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Corresponding Author:	Caitlin Murphy University of Wisconsin Madison, Wisconsin UNITED STATES
Corresponding Author's Institution:	University of Wisconsin
Corresponding Author E-Mail:	caitlin.murphy@wisc.edu
Order of Authors:	Caitlin A Murphy
	Aeyal Raz
	Sean M Grady
	Matthew I Banks
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TITLE:

Optogenetic Activation of Afferent Pathways in Brain Slices and Modulation of Responses by Volatile Anesthetics

AUTHORS AND AFFILIATIONS:

Caitlin A. Murphy[1], Aeyal Raz[1,2], Sean M. Grady[1], Matthew I. Banks[1]

[1]Department of Anesthesiology, University of Wisconsin, Madison, Wisconsin
[2]Department of Anesthesiology, Rambam Health Care Campus, the Ruth and Bruce Rappaport Faculty of Medicine, Technion - Israel Institute of Technology, Haifa, Israel

Corresponding Author:
Caitlin A. Murphy (caitlin.murphy@wisc.edu)

Email addresses of co-authors:
Aeyal Raz (ae_raz@rmc.gov.il)
Sean M. Grady (smgrady@wisc.edu)
Matthew I. Banks (mibanks@wisc.edu)

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SUMMARY:

Ex vivo brain slices can be used to study the effects of volatile anesthetics on evoked responses to afferent inputs. Optogenetics are employed to independently activate thalamocortical and corticocortical afferents to non-primary neocortex, and synaptic and network responses are modulated with isoflurane.

ABSTRACT:

Anesthetics influence consciousness in part via their actions on thalamocortical circuits. However, the extent to which volatile anesthetics affect distinct cellular and network components of these circuits remains unclear. Ex vivo brain slices provide a means by which investigators may probe discrete components of complex networks and disentangle potential mechanisms underlying the effects of volatile anesthetics on evoked responses. To isolate potential cell type- and pathway-specific drug effects in brain slices, investigators must be able to independently activate afferent fiber pathways, identify non-overlapping populations of cells, and apply volatile anesthetics to the tissue in aqueous solution. In this protocol, methods to measure optogenetically-evoked responses to two independent afferent pathways to neocortex in ex vivo brain slices are described. Extracellular responses are recorded to assay network activity and targeted whole-cell patch clamp recordings are conducted in somatostatin- and parvalbumin-positive interneurons. Delivery of physiologically relevant concentrations of isoflurane via artificial cerebral spinal fluid to modulate cellular and network responses is described.

INTRODUCTION:

Volatile anesthetics have been used ubiquitously in a variety of clinical and academic settings for more than a century. Distinct classes of anesthetics have unique, often non-overlapping molecular targets[1-3], yet nearly all of them produce unconsciousness. While their behavioral effects are quite predictable, the mechanisms by which anesthetics induce loss of consciousness are largely unknown. Anesthetics may ultimately influence both the level and contents of consciousness via actions on corticothalamic circuits, disrupting integration of information throughout the cortical hierarchy[4-9]. More broadly, modulation of corticothalamic circuits may play a role in experimentally[10] or pharmacologically[11] altered states of consciousness, and may also be implicated in sleep[12] and in pathophysiological disorders of consciousness[13,14].

The elusiveness of the mechanisms underlying loss and return of consciousness during anesthesia may be attributed partially to non-linear, synergistic actions of anesthetics at the cellular, network, and systems levels[15]. Isoflurane, for example, suppresses activity within the selected brain regions[16-18], impairs connectivity between distant brain regions[19-23], and diminishes synaptic responses in a pathway-specific manner[24,25]. Which effects of anesthetics, from the molecular to the systems level, are necessary or sufficient to effect loss of consciousness remains unclear. In addition to substantive clinical investigations of consciousness using non-invasive techniques[19,20,26], it is important that experimentalists seek to disentangle the distinct cellular and network interactions that subserve the conscious experience.

By simplifying the complex interactions found in the intact brain, ex vivo brain slices allow the study of isolated components of the brain's dynamic systems[9]. A reduced slice preparation combines the benefits of relatively intact anatomical structures of local neural circuits with the versatility of in vitro manipulations. However, until recently, methodological constraints have precluded the study of synaptic and circuit properties of long-range inputs in brain slices[27,28]; the tortuous path of corticothalamic fiber tracts made activation of independent afferent pathways all but impossible by electrical stimulation.

Investigating the effects of anesthetic agents on the brain slice preparations presents additional challenges. Absent an intact respiratory and circulatory system, anesthetic agents must be bath-applied, and concentrations carefully matched to estimated effect site concentrations. For many intravenous anesthetic agents, the slow rate of equilibration in the tissue renders traditional pharmacological investigations laborious[29,30]. Investigating the effects volatile gas anesthetics in ex vivo preparations is more tractable, but also presents challenges. These include converting inhaled partial pressure doses to aqueous concentrations, and the need for a modified delivery system of the drug to the tissue via artificial cerebral spinal fluid[31].

Here, methods are described by which investigators may capitalize on the well-documented physicochemical properties of the volatile anesthetic isoflurane for drug delivery to ex vivo brain slices, activate pathway- and layer-specific inputs to a cortical area of interest with high spatiotemporal resolution, and conduct simultaneous laminar recordings and targeted patch clamp recordings from select populations of neurons. Combined, these procedures allow

investigators to measure volatile anesthetic-induced changes in several observable electrophysiological response properties, from the synaptic to local network level.

PROTOCOL:

All procedures involving animals described in this protocol were approved by the University of Wisconsin-Madison School of Medicine and Public Health Animal Care and Use Committee.

1. Breeding mice to express fluorescent reporter protein in interneuron subpopulations

1.1. Pair homozyogous, Cre-dependent tdTomato male mouse with either homozygous SOM-Cre female or homozygous PV-Cre female mouse.

NOTE: Other specific neuronal populations may be targeted by using the appropriate Cre lines.

1.2. Allow heterozygous offspring to mature to at least 3 weeks of age before proceeding. For experiments described here, genotyping is not necessary, as homozygous parents produce offspring that are all heterozygous for both cell type-specific Cre recombinase and Cre-dependent reporter alleles.

2. Performing unilateral stereotaxic injection of viral construct

2.1. Adjust settings of the micropipette puller for injection pipettes as indicated in the instrument user manual (see **Table 1** for recommended settings). Pull the glass micropipette.

2.2. Break the tip of the sharp end of the pipette such that the tip diameter is approximately 30 µm with minimal taper over several millimeters.

2.3. Using previously documented procedures, decide on the appropriate titer and volume of virus to be injected. In the experiments described here, 1.0 µL (titer: 3.1-5.7 TU/mL) injected unilaterally produced good results.

2.4. Backfill the full volume of the pipette with mineral oil. Load the pipette onto the microsyringe and flow a small amount of mineral oil through the tip to ensure the tip is not clogged.

2.5. Frontfill at least 1.0 µL of viral construct. The recombinant adeno-associated viral vector used in these experiments was AAV2-hSyn-hChR2(H134R)-EYFP.

2.6. Arrange sterile drape in a surgical area. Sterilize tools for stereotaxic procedure and place it on the drape.

2.7. Anesthetize SOM-tdTomato or PV-tdTomato heterozygous animal using isoflurane (3% for induction, 1.5-2% for maintenance) and oxygen mixture. Periodically confirm surgical level of anesthesia with toe pinch throughout surgery. Ensure animal does not move beyond the surgical

plan of anesthesia by monitoring respirations every 10-15 min.

2.8. Shave the top of the animal's head. Apply 70% isopropyl alcohol and iodine-based solution liberally to surgical area and ophthalmic ointment to eye sockets to prevent drying of the membrane. Administer bupivacaine/lidocaine (1:1 ratio, 1.0 mg/kg) subcutaneously to surgical site for local anesthetic.

2.9. Fit the animal into stereotaxic frame.

2.10. Use scalpel to make incision along sagittal axis of skin overlying the dorsal surface of the skull. Retract skin using forceps. Hydrate skull with 0.9% saline as necessary.

2.11. On the surface of the skull, lightly mark the intersection of anterior and lateral coordinates with a cross in pencil. Drill a hole at the appropriate coordinates in the transverse plane (in mm relative to Bregma, for cingulate cortex (Cg) injection: anterior 0.2, lateral 0.3; for posterior thalamus (Po) injection: posterior 2.25, lateral 3.4).

NOTE: Markings should extend beyond the boundaries of the burr hole to provide guidance for accurate placement of pipette.

2.12. Turn on and balance the air table.

2.13. Reposition electrode manipulator of the stereotaxic frame at 0° for injections into Cg, or 45° in the coronal plane for injections into Po.

2.14. Attach the syringe pump to the electrode manipulator. Attach the microsyringe pump controller to the syringe pump.

2.15. Navigate pipette tip near (but not touching) the surface of the brain, at the intersection of markings created in Step 2.11. Advance the pipette at approximately 1 mm/s along its longitudinal axis into the brain either 0.9 mm (injection in Cg) or 3.1 mm (injection in Po). Wait for 10 min before proceeding.

2.16. Inject 1.0 µL of viral construct over a period of 10 min (100 nL/min). If welling of virus from pipette insertion site is observed, slow the injection rate to 50 nL/min.

2.17. After injection, wait for 10 min before slowly retracting the injection pipette.

2.18. Suture to close the scalp incision and administer 2-5 mg/kg meloxicam subcutaneously.

2.19. Discontinue isoflurane and monitor animal during emergence from anesthesia. Allow to recover according to procedures described by Institution's Animal Care and Use Committee, including further administration of analgesics.

3. Preparation of acute brain slices

3.1. Allow at least 3 weeks for the expression of viral construct before harvesting tissue.

3.2. Prepare 1 L of artificial cerebral spinal fluid for slicing procedure (slicing artificial cerebral spinal fluid, sACSF). See **Table 2** for ingredients.

3.3. Throughout the slicing procedure, supply sACSF with dissolved 95% O_2/5% CO_2 mixture, delivered via gas dispersion tube.

3.5. Prepare ice cold bath for vibrating blade microtome. Mount ice-cold specimen stage onto microtome and fix sapphire blade in place for tissue sectioning.

3.4. Anesthetize mouse with 3% isoflurane and oxygen until loss of righting reflex.

3.5. Decapitate the mouse using guillotine and immediately submerge head in 4°C sACSF. To preserve the health of the tissue, complete the following steps as swiftly as possible.

3.6. Open skull cavity by making a small incision at the base of the skull and gently removing each skull plate. Gently remove underlying dura mater.

3.7. While the brain is still in the skull cavity, use the razor blade to remove the cerebellum. Make a second vertical cut along the sagittal plane in the left hemisphere, just lateral to the midline.

