Video Article

A Guide to *In vivo* Single-unit Recording from Optogenetically Identified Cortical Inhibitory Interneurons

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Abstract

A major challenge in neurophysiology has been to characterize the response properties and function of the numerous inhibitory cell types in the cerebral cortex. We here share our strategy for obtaining stable, well-isolated single-unit recordings from identified inhibitory interneurons in the anesthetized mouse cortex using a method developed by Lima and colleagues[1]. Recordings are performed in mice expressing Channelrhodopsin-2 (ChR2) in specific neuronal subpopulations. Members of the population are identified by their response to a brief flash of blue light. This technique – termed "PINP", or Photostimulation-assisted Identification of Neuronal Populations – can be implemented with standard extracellular recording equipment. It can serve as an inexpensive and accessible alternative to calcium imaging or visually-guided patching, for the purpose of targeting extracellular recordings to genetically-identified cells. Here we provide a set of guidelines for optimizing the method in everyday practice. We refined our strategy specifically for targeting parvalbumin-positive (PV+) cells, but have found that it works for other interneuron types as well, such as somatostatin-expressing (SOM+) and calretinin-expressing (CR+) interneurons.

Video Link

The video component of this article can be found at https://www.jove.com/video/51757/

Introduction

Characterizing the myriad cell types that comprise the mammalian brain has been a central, but long-elusive goal of neurophysiology. For instance, the properties and function of different inhibitory cell types in the cerebral cortex are topics of great interest but are still relatively unknown. This is in part because conventional blind *in vivo* recording techniques are limited in their ability to distinguish between different cell types. Extracellular spike width can be used to separate putative parvalbumin-positive inhibitory neurons from excitatory pyramidal cells, but this method is subject to both type I and type II errors[2,3]. Alternatively, recorded neurons can be filled, recovered, and stained to later confirm their morphological and molecular identity, but this is a pain-staking and time-consuming process. Recently, genetically identified populations of inhibitory interneurons have become accessible by means of calcium imaging or visually guided patch recordings. In these approaches, viral or transgenic expression of a calcium reporter (such as GCaMP) or fluorescent protein (such as GFP) allows identification and characterization of cell types defined by promoter expression. These approaches use 2-photon microscopy, which requires expensive equipment, and are also limited to superficial cortical layers due to the light scattering properties of brain tissue.

Recently, Lima and colleagues[1] developed a novel application of optogenetics to target electrophysiological recordings to genetically identified neuronal types *in vivo*, termed "PINP" – or Photostimulation-assisted Identification of Neuronal Populations. Recordings are performed in mice expressing Channelrhodopsin-2 (ChR2) in specific neuronal subpopulations. Members of the population are identified by their response to a brief flash of blue light. Unlike many other optogenetic applications, the goal is not to manipulate circuit function but simply to identify neurons belonging to a genetically-defined class, which can then be characterized during normal brain function. The technique can be implemented with standard extracellular recording equipment and can therefore serve as an accessible and inexpensive alternative to calcium imaging or visually-guided patching. Here we describe an approach to PINPing specific cell types in the anesthetized auditory cortex, with the expectation that the more general points can be usefully applied in other preparations and brain regions.

In cortex, PINP holds particular promise for investigating the *in vivo* response properties of inhibitory interneurons. GABAergic interneurons comprise a small, heterogeneous subset of cortical neurons[4]. Different subtypes, marked by the expression of particular molecular markers, have recently been shown to perform different computational roles in cortical circuits[5-9]. As genetic tools improve it may eventually be possible to distinguish morphologically- and physiologically-separable types that fall within these broad classes. We here share our strategy for obtaining stable, well-isolated single-unit recordings from identified inhibitory interneurons in the anesthetized mouse cortex. This strategy was developed specifically for targeting parvalbumin-positive (PV+) cells, but we have found that it works for other interneuron types as well, such as somatostatin-expressing (SOM+) and calretinin-expressing (CR+) interneurons. Although PINPing is conceptually straightforward, it can be

surprisingly unyielding in practice. We learned a number of tips and tricks through trial-and-error that may be useful to others attempting the method.

Protocol

NOTE: The following protocol is in accordance with the National Institutes of Health guidelines as approved by the University of Oregon Animal Care and Use Committee.

