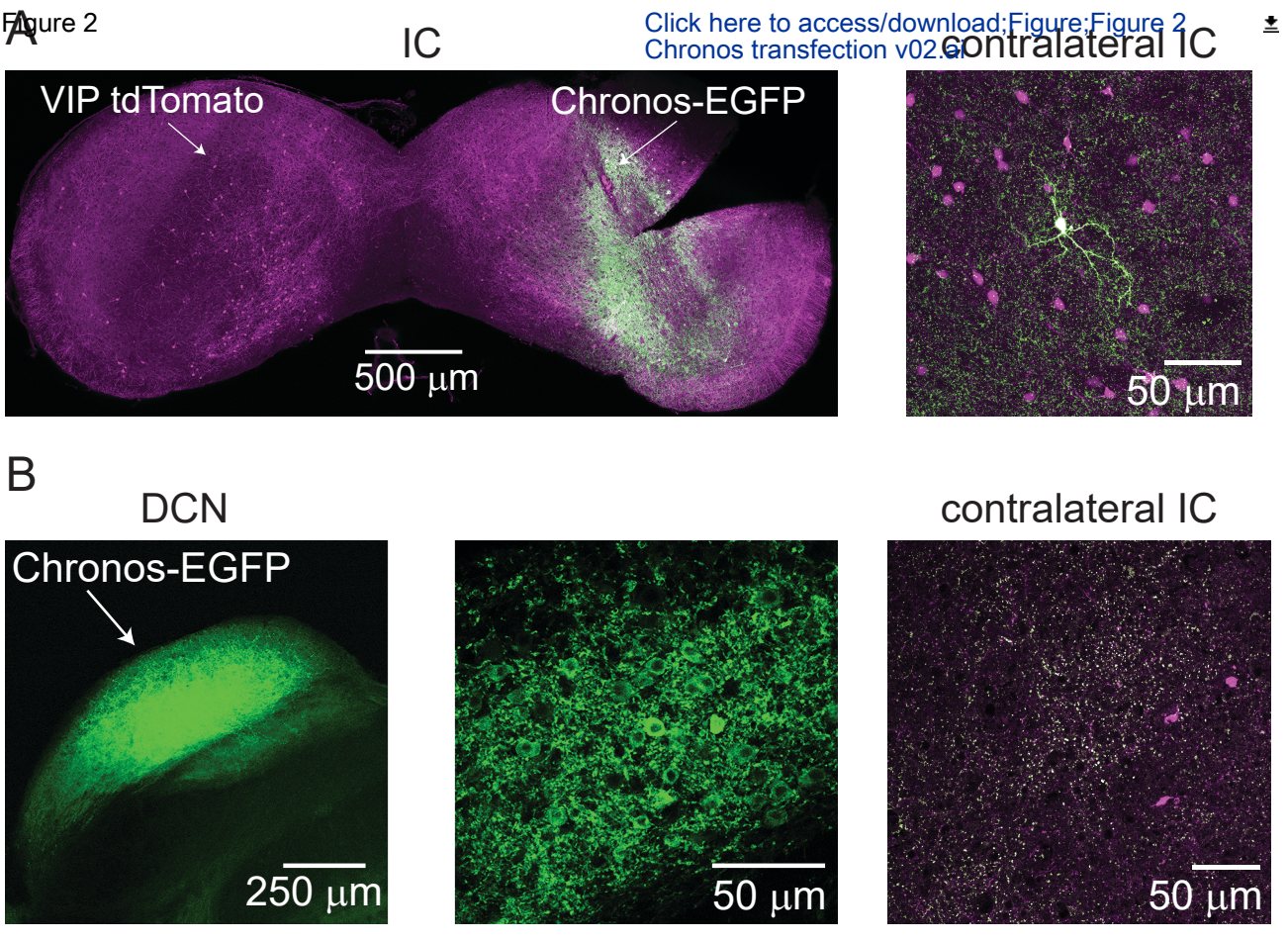


Figure 3