3.8. Prepare tissue block for sectioning.

3.8.1. Gently lift the brain from the skull cavity. Place the brain on the filter paper with the flat, sagittal plane down. Guide the filter paper over the blocking template and align the brain to the underlying template outline (**Supplementary Figure 1**).

3.8.2. Make two parallel cuts in the coronal plane as indicated by the lines on the template. Add a small drop of sACSF to keep filter paper wet, if necessary.

3.8.3. Place the tissue block in 4°C sACSF briefly while step 3.8.4 is conducted.

3.8.4. Apply a small amount of super glue to the ice-cold specimen stage.

3.8.5. Lift the tissue block from cold sACSF. Use the corner of an absorbent towel to wick away excess sACSF. Glue the posterior coronal plane of the tissue block to the specimen stage, with the dorsal surface of the brain facing the sapphire blade.

3.9. Collect 500 µm thick coronal brain slices. Place slices of interest on nylon mesh (**Supplementary Figure 2**) in 34 °C sACSF and allow the container to reach room temperature.

NOTE: For experiments described here, electrophysiological recordings were collected from a coronal section centered approximately 2.25 mm posterior to bregma to study a non-primary sensory area, medial secondary visual cortex (V2MM).

4. Preparation of experimental artificial cerebral spinal fluid (eACSF) bags containing dissolved volatile anesthetic isoflurane

4.1. Prepare 300 mL of a stock mixture of 3.0% isoflurane.

4.1.1. In a sealed polytetrafluoroethylene gas bag, add ~100 mL of 95% O_2/5% CO_2 gas mixture to 20-30 mL of liquid isoflurane and a small amount of 0.9% saline. Wait at least for 30 min to allow equilibration of isoflurane between liquid and gas phases.

4.1.2. Determine the amount of saturated isoflurane gas, V_{sat}, to add to the stock bag using the following equation:

$$V_{sat} = \frac{P\%_{stock} * V_{stock}}{\left(\frac{P_{isoflurane}}{P_{total}}\right) * 100} \quad (1)$$

where $P\%_{stock}$ is the target composition of the stock gas (3% in this case), V_{stock} is the final volume of the stock gas bag, $P_{isoflurane}$ is the partial pressure of isoflurane at room temperature (~240 mmHg), and P_{total} is the atmospheric pressure (~760 mmHg).

4.1.3. Add the calculated amount of saturated gas to an empty gas bag and fill the bag with a volume of 95% O_2/5% CO_2 gas mixture to bring the total volume of stock bag to 300 mL.

4.2. Prepare 2 L of artificial cerebral spinal fluid for perfusion of the slice during the experiment (experimental ACSF, eACSF). See **Table 2** for ingredients. Dissolve 95% O_2/5% CO_2 gas mixture into solution.

4.3. Prepare two separate bags of Control and Isoflurane solutions.

4.3.1. To an empty polytetrafluoroethylene gas bag, add 600 mL eACSF and 600 mL of 95% O_2/5% CO_2 gas mixture. Label this bag as Control.

4.3.2. To another empty polytetrafluoroethylene gas bag, add 300 mL of eACSF. Label this bag as Isoflurane.

4.3.3. Choose a physiologically relevant equilibrated gas phase concentration of isoflurane. Experiments were conducted using gas concentrations equivalent to 1.3% isoflurane. Mice lose righting reflex, and presumably consciousness, at 0.9% inhaled isoflurane.

4.3.4. Use the following equation to calculate the equivalent gas phase concentration at room

temperature, P%(T_{room}) [31]:

$$P\%(T_{room}) = P\%\left(T_{body}\right) * e^{\frac{-20.2(T_{body}-T_{room})}{273.15+T_{room}}} \quad (2)$$

where P%(T_{body}) is the physiologically relevant gas phase concentration chosen in Step 4.3.3, T_{room} is 25 °C, and T_{body} is 37 °C.

4.3.5. Use the following equation to determine volume of gas from stock gas bag, V_{stock}, to add to the Isoflurane solution.

$$V_{stock} = \frac{V_{solution} * P\%(T_{room}) * (1+\lambda)}{P\%_{stock}} \quad (3)$$

where $V_{solution}$ is the volume of eACSF in ISOFLURANE bag (300 mL), P%(T_{room}) is entered from equation (2), λ is the saline/gas Ostwald partition coefficient of isoflurane (λ = 1.2[32]), and $P\%_{stock}$ is the gas phase concentration of the stock gas bag ($P\%_{stock}$ = 3.0%).

4.3.6. To the Isoflurane solution bag, add the volume of gas from the stock gas bag, V_{stock}, calculated in Step 4.3.5.

4.3.7. To the Isoflurane solution bag, add a volume of 95% O_2/5% CO_2 gas mixture to bring the total volume of gas in the Isoflurane solution bag to 300 mL.

4.4. Shake both Control and Isoflurane bags on shaker for at least 1 h to allow isoflurane phase equilibration.

4.5. After all data has been collected, the correct concentration may be verified by using an anesthetic gas monitor to measure equilibrated gas concentration of isoflurane above the remaining solution in bag.

4.6. Report experimental concentrations of volatile gases in aqueous units, as millimolar concentrations are more robust to changes in temperature. Use the following equation to convert room temperature gas phase concentration, P%(T_{room}), to equivalent aqueous concentration ($C_{aqueous}$, in mM) [31]:

$$C_{aqueous} = 0.44614 * \alpha * P\%(T_{room}) \quad (4)$$

where α is the saline/gas Bunsen partition coefficient for isoflurane at 25°C[32].

5. Preparation of hardware and software for multi-channel recordings

5.1. Set up 16-channel data acquisition system according to manufacturer instructions.

NOTE: Several commercially available amplifiers and data acquisition systems can be used to collect multi-channel recordings. In the experiments described here, analog signals are delivered via an electrode reference panel to two amplifiers, where they are amplified (2000x) and filtered (0.1-10kHz). Analog inputs to the data acquisition system are digitized at 40kHz.

5.2. Fasten the appropriate 16-channel headstage adaptor to a microscope micromanipulator. Orient the adaptor such that the female connector ports are facing downward.

5.3. Adjust the angle of operation of this micromanipulator such that it is oriented downward toward the recording chamber, at an angle approximately 70° relative to horizontal.

5.4. Connect the headstage input to a 16 x 1 probe for in vitro electrophysiology via the headstage adaptor anchored to the micromanipulator.

5.5. Connect the headstage output connector to the data acquisition system.

5.6. Install appropriate software for data acquisition. Configure 15 input channels to correspond to input signals from the first 15 multi-channel probe contacts. Configure the remaining channel to receive input from the intracellular electrode.

NOTE: Take care to consider electrode and adaptor maps when collecting and analyzing data, to ensure the appropriate signal corresponds to the electrode contact from which it was collected.

6. Configuration of light stimulation protocols

6.1. Set up light delivery system and install the accompanying software.

6.2. Open the software. Choose hardware wiring configuration in which a Trigger Source (Digital/TTL Out) provides Trigger In signal to the light delivery system, and the light delivery system provides Trigger Out signal to a 470 nm LED.

6.3. Mount high-power objective lens. Using digital camera, calibrate high-power objective for use with light delivery system.

6.4. Create new profile sequence of light stimulation profiles.

6.4.1. Create a pattern of choice. In the experiments described here, a circle of diameter 150 µm is used to allow layer-specific activation of axon terminals.

6.4.2. To construct a profile sequence, copy and paste this profile for each of any number of trials.

6.4.3. Create a waveform list that contains waveforms of any light intensity, pulse duration, or pulse number.

6.4.4. Randomly assign waveforms to each profile. Each profile with its assigned waveform corresponds to one trigger pulse from a Digital TTL input, or one trial.

6.4.5. Save the profile sequence.

6.5. In the data acquisition software, create a new protocol.

6.5.1. Set the number of trials to equal the number of profiles in the profile sequence just created.

6.5.2. Choose signal inputs to match those configured in Step 5.6. Configure a protocol that provides a single digital TTL output, recording from these 16 input channels for an appropriate amount of time before and after the digital trigger.

7. Placing multi-channel probe in ex vivo brain tissue slice

7.1. Perfuse bubbled eACSF (not in sealed bags) at 3-6 mL/min.

7.2. Transfer the brain slice containing area of interest onto mesh grid in microscope perfusion chamber. Anchor with platinum harp (see **Supplementary Figure 3**).

7.3. Rotate mesh grid such that the line of electrode contacts on the distal end of the multi-channel probe is approximately perpendicular to the pial surface.

7.4. Under broadfield illumination and under fine control of the micromanipulator, lower the multi-channel probe toward the surface of the slice.

7.5. Rotate the filter cube turret to engage the appropriate filter cube for visualization of the fluorescent reporter protein expressed in axon terminals of cortical afferents. If necessary, rotate the slice to more precisely align the probe with the pial surface.

7.6. Position the probe just above the plane of the slice, ~200 µm short of the final target position along the x-axis, leaving at least one channel outside the boundary of the area of tissue being recorded

7.7. Slowly insert the probe into the slice by moving the manipulator along its longitudinal axis. To minimize damage to the tissue, only advance the probe to the extent that the sharp tips are just visible below the tissue surface. This will minimize damage to the tissue while still ensuring the electrode contacts are in contact with the tissue.

8. Patch clamping targeted neurons and obtaining whole-cell configuration

8.1. Switch eACSF source to bagged Control solution.

8.2. Identify fluorescently labeled cell for targeted patch clamp recording.

8.2.1. Restrict the aperture iris diaphragm to the smallest diameter. Engage a low-power objective lens and bring the tissue into focus.

8.2.2. Center the light over an area of tissue adjacent to (but not overlapping) the multi-channel probe.

8.2.3. Engage the high-power (40x or 60x) water immersion objective, using caution to avoid contact between the multi-channel probe and objective lens.

8.2.4. Rotate the filter cube turret to engage the appropriate filter set to allow imaging of cells expressing Cre-dependent fluorescent marker.

8.2.5. Identify a fluorescently labeled cell as a target for patch clamp recording. Raise the objective lens to create ample space to lower a patch pipette.

8.3. Load a patch pipette (see Table 1) with internal solution (Table 2) and mount pipette into electrode holder. Using 1 mL syringe, apply positive pressure corresponding to ~0.1mL air.

8.4. Lower patch pipette into the solution. Bring the pipette tip into focus under visual guidance.

8.5. Obtain whole-cell recording from the targeted cell using the steps previously demonstrated[33].

8.6. If planning to assess changes to intrinsic properties of the cell (e.g., input resistance, action potential firing rate in response to current steps), conduct these recordings. Otherwise, move to axon stimulation protocol in below.

