CHAPTER

2

Recording Day and Night: Advice for New Investigators in the Sleep and Memory Field

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1. INTRODUCTION

1.1 Research Perspective on Chronic Extracellular Electrophysiology

Investigation of the brain mechanisms underlying learning in freely behaving animals requires the monitoring of neural activity before, during, and after the acquisition and modification of memories (Pavlides and Winson, 1989; Wilson and McNaughton, 1994; Skaggs and McNaughton, 1996; Qin et al., 1997; Siapas and Wilson, 1998; Ji and Wilson, 2007; Peyrache et al., 2009; Benchenane et al., 2010; O'Neill et al., 2010; Csicsvari and Dupret, 2014; McNamara et al., 2014; Atherton et al., 2015; van de Ven et al., 2016). Ideally, such monitoring should include all spontaneous behavioral states exhibited by the animal model of choice, such as periods of active exploration, quiet waking, and specific sleep states during which memory consolidation takes place (Louie and Wilson, 2001; Jarosiewicz et al., 2002; Lee and Wilson, 2002; Pereira et al., 2007). Chronic electrophysiological extracellular recordings of local field potentials (LFPs) and/or action potentials (spikes) continue to be among the most useful methods for neurophysiology research (Ribeiro et al., 2010; Gomperts et al., 2015), being amenable to a variety of experimental designs (Chen et al., 2013; Gelinas et al., 2016) and capable of providing information about neural activity from multiple recording sites at excellent temporal resolution (Buzsaki, 2004; Tort et al., 2008, 2009; Wasilczuk et al., 2016).

1.2 Applicability to Sleep and Memory Research in Rodents

1.2.1 Importance of Chronic Electrophysiological Recordings Across the Sleep–Wake Cycle

Chronic electrophysiological monitoring is particularly advantageous for the simultaneous recording of LFP and spikes from large neuronal populations of rodents traversing waking, slow-wave sleep (SWS), and rapid eye movement sleep (Ribeiro et al., 2004; Montgomery et al., 2008). While the sorting of these major behavioral states can be greatly aided by the use of electromyographic (EMG) (Louis et al., 2004), inertial (Venkatraman et al., 2010) or piezoelectric (Flores et al., 2007) signals, it can be accurately performed based purely on the spectral analysis of LFPs (Gervasoni et al., 2004). The search for memory correlates, however, requires the analysis of spike patterns produced by neuronal assemblies (Lopes-dos-Santos et al., 2011, 2013; Almeida-Filho et al., 2014) and the determination of spike/LFP relationships within and across brain regions and cell layers (Fujisawa and Buzsaki, 2011). The wealth of information provided by chronic electrophysiology has been instrumental in the boom of the sleep and memory field in the past three decades, leading to the demonstration of memory processing during sleep. Chronic recordings can be very robust over time, typically yielding high-quality spike data for several weeks and high-quality LFP data for several months (Freire et al., 2011).

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1.2.2 Limitations of the Method

Neuronal sampling in chronic electrophysiological recordings is inherently constrained by the limited number of electrodes that can be implanted in the brain without disrupting behavior (Polikov et al., 2005). Surgical implantation is invasive, and great care is needed to obtain successful, lasting implants that do not interfere substantially with behavior (Kralik et al., 2001; Fu et al., 2016). The number of neurons recorded in a typical recording session is well below one hundred (Henze et al., 2000; Buzsaki, 2004), and therefore a great degree of subsampling is intrinsic to the method (Ribeiro et al., 2010). Some uncertainty about neuronal separation in spike recordings is a routine problem, derived from the extracellular positioning of single wire electrodes (Lewicki, 1998), but the use of tetrodes solves this issue (Buzsaki, 2004). It is also difficult to determine neuronal identity precisely following chronic recordings, a feat that is only performed in a few laboratories using juxtacellular recordings and stimulation, followed by dye injections (Pinault, 1996; Klausberger et al., 2003; Houweling and Brecht, 2008; Tang et al., 2014; Katona et al., 2016; Ebbesen et al., 2017).

2. METHODOLOGICAL CONSIDERATIONS

The beginner experimentalist, interested in chronic *in vivo* electrophysiology, must be attentive to a series of detailed procedures that contribute overall to four main goals: (1) chronic implantation of electrodes with minimal disruption of the neural milieu; (2) maximal preservation of spontaneous and learned behaviors; (3) high-fidelity recordings of neurophysiological signals (spikes, LFP, EMG, etc.); and (4) adequate computational analysis of the data (e.g., spike/spike, spike/LFP, LFP/LFP relationships).

Before delving into specific, step-by-step, guidance it is important to underscore that chronic electrophysiological recordings in rodents require many hours of surgical practice, precise organization of procedures and data acquisition, constant attention to the physiological and behavioral needs of the animals, prevention and resolution of electrical noise, standardization of learning tasks, and adequate monitoring of associated behaviors through video and audio recordings.

2.1 Electromyogram Electrodes

To manufacture EMG electrodes, you may use stainless steel wires of 50–100 μm in diameter and 5 cm long. They are cheaper and more flexible than tungsten. Remove 5 mm of the wire insulation on both ends. Roll one end of the wire using tweezers with rubber tips to make a small coil with it and reserve. Over a thermoresistant surface, melt ~2 g of tin (you can reuse it to assemble several EMG electrodes). Dip the "coil" side of the wire into the melted tin and pull it off slowly. The surface tension of the melted tin will produce a perfect tin ball. If you experience a lack of adherence of the tin to the electrode coil, apply a small droplet of soldering flux (e.g., phosphoric acid) to the coil before dipping it into the melted tin. Apply the flux with a swab, and start soldering before it dries. Once you get sufficient metal (it may depend on whether you will use rats or mice), you can shape it by touching the small ball on the solder stalk until it melts and turns the surface smooth and uniform. Pay attention to the ball surface. It should be perfectly smooth, otherwise it might hurt the animal's muscle or skin, produce inflammation, and disrupt normal behavior. After semi or full sterilization using alcohol (70%) or an autoclave, respectively, the EMG electrode should be placed under the skin and above the nuchal muscle. Use thin tweezers to introduce the electrode and certify that the metal ball is well positioned on the nuchal muscle. If you are using two EMG electrodes, place one ball on each side of the animal. The other edge of the EMG wire should be uncovered and soldered on a printed circuit board (PCB).

2.2 Design of Multielectrode Implants

The experimenter must begin by having clear neuroanatomical aims in mind before designing the array, bundle, or drive of electrodes. The multielectrode implant and the surgical process must be planned as a whole, with the aid of a stereotaxic atlas (Paxinos and Watson, 2005) and occasionally the use of trigonometric calculations to ensure the proper positioning of the electrodes at an angle. In the case of multisite electrode arrays and bundles, the electrode configuration built in the device to be implanted may not precisely fit the stereotaxic opening of multiple holes in the skull. Extra care is required when opening the bone and determining fixation anchors.

2.2.1 Single Wire Arrays

For most applications, single wire electrode arrays must be built from hard materials (e.g., tungsten) so that the wires do not bend while penetrating the brain (Hubel, 1957). Spike recordings are typically favored by small rather than large wire diameter, whereas for LFP recordings the opposite is usually observed. For extracellular electrophysiological purposes comprising both spike and LFP recordings, 35 or 50 μm Teflon-coated tungsten wires are suitable. Other metals and

insulations can be used, provided that they are hard enough and noncytotoxic (Polikov et al., 2005). As explained later, the construction of electrode arrays can be divided into four main steps: 1. soldering, 2. wire cutting and alignment, 3. assembling, and 4. closing electrical contacts. All these steps should be performed under a 10−100× magnifier.

2.2.1.1 Soldering

This first step consists of soldering the connector to the PCB. Under the magnifier, use a holder to mount the plastic connector and the PCB together, using adhesive tape to keep them in place while soldering. To avoid inhaling toxic fumes, use a protective mask and work under a ventilated hood. Plastic tape should be used with care because it can melt. It is advised to close the connector holes with tape to prevent molten tin drops from accidentally blocking the holes. If the holes get sealed with tin, wiping it out to reopen them will most likely damage the connector and its electric contacts. The use of flux is highly recommended to improve the quality of the soldering. If adjacent contacts become short-circuited, carefully remove the tin excess upward using the soldering tip. Keep the soldering contacts very brief so as not to melt the plastic connector nor break the connector's metal claw. After soldering, use a multimeter to check the connections for zero impedance. Now is the last opportunity to eliminate any short circuit among channels. Small pieces of standard wire attached to the tip of the multimeter will help in this task. An overview of the connector attached to a PCB is shown in Fig. 2.1A−C. Note the contacts after soldering in panels B, C, and E, indicated in blue.