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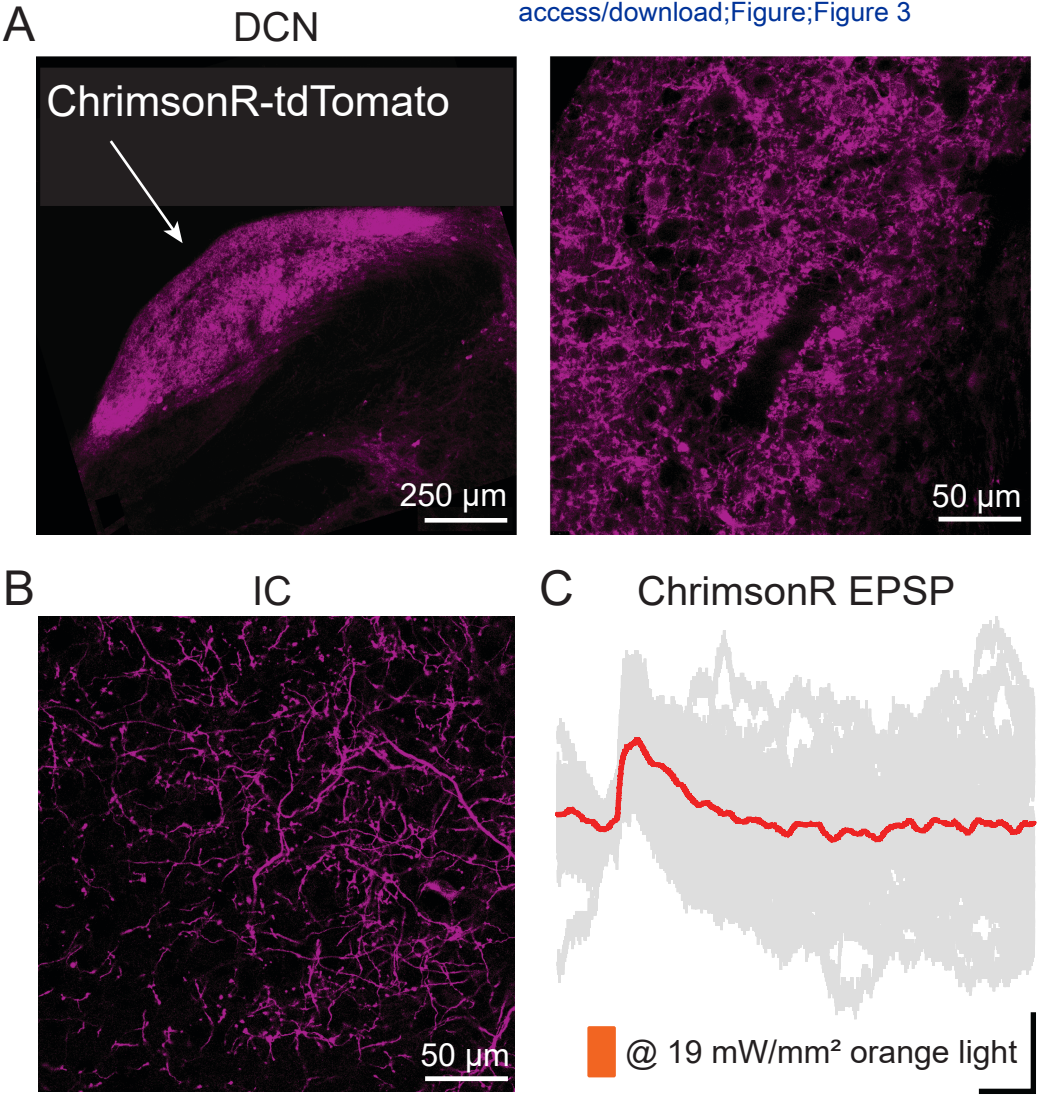

