Video Article

A Procedure for Implanting Organized Arrays of Microwires for Single-unit Recordings in Awake, Behaving Animals

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Abstract

In vivo electrophysiological recordings in the awake, behaving animal provide a powerful method for understanding neural signaling at the single-cell level. The technique allows experimenters to examine temporally and regionally specific firing patterns in order to correlate recorded action potentials with ongoing behavior. Moreover, single-unit recordings can be combined with a plethora of other techniques in order to produce comprehensive explanations of neural function. In this article, we describe the anesthesia and preparation for microwire implantation. Subsequently, we enumerate the necessary equipment and surgical steps to accurately insert a microwire array into a target structure. Lastly, we briefly describe the equipment used to record from each individual electrode in the array. The fixed microwire arrays described are well-suited for chronic implantation and allow for longitudinal recordings of neural data in almost any behavioral preparation. We discuss tracing electrode tracks to triangulate microwire positions as well as ways to combine microwire implantation with immunohistochemical techniques in order to increase the anatomical specificity of recorded results.

Video Link

The video component of this article can be found at https://www.jove.com/video/51004/

Introduction

Electrophysiological recordings allow scientists to examine the electrical properties of biological cells. In the central nervous system, where electrical impulses serve as a signaling mechanism, these recordings are of particular importance for understanding neural function[1-2]. During single-unit recordings in behaving animals, a microelectrode that has been inserted into the brain is able to record changes in a neuron's generation of action potentials over time.

While many techniques allow one to record brain activity, single-unit electrophysiology is one of the most precise methods by allowing resolution at the single neuron level. When a high degree of spatial specificity is desired, microwires can be used to target discrete sub-nuclei or ensembles of cells within the brain3. Single-unit recordings also benefit from high temporal resolution as recordings are accurate at the microsecond level. And, *in vivo* awake recordings allow intact circuit interactions, with the natural milieu of afferent and efferent projections, systemic chemical and hormonal influences, and physiological parameters. Neural signals are derived from sensory input, motor behaviors, cognitive processing, neurochemistry/pharmacology, or some combination. Accordingly, the segregation of sensory, motor, cognitive, and chemical influences necessitates well-conceived experiments with effective contingencies and controls that may allow for the assessment of each of the aforementioned influences. All in all, recordings in behaving animals allow experimenters to observe the integration of multiple sources of information within a functioning circuit and to derive a more comprehensive model of circuit function.

Single-unit recordings also suffer from a number of disadvantages of which any experimenter should be aware. First and foremost, recordings can be difficult to conduct. Indeed, properties of the headstage amplifiers and the implanted microwires that allow for spatial and temporal specificity in these recordings also makes recordings susceptible to the influence of extraneous electrical signals (*i.e.* electrical "noise"). Accordingly, the ability to troubleshoot problems in an electrophysiological system necessitates a well-developed technical understanding of electrophysiological principles and apparatus. It is also important to note that, under certain circumstances, recorded electrical signals in extracellular recordings can represent the summation of multiple neural signals. Moreover, the generalizability of single-unit activity to population activity within a target region can often be limited by the degree of cellular heterogeneity within the target region (but see Cardin[4]). For example, electrodes might be biased towards recording high amplitude output neurons in lieu of other cells. The interpretability of single-unit recordings is increased by combining recordings with other techniques including, but not limited to, electrical (orthodromic or antidromic), chemical (*e.g.* iontophoretic or designer receptor) or optogenetic stimulation[4], temporary neural inactivations, sensorimotor examinations[5], disconnection procedures, or immunohistochemistry[3].

In the protocol that follows we will enumerate the materials and steps necessary to implant an organized microwire array in the rat (although the protocol can be adapted for use in other species). The procedure and style of fixed arrays used in our laboratory have proven reliable

for longitudinal recordings and can sustain recordings of the same neuron for over one month's time [6-8]. This makes this procedure ideal for examining phasic responses to experimental stimuli, plastic changes in neural responses, or mechanisms of learning and motivation.

Protocol

The utmost care must be taken to maintain aseptic conditions (as described in the Guide for the Care and Use of Laboratory Animals[9]) while preparing for and conducting the following procedure. The following protocol is in compliance with the Guide for the Care and Use of Laboratory Animals and has been approved by the Institutional Animal Care and Use Committee, Rutgers University. It is estimated that the subsequent procedures will require 3-6 hr to complete.