2.2.1.2 Wire Cutting and Alignment

In this second step, use very sharp scissors (for tungsten wires, use tungsten carbide scissors) to cut the wires into pieces of 3−5 cm. If you are using movable arrays, be sure to cut the wire to a length that allows for wire displacement. Check under the magnifier whether the coating is intact, and discard wires with damaged coating. Use two identical templates for aligning the electrodes. The templates can be made of cardboard, stiff plastic, or acrylic, and their holes can be drilled using precision drilling. Make sure to manipulate the coated wires using tweezers with rubber tips so as not to damage the coating. This step requires a completely clean table because it is very easy to lose wires; for instance, they can "jump" at the moment you cut. Cover the table with a sheet of white paper, and mark the length of the electrodes on the paper with a pen to standardize the cutting.

Once the wires are cut, place the two templates on a support so as to align the wires parallel to each other, ∼7 mm apart from each other on the horizontal axis. Make sure that the holes match between templates in the vertical axis. Aligning the wires is one of the most delicate steps and requires patience. Using the rubber tipped tweezers, grab each electrode and pass it through the corresponding holes in both templates. Be careful not to use too much force, as it may bend the wire irreversibly. Start passing the farthest electrode line from you, and if you are right-handed start by passing wires from left to right; if you are left-handed, start the other way around. Once all wires are in place, align their length using any object with a flat surface (e.g., plastic chip). Depending on the brain structures targeted, you may want to vary wire lengths within the array (for example, staggered electrodes with a "staircase" configuration).

Once all the electrodes have been properly placed and aligned, it is time to glue them together with photopolymerizing dental resin. Calculate where to put the resin drop by adding 2.5 mm to your dorsoventral (DV) coordinate. This will give you enough space to implant the array and to have a good visualization of the procedure. Be careful to estimate the amount of resin used, as it will spread both downward and upward. Align the length so that the target spot is placed right between the templates, but be careful not to let the resin spread into the templates lest they be damaged. Also, once you put the resin, make sure that all the electrodes are covered. Looking at it from different angles, compare it with surrounding straight borders, with and without using a magnifier. A very common error is to let one or two wires be untouched by the resin. Check the center and, if necessary, apply more resin carefully. Once you confirm that all electrodes are covered, apply light on it as fast as possible. If you take too long, the resin drop can move into the support or table, and you will need to reapply it. An illustrative example of array design is shown in Fig. 2.1B−D. Note that in this case (Fig. 2.1B and D, red arrow), there are four different regions of interest, and in two of them the electrodes are distributed in a bundle, with a fixed distance between each tip, which could be useful to record a laminar profile in the DV axis.

2.2.1.3 Assembling

This third step consists of assembling the products of the previous two steps. For that, one must first plan the orientation of the connector over the animal's head, considering the anteroposterior axis (from the nose to the back) and the mediolateral axis. If the brain areas of interest are anatomically too lateral, be careful to optimize the connector's positioning, choosing an orientation that both facilitates the implantation and avoids animal discomfort during subsequent behavioral tasks and recordings. Remember that you need to make room for the recording cable and connector. To achieve

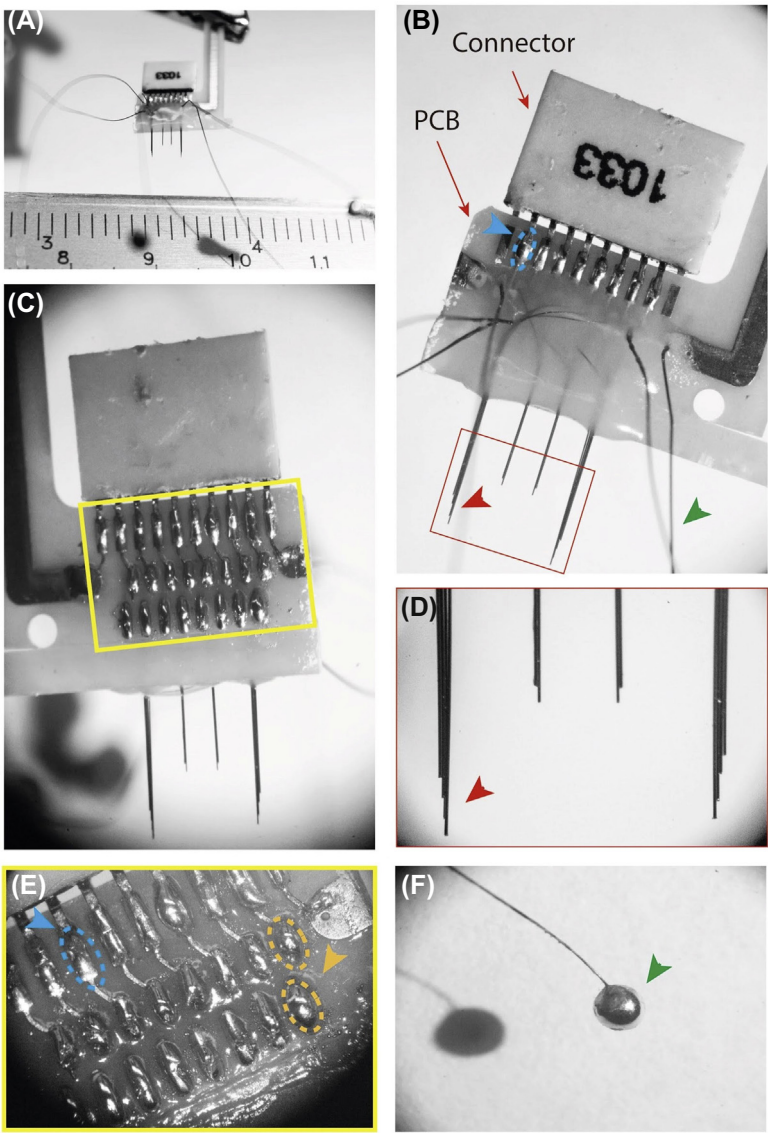

FIGURE 2.1 Important checkpoints for the manufacture of multielectrode arrays. (A) Overview of size and final arrangement of the steps illustrated in the following panels. (B) Side view. Note how the connector and the printed circuit board (PCB) are attached. Soldering contacts are indicated by the blue arrowhead and dashed line. The four arrays display a 90° angle of alignment with the PCB (red square). One electromyogram electrode is soldered to the PCB (green arrow). (C) View of the other side, where electrodes are either soldered to the PCB or covered with silver paint to close contacts. (D) Detailed view of array tips, arranged as a bundle of staggered electrodes (indicated by the red arrowhead). (E) Zoom-in of the soldering shown in (C). Blue indicates the bond between the connector and the PCB; orange indicates the soldering of the electrodes to the PCB holes. Note the absence of any short circuits between channels. After visual inspection, a multimeter must be used to confirm that the conducts are adequate. (F) Electromyogram electrode (green arrowhead) connected to a channel of the PCB. Note that the tip is quite round, and there are no sharp edges at the end.

an optimal design, you can use small plastic chocks (leftovers from previously used PCBs) to make "bridges" linking the array(s) to the PCB. Use photopolymerizing dental resin to glue the parts together. Your choices here will have major consequences during the implantation. Try your best to fix the pieces so that the array and the PCB form a 90° angle between each other in one axis. Also, make sure the other axis is parallel (check it by looking from different perspectives). To hold the parts in place while gluing you can use tape on the wires in the opposite end of the electrode tips to be implanted. Usually, the PCB will be laying down on the magnifier's specimen surface, and you will adjust the array's position according to the PCB, always using the rubber tip tweezers. Toothpicks can help you further build support under the array, but attention is needed to avoid damage to the electrodes. Finally, when all the parts are precisely aligned, you may shine light to polymerize the resin. Because this is the most fragile step in the construction of multielectrode arrays, it is recommended to reapply the resin involving the plastic part of the PCB, paying particular attention not to close the holes with resin.

2.2.1.4 Closing Electric Contacts

Once the arrays are fixed to the PCB, it is time to proceed with making the electrical contact between the wires and the connector. For that, pass each wire through the respective hole of the PCB. Make sure to take notes identifying which channel is assigned to each electrode. It is preferable to pick them in a predetermined order according to a standard map of correspondence. This map must contain information linking wires and PCB holes and identifying which electrode corresponds to which output channel in the connector.