Figure 4

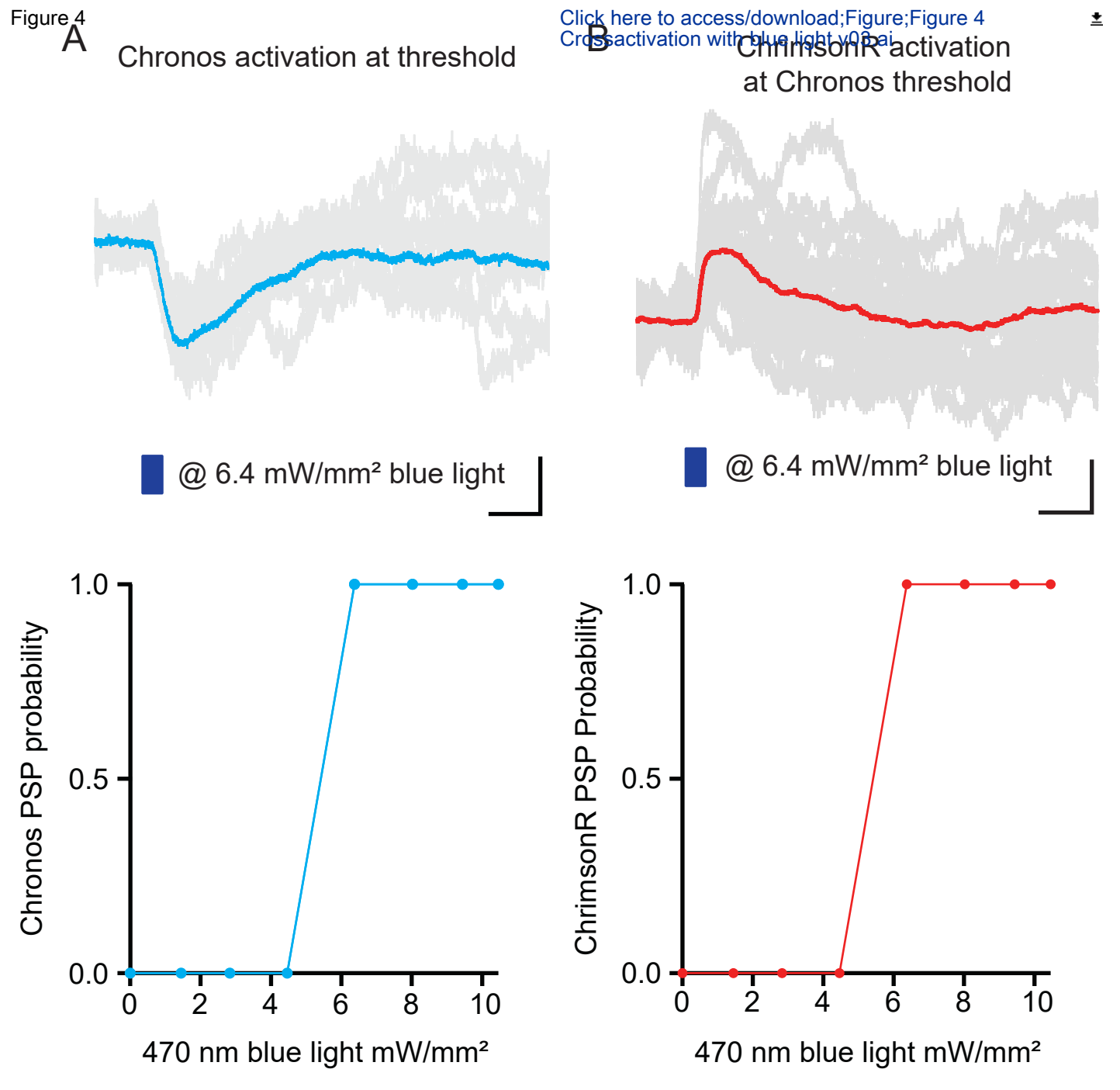

Figure 5

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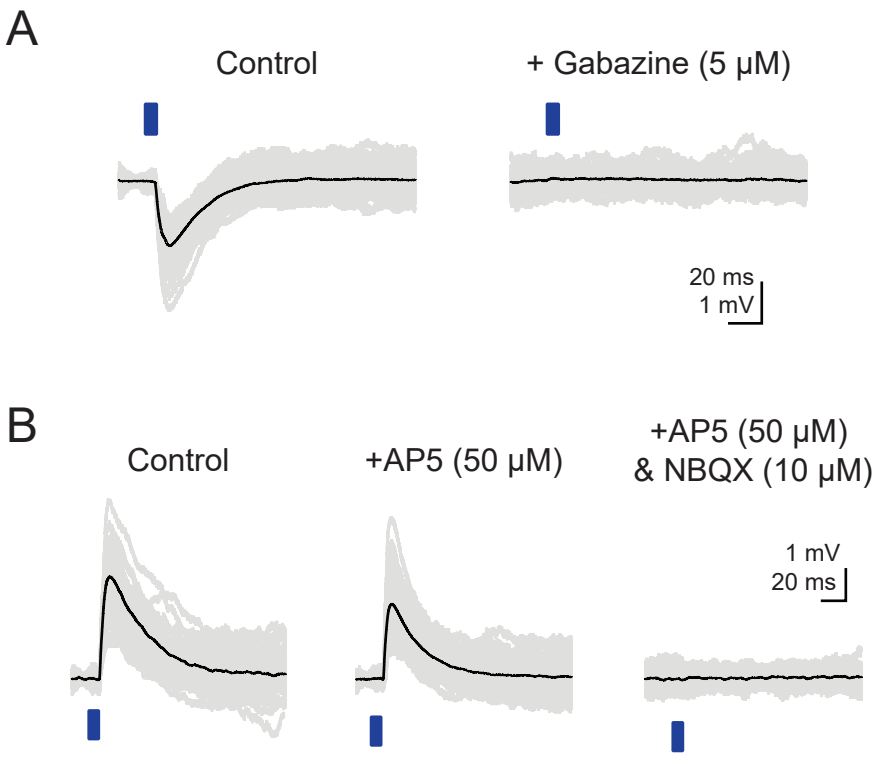

Table 1

	X (lateral)	Y (caudal)	Z (depth)
Right IC penetration 1	1000 µm	-900 µm	2250 – 1500 µm (250 µm increments)
Right IC penetration 2	1250 µm	-900 µm	2250 – 1750 µm (250 µm increments)
Right DCN	2155 µm	-1325 µm	4750 µm, 4550 µm

Name of Material/Equipment	Company	Catalog Number
AAV1.Syn.ChrimsonR-tdTomato.WPRE.bGH	Addgene	59171-AAV1
AAV1.Syn.Chronos-GFP.WPRE.bGH	Addgene	59170-AAV1
Ai14 reporter mice (B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J)	Jackson Laboratory	stock #007914
Amber (590nm) LUXEON Rebel LED	Luxeon Star LEDs	SP-01-A8
Blue (470nm) LUXEON Rebel LED	Luxeon Star LEDs	SP-01-B4
Carproject (carprofen)	Henry Schein Animal Health	59149
Drummond glas capillaries	Drummond Scientific Company	3-000-203-G/X
Drummond Nanoject 3	Drummond Scientific Company	3-300-207
Electrode beveler	Sutter Instrument	FG-BV10-D
Ethilon 6-0 (0.8 metric) nylon sutures	Ethicon	local pharmacy
Fixed stage microscope	any	n/a
Gas anesthesia head holder	David Kopf Instruments	933-B
General surgery tools	Fine Science Tools	N/A
Golden A5 pet clipper	Oster	078005-010-003
Heating pad	Custom build	N/A
Hooded induction chamber w/ vacuum system	Patterson Scientific	78917760
Hot bead sterilizer Steri 250	Inotech	IS-250