Implanting the Microwire Array:

1. Place animals under anesthesia using 50 mg/kg sodium pentobarbital (i.p) and administer 10 mg/kg of atropine methyl nitrate (IP; Glycopyrrolate may be substituted) and 0.25 mg penicillin (300,000 U/ml i.m.) to maintain respiratory function and prevent infection, respectively.
   Note: With the 3-6 hr length of this implantation surgery, sodium pentobarbital is used because it is cost-effective and limits human exposure to anesthetics (as might occur with prolonged use of gas anesthetics) while still providing long-lasting anesthesia. Substitution of other anesthetics is acceptable.
2. Verify that the anesthesia has taken effect using the tail pinch test before proceeding.
3. As necessary, give alternating injections of ketamine hydrochloride (60 mg/kg IP) and sodium pentobarbital (5-10 mg/kg IP) to maintain anesthesia throughout the surgery.
4. Shave the scalp using a #22 scalpel blade.
5. Disinfect the shaved scalp with povidone iodine.
6. Give subcutaneous injections of bupivacaine (~1 mg/kg SC spread over 4 injection sites) to locally anesthetize the scalp. Allow 5-10 min for the local anesthetic to take effect.
7. Place an ophthalmic lubricant over the eyes to maintain moisture during anesthesia.
8. Secure the animal into the ear bars and nose clamp of a stereotaxic apparatus.
9. Use visual landmarks (*e.g.* a horizontal bar on the stereotaxic frame) to approximately level the animal's head. This step is only meant to *approximately* level the skull.
10. Make an incision along the midline of the scalp using a #11 scalpel blade mounted on a scalpel holder. The incision must extend from just behind the ears to the posterior portion of the nasal bone.
11. Using a dissection spatula, clear the skull of all remaining tissue until both lateral skull ridges and the posterior skull ridge have been reached.
12. Pull back the skin around the incision using a number of hemostats (6x).
13. Clean the skull of any blood and allow it to dry. If any remaining bleeding occurs, terminate the residual bleeding with a small cauterizing tool. Ensuring that the skull remains clean and dry allows the dental acrylic used in subsequent steps to bind to the skull permanently.
14. Mark bregma and lambda (**Figure 2D**) by interpolating the intersection of the skull sutures. A dissecting microscope is necessary to accurately mark these positions.
15. Attach a small pointed item (*e.g.* a pin or dental drill bit) to a stereotaxic arm and lower it to determine the dorsal/ventral (DV) coordinate of bregma and lambda.
16. Adjust the nose clamp until the DV coordinates for bregma and lambda are within 100 µm (0.1 mm) of each other.
17. Once leveled, record the anterior/posterior (AP), medial/lateral (ML) and DV coordinates of bregma along with the position of the nose clamp. Double check the coordinates for accuracy, as the remainder of the surgery depends on the precision of these coordinates.
18. Use the measured coordinates to calculate the ML and AP coordinates for the four corners of the "skull window" relative to bregma (*i.e.* craniotomy; **Figure 1**). The skull window is a precisely positioned rectangle through which the microwire array will pass.
19. Use the calculated coordinates to mark the skull window coordinates on the skull using a pointed stereotaxic attachment and fountain pen ink. To obtain precise marks, apply only a small amount of ink to the tip of the marking tool using a cotton applicator.
20. Drill out the skull window by removing the bone in a series of small layers.
    1. Start by drilling the marked corners of the window where the marks have been placed.
    2. Next, connect the corners and outline the window.
    3. Finally, clear out the area within the outline down to the depth of dura mater. The hole must be wider (*i.e.* beveled) below the superficial layers of the skull to ensure that the window maintains an appropriate width from top to bottom.
21. Use microforceps to carefully remove any remaining bone chips, debris, or dura mater inside the skull window. This step is extremely important, as these bits of material can compromise the integrity of the array during lowering. Once cleared, one must keep the window moist with bacteriostatic saline for the remainder of the surgery.
22. Place markings on the skull for 5 skull screws and 1 ground wire (**Figure 1**). These positions will vary depending on the targeted brain region. The headstage will be most secure if one screw is placed on each of the 5 skull bones. Place both the skull screws and ground wire in locations that will not interfere with the microwire array placement.
23. Drill holes for the skull screws and secure the screws in place. Screws must be lowered until only 3-4 threads are showing (or, if using screws other than those recommended, deep enough to traverse the thickness of the skull to promote array stability but not too deep so as to damage cortex). Clean the threads of the screws after placement.
24. Drill a hole for the ground wire. Lower the wire slowly (over 1-2 min) to the target DV coordinate and fix the wire in place using dental acrylic.
25. Add acrylic around the threads of the skull screws. Before the acrylic dries, remove any excess cement that flows away from the ground wire or skull screws. Allow 15 min for the dental acrylic to dry/harden.
26. Attach the array to the stereotaxic arm. Level the array and orient it so that it will squarely pass through the confines of the skull window.
27. Place saline in the skull window so that it is level with the skull. Lower the array until it creates a dimple in the saline. This coordinate is used as skull level for the array. Use this value to calculate the final DV coordinate of the array.