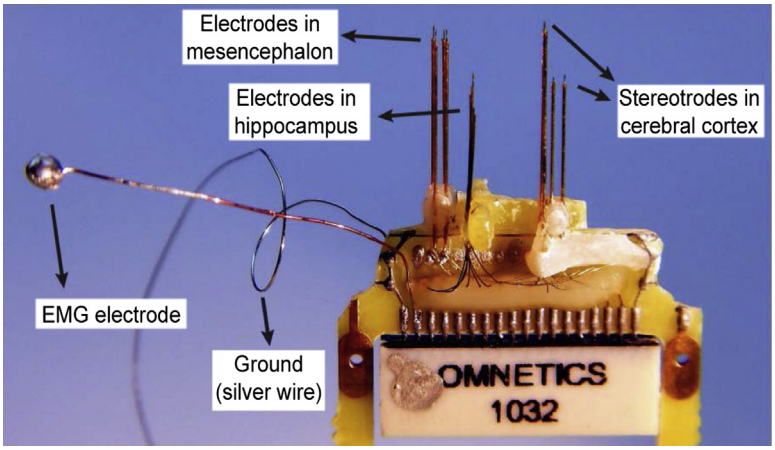

FIGURE 2.2 **Multielectrode array.** An example of multisite, multielectrode arrays capable of recording neural activity from four distinct brain regions. The configuration also includes two channels of electrocorticogram (ECoG) and two channels of electromyogram (EMG). Signals from the hippocampus were recorded with 50 μm electrodes. Stereotrodes (2 bonded electrodes, 35 μm diameter each) were used for cortical structures, whereas mesencephalic structures received tetrodes (4 bonded electrodes, diameter 12.5 μm each, within a silica tube to increase rigidity).

This step ensures you will be able to make sense of the recorded signals according to the targeted brain structures. Again, manipulate all the wires using the rubber tip tweezers. After passing all electrodes, pull them carefully from the other side of the PCB to make them tight. Again, if you are planning to use a microdrive, remember to estimate the length of the wires to allow the complete displacement of the array. Then, apply the photopolymerizing resin on the entering side of the holes and cover them completely (in Fig. 2.1B, note the semitransparent resin covering the wires that pass through the PCB). Apply light and, once the piece is firm, cut the electrode tips on the other side of the PCB. This is a key procedure that can greatly impact your recordings. Use tungsten carbide scissors to cut the wires as short as possible. Inspect the electrode tips to make sure they are straight and have no debris around them. Do not bend the wires, for they can easily break. Remove the insulation from the electrode tips with a sharp and tiny blade. This is one of the most critical steps because the wires can break or the electric contact can fail due to insufficient removal of the coating. Pay attention to the color of the electrode's surface before and after removing the insulation because the metal and the coating material have different reflection characteristics. After finishing this step, apply silver paint on each tip, letting it spread downward through the corresponding hole. One can use a 30Ga (gauge) needle to apply the silver paint. Do not apply a big drop to avoid spreading it sideways and increasing the chance of short circuits. Silver paint is also very volatile, so you must manipulate small aliquots and work as fast as you can. If short circuits occur, wait until everything is dry to gently remove the silver paint between holes, scraping the excess with the needle's tip. If there are too many short circuits, consider removing all the silver paint, cleaning up the connections, and starting over again. Use a soft brush to clean the silver paint debris.

As an alternative to the application of silver paint, you can solder the wires' tips to the PCB (Fig. 2.1C and E, orange arrowhead). Perform this method only if you have excellent soldering skills. Remember that the soldering tip should be thin and that the soldering should be restricted to the edge of the PCB hole, avoiding electric contact between adjacent holes. Finally, use a multimeter to verify that your electrodes are not short-circuited, measuring from the connector output (at the last section, where it closes contact with the recording cable). Once all pairwise combinations of electrodes have been successfully tested, proceed to cover the connections with dental resin. Make sure to include in the final assemblage the reference and ground electrodes and the electrocorticogram (ECoG) (Fig. 2.1A) and electromyogram electrodes (Fig. 2.1B and F, green arrow). These electrodes can be made of Teflon-coated stainless steel wires, which are less rigid and bend without breaking. The electrode tips can be soldered to a small screw (ECoG, ground, and reference) or a tin ball (EMG) (Fig. 2.1F). Make sure to allow sufficient length for the electrodes to reach comfortably the surface of the skull or the neck. Use an impedometer to measure the impedances inside saline. An example of the final configuration of a multisite, multielectrode array is shown in Fig. 2.2.

2.2.2 *Tetrode Arrays and Microdrives*

Detecting and classifying spike activity in a large population of neurons during the sleep–wake cycle is a major technical challenge. While the use of single wires can increase the spatial sampling of the target neural population, the identification of unitary events can be compromised. This is particularly the case for structures with high cellular density, as the principal layers of the hippocampus (Amaral and Witter, 1989). In these structures, extracellular recordings from a single electrode may reflect action potentials from dozens of units (Henze et al., 2000). Although tissue anisotropy and

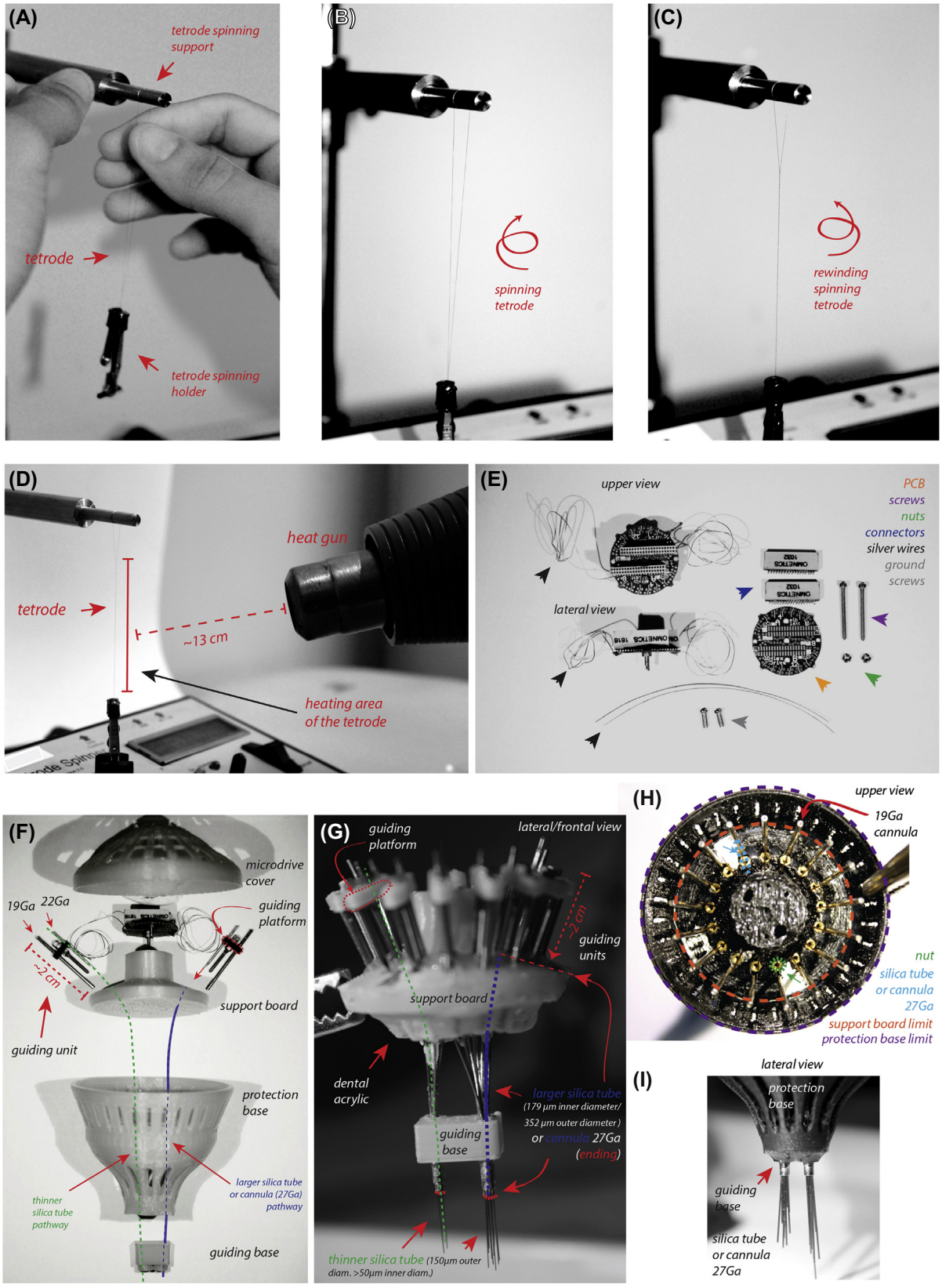

FIGURE 2.3 **Tetrode and microdrive manufacturing process.** (A) For tetrode manufacturing, cut two pieces of coated wires 20 cm long (nickel-chrome or tungsten, 12.5 µm diameter), fold them together in a U shape, hang the resulting lace on the spinning support, and grab the four tips with the holder. (B and C) Lower the support to gently attach the holder on the spinner's magnet and start spinning protocol. (D) After spinning, use a heat gun to slightly melt the coating and glue the wires. Warning: Apply heat only in the twisted area; in the upper part

damage by electrode positioning may contribute to this number never being reached, sorting spike waveforms from more than 2 or 3 units can be very difficult. To circumvent this limitation, one can make use of stereotrodes (McNaughton et al., 1983) or tetrodes (O'Keefe and Recce, 1993; Wilson and McNaughton, 1993), which consist, respectively, of two or four wires bonded or twisted together. This strategy, which uses the relative amplitudes on independent recording channels at specific time points to assist in the task of unit isolation (Gray et al., 1995) (see "spike sorting" below), has been reported to identify and segregate from 5 to 20 spike waveforms from hippocampal neurons per tetrode (Wilson and McNaughton, 1993). Stereotrodes and tetrodes can be combined in fixed-position arrays or loaded in movable microdrives. A detailed description of how to build tetrodes and microdrives and how to assemble them together (Kloosterman et al., 2009; Nguyen et al., 2009) are discussed later. Stereotrodes can be built by downscaling the tetrode manufacture procedure to two wires.