9. Layer-specific optogenetic activation of axon terminals

9.1. Manipulate field of view in the x-y plane to align light stimulation profile with desired location on slice.

9.2. Load light stimulus protocol and prepare the light delivery system to receive a digital TTL pulse.

9.3. Optogenetically activate axon terminals while simultaneously recording extracellular field potentials and intracellular membrane fluctuations.

9.4. Switch eACSF source to Isoflurane solution and wash drug in for 15 min. If necessary, collect spontaneous recordings during the wash-in.

9.5. Repeat step 9.2-9.3.

9.6. Switch eACSF source to Control solution and wash drug out for 20 min. If necessary, collect spontaneous recordings during wash-out.

9.7. Repeat step 9.2-9.3.

REPRESENTATIVE RESULTS:

A timeline of steps described in the protocol is shown in **Figure 1**. Cortical inputs arriving from higher order cortical areas or from non-primary thalamic nuclei have partially overlapping terminal fields in layer 1 of non-primary visual cortex[24]. To isolate independent thalamocortical or corticocortical afferent pathways, a viral vector containing ChR2 and an eYFP fluorescent reporter into either Po or Cg was injected. Cells within the injection radius take up the viral vector and, after 2-4 weeks, express the non-specific cation channel ChR2 and the reporter in both the soma and projecting axons (**Figure 2A**). Coronal slices were collected. With the appropriate filter cube engaged, axons expressing the viral construct were imaged (**Figure 2B**). The use of ChR2 to activate axon terminals allows for activation of afferents without the prerequisite for an attached soma.

The animals used in the experiments described here were SOM-tdTomato or PV-tdTomato hybrid animals, which express the fluorescent reporter protein tdTomato in either somatostatin- (SOM+) or parvalbumin-positive (PV+) interneurons, respectively. SOM+ or PV+ interneurons in layer 2/3 were targeted for patch clamping under visual guidance with the appropriate filter cube engaged (Layer 1C). These interneurons have dendrites in layer 1 and are targets of corticocortical inputs (**Figure 3A**).

Addition of 125 mL of 3.0% isoflurane gas and 175 mL of 95% O_2/5% CO_2 to a sealed bag resulted in a pre-equilibrium concentration of gas of 1.3%. Gas dissolved into eACSF according to its partition coefficient; the predicted gas phase equilibrium concentration of isoflurane at room temperature was 0.6% (**Figure 2D**). This was confirmed via gas monitor.

The tissue slice was transferred to the recording chamber and the 16x1 multi-channel recording probe was placed orthogonally to the cortical laminae (**Figure 2E**). A 150 µm circle of 470 nm light centered over cortical layer 1 was delivered via the objective light path, while extracellular field potentials were collected using the 16 x 1 multi-channel probe and targeted whole-cell patch clamp recordings were conducted in interneurons. A schematic of the recording set-up is shown in **Figure 2F**.

Post-synaptic potentials (PSPs) were observed in interneurons in response to a train of four 2 ms pulses of light (10 Hz; **Figure 3A**). Local field potentials were also recorded (**Figure 3B**). Current source density (CSD; **Figure 3C**) and multi-unit activity (MUA; **Figure 3D**) were extracted from local field potentials. Ten trials at several different light intensities were used to conduct post hoc analyses. The amplitude of current sinks extracted from the CSD increased as a function of light intensity (**Figure 4A**). A three-parameter nonlinear logistic equation was fit to the data for comparisons across pathways. PSP amplitude also increased with current sink amplitude (**Figure 4B**).

Synaptic responses to thalamocortical and corticocortical inputs were measured during control, isoflurane (0.28 mM), and recovery conditions. Post-synaptic responses of somatostatin- (**Figure 5A**) to corticocortical stimuli were suppressed during isoflurane, as were evoked current sinks (**Figure 5B**).

FIGURE AND TABLE LEGENDS:

Figure 1: A schematic outlining timeline of important steps in protocol. Top: Describes timeline of steps necessary for breeding of transgenic animals and expression of viral vector. **Bottom:** Depicts steps and timeline for preparing materials and conducting experiment on the day of slice preparation.

Figure 2: Injection of viral vector and preparation ex vivo coronal brain slices. (**A**) Schematic representation of injection of viral vector into SOM-tdTomato or PV-tdTomato hybrid mice. (**B**) Coronal slices of the medial parietal association area (mPtA) were harvested, and thalamocortical (top) or corticocortical (bottom) afferent fibers were identified by their eYFP reporter in layer 1. This figure is modified with permission from [24]. (**C**) Overlay of eYFP-labeled axon terminals in layer 1 (green) and tdTomato-labelled SOM+ interneurons (red) in superficial layer 2/3. (**D**) Sealed bags were prepared with a 50:50 solution-to-gas mixture. (**E**) Placement of a 16 x 1 probe into mPtA (black outline). (**F**) Schematic of the recording set-up in the cortical slice.

Figure 3: Simultaneous intracellular and multi-channel extracellular recordings in cortical slice. (**A**) Whole-cell current clamp patch recording from the soma of a layer 2/3 PV+ interneuron. Four pulses (2 ms each, blue arrows) of blue light (2.2 mW) at 10 Hz were delivered to corticocortical axon terminals in L1. Average (red trace) of ten trials (grey traces) are shown. (**B**) Raw data from 16 channels of extracellular 16 x 1 probe. Channels placed in cortical tissue are shown in black, and those lying outside of cortex in grey. (**C**) A current source density diagram, extracted from the local field potential signal, shows synaptic current sinks (blue) in layer 1. (**D**) Multi-unit activity, generated by applying a high-pass filter to the local field potential signal, isolates spiking activity evoked in lower layers.

Figure 4: Comparison of responses from recordings in two different slices. Multiple light intensities were used to evoke synaptic responses in cortical layer 1. For each trial, the peak amplitude of the evoked response was extracted from the layer 1 extracellular current sink and EPSPs in layer 2/3 PV+ interneurons. (**A**) Extracellular response profiles of thalamocortical and corticocortical afferents are compared as a function of light intensity. (**B**) The relationship between current sink amplitude and EPSP amplitude is pathway dependent. Within each stimulus pathway, data from (A) and (B) were collected simultaneously.

Figure 5: Bath application of isoflurane dissolved in eACSF during simultaneous recordings. (**A**) Intracellular whole-cell current clamp recording from layer 2/3 SOM+ interneuron upon activation of corticocortical afferents during control, isoflurane, and wash conditions. Vertical blue lines indicate light stimuli (2 ms; 1.65 mW). (**B**) Current source density trace extracted from electrode in layer 1. Data were collected simultaneously with those collected in (A). Recovery of

responses upon wash demonstrates depression of synaptic responses by isoflurane.

Table 1: Recommended glass and parameters for pulling micropipettes for viral injections and whole-cell patch clamp recordings. Glass used for viral injections and whole-cell patch clamp recordings is described, as well as the parameters for pulling micropipettes using the micropipette puller. Consult instruction manuals for micropipette puller for further recommendations or fine-tuning of settings.

Table 2: Composition of artificial cerebral spinal fluid and intracellular solution. Reagents and concentrations for sACSF, eACSF, and intracellular pipette solution for patch clamp recordings are listed.

Supplementary Figure 1: Template for preparing block of tissue to collect brain slices. The template is adjusted to the appropriate size, printed, and glued to a microscope slide. A cover slip is glued over the template to prolong its use. The tissue block is placed on a piece of filter paper with the sagittal plane down, aligned to the pink background, and a vertical cut is made in the coronal plane along the black line.

Supplementary Figure 2: Incubation chamber for harvested brain slices. The chamber is filled with sACSF and bubbled with 95% O_2/5% CO_2 gas mixture via a bent needle attached to tubing. Incubation platform is made of nylon stretched over a plastic circular fitting.

Supplementary Figure 3: Platinum structures for slice in recording chamber. Brain slice is transferred to recording chamber via pipette and placed on top of nylon mesh, which is stretched over a horseshoe-shaped piece of flattened platinum wire and super glued in place. Platinum harp is placed over brain slice to anchor it in place during recording.

Supplementary Table 1: Ostwald (λ) and Bunsen (α) coefficients for other volatile anesthetics. Adapt this protocol for study of other volatile gas anesthetics, such as halothane, sevoflurane, or desflurane. Substitute the equations described in the protocol with the appropriate coefficients as listed in this table.

DISCUSSION:

In this manuscript, a protocol for evaluating intra- and extracellular responses to selectively activated afferent pathways in ex vivo brain slices is described.

The use of optogenetic tools and parallel recording schemes allows investigators to probe responses of local populations to afferent inputs from distant brain regions, while recording simultaneously from targeted populations of interneurons. The use of optogenetic technology allows for axon terminals of afferent projections to be preserved and activated even though their cell bodies are no longer attached. This relieves geometric restrictions previously imposed upon *ex vivo* slices, as preservation of long-range electrical connections is no longer paramount. Still, care should be taken to prepare slices in a geometrical plane that preserves any remaining connections of interest. For example, pyramidal cells are oriented vertically along the cortical

column, and evoked network activity measured by the multichannel probes in these experiments requires such local connections to be preserved as much as possible. Thus, coronal slices were prepared to keep local connectivity intact.

When choosing optogenetic constructs and relevant fluorescent reporter proteins, properties of their excitation/emission spectra and microscopic optics must be considered. Persistent light stimulation may result in partial inactivation of many channelrhodopsin variants[34], which can be avoided by choosing reporter proteins whose excitation spectra do not overlap with that of the opsin. Alternative variants with different kinetics or light sensitivities may also be chosen depending on the experimental paradigm[35], including manipulations using alternative excitatory or inhibitory opsins. Filter cubes must also be appropriately aligned with the chosen fluorescent reporters, such that afferent axon terminals or interneurons may be imaged independently and without activating expressed opsins. To account for the variability in virus expression, it may also be pertinent for investigators to normalize any optogenetically-induced activity to the expression level of the viral construct, measured by the fluorescent output of the reporter protein.

Delivery of pre-calculated concentrations of volatile anesthetics to slice tissue is also possible using the methods outlined here. When choosing appropriate physiologically relevant gas equilibrium percentages, investigators should account for 10-15% loss of dissolved isoflurane gas between the perfusion line and tissue[36]. The methods applicable to isoflurane have been presented, but other drugs such as halothane, sevoflurane, or desflurane can be handled similarly using the appropriate Ostwald and Bunsen coefficients (**Supplementary Table 1**). The partitioning properties of volatile anesthetics assure that they will predictably dissolve into ACSF. However, because partial pressures are more sensitive to changes in temperature than aqueous EC_{50} concentrations[37], gas equilibrium volume percentages of volatile anesthetics must be converted to predicted room temperature millimolar concentrations to compare observed effects to physiologically relevant doses *in vivo*. If opting to study intravenous anesthetics such as etomidate or propofol in brain slices, investigators must consider diffusion profiles of the drugs under study, as equilibration times and physiologically relevant concentrations may vary greatly[30].