1. Acute Surgery

1. Anesthetize the animal with a ketamine-medetomidine cocktail, via intraperitoneal (i.p.) injection (**Table 1**).
   NOTE: The mice used in these experiments are generated by crossing a cre-dependent ChR2-eYFP transgenic line10 to interneuron driver lines (Pvalb-iCre11, PV+; Sst-iCre12, SOM+; Cr-iCre12, CR+). Viral delivery of ChR2 or related opsins should work equally well, assuming similar expression levels are obtained.
2. Before beginning surgery, ensure that the animal exhibits no response to a gentle toe pinch. Re-administer anesthetics throughout the experiment as necessary to maintain this depth of anesthesia. If using injectable anesthetics, optionally implant an i.p. catheter for maintenance injections.
3. Keep the animal hydrated with saline or lactated Ringer's solution throughout the experiment (approximately 3 ml/kg/hr), for example by using an appropriately diluted anesthetic cocktail for maintenance injections (**Table 1**).
4. Place the animal in a stereotaxic or other head-holding apparatus. Ensure that the skull is well-secured. This is essential for maintaining stable single cell recordings.
5. Apply opthalamic ointment to the eyes to prevent dryness. Maintain body temperature at 36.5-37 °C.
6. Perform a cisternal drain for additional recording stability. Using a scalpel blade, remove tissue from the posterior face of the skull to expose the cisterna magna. Make a small nick in the dura to drain the cerebrospinal fluid. Use only the very tip of the blade, and avoid contacting the cerebellum or brain stem.
7. Using the scalpel, perform a small craniotomy (~2 mm^2) over the cortical area of interest (for auditory cortex, roughly -2.3 mm posterior of bregma, 4.5 mm lateral of the midline).
8. Remove the dura and cover the exposed cortex with a layer of warm agarose 0.5 - 1 mm thick (1.5% agarose in saline, 0.9% NaCl; apply at ~37 °C). Keep the agarose moist throughout the experiment by periodically applying several drops of saline.

2. Recording Set-up

1. Prepare a tungsten electrode (7-14 MΩ, 127 µm diameter, 12° tapered tip, epoxy-coated). Superglue the electrode to a glass capillary tube and add heat shrink tubing for grip (**Figure 1**).
2. Mount the electrode on a motorized (or hydraulic) micromanipulator, set to travel orthogonal to the cortical surface. Slide a wire ground under the skin, against the skull. Avoid contact with the muscles on the side of the head and the back of the neck as they can generate electromyographic artifacts.
3. Amplify the electrical signal using an extracellular amplifier suitable for single-unit recording, preferably equipped with an impedance check mode. Monitor ongoing spiking activity (band pass 300 - 5,000 Hz) with two oscilloscopes and a set of powered speakers. Here, the recorded data is digitized continuously at a sampling rate of 10 kHz; spikes are extracted offline.
4. Advance the electrode through the agarose until the tip reaches the surface of the cortex. Observe this step through a microscope. "Zero" this position on the micromanipulator.
5. To best approximate the depth of recording, visually confirm that the electrode tip exits the cortical surface at a depth of zero when withdrawing the electrode at the end of a penetration.
   NOTE: A discrepancy between the manipulator reading and the observed electrode position indicates that it has drifted relative to the tissue. This can be caused by instability of the brain or animal, or manipulator drift.
   1. As an additional check to ensure that this zero set-point corresponds to the surface of the cortex, monitor stimulus-evoked field potentials while advancing through the first several hundred micrometers of tissue. When monitoring field potentials, lower the lower band-pass filter cutoff of the extracellular amplifier to 10 Hz. Confirm that the polarity of the local field potential reverses around a depth of ~100 µm, near the layer I/II border[13]. This is a reliable reference point for auditory cortex, but it may differ for other cortical areas.
6. Mount an optical fiber coupled to a blue light source (**Figure 2**) onto a manual micromanipulator. Using the microscope, position the tip of the fiber as close to the surface of the agarose as possible. Center the beam where the electrode will enter the tissue.
7. Regulate the output of the light source with a control unit capable of delivering a TTL (transistor-transistor logic) pulse train of specified width and duration– *e.g.,* an Arduino (**Figure 3**), a computer I/O card (as shown), or a commercially available pulse generator.
   1. Monitor the signal on both oscilloscopes.
8. Measure total light power (mW) at the tip of the optical fiber using a power meter. Use this value to calculate irradiance (mW/mm^2) by dividing by the cross-sectional area of the fiber core. Begin with an intensity value in the range of 10 - 15 mW/mm^2 and adjust downward if necessary, until artifacts are eliminated.
9. Check for light artifacts with the electrode in the agarose, poised to enter the tissue. Start the search pulse train (*e.g.,* 30 msec light pulses, 500 msec interstimulus interval (ISI)).
   1. Eliminate transient light artifacts (**Figure 4**) by repositioning the optical fiber relative to the electrode to change the angle of incident light. If artifacts persist, try decreasing the light power. In the rare case that artifacts cannot be completely eliminated (only minimized), extend the duration of the search pulse. This can aid in identifying true neuronal spikes.