2.2.2.1 Tetrodes

Just like single wires, tetrodes can be arranged into templates designed to target different brain regions of interest. Because of the fragility of tetrodes, it is desirable to use a support conduit (e.g., silica tubes) to guide them into the brain. As mentioned earlier, tetrodes are made of four wires spun around their longitudinal axes. To minimize brain damage, it is key to choose well the diameter of the wires used. As a rule of thumb, the total diameter of the tetrode, i.e., the diameter of the four wires together, should not exceed 70 μm. The diameter directly affects the impedance of the electrode, which should ideally be around 70–100 kΩ to record from single units (spikes). A description of a typical procedure to build tetrodes based on tungsten or nickel-chromium 12.5 μm diameter wires will be discussed later. Using tweezers with a rubber tip under a magnifier, carefully manipulate the reel to cut the wire pieces needed. Make sure you avoid scraping the insulation coating from the electrodes. Cut two pieces of wire 20 cm long, align both in a parallel way, and apply water to stick them together. Then, hold all tips together to make a loop shape and place the wires on a tetrode spinner. The tips will be held by the spinner itself on the bottom, whereas the loop part is attached to a top support to hang the tetrode for spinning (Fig. 2.3A). One can build the tetrode with as many turns as needed; we recommend 90 turns in one direction (Fig. 2.3B), followed by 30 turns of relaxation in the opposite direction (Fig. 2.3C). Tetrodes get stiffer in proportion to the net number of turns spun in one direction, but keep in mind that overly rigid tetrodes are more likely to break. Finally, use the heat gun to melt the insulation a bit (Fig. 2.3D) to keep the wires attached to each other after the tetrode is removed from the spinner. Be careful not to apply the heat for too long or from too close a distance nor on the nontwisted part of the tetrode (i.e., the uppermost part attached to the holder). For a reference, apply the heat from at least 13 cm away from the wires, for up to 5 s continuously.

2.2.2.2 Microdrives

Microdrives allow for electrodes to be repositioned multiple times after surgery in awake animals. Depending on the needs, electrodes can be moved together or individually. The use of microdrives also allows the experimenter to target different brain regions using a single movable system. The first microdrives were built using metal components, but nowadays their main building blocks usually consist of plastic components produced in 3-D printers. The main advantages of plastic components are their low weight, good mechanical precision, and relatively high assemblage speed (in comparison with original approaches). The materials used are typically polylactic acid and acrylonitrile

(near the support), wires must not be glued. Heat should be applied from various directions through vertical movements. Subsequently, cut the tetrode next to the holder and remove it gently from the spinning support. (E) Materials used to build the interface of tetrodes/microdrive with the acquisition system. Weld the *connectors* on the *printed circuit board (PCB)* using solid weld. Warning: protect the holes and pay attention not to melt the plastic parts of the *connectors* and *PCB* while welding. *Screws* and *nuts* are used to attach *connectors* + *PCB* to the *support board*. *Ground wires* (silver or stainless steel) are also welded on the *PCB* and then to implant screws. (F) 3-D printed microdrive parts: Microdrive *cover, support board, guide platforms, protection base,* and *guiding base.* Each of the 16 *guiding units* is composed of a 19 Ga guiding pole, a screw and nut, a 22 Ga cannula, and a 3-D-printed guiding platform. For each *guiding unit,* there are three radially aligned holes in the *support board* for the guiding pole, screw, and 22 Ga cannula. A thin silica tube (which will carry the tetrode) is inserted inside the 22 Ga cannula and glued to it at the height of the *guide platform*. The *guide platform* slides down through the 19 Ga guiding pole on turning the *screw,* carrying the 22 Ga cannula and the thin silica tube across the *support board*. Inside the *support board,* the thin silica tube slides into a 27 Ga silica tube. The *support board* fits on the *protection base* and connects to the *guiding base* through the 19 Ga guiding pole. (G) A further step in the microdrive assembly, in which all the *guiding units* and the 27 Ga silica tube (blue) are docked into the *support board*. Note that the thin silica tube (green) goes inside the 27 Ga silica tube (blue). In further steps, the *guiding units* are lowered through screw turns, and the tips of the both silica tubes are cut at the bottom. Next, they are moved back upward, and tetrodes are loaded and glued into the thin silica tubes. (H) Upper view of the microdrive without the *guiding units*. The *support board* has three circular rows of holes through which the 22 Ga cannula (inner row), the screw (middle row, notice nuts at this row), and the 19 Ga guiding pole (outer row) are inserted. (I) Frontal/lateral view with the *protection base*, *guiding base* (fixed inside with dental acrylic), and the 27 Ga silica tubes not yet cut.

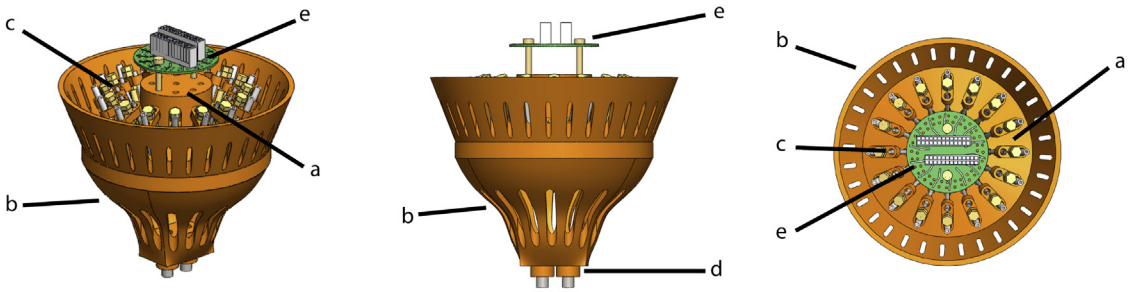

FIGURE 2.4 **Microdrive for 16 movable tetrodes.** A 3-D prototype of a microdrive, shown from angled (left), frontal (center), and top views (right). Letters indicate components (a) support board, (b) protection base, (c) guide platform, (d) guiding base, (e) PCB. In this case, the guiding base (d) is designed to target two regions. The support board (a) lays on the upper part and fits into the protection base (b). It also receives a closing cap (not illustrated). The support board has holes for guiding poles, screws, and cannulas of the guiding units (see Fig. 2.3). The upper part of the support board holds the PCB and connectors; tetrode wires connect with the PCB through gold pins. The required files for printing the 3-D parts of this microdrive are freely available at https://github.com/tortlab/Open-Source-Microdrive. *PCB*, printed circuit board.

butadiene styrene. A schematic example of a microdrive is shown in Fig. 2.4.

The first step in the manufacture of a microdrive is to build its mounting parts (by 3-D printing): (1) *Support board*—central board on which the microdrive is mounted; (2) *Protection base*—cone-like structure placed underneath the support board and which provides side protection; (3) *Guide platforms*—components for carrying the tetrode, guiding pole, and positioning screw; (4) *Guiding base*—placed at the bottom of the protection base and used to spatially distribute tetrode bundles in the anterior–posterior and mediolateral axes; (5) *Microdrive cover*—upper cap to close and protect the microdrive when not in use.