In this manuscript, a protocol is described for testing the effects of volatile anesthetics on distinct components of thalamocortical circuits in ex vivo brain slices. Many of the variables and parameters in the methods described may be manipulated for further investigations. For example, different brain areas, afferent pathways, cell targets, or volatile anesthetics may be studied by adapting the outlined methods to answer novel questions. Combined with other theoretical and experimental methods, study of unique cellular and network components using ex vivo brain slices will advance our understanding of the dynamic brain, and the changes it undergoes during pharmacological and pathophysiological changes in consciousness.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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707

Figure 1

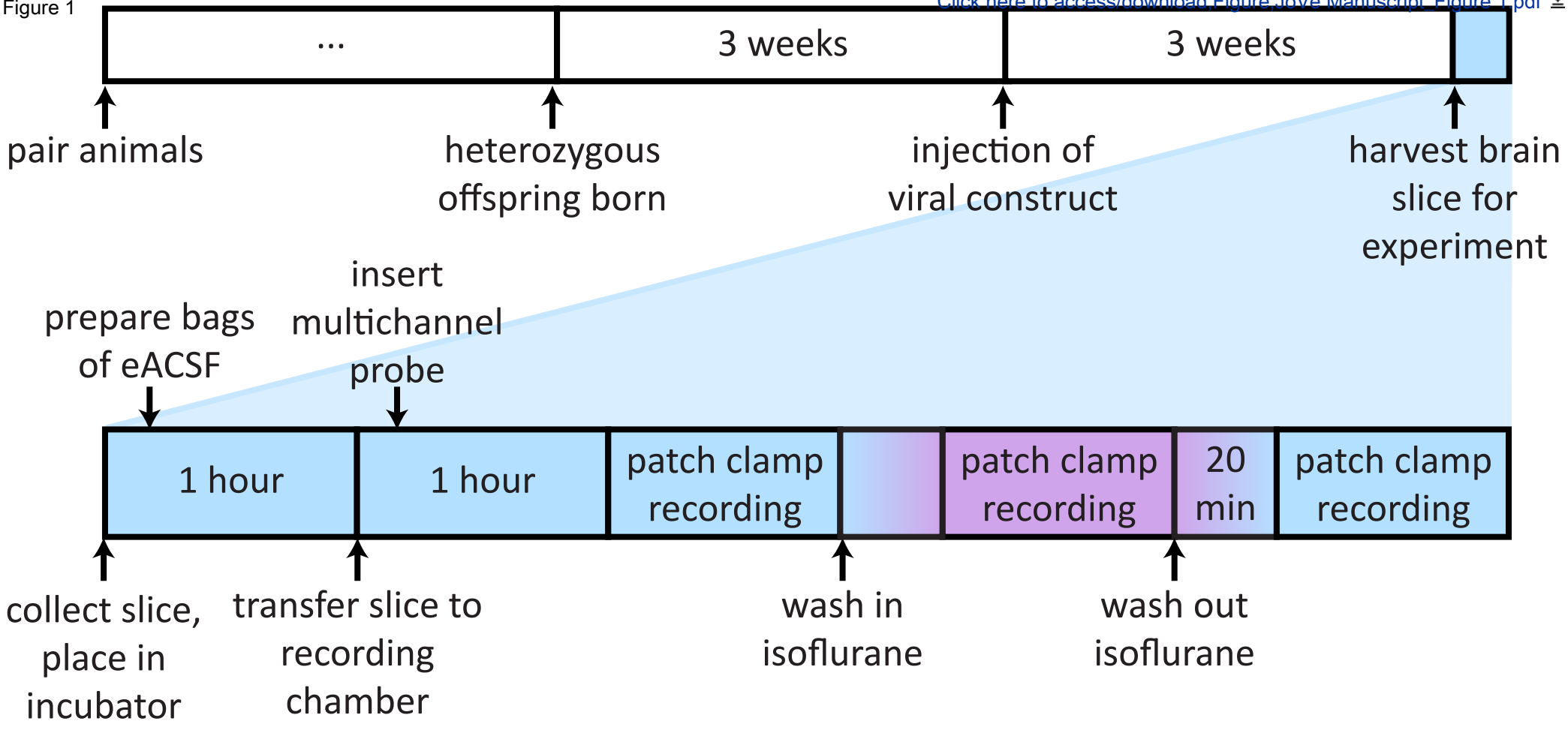
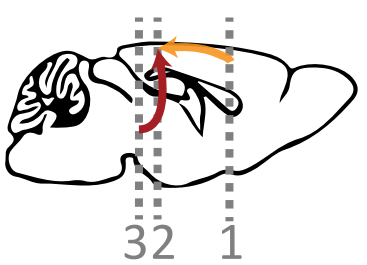


Figure 2

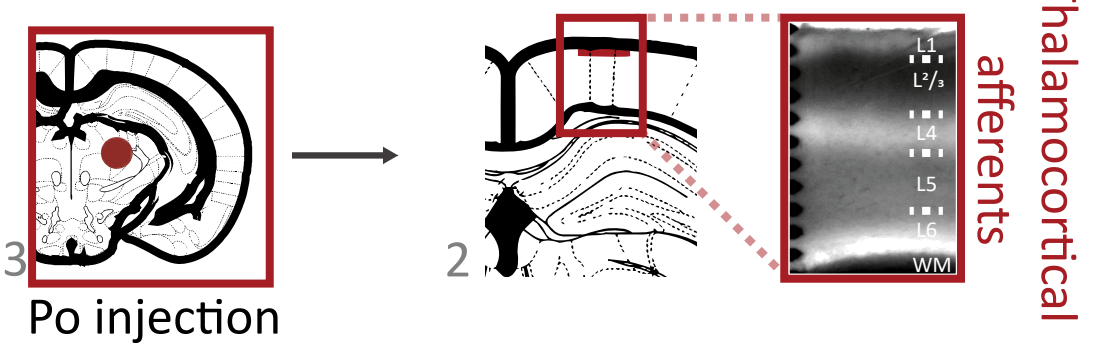
A

3-4 week-old mice:
SOM-tdTomato
or
PV-tdTomato

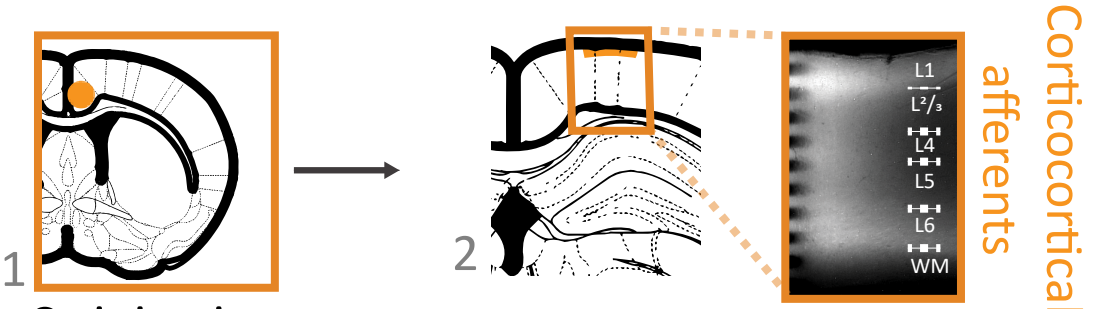


Inject channelrhodopsin* in cingulate cortex (1) or posterior thalamus (3).

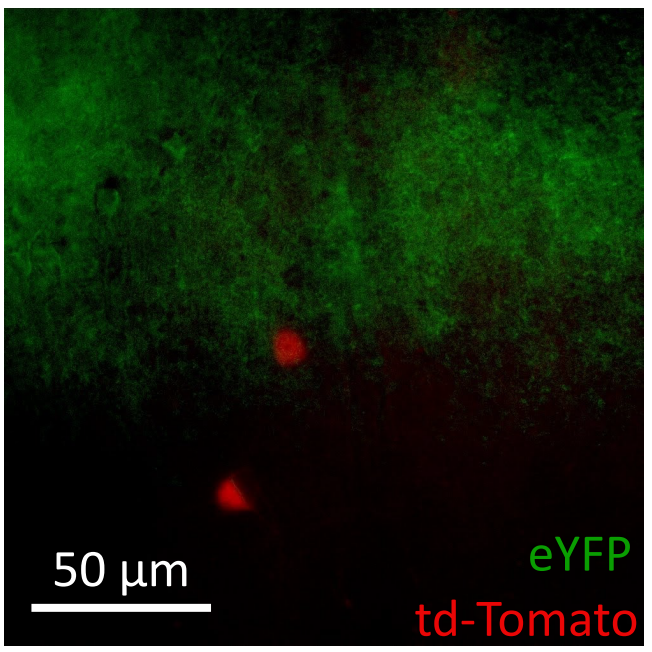
B



Express ChR2/eYFP in coronal slice in mPtA.

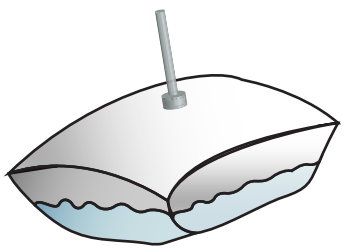


C



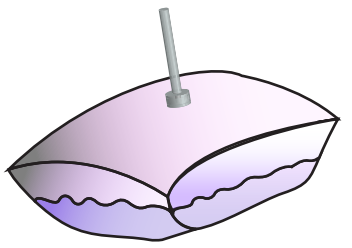
ChR2-expressing axon terminals and td-Tomato-labelled interneurons fluoresce.