Iodine solution 10%	MedChoice	local pharmacy
Isoflurane vaporizer	Patterson Scientific	07-8703592
Lidocain topical jelly 2%	Akorn	local pharmacy
Micro motor drill 1050	Henry Schein Animal Health	7094351
Micro motor drill bits 0.5 mm	Fine Science Tools	19007-05
Motorized Micromanipulator	Sutter Instrument	MP-285/R
Ophthalmic ointment Artificial Tears	Akorn	local pharmacy
P-1000 electrode puller	Sutter Instrument	P-1000
Patch clamp amplifier incl data acquisition software	any	n/a
Portable anethesia machine	Patterson Scientific	07-8914724
Small animal steroetaxic frame	David Kopf Instruments	930-B
Standard chemicals	local vendors	N/A
standard imaging solutions		

Sterile towel drapes	Dynarex	4410
Surgical marker	Fine Science Tools	18000-30
Temperature controller	Custom build	N/A
Vibratome	any	n/a
VIP-IRES-Cre mice (Viptm1(cre)Zjh/J)	Jackson Laboratory	stock # 010908
Water bath	any	n/a

Comments/Description

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October 8, 2019

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
Assistant Professor
Kresge Hearing Research Institute
Department of Otolaryngology-Head & Neck Surgery
University of Michigan

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.

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Author(s):	David Goyer & Michael T. Roberts

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