3. Straight PINP-in'

1. Advance the electrode slowly through the brain at a rate of approximately 1 µm/sec. Use one oscilloscope to monitor ongoing activity. On the other, trigger off the laser pulses.
2. Listen for the faint 'hash' of light-evoked spikes on the audio monitor, which indicates that the electrode is approaching a ChR2+ cell and can often be perceived well before the electrical signal is apparent on the oscilloscope. Slow the rate of advance.
   NOTE: If the cell lies directly in the path of the electrode, the light-evoked activity will grow larger (and louder). As the electrode approaches a single interneuron, the hash will resolve into small but well-defined spikes of uniform size and shape.
3. As soon as light-evoked spikes are large enough to trigger off of individually on the oscilloscope, begin doing so. Adjust the scaling on the horizontal axis to observe the exact shape of the spike waveform.
4. Stop and wait for the tissue to settle. Resist the temptation to move the electrode closer to the cell. This step is critical for a stable recording. If after several minutes the signal-to-noise ratio has not improved – that is, the tissue has settled, but it hasn't brought the cell any closer to the tip of the electrode – advance 5 µm further and wait again.
   1. Repeat this process until either the peak or the trough of the action potential can be reliably captured with a voltage threshold set well above the noise floor (*e.g.,* +/-300 µV or greater).
      NOTE: There's little risk of false-positives or ambiguity when PINPing inhibitory interneurons. Most cells can easily follow a train of pulses with duration of 30 msec and inter-pulse interval 500 msec, or faster, with a reliable first spike latency of 2-5 msec (**Figure 5**). The majority of PV+ cells, in particular, can sustain firing for a full 1 sec (**Figure 6**). Because these are inhibitory neurons, disynaptic (indirect) activation isn't a major concern.

5. While recording, monitor ongoing activity on the first oscilloscope. Keep a close eye on the size and shape of spikes using the second oscilloscope. Note the quality of their sound on the speaker.
   1. Listen attentively for abrupt changes, which indicate that the cell is either drawing too close to the electrode (where it risks being impaled or damaged), or is drifting away. If the spikes become large and distorted, back out; if they grow smaller, advance the electrode. Move slowly in 2 µm steps.
   2. Confirm the quality of the recording post-hoc by superimposing all spike waveforms, aligned to peak or trough. Vary the voltage threshold. Across a wide range of threshold values, there should only ever be one consistent spike shape of uniform height (**Figure 5a**).

6. If at any point the signal becomes contaminated with spikes from a neighboring neuron, move on. Advance slowly, on the off-chance that the electrode will pass out of range of the neighboring cell, but remain in range of the target neuron. (This is usually unsuccessful.)
7. Move the electrode through the entire depth of cortex (900+ µm) in each penetration. If no light-responsive neurons are encountered after many penetrations or, conversely, recordings are routinely contaminated with spikes from neighboring ChR2- cells, try a new electrode.
   NOTE: Even within a single batch, the impedance and tip geometry of individual electrodes can vary considerably. Both factors contribute to the effective "listening radius" of the electrode, which must be large enough to detect light-evoked spikes while searching, yet restricted enough to enable recording a single interneuron in isolation.
8. Test the impedance of electrodes as desired. To avoid damaging the tissue, do this with the tip of the electrode in the upper part of the agarose layer, well outside the brain. Within the range of 7-14 MΩ, the exact impedance is not a sure predictor of performance, or yield, for this application. That said, it's a reasonable proxy for listening radius: lower-impedance electrodes pick up more units; higher-impedance, fewer.
9. At the end of the experiment, euthanize the animal by institutionally-approved means, such as anesthetic overdose, or cervical dislocation under a deep plane of anesthesia.

Representative Results

We here share our strategy for obtaining single-unit recordings from genetically-classified inhibitory interneurons in the anesthetized mouse cortex, using an optogenetic method developed by Lima *et al.*[1]. **Table 1** details the suggested anesthetic cocktail, Ketamine-Medetomidine-Acepromazine ("KMA"). **Figure 1** depicts a tungsten microelectrode, prepared for recording. **Figure 2** contains a circuit diagram for a simple LED control unit. **Figure 3** contains the configuration and code for gating light output with an Arduino microcontroller. **Figure 4** shows an example of the light-induced electrical artifacts discussed in the Protocol. Artifacts are occasionally encountered (left panel), but easily corrected (right panel). **Figure 5** shows example recordings from three types of optically-identified interneurons (PV+, CR+, and SOM+). The left panel shows raw voltage traces and aligned waveforms, representative of the kind of signal-to-noise ratio one can expect to obtain with this strategy. Spikes are reliably captured with a fixed voltage threshold of several hundred microvolts. A raster plot (right panel) shows the short latency and reliability of light-evoked responses, which allow for unambiguous identification of ChR2-positive interneurons. **Figure 6** shows voltage traces from three PV+ interneurons, responding to a 1 sec light pulse. The majority of PV+ cells can sustain high-frequency firing for hundreds of milliseconds, making them particularly easy to identify.