D



CONTROL

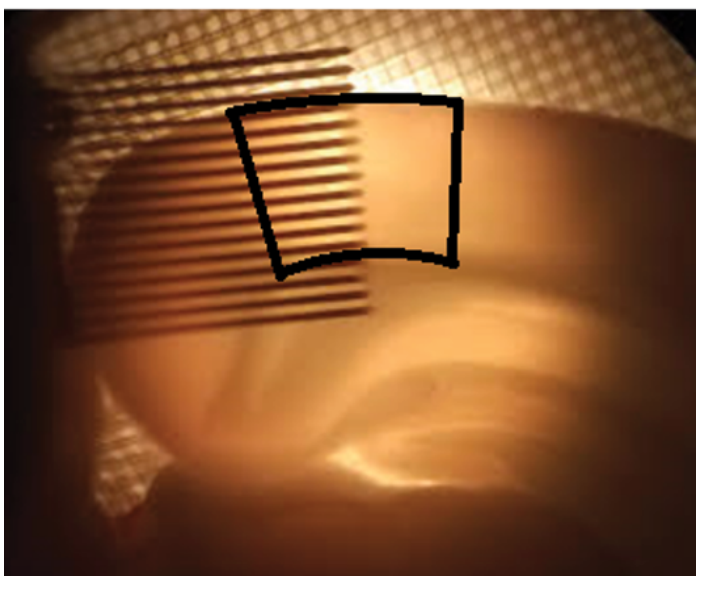
ACSF (600 mL)
+
95% O_2/5% CO_2 (600 mL)



ISOFLURANE
(0.28 mM)

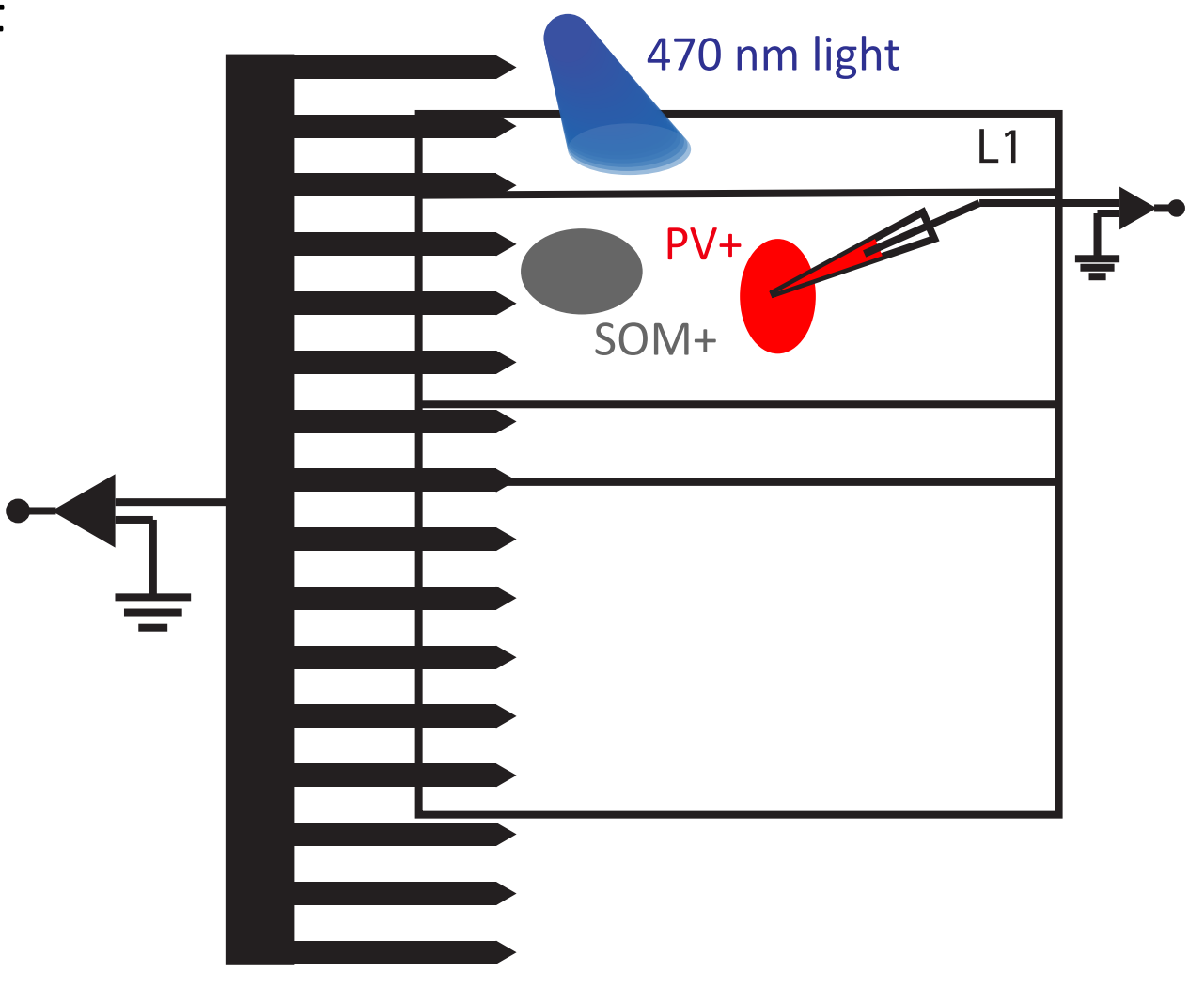
ACSF (300 mL)
+
95% O_2/5% CO_2 (175 mL)
+ isoflurane (125 mL)

E



Insert 16x1 extracellular probe spanning cortical column.

F



Collect simultaneous extra- and intracellular recordings.

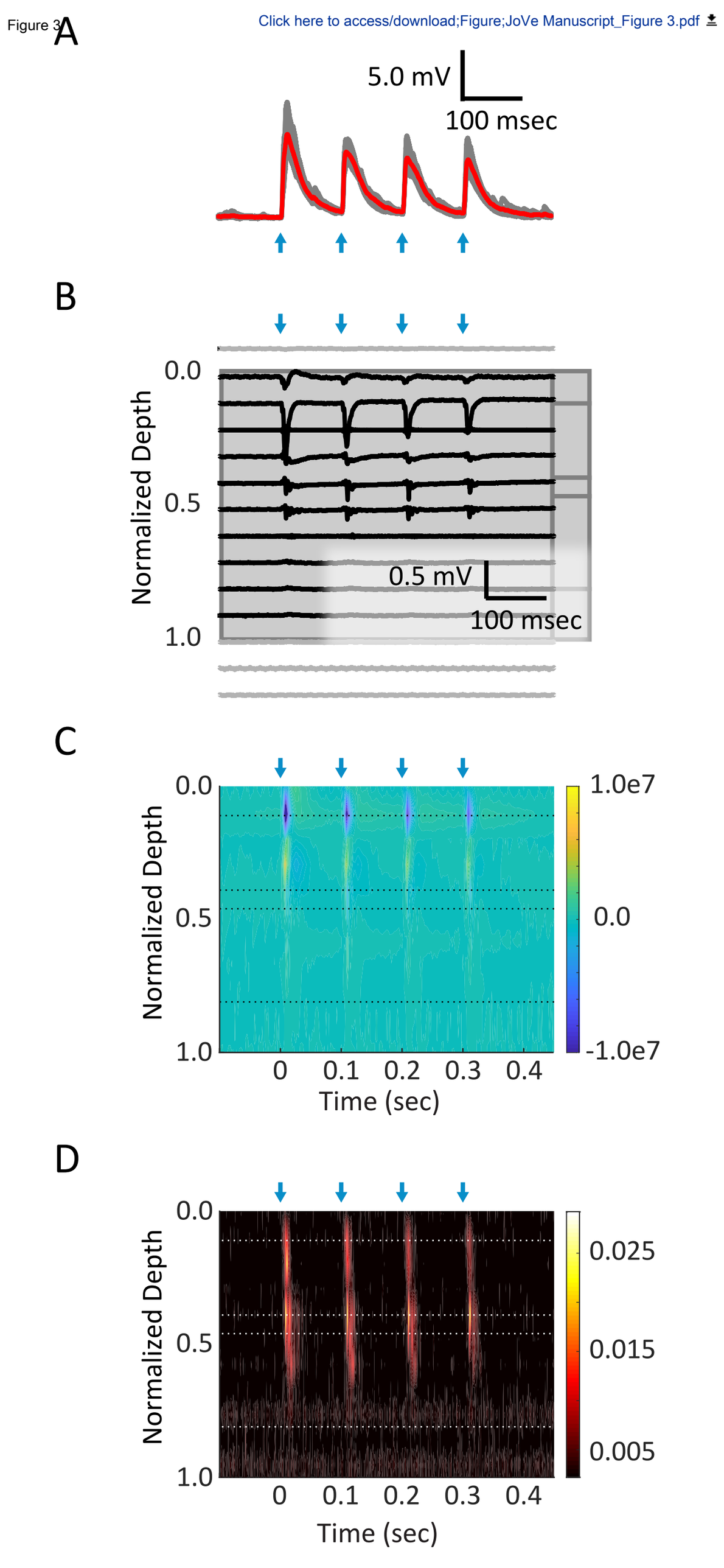
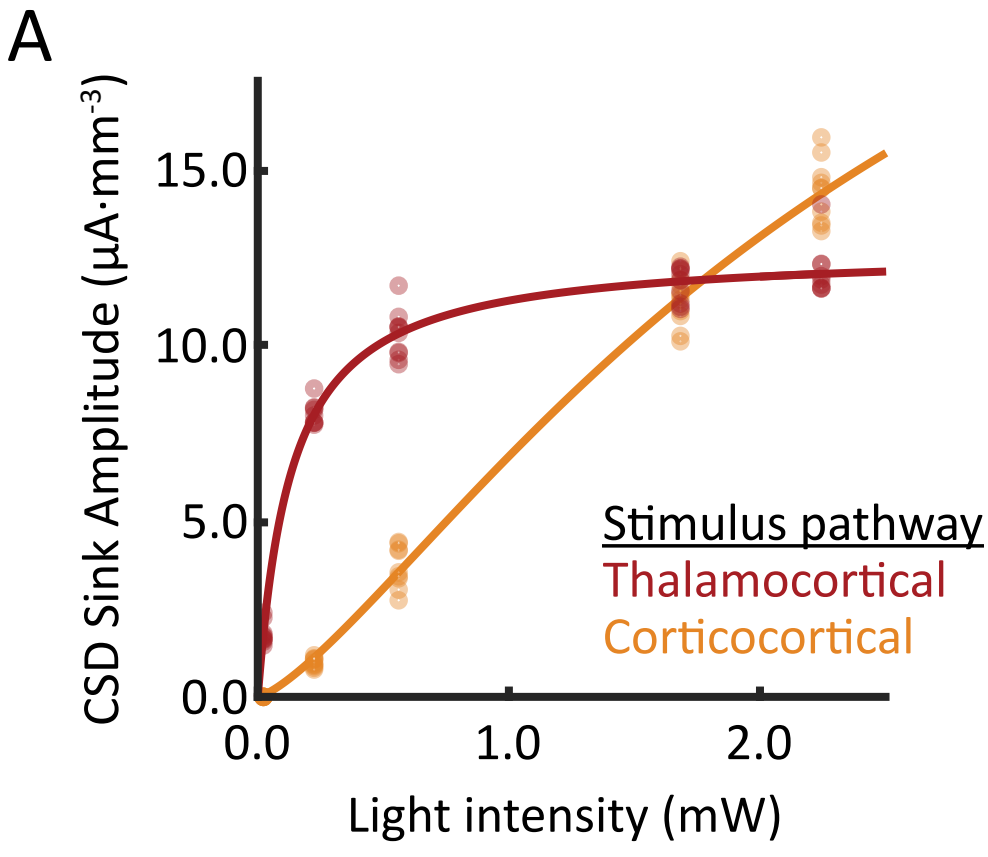