28. Lower the array slowly until it reaches its final DV coordinate. Stop every 1mm of lowering and wait for several minutes in order to allow brain tissue to recover from dimpling and to dissolve the bottom portion of the polyethylene glycol (PEG) on the array (PEG is used to temporarily keep the wires in their conformation while lowering, and dissolves slowly in saline).
29. When the array is 1mm away from the target, lower it more slowly until the final target is reached. Lowering at 0.1-0.2 mm at a time before allowing the tissue to rest for a period of 5 min is intended to preserve the tissue at the target site as well as proximal synaptic connections. If the equipment is available, precision lowering of the array can be assisted using a motorized manipulator.
30. Dissolve the remaining PEG and use dental acrylic to cement the microwires in place. Add multiple layers of cement to ensure that the wires are secure and then allow 15-20 min for the cement to harden before proceeding. This ensures that the wires will not be jostled from their final placement.
31. Build the remainder of the animal's headstage using dental acrylic. Use the acrylic to encase the skull screws, ground wire, and connector for the microwires. Allow the acrylic hat to harden sufficiently before proceeding.
32. Suture the scalp incision using absorbable sutures and administer 2 ml of bacteriostatic saline (s.c.) to restore hydration from surgery.
33. Remove the animal from the ear bars and place the subject in a clean area. Observe the animal frequently during the post-operative recovery until thermoregulation and locomotion have recovered. Following recovery from anesthesia, move the animal into single-housing for the remainder of post-surgical recovery.
34. Give animals daily postoperative monitoring and care in the days following the procedure. Recovery from this procedure is optimal when seven or more days are allowed.
35. Give animals injections of Carprofen (5mg/kg) and Enrofloxacin (5-10 mg/kg) or their equivalents during recovery per the schedule recommended by the attending veterinarian.

Representative Results

A list of Equipment used by this laboratory for recording electrophysiological signals can be found in **Table 3**. Following recovery from surgery, single-units are recorded by plugging a unity-gain headstage into the implanted connector. This headstage is connected via a cable to a commutator, which is capable of free rotation without breaks in the electrophysiological recording through the use of electrical slip rings. The commutator allows subjects to freely move while recording during behavior, which is one of the principle advantages of this preparation. Signals are then fed through a preamplifier (10x gain) that is used to differentially amplify the signal on each electrode against the ambient noise on an electrode selected because it does not exhibit a unit signal. Differential recordings are used to amplify the instantaneous difference between the two voltages. Because both wires record ambient noise approximately equally, their opposite polarities effectively enable subtraction of ambient noise from neural recordings (**Figure 2**). Finally, signals are band-pass filtered (450 Hz to 10 kHz; roll off -1.5 dB/octave at 1 kHz and -6 dB/octave at 11 kHz) and amplified 700x before being digitized using a computer with an A/D card (50 kHz sampling frequency/wire) and stored for offline analysis.

In software, waveforms are often sampled using a threshold detection method. Thus, signals which pass a given voltage threshold are digitized and stored. Still, there is an inherent rate of false-positives, in which electrical artifacts pass through the threshold, filtering and differential amplification, and are sampled. These non-neural waveforms are subtracted post-hoc using spike-sorting software which allows sampled discharges to be sorted based on their parameters (*e.g.* amplitude, peak and valley voltages, *etc.*) and subsequently allows for the elimination of electrical "noise" and the selection of the neuronal signal (**Figure 3**). Waveforms are included for analysis if 1) Waveforms present with canonical patterns of neural activity including a clearly defined action potential and after hyperpolarization (**Figure 3**; right panel); 2) the amplitude of a putative neural waveform exhibits at least a 2:1 signal:noise ratio (*i.e.* is clearly separable from the channel's noise band; amplitude of noise band = approx.. 50 μV); 3) Parameters remain stable throughout the entire session and 4) an interspike interval (ISI) histogram shows that no discharges occurred during the neuron's natural refractory period (*i.e.* ~2 msec; **Figure 3** left panel).

Neural discharges can then be correlated with behavioral events (*e.g.* lever responding) that are recorded as timestamps via the use of a digital input-output (I/O) card. These time-stamps can be used to create peri-event time histograms (PETHs; **Figure 4A**), which are used to display neuronal discharges that occur in a specified time range around a particular behavioral event. Our laboratory has typically examined neural firing on the order of hours (Tonic[10]), minutes (Slow-Phasic[11]), and seconds/milliseconds (Rapid-Phasic[12]). Tonic firing is typically used to examine the most global events in a session. For example, we often use this to compare firing rates of neurons when animals are self-administering drug, *i.e.* predrug versus on-drug firing rates. Slow phasic analyses are often used to examine firing during slow changing events like the pharmacological decay of a drug over minutes. Finally, rapid phasic analyses are used for more instantaneous events like an operant response or the onset of an experimental cue.