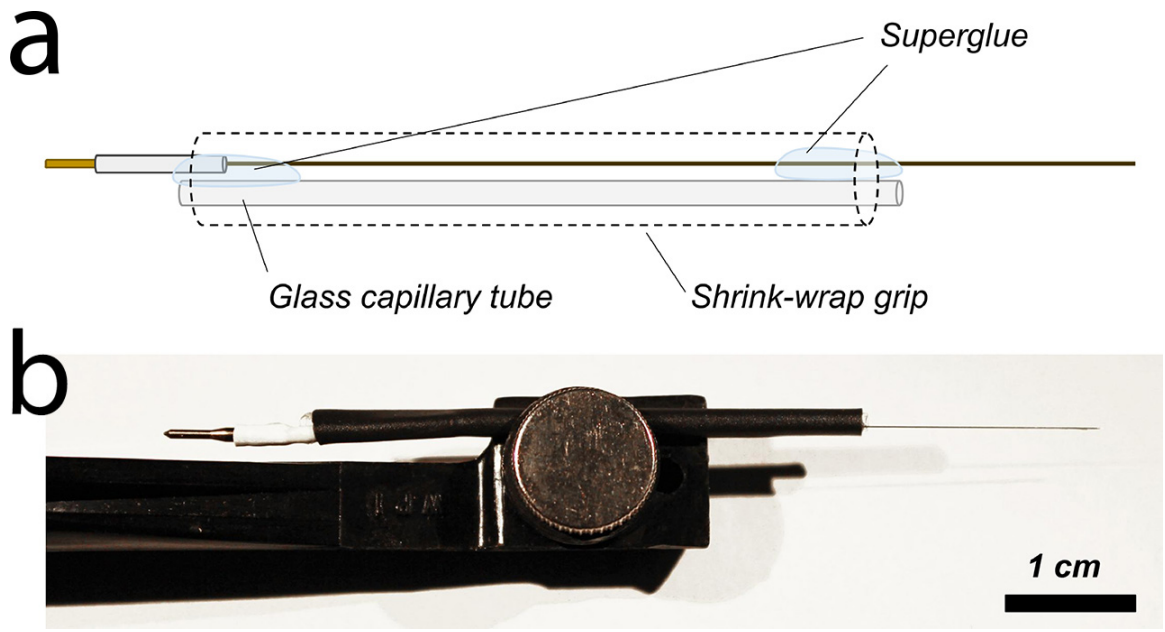


Figure 1. Preparing an electrode. (A) Diagram of a prepared electrode. The electrode is affixed to a glass capillary tube using two small drops of Super Glue and a minimal amount of accelerator (such as Zap-It). Heat shrink tubing is added for grip, using very low heat. Electrodes typically ship with a connector pin pre-attached. **(B)** Photograph of a prepared tungsten microelectrode mounted in an electrode holder.

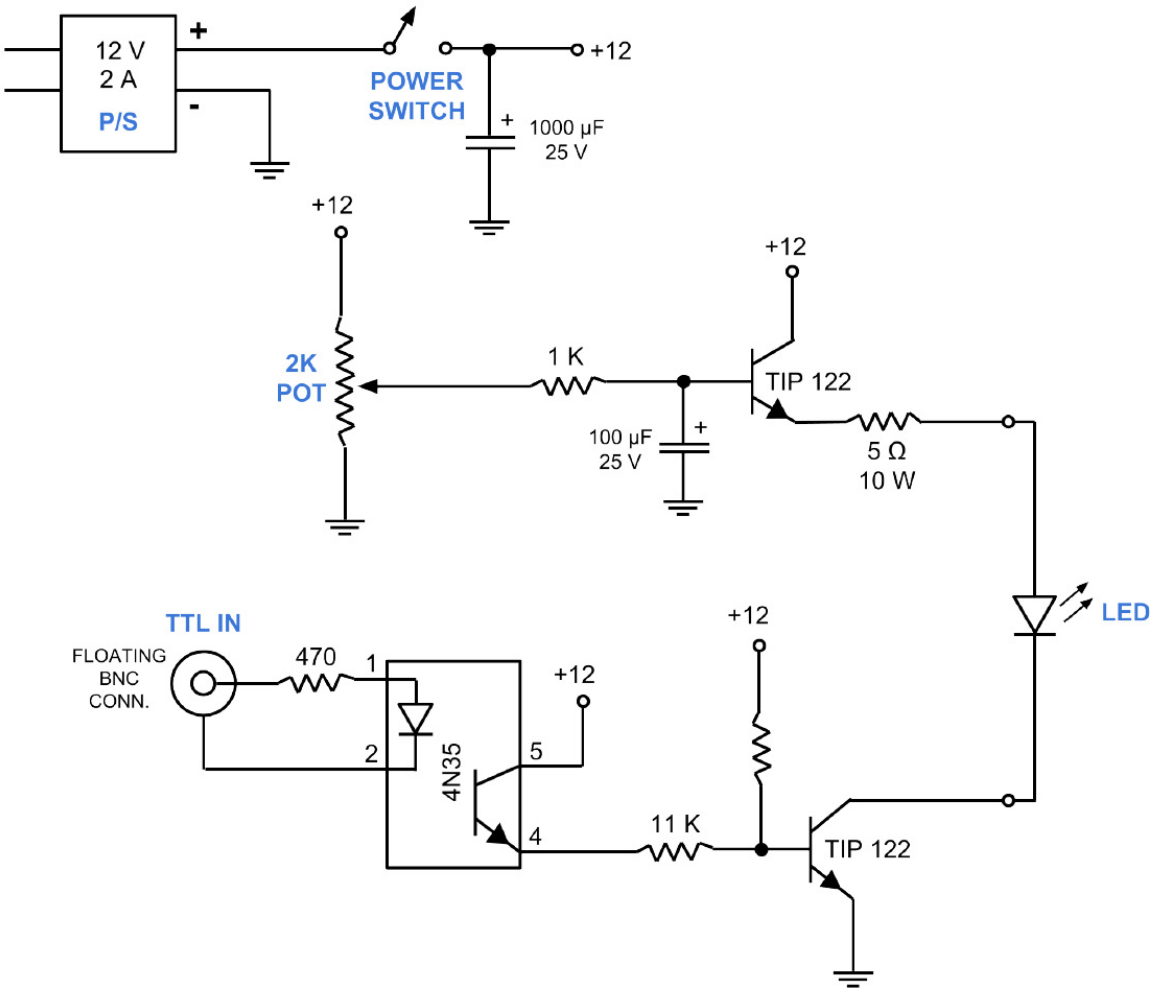
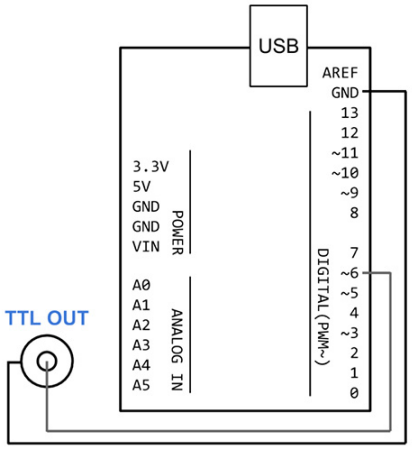