Figure 4



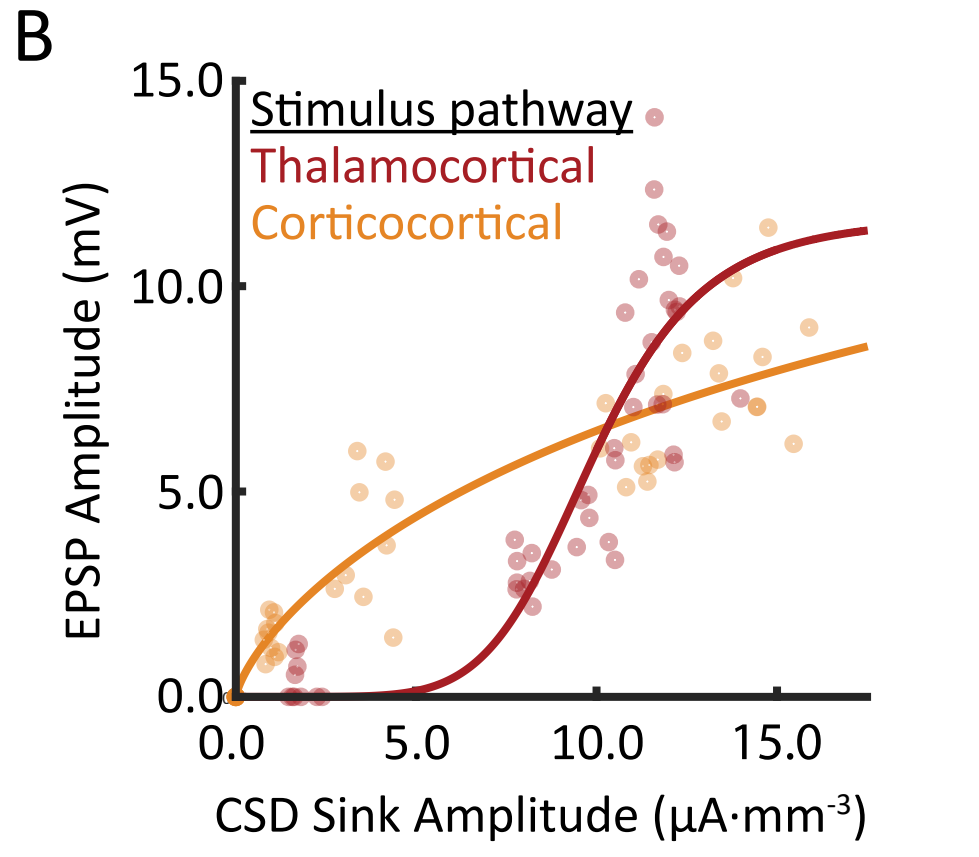


Figure 5

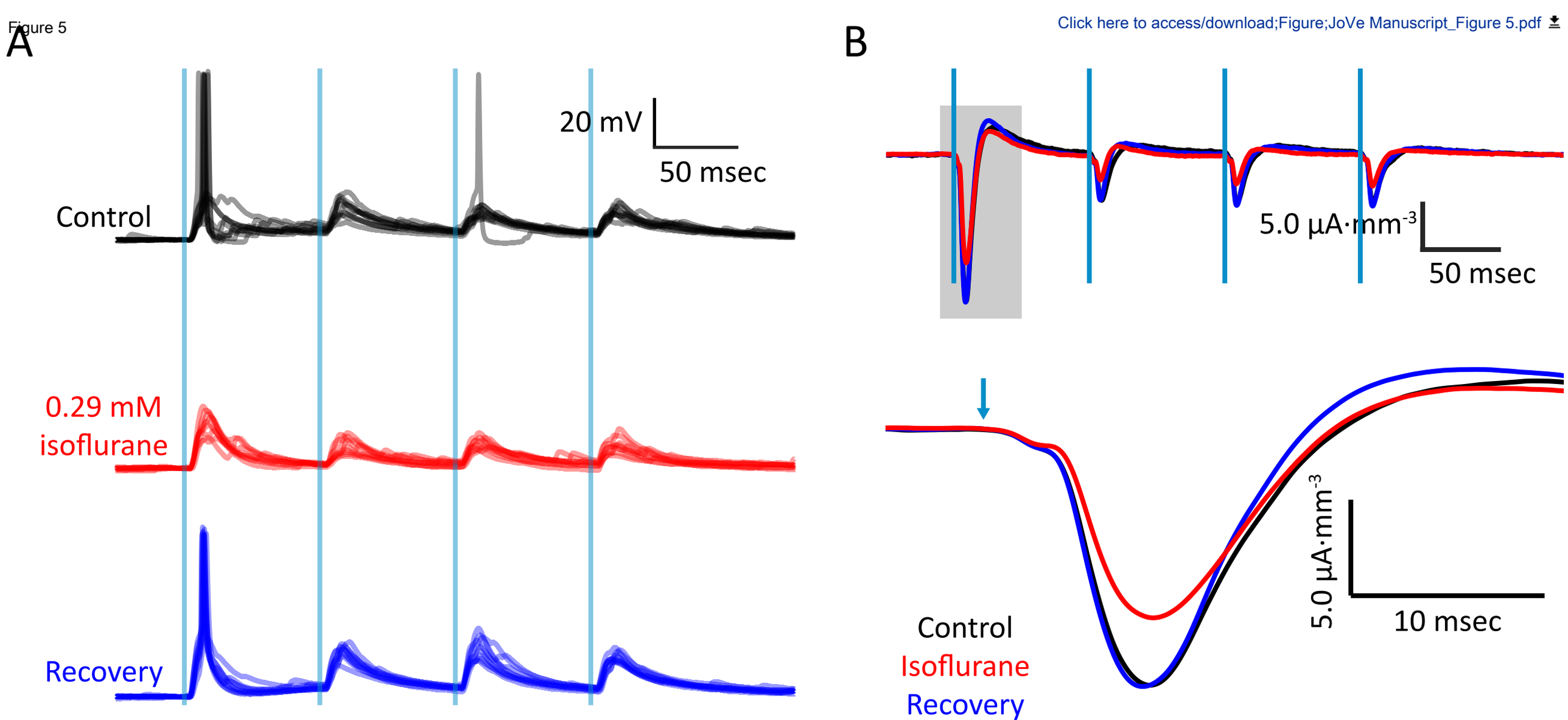


Table 1

Micropipette for virus injection

Glass	ID: 0.05 mm, OD: 0.11 mm
Loops	1

Heat	Pull	Vel	Time	Pressure
Ramp + 10	20	40	200	300

Micropipette for whole-cell patch clamp recordings

Glass	ID: 1.1 mm, OD: 1.7 mm
Loops	4

Heat	Pull	Vel	Time	Pressure
Ramp	0	25	250	500

Table 2

	Slicing ACSF, sACSF (in mM)	Experiment ACSF, eACSF (in mM)
NaCl	111	111
$NaHCO_3$	35	35
HEPES	20	20
KCl	1.8	1.8
$CaCl_2$	1.05	2.1
$MgSO_4$	2.8	1.4
KH_2PO_4	1.2	1.2
glucose	10	10

	Internal Solution
K-gluconate	140
NaCl	10
HEPES	10
EGTA	0.1
MgATP	4
NaGTP	0.3
	pH = 7.2

Table of Materials

Click here to access/download;Table of Materials;Table of Specific Materials + Equipment.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2.5x broadfield objective lens	Olympus	MPLFLN2.5X	
40x water immersion objective lens	Olympus	LUMPLFLN40XW	
95% O_2/5% CO_2 mixture	Airgas	Z02OX95R2003045	
A16 probe	NeuroNexus	A16x1-2mm-100-177-A16	16-channel probe
AAV2-hSyn-hChR2(H134R)-EYFP	Karl Deisseroth Lab,		
Anesthetic gas monitor (POET II)	Criticare	602-3A	
ATP, Magnesium Salt	Sigma Aldrich	A9187	intracellular solution
B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J	The Jackson Laboratory	007914	Cre-dependent tdTomato mouse
B6;129P2-Pvalbtm1(cre)Arbr/J	The Jackson Laboratory	008069	PV-Cre mouse
Belly Dancer Shaker	Thomas Scientific	1210H86-TS	for equilibration of sealed gas bags
Betadine solution	Generic brand		
Bleach	Generic brand		for silver chloriding patch clamp electrode
Bupivicaine			
Calcium Chloride ($CaCl_2$)	Dot Scientific	DSC20010	ACSF
Capillary glass (patch clamp recordings)	King Precision Glass, Inc.	KG-33	Borosilicate, ID: 1.1mm, OD: 1.7mm, Length: 90.0mm
Capillary glass (viral injections)	Drummond Scientific Company	3-000-203-G/X	3.5"
Control of junior micromanipulator	Luigs and Neumann	SM8	for control of junior micromanipulator

Control of manipulators and shifting table	Luigs and Neumann	SM7	for control of multichannel electrode and shifting table
Digidata 1440A + Clampex 10	Molecular Devices	1440A	Digitizer and software
E-3603 tubing	Fisher Scientific	14171208	for delivery of 95% O2/5% CO2 gas mixture to incubation chamber + application of pressure during patch clamping
EGTA	Dot Scientific	DSE57060	intracellular solution
ERP-27 EEG Reference/Patch Panel	Neuralynx	Retired	
Filling needle	World Precision Instruments	50821912	for filling patch clamp pipettes
Filter cube for imaging EYFP	Olympus	U-MRFPHQ	
Filter paper	Fisher Scientific	09801E	lay over slice template during preparation of tissue block
Flaming/Brown micropipette puller	Sutter Instrument	P-1000	2.5x2.5 Box filament
Gas dispersion tube	Sigma Aldrich	CLS3953312C	
Glass syringe (100 mL)	Sigma Aldrich	Z314390	for filling gas-sealed bags
Gluconic Acid, Potassium Salt (K-gluconate)	Dot Scientific	DSG37020	intracellular solution
Glucose	Dot Scientific	DSG32040	ACSF
GTP, Sodium Salt	Sigma Aldrich	G8877	intracellular solution
Headstage-probe adaptor	NeuroNexus	A16-OM16	adaptor to connect 16-channel probe to headstage input
Hemostatic Forceps	VWR International	76192-096	
HEPES	Dot Scientific	DSH75030	ACSF,intracellular solution
HS-16 Headstage	Neuralynx	Retired	
Isoflurane	Patterson Veterinary	07-893-1389	