Once all the parts are printed, the guiding poles that support the guide platform and the cannulas that direct the tetrodes can be obtained by cutting needles. Use needles of 19 and 22 Ga, with lengths of 2 and 1.7 cm, respectively. Each guiding unit is built by traversing the 22 Ga cannula through the guide platform, followed by the screw (use a nut below the platform to keep the screw fixed). The guiding pole is next inserted into the support board and fixed to it with dental acrylic. After that, the guide platform must be attached to the guiding pole. The 27 Ga silica tube, measuring 5 cm approximately, should then be placed and glued into the support board. Note that the length of the tubes must match the protection base size to guarantee that all tetrodes will pass through. Surface-mounted soldering to connect the PCB to the connectors requires special attention and must be done very carefully. The contacts are minimal and, therefore, it is highly recommended to use a magnifier. As described earlier, one must also check all the contacts for possible short circuits. To finish, apply epoxy resin on the soldered contacts. Be careful not to let the resin spread to the PCB holes. Drying time is approximately 10 min.

Next, attach the PCB to the support board. Preferentially use screws (Fig. 2.3E and F), which makes it easier to recycle the PCB with connectors afterward (usually the most expensive part of the microdrive), although dental acrylic can also be used to attach the parts. Next, put together the support board and the guiding base. The sixteen 27 Ga silica tubes crossing the support board (top part) must be passed through the guiding base (bottom part). Glue with cyanoacrylate (instant glue); cut the exceeding parts as necessary. Wait 2 min for the glue to dry and apply dental acrylic at the bottom part of the support board to hold all the tubes in place. Again, be careful not to let the acrylic spread into the holes of the support board. Then, fit the protection base around the support board and guiding base and glue it.

Next, cut sixteen 5 cm long pieces of the thin silica tubes that will carry the tetrodes (150 μm outer diameter; >50 μm inner diameter to ensure that the tetrodes will fit). Insert and glue the thin silica tubes in the 22 Ga cannulas of the guiding unit; this will make the thin silica tubes move together with the guide platforms when the screws are turned. Let all parts dry and screw downward the guide platform so that the thin silica tubes cross the support board and guiding base. Cut the exceeding parts as necessary. Pass a silver or tungsten wire through each tube to clean possible debris. Then, pass one tetrode through each thin silica tube and connect the nontwisted part of the wires to the PCB contact using gold pins. After finishing "loading" the microdrive with all tetrodes, glue them together to the thin silica tubes. Make sure they are at least 7 mm long from the guiding base.

To cut the tetrodes, first raise all guide platforms to the maximum possible. Then bring each one down individually, cut the tetrode at a 90° angle, and subsequently bring the guide platform back up to keep the tetrode protected inside the microdrive. All tetrodes must be of the same length, and make sure the wire is sufficiently long for reaching the areas of interest; pay attention to the length of the nontwisted part of the wires, which may stretch when tetrodes are lowered.

The impedance of the tetrodes must be checked and brought to 100–200 kΩ with a gold plating solution. After that, the microdrive is ready to be implanted, although it is advisable to check the impedance once again right before surgery. Be certain that all tetrodes are positioned right at their exit from the guiding base. Also, measure under a magnifier how much the tetrode goes down with a single turn of the screw. Use this to estimate the location of the tetrodes as you move them between recording sessions. After surgical implantation, the microdrive cover should be placed on top and fastened to the protection base with a piece of tape. Wrapping up the whole assembly is crucial to avoid tetrode damage and the entrance of debris into the mechanical part of the microdrive.

2.3 Combining Multielectrode Arrays With Optical Fibers or Cannulas

Many researchers seek to relate electrophysiological correlates to the action of a drug (Meyer and Quenzer, 2005) or to the stimulation/inhibition of specific neurons by optogenetic techniques (Deisseroth et al., 2006; Kim et al., 2017). Both methods involve the need of a second device in addition to the array, respectively a cannula for the intracerebral injection of the drug or an optical fiber. This type of experiment requires total care and attention to the use of both techniques so as not to jeopardize the performance of the array. Such deleterious influence can derive from physical impediment at the moment of the implant in the case of wanting to record and stimulate brain areas that are too close to each other or from interference of electrophysiological artifacts at the moment of stimulation or drug injection.

2.3.1 *Optical Fibers*

If your need is to combine optical fiber and arrays in your experiment, the first thing to do is to determine which brain regions you want to stimulate and record from. In case you want to target overlapping brain areas, the following are two main ways to perform the implant:

1. To separately implant the array and the optical fiber in the regions of interest with distinct angles.
2. To attach the optical fiber to the array so that they are fixed and implanted together.

In the first case, one must be careful about the spread of the optical fiber in the brain. If the light hits the electrodes, it will induce a photovoltaic artifact at the same frequency of the light stimulation (Mikulovic et al., 2016). In the second case, there is a greater chance of success in surgery because the fiber will be attached to the array at the right height, thus ensuring greater accuracy in the final target. The fiber, with a diameter of approximately 100 μm, is easy to fit between the electrodes, thus making the procedure easier.

2.3.2 *Cannulas*

The combined implantation of cannulas and arrays is very similar to the procedure just described regarding optical fibers, with the difference that in the case of a cannula one does not worry about the spread of the laser but about the diffusion of the drug. When the drug is applied manually via cannula during electrophysiological recordings, usually no artifact is observed in the signal. The use of an infusion pump will enhance precision, but one must be careful not to record electrical artifacts produced by the pumps.

2.4 Successful Stereotaxic Surgery

Sterilize all the surgical tools in an autoclave. On the day of the surgery, preferably induce anesthesia with an inhaled anestheticl such as isoflurane 5%. Once the animal is sedated, maintain this status using isoflurane 1–2% delivered through a mask. Alternatively, inject liquid anesthetics such as ketamine/xylazine (80–100 mg/kg and 10 mg/kg i.p.), or sodium pentobarbital (50 mg/kg i.p.) (van Zutphen et al., 2001). Keeping the animal in a quiet place immediately before and after anesthesia induction significantly helps the sedation to kick in and last longer. Atropine (0.02 mg/kg i.m.) should be injected to prevent mucous secretion.

Systematically check the animal's breathing throughout the surgery. If atropine is not used and mucous secretion begins to occlude the respiratory airways, use a syringe with a perforated tube to extract the secretion. Assess the pain threshold regularly by gently squeezing a paw with a forceps. In the case of liquid anesthetics, supplement as needed during the surgery with 25% of the dose of ketamine, or sodium pentobarbital. Avoid supplementing xylazine, which can kill the animal after the initial dose.

Shave the head carefully, so as to preserve the integrity of the mystacial vibrissae. The area shaved should be large enough to allow all the stages of the implant to take place without contact with the animal's fur. Spread iodine on the shaved regions with a cotton swab. Position the animal on the stereotaxic apparatus. Make sure that the head is tightly secured, otherwise it can move during the surgery, causing coordinates to change. Put a drop of aqueous gel on each eye to prevent corneal dehydration. If the surgical scope has a strong lamp, position it at least 30 cm away from the animal to avoid overheating it. Place a warm pad under the animal to keep it at the right core temperature.

Use a sharp blade to open the skin from the point between the eyes until the posterior end of the cranium. The skull surface should be exposed, but the neck

muscles should be kept intact. Use the blade to scrape off all the connective tissue above the skull. Apply hydrogen peroxide with a cotton swab to clean and dry the skull's surface. Check the head alignment (DV and mediolateral), measuring the bregma and lambda anatomical landmarks. Keep their DV disparity at a maximum of 200 μm. Using the stereotaxic rulers, mark the stereotaxic coordinates of interest on the skull with a pen. Draw the windows and holes that should be drilled for electrodes and screws, respectively, according to the design of the array to be implanted.

Do not forget the screw anchors to help fix the helmet. They should preferably be positioned away from the regions to be implanted to balance the anchorage of the helmet. Once the windows and holes are drawn, start drilling the holes for the screws (including those for ground and ECoG). Be careful not to go too deep. The skull bone must be perforated, but the meninges should remain intact. Keep the skull's surface clean. Saline applied with cotton swabs will help to remove blood and bone powder. To open the windows through which the arrays will be introduced, start at the sides. Try to cut out a bone square without producing small bone fragments. The best option is to etch with a fine-tip drill the exact perimeter required. Check under the surgical scope that there are no loose bone fragments. Explore the window with small tweezers or needles to remove debris. It is important to lapidate the window's sides and not leave irregular bone edges, so as not to damage the electrodes when passing through. You may dry liquids with absorbent filter paper.

With the help of a needle with the tip bent at 90-degrees, remove the dura mater (Fig. 2.5A). Start at one end and try to rip three sides of a square, leaving the fourth one in place to allow the dura mater to fold over; this will increase protection for the electrodes' passage. Use the maximum magnification on the surgical scope to perform this step and examine well the dura mater before you begin to rip it. Cerebrospinal fluid and maybe blood will spread in the window when the procedure starts, hindering the visualization. Keep the window clean and dry.