Figure 2. Circuit diagram for an LED control unit. A simple and inexpensive circuit for light delivery. A super-bright LED (475 nm) is attached to a heat sink and coupled to the other end of a fiber optic cable (800 µm diameter). The output of the LED is gated with a TTL pulse. The control unit incorporates a voltage-mode power supply for adjustable light intensity. 2K POT: potentiometer to control light intensity. P/S: DC power supply (*e.g.*, laptop charger). TIP 122: Darlington transistor. 4N35: Optical isolator. The parts listed here cost <$10.




```
const int LED = 6;
int pulseDuration = 1000; // Light pulse duration & ISI in ms.
int ISI = 10000;
int on=0; // Pulse train looping (1), pulse train off (0).

void setup()
{
  pinMode(LED, OUTPUT); // Specify pin for digital output.
}

void loop()
{
  if (on==1)
  {
  digitalWrite(LED, HIGH); // LED on.
  delay(pulseDuration);
  digitalWrite(LED, LOW); // LED off.
  delay(ISI);
  }
}
```

Figure 3. Arduino code and configuration. A TTL pulse train for gating light output can be generated using an Arduino microcontroller. The program specifies a pulse duration and ISI for a looping pulse train. Instructions for loading the program onto the board can be found on Arduino's "Getting Started" page (http://arduino.cc/en/Guide/HomePage). Please click here to view a larger version of this figure.