Isopropyl alcohol (70%)	VWR International	101223-746	
Junior micromanipulator	Luigs and Neumann	210-100 000 0090-R	for manipulation of patch clamp electrode
LED Light Source Control Module	Mightex	BLS-PL02_US	optogenetic light source control
Lidocaine			
Lynx-8 Amplifier	Neuralynx	Retired	
Lynx-8 Power Supply	Neuralynx	Retired	
Magnesium Sulfate ($MgSO_4$)	Dot Scientific	DSM24300	ACSF
mCherry, Texas Red filter cube	Chroma	49008	for imaging tdTomato fluorescent reporter
Meloxicam			
Micropipette holder	Fisher Scientific	NC9044962	
Microsyringe pump	World Precision Instruments	UMP3-4	
Mineral oil	Generic brand		
MultiClamp 700A	Molecular Devices/Axon Instruments	700A	Amplifier
Nitrogen (for air table)	Airgas	NI200	
Nylon mesh	Fisher Scientific	501460083	stretched over horseshoe of flattened platinum wire, slice rest on top of this during recordings
Nylon, cut from pantyhose	Generic brand		small piece to create slice platform in incubation chamber, single fibers to create platinum harp
Ophthalmic ointment	Fisher Scientific	NC1697520	
Pipette	Dot Scientific	307	For transferring tissue to rig
Platinum wire	VWR International	BT124000	2 cm, flattened, to make platinum harp

Polygon400	Mightex	DSI-E-0470-0617-000	optogenetic light delivery system, comes with PolyScan2 software
Potassium Chloride (KCl)	Dot Scientific	DSP41000	ACSF
Potassium Phosphate (KH_2PO_4)	Dot Scientific	DSP41200	ACSF
Razor blade	Fisher Scientific	12-640	
Sapphire blade (for vibratome)	VWR International	100492-502	
Scalpel blade	Santa Cruz Biotechnology, Inc.	sc-361445	
Sealed gas bag	Fisher Scientific	109236	
Shifting table for microscope	Luigs and Neumann	380FMU	
Sodium Bicarbonate (HCO^{3-})	Dot Scientific	DSS22060	ACSF
Sodium Chloride (NaCl)	Dot Scientific	DSS23020	ACSF, intracellular solution
Ssttm2.1(cre)Zjh/J (SOM-IRES-Cre)	The Jackson Laboratory	013044	SOM-Cre mouse
Stereotaxic instrument	Kopf	Model 902	Dual Small Animal
Super glue	Staples	886833	to fix tissue block to specimen stage during slice preparation
Surgical drill	RAM Products Inc.	DIGITALMICROTORQUE	Microtorque II
Syringe (1 mL) with LuerLock tip	Fisher Scientific	309628	for application of pressure during patch clamping
Syringe (1 mL) with slip tip	WW Grainger, Inc.	19G384	for filling patch clamp pipettes
Syringe Filters	VWR International	66064-414	
Upright microscope	Olympus	BX51	
Vibrating microtome	Leica Biosystems	VT1000S	
Wypall towels	Fisher Scientific	19-042-427	

University of Wisconsin
SCHOOL OF MEDICINE
AND PUBLIC HEALTH

Caitlin A. Murphy, BSc
Graduate Research Assistant
Department of Anesthesiology

31 March 2020

Dear Dr. Bajaj,

We thank the editor and reviewers for their insightful questions and constructive feedback regarding our manuscript. Enclosed please find our responses the assessors' comments. We have made changes to the manuscript, as described below, and uploaded the revised version. Please note that all line references in our responses below are line references to the document showing tracked changes.

We would also like to note that we have added an author, Sean M. Grady, who has provided physical and intellectual contributions to the manuscript since the first submission.

Please contact me at caitlin.murphy@wisc.edu if you have any questions about our submission.

Sincerely

Caitlin A. Murphy
Graduate Research Assistant
Advisor: Matthew I. Banks (mibanks@wisc.edu)

Dear Dr. Murphy,

Your manuscript, JoVE61333 "Optogenetic activation of afferent pathways in brain slices and modulation of responses by volatile anesthetics," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **Mar 18, 2020**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Vineeta Bajaj, Ph.D.
Review Editor
JoVE
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_____

Dear Murphy,

The revision due date for your JoVE submission JoVE61333R1 Optogenetic activation of afferent pathways in brain slices and modulation of responses by volatile anesthetics, has now been updated to **Apr 01, 2020**.

When you are ready to submit your revision, please go to the JoVE submission site and log in using the credentials below. We look forward to reviewing your revised manuscript. Please feel free to contact me if you have any further questions.

username: Caitlin Murphy
password: click here to reset your password

Best,

Vineeta Bajaj, Ph.D.
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Please note that all line references in our responses below are line references to the document showing tracked changes.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been thoroughly proofread for spelling, grammar, and syntax.

2. Please define all abbreviations during the first time use.

This addition has been made to the manuscript (line 257, line 304).

3. JoVE cannot publish manuscripts containing commercial language. Please remove all 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: Digidata 1440A, Lynx-8 amplifiers, NeuroNexus probe, Clampex 10, Polygon400, PolyScan2, etc. Please remove th commercial term from the figure as well e.g., PolyScan 2.

All references to commercial products have been removed, and language has been adjusted to apply generically. Supplementary Figure 4 has also been removed.

4. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

All use of the active voice, including personal pronouns, has been changed accordingly throughout the manuscript.

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

An ethics statement has been added (line 105).

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions

should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

The protocol steps have been reviewed to ensure all verbs are written in the imperative form. A note has been added to Step 1.1 on line 113.

7. Please ensure you answer the "how" question, i.e., how is the step performed?

Steps have been reviewed to ensure each answers "how" question.

8. 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 headings 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.

The limitations outlined here have been fulfilled as instructed. The protocol is ~9 pages, and the highlighted portion just under 2.75 pages total.

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Figure 2 (previously Figure 1, see Reviewer 2 comments) has been modified with permission from a previous publication from our lab in the British Journal of Anaesthesia. Permission has been granted as instructed and uploaded to the Editorial Manager.

10. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol

Critical steps within the protocol are emphasized in the discussion: correct calculation and preparation of anesthetic bags (line 791), care in preserving relevant local connections when preparing slice (line 773)

b) Any modifications and troubleshooting of the technique

Modifications and troubleshooting at multiple points during the protocol are described in the discussion: properties of opsins (line 780), slice geometry (line 773), accounting for viral expression (line 787)

c) Any limitations of the technique

Limitations of the technique are described in the discussion: choice of opsins and reporters with respect to their excitation/emission spectra (paragraph beginning with line 779), diffusion profiles of intravenous anesthetics (line 801).

d) The significance with respect to existing methods

Significance with respect to existing methods is described in the discussion: activation of long-distance afferents, simultaneous recording of intra- and extracellular activity (paragraph beginning with line 748).

e) Any future applications of the technique

Future applications are described in the discussion: alternative opsins, cell targets, and anesthetics (described throughout discussion section, reiterated line 807).

Reviewers' comments:
Reviewer #1:

Manuscript Summary:

This is a well written and beautifully illustrated paper describing state-of-the-art techniques for studying anesthetic effects on selected circuit pathways and cell populations using brain slice preparations. I could only find a few minor improvements:

"even for a careful geometer" should be deleted, since 'geometer' is not commonly used and this statement is not needed.

This statement has been removed from line 80.

"demonstrates block of synaptic responses" should be "demonstrates depression of synaptic responses"

This change has been made to line 712.

"changes in temperature than aqueous EC50 concentrations31" Ref 31 Franks, N. P. & Lieb, W. R. is a general review, you should cite Hagan et al 1998 for actual temperature, aqueous concentration and electrophysiologic measures using brain slices.

This change has been made accordingly (line 799).

Major Concerns:
None

Minor Concerns:
None

Reviewer #2:

Manuscript Summary:
The authors demonstrate an experimental approach in which they combined optogenetics with brain slice electrophysiology to study the neural circuits involved in anesthetic action. Although anesthesia is a behavioral state that can be best studied in normal intact subjects, brain slices offers a much simpler models system that compliments the behavioral studies.

Major Concerns:
None

Minor Concerns:

This is a very well-written manuscript and outlines complex procedures that can be of immense help to new users. One of the main suggestions I have is that addition of a schematic to illustrate the major steps/timelines (starting from breeding to end of recording session) would be helpful to the readers and users.

This is a helpful recommendation. A figure depicting the timeline of important steps in the protocol has been included as Figure 1. Figures originally titled 1-4 have been shifted to Figures 2-5.

Some of the other comments I have are as follows:

1. Line 98-100: It will be helpful to mention some sources of cre mice so that researchers who want to use a different phenotype know where to get these. Does the author perform genotyping? Tail snip or any other method?

Animals used are bred from parents homozygous for both cell-specific Cre recombinase and Cre-dependent tdTomato reporter (ordered from Jackson labs). As such, all offspring are heterozygous for both alleles and genotyping is unnecessary. A slight addition to the protocol (lines 115-118) has been made to clarify this.

As per JoVE policy, we have avoided making commercial references to sources of mice, but have included their sources in the Excel spreadsheet of materials. We can make adjustments accordingly if the editors see fit.

2. Line 106-108: What's the diameter of the glass tube? Is it smaller than a 30 or 32 G steel tubing? If not, perhaps it's much easier and less damaging to use a steel cannula that can be cut using any regular drill with a circular blade. The authors may have standardized the pulled glass methodlogy for their experiments but I think they can at least allude to the possibility of using a steel cannula.

We agree that the details of the injection pipette should be included; they have been added (line 125-126).

However, given the size of the micropipette used in our experiments and the acute nature of the viral injections, we believe steel canulae are almost 10 times larger in diameter and would be much more damaging than the current method; it is likely best to avoid their use for this purpose.

If the editors prefer mention of steel canulae be included, we are happy to revisit this comment.

3. Line 117-119: It is also of value to confirm periodically (every 15 min) if the anesthesia is too deep. The color of mucosal membranes and breathing is generally a good indicator and should be listed as such in any surgical procedure.

Comments describing evaluation of maintenance of appropriate levels of anesthesia have been added (line 156-157).

4. Line 128-129: I would assume that perioxide and saline won't be applied at the same time and likely be alternated. Can the authors please include if this is the case and how many

time they alternate peroxide with saline and if saline step is always the last one?

The text has been changed to more accurately reflect protocol for these surgeries (line 167).