Figure 4 shows a histogram of tonic firing with a count of discharges in 30 second bins across an entire recording session during cocaine self-administration. The example cell shown is sensitive to changes in body levels of cocaine and becomes inhibited (**Figure 4A**) as the animal's body level of cocaine increases (**Figure 4B**), but returns to its original firing rate as the level of cocaine falls after the self-administration contingencies have ended. Depending on the target region, neurons may be sensitive to pharmacological effects, or any number of behavioral events including reward-associated cues[6], sensorimotor input[13], or motivated approach[3].

Under the correct conditions, it is possible to record the same neuron for many sessions. In the striatum, neurons are homogeneously distributed (80% of wires yield only a single unit and cells are not layered or tightly grouped) and produce relatively small signals that quickly decay over distance. Under these conditions, our laboratory has been able to record the same neuron in the striatum for multiple weeks. Importantly, not all neurons can be verified across sessions and not all neurons are maintained over time. Determining one's ability to obtain these types of longitudinal recordings requires careful testing of the electrophysiological system. For example, it is possible to estimate the recording distance of microwires by using drivable electrodes. When considered alongside the physical properties of the wires, regional anatomy, and the waveform parameters of the neuron across sessions, one can determine with reasonable confidence that a waveform has been maintained across sessions[7,8,13].

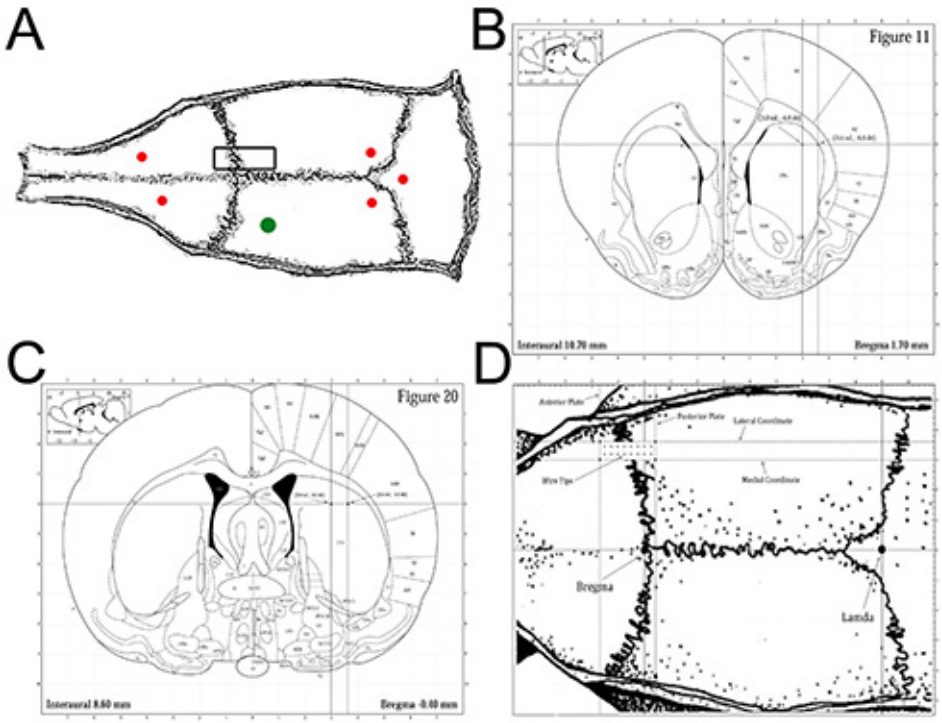

Figure 1. Representative placements for the "skull window" (*i.e.* craniotomy), skull screws, and ground wire. A) A dorsal view of the skull showing the placement of a skull screw on each plate of the skull (red), a skull window (square), and a contralateral hole for the ground wire (green). When planning the skull window, the coordinates should be defined by determining the **B**) anterior and **C**) posterior medial and lateral coordinates for the target region (*e.g.* dorsolateral striatum). **D**) These coordinates can then be used to determine the placement of the window on the dorsal surface of the skull. Note that the skull window corresponds to the medial and lateral targets identified in **B** and **C** and also spans the specified range in the anteroposterior plane between **B** and **C**. These coordinates will be specific to each individual target region. Representative locations of the microwires in a 2 x 8 array are shown using small dots within the skull window shown in **D**. The final dorsoventral coordinate of the wires will depend on the depth to which they are lowered. Figures modified from Paxinos & Watson[14]. Click here to view larger image.