Once all the holes and windows are cut, start placing the screws (Fig. 2.5B–E). Perform 1.5 turns inside the bone for each screw. This will avoid going too deep and damaging the tissue while keeping the screw firm and stable. One drop of instant glue can be placed around the screw using a needle to enhance stability. Finally, check whether there is any clogging of the window, remove it and dry if necessary, and then position the array above the window using the stereotaxic apparatus. Inspect the position from several perspectives to verify whether it is centralized in the window and to guarantee that no wire will be bent during the procedure. Go down with the array until it touches the brain surface and then measure the DV coordinate. Descend very slowly (about 0.1 mm per minute) to allow the brain tissue to gradually settle down (Fig. 2.5C). It is advisable to perform this step in combination with electrophysiological recording. During electrode implantation, the ability to visualize neural activity on the oscilloscope, or to hear it using sound speakers, allows one to estimate the depth of neuronal layers and white matter. This is very important for the precise positioning of the electrodes.

Once the planned coordinate has been reached, it is time to build the helmet. If the array is not movable, use a small amount of vaseline paste (~50 μg) to fill the window around the electrodes before building the helmet. This will reduce the postoperative intracranial pressure. If you are using a microdrive, it is critical that you leave enough space for the moving parts to reach the maximum range of movement. The space should also be filled with vaseline paste. Be careful not to spread vaseline on the bone, for this will impair the cement adhesion.

The next step to build the helmet is to fix the array with dental cement or instant glue. Make sure that the array is fixed before releasing it from the stereotaxic apparatus. It is important that the array does not slip to avoid damaging the encephalic tissue around the target area. Once the array is securely attached, remove the stereotaxic handle. Using a small paintbrush wet in acrylic, capture small droplets of cement powder and spread dental cement all over the skull bone, making a thin layer. Apply gradually to build a helmet with as little cement as possible. First put cement around the arrays to keep them in place. Again, do not forget to leave room for the microdrives to move. Pay attention to the borders of the implant, not letting the cement spread to the surrounding muscle tissue and avoiding the solidification of sharp edges. Cover the entire array with cement, including the ground wire, leaving only the top part of the connector cement-free. A piece of tape can be used to prevent the cement from entering the connector holes. The helmet must have its entire surface finished round, as small and light as possible.

2.4.1 *Preoperative Care*

Before the surgery, plan the target areas, check the feasibility, and calculate the coordinates using a reliable neuroanatomical atlas (Paxinos and Watson, 2005). Coordinates above 6 mm laterally, or 9 mm posterior, from bregma are difficult to reach perpendicularly and require angling the cannula or electrode. Weigh the animal on the day of the surgery. Ideally, at the time of surgery, a male rat should weigh 250–300 g and a male mouse, 18–23 g.

The animal's health must be excellent before the surgery. Animal preparation for the surgery may include

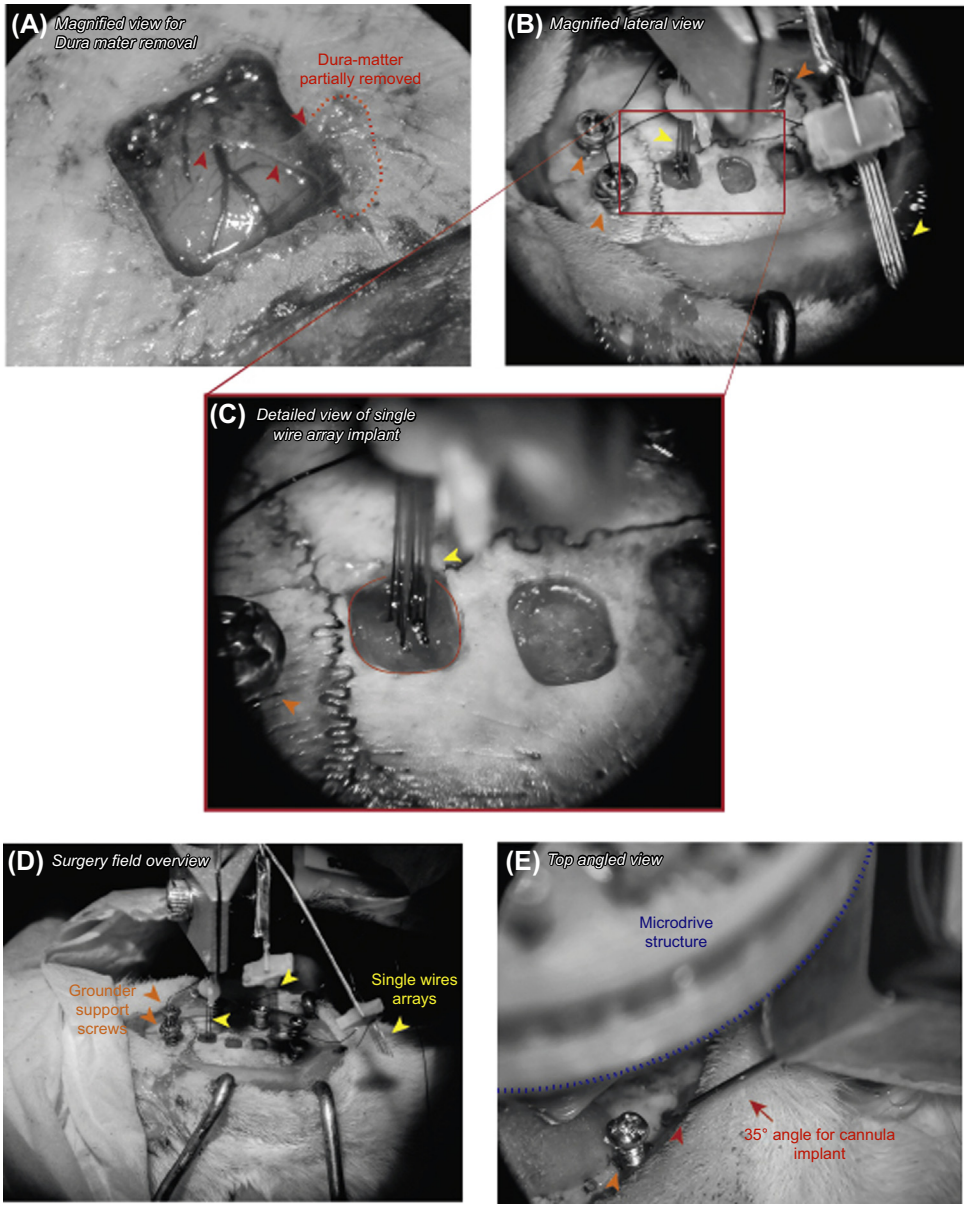

FIGURE 2.5 **Overview of surgery for electrode implantation.** (A) Dura mater partially removed. Note the semitransparent tissue indicated by red arrows and dashed line. The bottom part of the window shows exposed cortical surface and blood vessels. The dashed line outlines the dura mater already removed. (B) Overall view of the surgical field. Orange arrowheads indicate support and ground screws. (C) Detailed view of electrodes penetrating the cortex. The craniotomy window must be wider than the array dimensions (red line) because removal of the dura mater requires extra space. (D) Three different arrays being implanted, targeting different regions of interest (yellow arrowheads). (E) Cannula implant at an angle of 35° in addition of the microdrive (already implanted, delimited by the dashed blue line). Note the support screw secured next to the implant to guarantee that the cannula holds steady.

dietary supplementation to facilitate recovery. Handle each animal several times and for days; this will facilitate manipulation after the implant. A clean surgery room will help to avoid opportunistic infections. Spray alcohol 70% over the surgery table and stereotaxic device. Lay all instruments within reach of your hand, ideally in the order in which they will be used. Position and prepare the recording device. Keep an extra array at hand for immediate substitution in case the first one is damaged by accident during the surgery. Always use a checklist to ensure that nothing is missing.

2.4.2 *Postoperative Care*

After surgery, wait for the animal to wake up, ideally staying in the same environment to be able to check the animal's state frequently. The animal should be returned to the vivarium only after being fully awake, moving, and preferably feeding itself. Apply a dorsal

subcutaneous injection of an antiinflammatory (carprofen 5 mg/kg) diluted in saline (~3 mL for rats, ~0.3 mL for mice). Feed the animal with wet chow or a piece of banana to avoid friction when eating. Leave the food inside the cage to prevent the animal from raising the head too much. A warming lamp can be used, but be careful not to overheat the animal.

Examine the animal carefully on every postsurgical day. Observe the posture for signs of pain or injury, assess food and water intake, check around the eyes for secretion, inspect the wound surrounding the implant for signs of inflammation and/or infection (redness, swollen tissue, pus), and verify that there is no blood in the urine. Body weight typically decreases up to 10% in the first 48 h after surgery, increasing thereafter. Persistent weight loss on the days after the surgery may indicate the need to administer antibiotics.