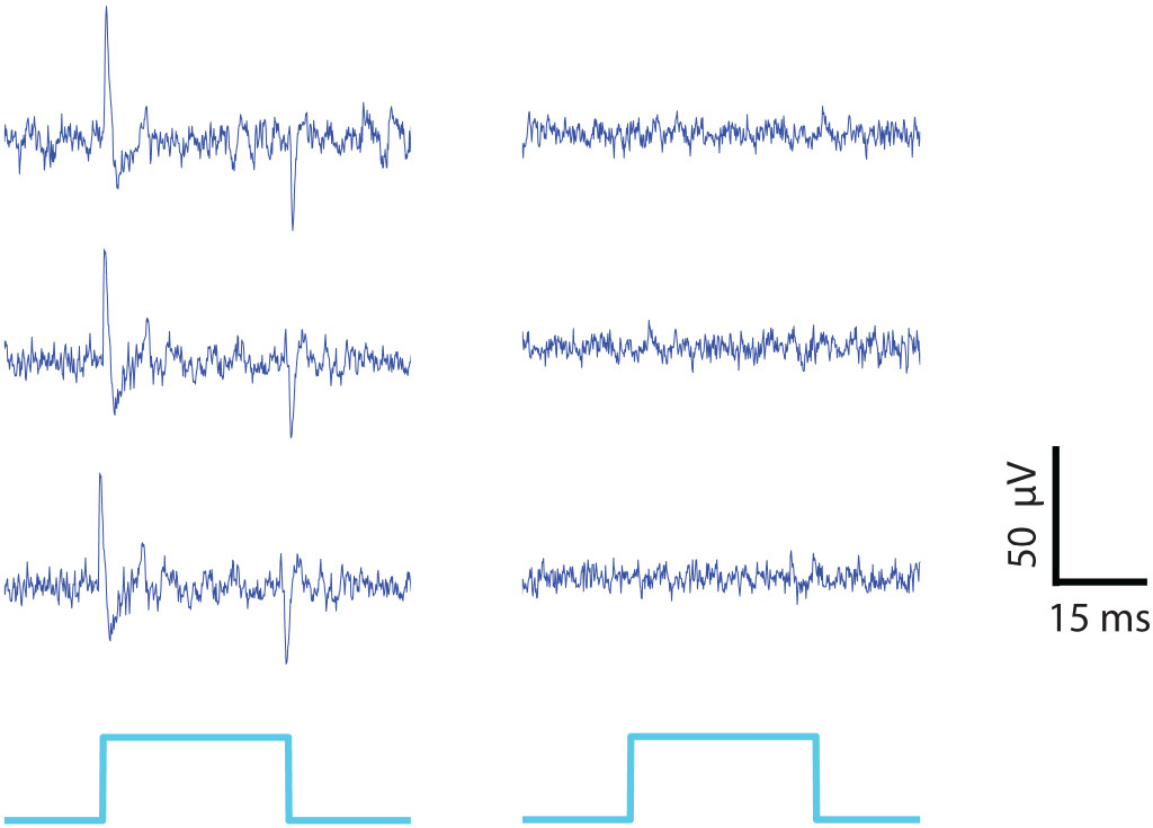


Figure 4. Light artifact. Metal electrodes are susceptible to light artifacts, present at the onset and the offset of pulses (left panel; filtered signal, 300 - 5,000 Hz). They can usually be eliminated completely (right panel) by repositioning the optical fiber relative to the electrode to change the angle of incident light, and/or decreasing the light power.

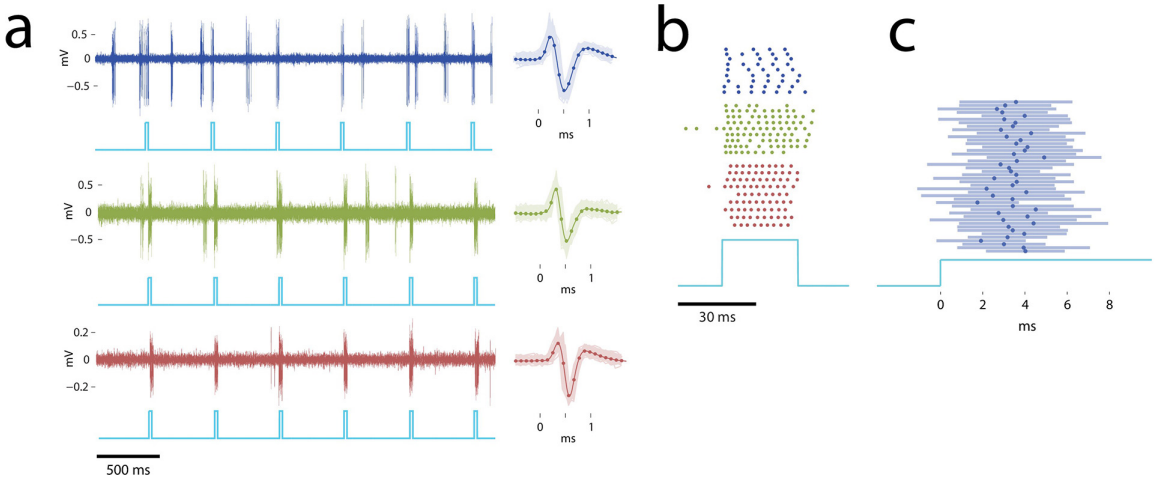


Figure 5. Example cells. (A) Typical example recordings from optically-identified interneurons, PV+ (purple), CR+ (green), SOM+ (red). Cells respond to the search pulse train (pulse duration 30 ms, ISI 500 ms) with a reliable burst of spikes. The panel to the right shows 100 trough-aligned spikes and the average interpolated waveform (10x oversampled from a sampling rate of 10 kHz). **(B)** Raster plots for the same files show the short latency (~2 - 5 msec) and consistent timing of light-evoked spikes. **(C)** Mean and standard deviation of first-spike latencies (5 - 10 pulses) for a sample of 44 PV+ interneurons (average mean and standard deviation: 3.4 +/- 2.6 msec). Please click here to view a larger version of this figure.