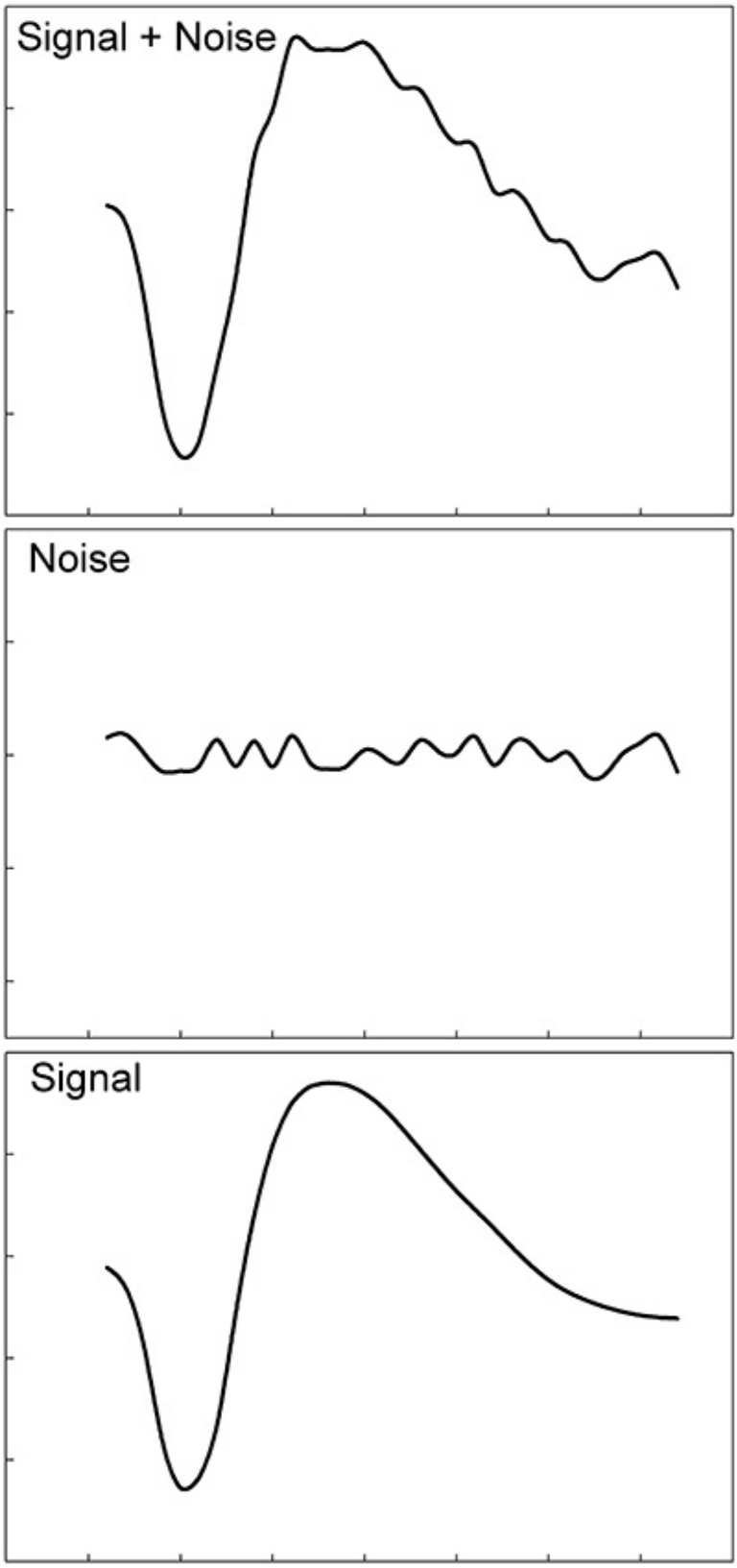

Figure 2. An illustration of the process of differential amplification. The topmost panel represents a neural signal prior to differential amplification (Signal + Noise). Following differential amplification, extraneous electrical signals that are found on all channels (Noise; middle panel) can be effectively subtracted from recording wires via the use of a specified differential electrode in order to improve isolation of the neural waveform (Signal; bottom panel).