To mitigate loneliness and social stress, you can enrich the home cage environment with paper balls made from absorbent tissue, or cartoon polygons. If the animal had a companion in the cage before the surgery, put them in daily contact to reduce social stress.

2.5 Chronic Extracellular Recordings

Chronic extracellular recordings require substantial effort to achieve success and produce reliable results. Before any experimental manipulation or surgical intervention, the investigator must prepare the recording setup. Ensure that the acquisition system is well connected to all complementary devices. If the experimental paradigm requires controlling, synchronizing, or manipulating any device (e.g., a laser, a drug delivering device, a running wheel, etc.), one must be aware of all the related technical issues. For instance, laser onset/offset delay, light intensity, whether actual running wheel speed coincides with programmed speed, etc. Experimental details as such are usually tricky and deserve special attention.

Once all the setup issues are solved and well known by the experimenter, establish a general recording schedule. With rare exceptions, it is crucial to habituate the animal to the experimental setting and routine manipulation by the investigators. It is important to handle the animal mildly and consistently across manipulations. For instance, avoid using strong odorants, or always use the same one, so that the animal will be able to recognize you every session. Also, if during the experimental session you will need to plug a cable or cannula over the animal's head, it is highly recommended that you habituate the animal to this procedure, restraining the head to mimic the procedure, or actually plugging the cable or cannula. It is also important to habituate the animal to the recording box, unless the experimental paradigm includes placing the animal in a nonfamiliar environment.

An alternative option for plugging the animal's implant to the recording cable is to use isoflurane, but keep in mind that this will be a new variable to be dealt with. After using isoflurane, wait for a washout of at least 1 h before starting the experiment. Never forget to make a detailed log of the experiment, noting all the specific manipulations and possible external perturbations. It is highly recommended to perform preliminary analysis of the data soon after acquisition, on an ongoing basis. Such preliminary analysis is essential to prevent data acquisition problems to linger and can give insights on future directions not considered before the experiment.

2.6 Local Field Potentials

The LFP is a very conspicuous signal, relatively easy to acquire and quite robust across recording sessions. Large electrode diameter (e.g., 50 μm) leads to low electrode impedance, which favors LFP acquisition. Typically, LFPs are preamplified (e.g., 500 times), filtered (e.g., 0.5–100 Hz), digitized using a digital acquisition card, and further processed in both online and offline software, using custom-made (e.g., http://www.open-ephys.org/) or commercial neural signal acquisition systems (e.g., Plexon, Neuralynx, Blackrock Microsystems). According to the Nyquist Theorem, the LFP sampling rate should, at a minimum, be twice as large as the maximum frequency of interest. For most applications in systems neuroscience, the LFP frequencies of interest are below 250 Hz. Nevertheless, to be on the safe side and preserve the ability to analyze faster signals, a sampling rate of 1000 Hz is recommended for digitalization of the LFP signal. Depending on the research scope, this sampling rate can be then subsampled offline to yield lighter, easier to handle files. Be careful not to overamplify the signals, leading to the recording of LFP clipped at the peaks and troughs. Noise due to poor isolation from the power line, or caused by animal movements, can be minimized by digitizing the signals right on top of the animal's head (e.g. http://intantech.com/). Be careful with the possible "spectral leakage" of spiking activity into the frequency band of high-frequency oscillations >100 Hz (Ray et al., 2008; Colgin et al., 2009; Jackson et al., 2011; Ray and Maunsell, 2011; Scheffer-Teixeira et al., 2012). "True" fast oscillations and "false" spike-leaked activity can be distinguished by preferred coupling phase with regard to the theta rhythm and by specific spectral signatures (Scheffer-Teixeira et al., 2013).

2.7 Spike Sorting

Extracellular recordings of action potentials (spikes) can be performed continuously for dozens of minutes to several hours, and even multiple days, using commercial or customized software for supervised real-time

spike sorting. To detect spikes, the raw signal must first be high-pass filtered (e.g., >500 Hz) to allow only fast frequency components to pass through; this filtering thus separates the extracellular spike waveforms from the slow frequency components that compose the LFP. Depending on electrode geometry, impedance, and location, the spikes will then be observable in the filtered signal as sharp voltage variations from the background activity. In this field, a neuron is typically referred to as a "unit." The term "multiunit activity" (MUA) refers to the combined spike times of all recorded neurons in the high-pass filtered signal. However, in extracellular recordings, different neurons may have different spike waveforms (as opposed to the stereotyped shape of action potentials when recorded inside the cell). This allows researchers to classify ("sort") spike waveforms into similarity groups ("clusters") in which each group of spikes corresponds to an individual neuron, also referred to as a "single unit" (SU).

Typically, 1–4 units can be sorted online from a single wire with spikes, with an average typically below 1.5 per electrode (Kralik et al., 2001). Tetrodes have been reported to record in average 5.4 units per tetrode and a maximum of 9 units (Gray et al., 1995). The offline validation of detected spikes should use the following cumulative criteria: (1) voltage thresholds >3 standard deviations of amplitude distributions; (2) signal-to-noise ratio >2.5 (defined as the amplitude of the spike over the background amplitude), as verified on the oscilloscope screen; (3) less than 0.5% of interspike intervals (ISIs) smaller than 1.0 ms; (4) stereotyped waveform shapes, as determined by a waveform template algorithm and principal component analysis (Fig. 2.6). Spike and LFP data can be investigated concurrently to distinguish different states of the sleep–wake cycle (Fig. 2.7).

2.8 Histological Confirmation of Electrode Placement

Once the recordings have been performed, the animal must be euthanized for histological confirmation of correct electrode positioning. This is done by means of an overdose of sodium pentobarbital (100 mg/kg, i.p.) after treating the animal with lidocaine (10 mg/kg, i.p., 10 min in advance). The brain can be removed and fixed fresh, to be sectioned, thaw-mounted on glass slides, fixed, and stained subsequently. Alternatively, to remove all the blood and fix the tissue before freezing, perform cardiac perfusion with 0.1 M phosphate buffer saline (PBS) at room temperature, followed by 4% fresh paraformaldehyde in PBS at 4°C (refrigerator temperature). Next, the brain should be removed from the skull and washed for 24–48 h at 4°C in PBS with sucrose 20–30% (~200 mL per brain). The brain should then be dried using a paper towel and cut into appropriate chunks using a blade (e.g., a frontal cut at the level of the optic chiasm will generate anterior and posterior chunks). Each brain chunk should then be positioned with the cut face down inside a small plastic mold, covered in precooled embedding medium (e.g., Tissue Tek at 4°C) without forming air bubbles, and quickly frozen by inserting ~75% of the mold into a mushy mixture of powdered dry ice and alcohol (ideally isopropylic alcohol, otherwise ethylic alcohol). Frozen brains should be stored at - 80°C until the moment of sectioning. On the day of the sectioning, place the frozen brain chunks inside the cryostat (~−20°C) and wait for 1 h for the tissue temperature to equilibrate with the surrounding. Then section the tissue at a thickness suited to the problem at hand (usually frontal sections of 20–50 μm). The brain sections can be thaw-mounted over glass slides following a serial distribution. For example, separate and label 10 slides and mount the 1st section on the first slide, the 2nd section on the second slide, etc. until the 11th section, which should be mounted on the first slide again, below the 1st section, and so on; this arrangement creates 10 "equivalent" slides that sample the brain similarly. In the case of brains that were frozen fresh, the slides should be post-fixed after sectioning (5 min in fresh 4% paraformaldehyde in PBS at 4°C). The brain sections can then be stained with cresyl violet or another staining able to reveal the glial scars surrounding the electrode tracks, quite useful to determine electrode position.

3. TROUBLESHOOTING

As in any experimental method, many things can go wrong during the various stages of chronic electrophysiological recordings for sleep and memory research. While some problems have no solution other than discarding the data and animal, other problems can be prevented or circumvented by a series of practices detailed later.

3.1 Avoiding Pitfalls

To succeed in the chronic implant, the following should be considered:

- In manufactured arrays, avoid movable parts as much as possible. The movable or untied parts are extremely delicate and may constitute rupture points during handling or implanting. Try to avoid loose parts and attach most of the elements to the PCB board or directly into the connector.
- Use very sharp scissors. The edge is crucial if you wish to detect neuronal spikes.