5. Line 131-135: Do the authors use Brema? If yes, then please include the description.

This addition has been made (line 171).

6. Line 137-138: The "dorso-ventral arm" of the stereotax is called Electrode Manipulator. Please use this term.

This change has been made (line 179, 182).

7. Line 144-147: Is there a pace (e.g., 1mm over a few minutes or so) at which the electrode is advanced?

This information has been added (line 186).

8. Line 149-150: Bilateral or unilateral injection?

This detail has been added to Step 2 (line 120).

9. Line 202: Is it 34 degrees or 4 degrees?

This step is written correctly – tissue is placed very briefly in cold sACSF before collecting slices. Then, tissue slices are transferred to warm sACSF. The language “briefly while step 3.8.4 is conducted” has been added for clarity (line 286).

10. Line 207: Please expand eACSF at the first use. Same for sACSF.

This change has been made (see #2 from editorial comments above).

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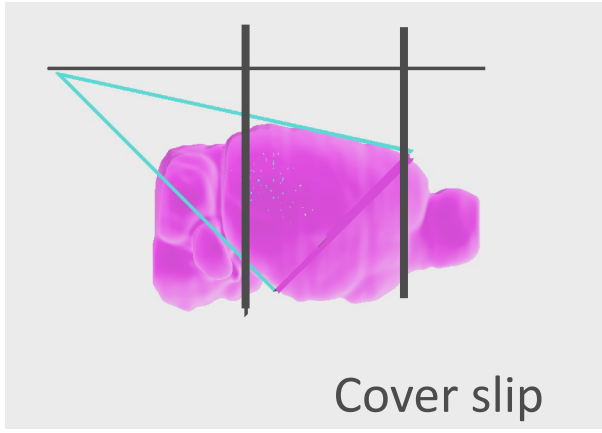

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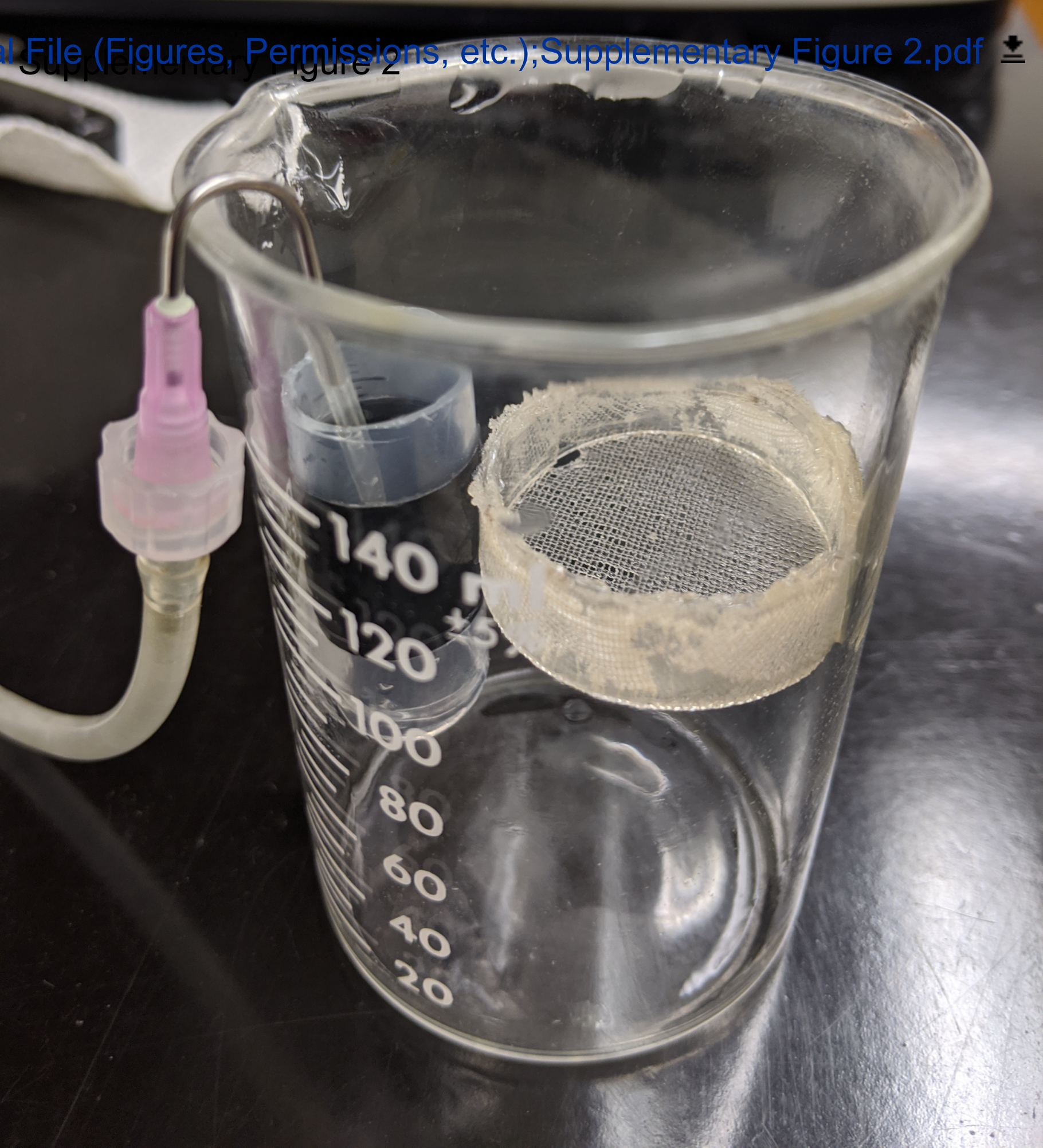
Supplementary Figure 2



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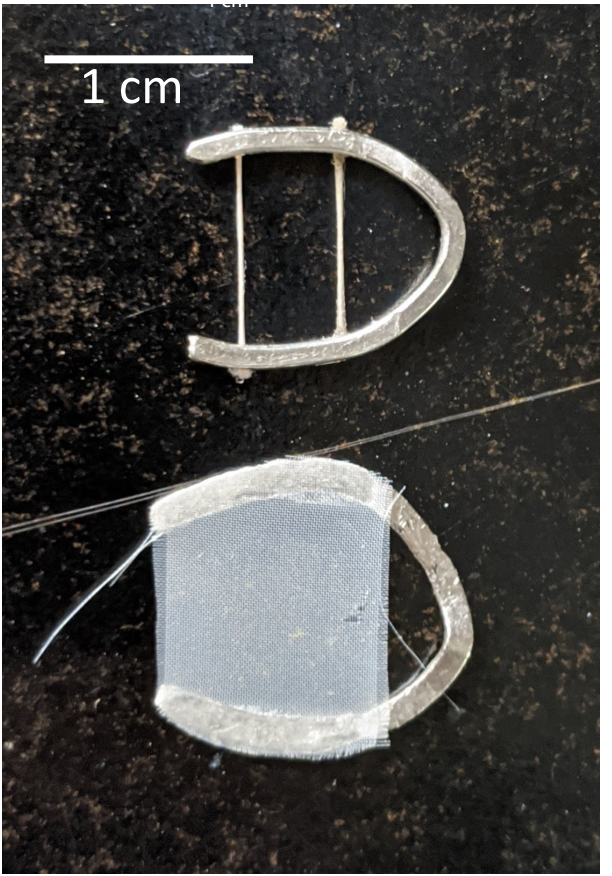

Supplementary Table 1

	λ	α
Halothane	1.45	1.34
Sevoflurane	0.45	0.42
Desflurane	0.32	0.30

Caitlin Murphy is a graduate research assistant in the lab of Dr. Matthew Banks in the Department of Anesthesiology at the University of Wisconsin.

Caitlin earned her bachelor's degree in biology and science education from the University of Wisconsin in 2014. During this time, Caitlin conducted research under the guidance of Dr. Michael Hammer, exploring the relationship between sensorimotor deficits in the larynx and dysphagia in people with Parkinson's Disease. Upon graduation, she taught an accelerated biology course for rising 9th graders with the PEOPLE program, a pre-college pipeline for students of color and low-income students. Caitlin also worked on an interdisciplinary collaboration between the LaFollette School of Public Affairs and the Department of Psychiatry, where she conducted a preliminary literature review in economics and the neurobiology of addiction to inform future policy-making decisions. Caitlin joined the lab of Dr. Matthew Banks in 2015, and she is currently working on her PhD thesis investigating the modulation of evoked synaptic and network responses by isoflurane in *ex vivo* brain slices.

Dr. Aeyal Raz is an Attending Physician in the Department of Anesthesiology at Rambam Health Care Campus.

Dr. Raz completed his medical studies at the Hebrew University in Jerusalem, where he also earned a PhD in neurobiology, both in 2001. Following an internship at the Tel-Aviv Sourasky Medical Center, he started his residency in the Department of Anesthesiology at Beilinson Hospital. During his residency he was awarded the prize for outstanding resident, received a top grade in the national step A board exams, and served as a chief resident. Following his graduation, he remained in the same department as a faculty anesthesiologist, and went on to become director of the residency program, where he was involved in teaching residents and medical students. In 2011, Dr. Raz began a research sabbatical at the University of Wisconsin-Madison, where he later joined the neuroanesthesia team, dividing his time between clinical work and neuroscience research.

In 2016 Dr. Raz returned to Israel, and joined the Department of Anesthesiology at Rambam Health Care Campus. He divides his time between clinical duties and his new research laboratory, investigating the effects of anesthetic drugs on the brain - The Neural Basis of Anesthesia Laboratory.

Dr. Matthew Banks is a Professor of Anesthesiology in the Department of Anesthesiology at the University of Wisconsin's School of Medicine and Public Health.

Dr. Banks earned his master's degrees in biomedical engineering at John Hopkins University in 1988. He earned his PhD in the lab of Dr. Phillip Smith at the University of Wisconsin, where he studied brainstem mechanisms of auditory processing and sound localization. Following his graduation in 1992, he remained at the University of Wisconsin as a postdoctoral fellow in the Department of Physiology under Dr. Meyer Jackson and in the Department of Anesthesiology under Dr. Robert Pearce. Dr. Banks was awarded a National Research Service Award from the National Institute of Neurological Disorders and Stroke in 1992 and 1994, for his study of inhibitory circuitry in hippocampus. Following his postdoc, Dr. Banks became a professor in the Department of Anesthesiology at the University of Wisconsin, where he now runs his own lab. His research interests involve understanding the neural circuitry underlying sensory processing, as well as the mechanisms underlying disruption of cortical processing during a variety of pharmacological and pathophysiological conditions, such as anesthesia, psychedelics, and delirium.

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