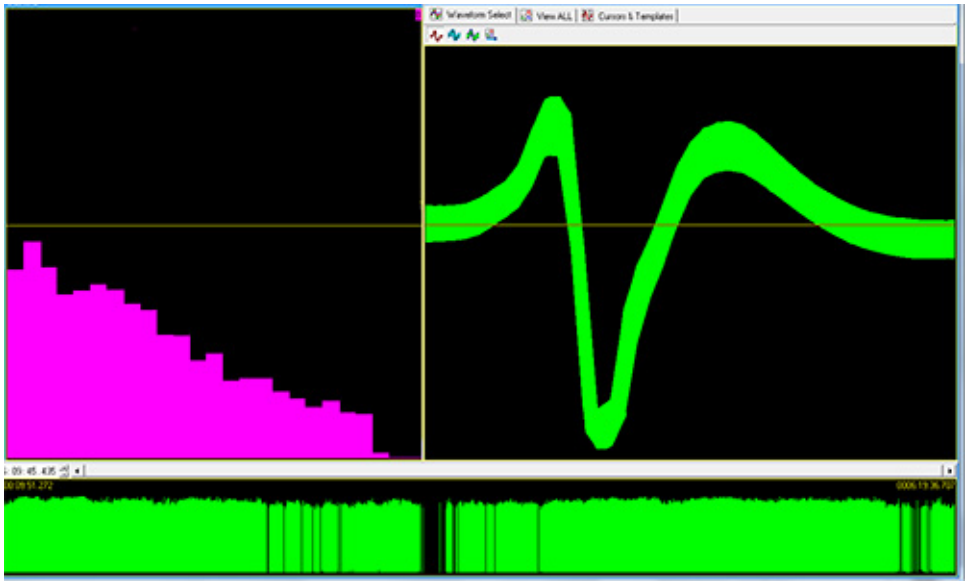

Figure 3. An example neural waveform (right) recorded from one wire of a microwire array. The histogram (left) shows the number of action potentials in the milliseconds preceding each observed neural discharge in the session. Each bin represents a 1 millisecond period. The histogram demonstrates that no action potentials occurred in the neuron's natural refractory period (2 msec).