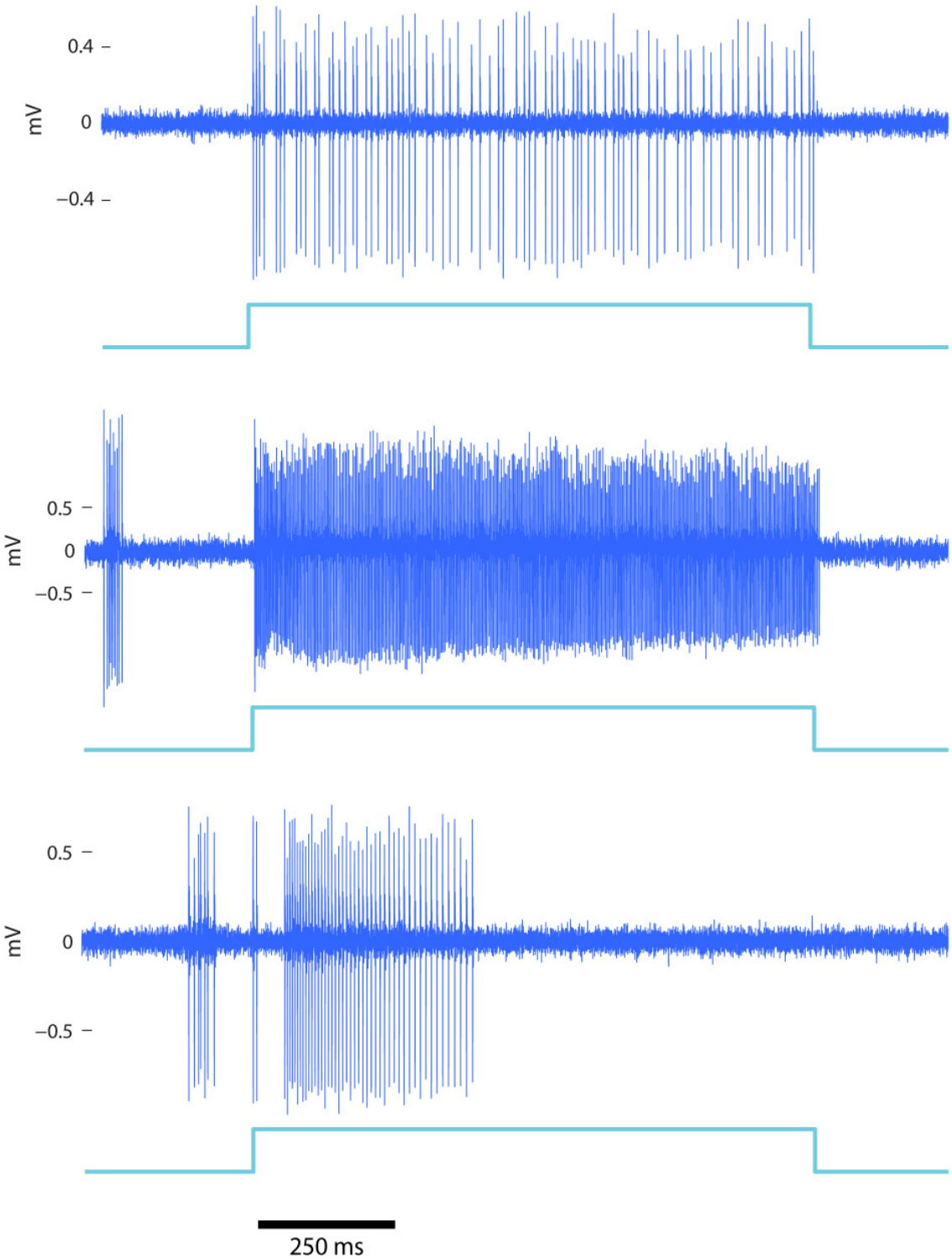


Figure 6. Examples of sustained responses. One can be especially confident in identifying PV+ interneurons as the majority of them can sustain high-frequency firing for hundreds of milliseconds – reminiscent of their responses to current injection *in vitro*[14]. Three ChR2/PV cells responding to 1 sec light pulses: two that fire regularly throughout the entire duration of the pulse, and one unusual example of a cell that does not (<10% of encountered cells).

KMA (20.0 ml)
2 ml of 100 mg/ml Ketamine (10 mg/ml, once diluted)
0.4 ml of 1 mg/ml Dexdomitor (medetomidine; 0.02 mg/ml, once diluted)
0.05 ml of 100 mg/ml Acepromazine (0.25 mg/ml, once diluted)
17.55 ml of 0.9% saline
Dosage, adult mouse (15 - 40 g)
Knock-down: 0.012 ml/g * body mass (Ketamine = 120 mg/kg)
Maintenance: 0.0025 ml/g * body mass (Ketamine = 25 mg/kg)

Table 1. **Ketamine-Medetomidine-Acepromazine ("KMA").** Knock-down and maintenance dose for an adult mouse, diluted to maintain hydration. The cocktail is re-administered every 40 - 90 min, as necessary.