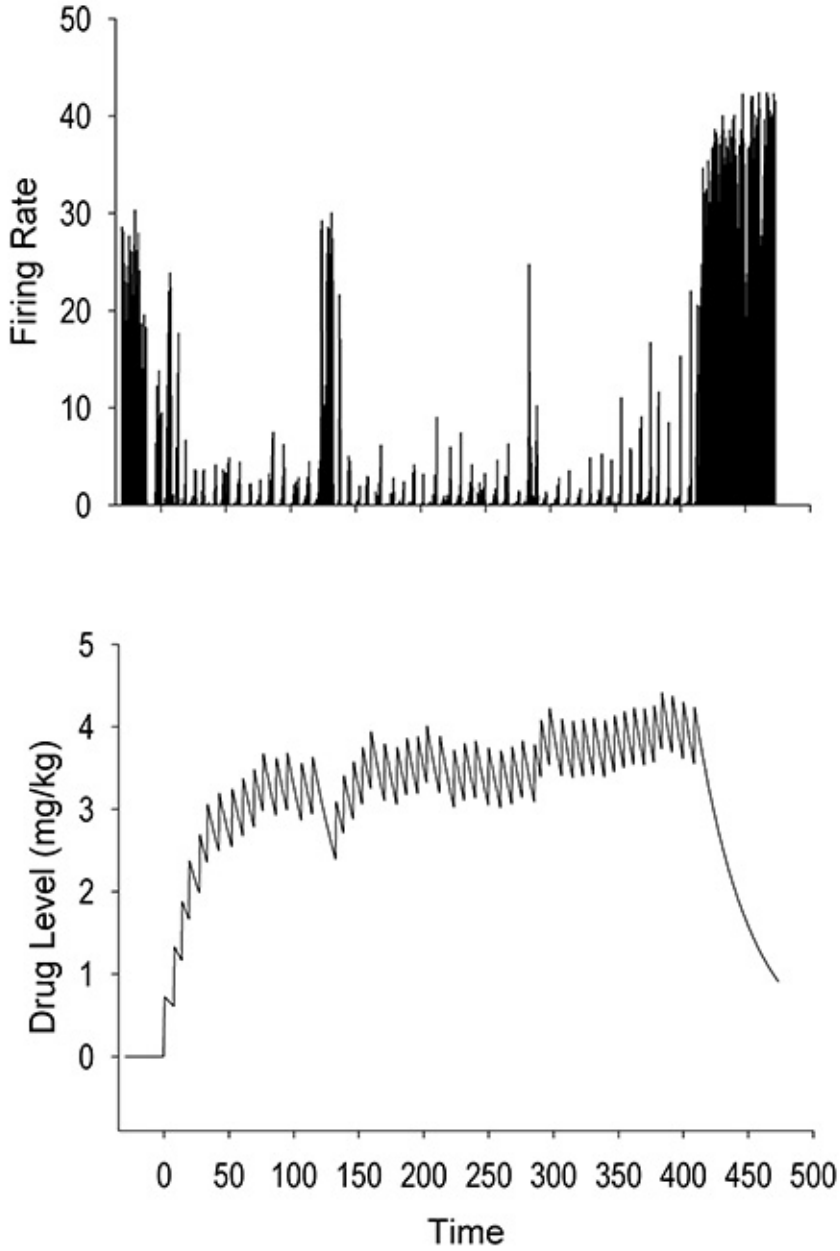

Figure 4. An example of a neuron that is tonically inhibited during cocaine self-administration. **A**) The firing rate (Spikes/30 sec) of a neuron across a long-access cocaine self-administration session in 30 second bins. **B**) The corresponding calculated drug level across the same session. In combination, increases in drug level are correlated with a depression in neural firing. Accordingly, when the session ends at ~425 min, neural firing recovers as body levels of cocaine fall.