Discussion

Although PINP is conceptually straightforward, it can be challenging in practice. A major determinant of success is the choice of electrode. The electrical listening radius is the critical parameter. It must be sufficiently large to detect light-evoked spikes when the tip is still some distance away from a ChR2+ cell, so that one can adjust the rate of advance accordingly. At the same time, it must be restricted enough to enable good single-unit isolation. That is, the electrode must not also pick up spikes from neighboring ChR2- units. Striking the right balance in terms of listening radius will be a challenge for any targeted cell type, but it is especially true for inhibitory interneurons, which are sparse, small, and often found in close proximity to pyramidal cells, which that have comparatively large extracellular spikes. Even using the strategies suggested here, the expected yield is not high. Our typical yield was 0-2 PV+ neurons/animal, given our criteria of excellent single-unit isolation and recordings lasting over half an hour.

When PINPing inhibitory neurons, disynaptic (indirect) activation isn't a major concern, because inhibitory neurons are relatively unlikely to indirectly activate postsynaptic cells. For PINPing excitatory neurons, disynaptic activation is common; many neurons that fire in response to light do not express ChR2, but rather receive powerful synaptic excitation from those that do. Strategies for discriminating directly- from indirectly-activated neurons are discussed in detail in Lima *et al.*[1].

Electrode Considerations

We systematically explored a variety of electrodes and recording techniques: glass patch pipettes with tips of different sizes, tungsten microelectrodes varying in impedance and tip geometry, and multi-channel arrays (*e.g.*, tetrodes). Angular, high-impedence tungsten microelectrodes (as suggested here) gave, by far, the best yield. A discussion of the issues encountered with each of these techniques follows.

Using low-impedance tetrode arrays and multi-channel probes, we routinely detected light-evoked spikes; however, they were rarely large enough to sort reliably. In other words, the signal-to-noise ratio (SNR) was unsatisfactory. We did occasionally get excellent recordings with tetrodes, but the asymptotic yield was higher with single tungsten microelectrodes, and the quality of recordings was much better. Tetrode tips are comparatively blunt as well, which limited the total number of penetrations possible per animal. The issues we encountered with multi-channel probes were probably exacerbated by the use of broad surface illumination. Unlike targeted, low-power stimulation from an implanted fiber, surface illumination will cause synchronous firing in ChR2+ neurons beyond the recording site, leading to superimposed spike waveforms that are more difficult to sort.

Glass patch electrodes, like those used for standard loose cell-attached recordings, were also not well-suited to the task, but for different reasons. Low resistance (<5 MΩ) patch electrodes are strongly biased toward pyramidal cells, which makes it difficult to target inhibitory interneurons. While this can be corrected to some extent by using higher resistance (smaller tip) electrodes, the greater problem with patch electrodes is their extremely restricted listening radius. Although cell-attached patch recordings provide a very high SNR, light-responsive cells cannot be detected outside of cell-attached mode. The strategy with patch electrodes is, inevitably, to randomly and sequentially obtain cell-attached recordings, very few of which are ChR2-expressing inhibitory interneurons.

With high impedance tungsten electrodes one can both detect light-evoked spikes from a distance, and achieve good single-unit isolation. The geometry of the tip is probably the most important factor. We tried tungsten electrodes from two manufacturers, with impedances ranging from 2 - 14 MΩ, and tip angles from 5-12°. We found that 12° tip angles were superior, but that the precise impedance was less important, within the range of 7 - 14 MΩ. We found that glass-coated electrodes were more susceptible to light artifacts compared to epoxy-coated electrodes, so we recommend the latter. We did not attempt to make our own custom tungsten electrodes. As with any type of electrode, common modes of failure are to lose isolation or injure a neuron. Different tip parameters do not appear to have much influence on this. Rather, waiting for the tissue to settle is the more important factor in maintaining high-quality recordings.

Light Delivery

Because light is used only as a means of identifying ChR2-positive cells – rather than regulating their output in a specific manner – the range of usable intensities for this experiment is wide. A lower limit is set by the fact that the intensity must be sufficient to activate ChR2-positive cells at the maximum depth of your cells of interest. We found that tip irradiances >5 mW/mm^2 (measured at 470 nm) were sufficient to elicit robust responses from PV+ cells in deep Layer 6. Irradiance at specific depths within the brain can be estimated using a calculator based on direct measurements in mammalian brain tissue (*e.g.,* http://www.stanford.edu/group/dlab/cgi-bin/graph/chart.php), but we strongly recommend determining the minimum for your own experiments empirically, using a low impedance microelectrode (1 - 3 MΩ, for multiunit activity). An upper

limit is set by the fact that high intensities can cause light artifacts, which can complicate the detection of evoked spikes. In transgenic animals, high intensities can also cause electromyographic artifacts by activating ChR2 in exposed PV+ muscle on the neck and head.

In building our own light delivery system, we found that a coupling an LED to an optical fiber is ultimately more convenient than placing the LED directly over the brain. The latter is a very effective way to deliver light, but has some practical disadvantages. The current flowing through the device produces electrical artifacts that must be shielded, and the LED generates substantial heat that must be dissipated with a large heat sink. Moreover, light artifacts (both photovoltaic and electromyographic) are much more difficult to control with the broad illumination pattern of LEDs. Fiber coupling solves all of these issues.

Disclosures

The authors have no competing financial interests.

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