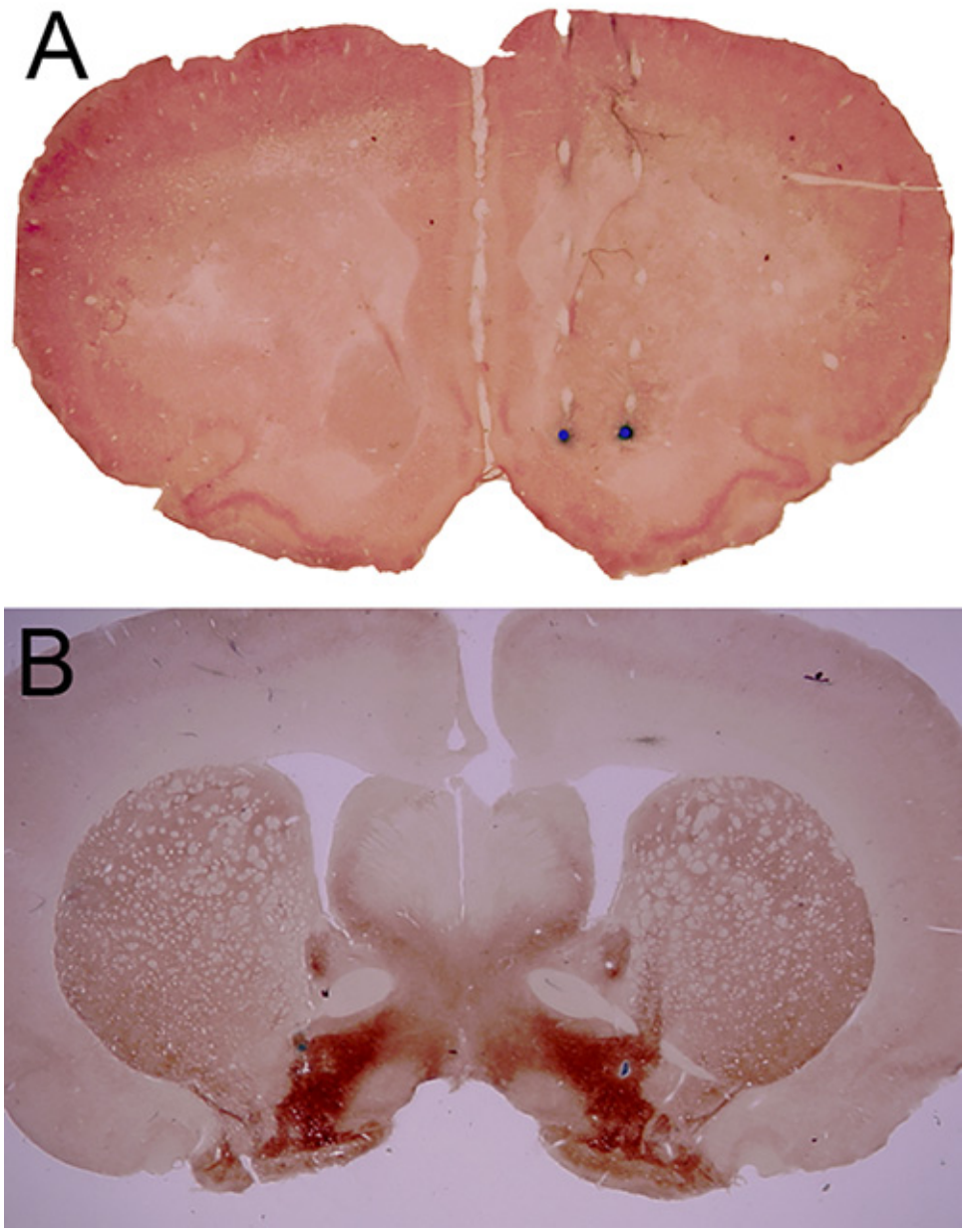

Figure 5. Histological slices from two animals with microwire implants. A) A Nissl stained section using neutral red combined with a potassium ferrocyanide counter stain revealing lesioned microwire tips (blue). A microwire can be traced from the cortex down to the wire tips (blue) by following the wire tracks in the tissue. Tracks may be traced in a single slice or a series of adjacent slices. **B**) A section stained for Substance P, which reveals the ventral pallidum, coupled with a potassium ferrocyanide counter stain. Using immunohistological techniques, individual micro-wires can be localized within specific brain regions, or even within specific sub-regions of a particular nucleus. Figure shows a microwire tip (blue) localized within the ventral pallidum using the Substance P stain.

Discussion

Extracellular recordings represent a powerful experimental technique that can be incorporated into nearly any experimental preparation in neuroscience. Wires that have been implanted in organized arrays can be tracked as their shafts pass through the brain and into their target region (**Figure 5A**). When a small, post-experimental lesion is created at the noninsulated microwire tip to create a small iron deposit from the stainless steel wire, one can precisely mark the location of the uninsulated microwire tip (where the single-unit was recorded) using a solution of 5% potassium ferrocyanide and 10% HCl (**Figures 5A** and **5B**; blue dots). Thus, one can readily use a series of subsequent brain slices to recreate the position of the entire array within the brain. Finally, the above mentioned techniques can be readily combined with immunohistochemistry in order to increase the spatial specificity of recordings, in order to verify target placements, and even in order to study subregional differences within a single target nucleus[3] (**Figure 5B**).

To achieve the desired spatial specificity, microwire arrays need to be designed and implanted with care. First and foremost, the spacing of the rows and columns within the array must be designed specifically for the region of interest. Arrays that are too long or wide for the desired target

can decrease accuracy or produce a large number of wires that fall on the border between two adjacent nuclei. At the same time, microwires spaced too closely together can prohibit a separate demarcation of each electrode. Second, microwire arrays must be handled with care in order to maintain the integrity of the array. Single electrode arrays are produced using thin, hair-like wires that can easily be damaged or jostled from their configuration when contacted. The qualities of these wires are ideal for reducing damage to tissue on the path of approach to one's target and also allow the wires to shift in concert with small movements of the brain. Thus, it is typically advisable to store arrays in a safe location and avoid removing arrays from their protective cases until just prior to implantation. Also, care must be taken when sterilizing microwire arrays. In some cases, autoclaving of electrodes may be acceptable. However, ethylene oxide or UV sanitization may be preferable to protect fragile arrays. Finally, the electrode metal and insulation, and the diameter of each, should be carefully considered. For instance, only electrodes that contain iron (*e.g.* stainless steel) will be able to produce Prussian blue reactions for individual demarcation of each microwire in the array.

On occasion, microwires in the array will stray from their configuration during lowering due to unnoticed obstructions (*e.g.* skull fragments in the skull window) or poor handling. In these instances, such a microwire can often still be tracked to its tip (although it will likely be outside of the target region). Should an instance arise where any numbered microwire in the array cannot be verified, it is important to the integrity of the experiment that this animal be removed from the dataset. Misinterpretation of an individual microwire's position can enable its neural signals to complicate or corrupt interpretation of the data.

During implantation, the precision of the skull window and array alignment are also critical to target accuracy. Skull windows must be made wide enough to allow the array to pass without bending or damaging wires. On the other hand, the window is also used to accurately guide the array to the target position and must therefore be drilled with precision in every dimension. When ready for implantation, one must also be certain that the array is plumb with the skull window in all dimensions. That is, a slight tilt of the array in any dimension can cause the array to either partially or fully miss the target nucleus. Lastly, special care should be taken when lowering the first 1-3 mm. It is during the initial lowering that pieces of debris that have gone unnoticed within the skull window can compromise the integrity of the array and upset the path of microwires. If the path of the microwires is obstructed while they are slowly lowered, one can see microwires bend or bow under slight magnification before the array incurs any damage (*e.g.* using a magnifying glass). At this point, microwires can be retracted and debris can be cleared from the window before continuing with the implantation.

Last but not least, successful implanting of microwire arrays requires special attention to maintaining an aseptic operating room and providing thorough post-operative care. When combined with the above mentioned precautions, viable recordings can be obtained from regions of interest for upwards of one month; we have verified recordings up to 40 days following surgery[6]. Perhaps most importantly, the longevity of these recordings provides the opportunity to study discrete functional circuits within the complex milieu of electrical, chemical, and hormonal influences and answer vital questions about the role of these circuits in learning and motivation.

Disclosures

The authors have no competing financial interests to disclose.

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