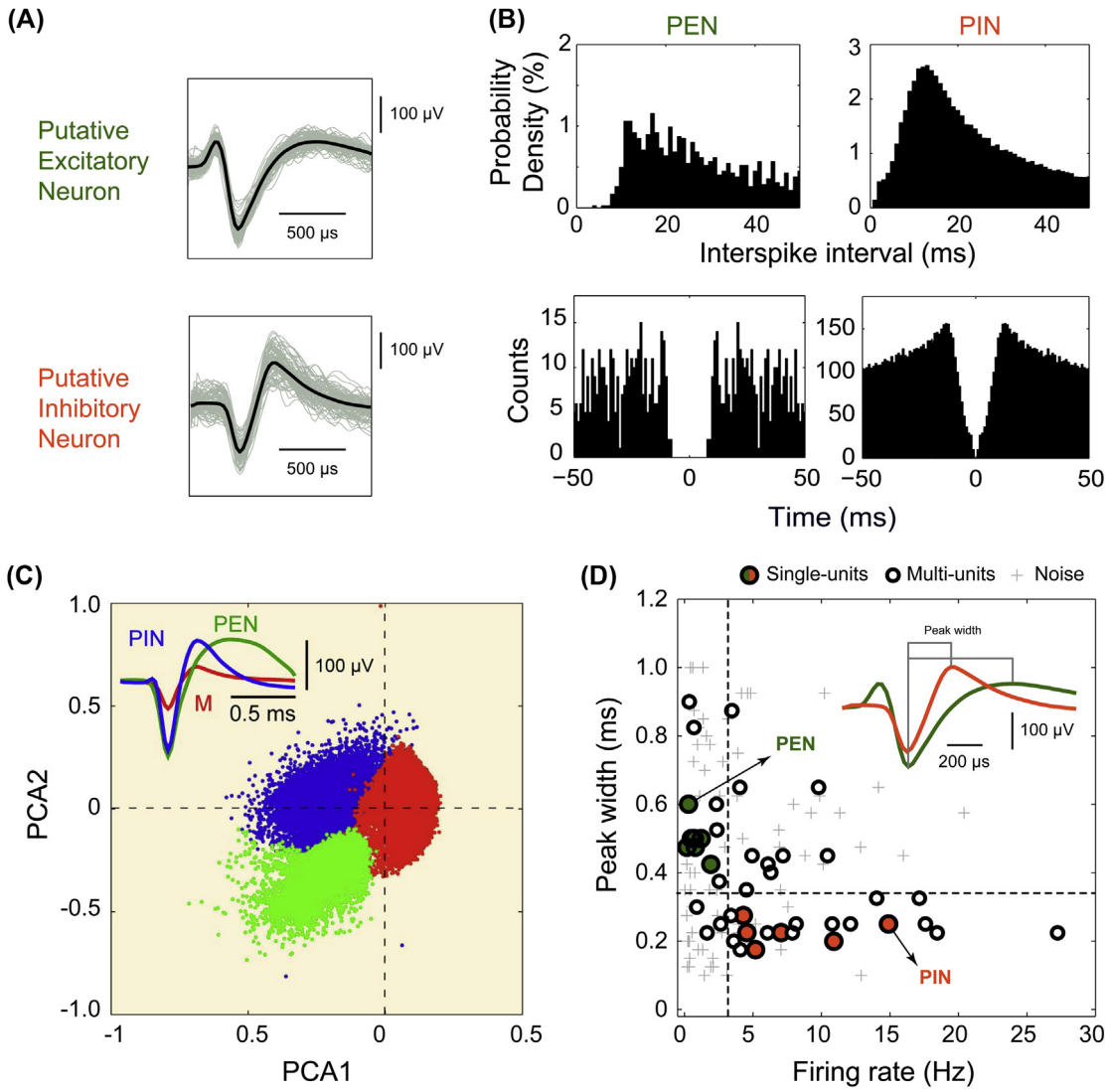

FIGURE 2.6 **Spike sorting.** (A) Multiple potentials of similar shape (gray waveforms) are superimposed to generate a mean profile of the unit (black waveform). (B) Probability density (top) and autocorrelations (bottom) for a putative excitatory neuron (PEN) and a putative inhibitory neuron (PIN). (C) Principal component analysis (PCA) is a commonly used method to disentangle distinct units recorded from the same electrode. M stands for multiunit signal. (D) PEN and PIN can be disambiguated in a plot of peak width versus firing rate.

- After building the array, keep it protected by a hard transparent cover (falcon tube with modeling clay). It will avert possible damage of the very delicate array.
- If the array ended up faulty, discard it and build another one. It is better to reconstruct the array than implant a bad one and acquire bad signals. You will regret later.
- Measure the impedance. It will detect imperfections in the welding. You can fix it before the surgery.
- Adequate preoperatory handling is very important to reduce stress immediately before the surgery and facilitate recovery. Daily handling sessions of 10 min for three consecutive days are usually enough to achieve the goal of habituating the animal to the experimenter. Put a laboratory coat on and let the animal walk on your lap and arms, gently touching the animal until it loses its fear.
- Nutrition is very important for adequate recovery after surgery. If your animals are experiencing poor recovery after surgery, diet supplementation for a couple of days with caloric foods (e.g., cat food) is a good idea.
- Usually, the surgical implant of electrode arrays takes more than 2 h, potentially running for several hours. Therefore, animals must be well nourished and hydrated before the surgery to ensure a fast recovery afterward. Subcutaneous injections of glucose-saline can be used to mitigate this problem.
- It is highly recommended to use only inhalation anesthetics, which can be quickly reverted. Note,

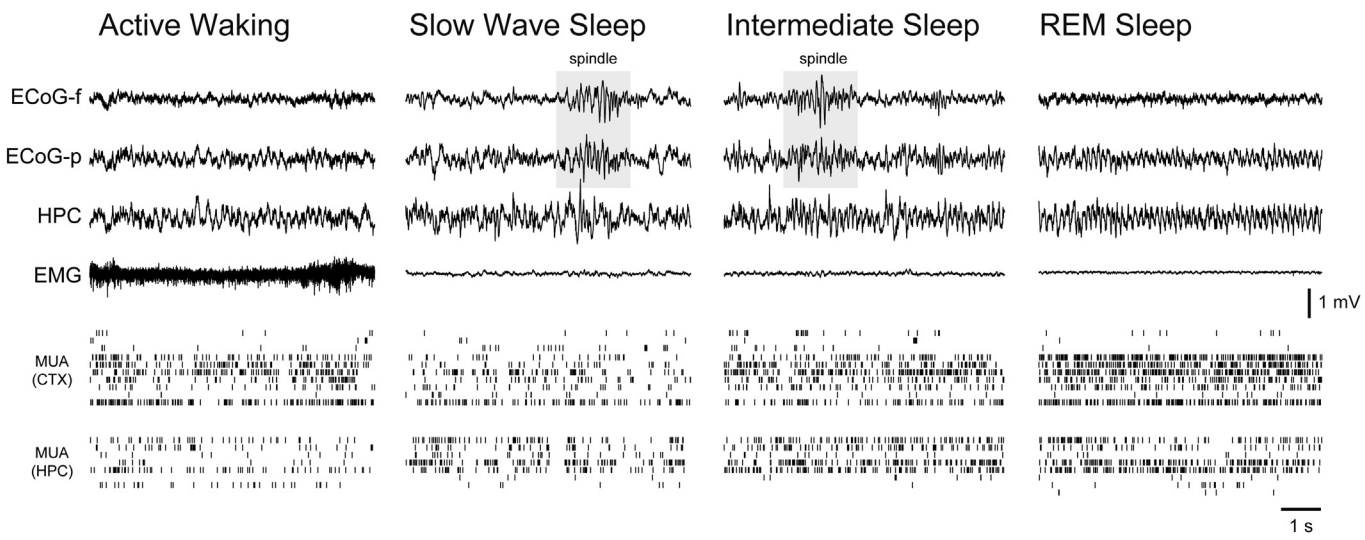

FIGURE 2.7 **Continuous recordings of multiple neurophysiological signals.** Frontal (f) and parietal (p) electrocorticogram (ECoG), intra-hippocampal local field potential (LFP) (HPC), electromyographic (EMG), and raster plots of multiunit activity (MUA) in the medial cortex (CTX) and hippocampus (HPC). Note the presence of cortical spindle activity during slow-wave sleep (SWS) and intermediate sleep.

however, that it is crucial to follow the clinical signs of the animal throughout the surgery because a fatal inhalatory overdose can occur quite fast.

- Entirely avoid crushing muscles or skin during the surgery. Use four small hemostats to pull the skin away from the cranium, making sure to clamp only the connective tissue beneath the skin.
- Avoid bleeding as much as possible during the surgery. If it happens, stop it by compressing the source with cotton swabs, using hemostatic powder, or scraping the bone over ruptured vessels. Wait until the bleeding ceases completely to continue the implant. Wash all the blood away with saline and dry the bone surface before going down with the electrodes. Some drops of hydrogen peroxide directly on the skull bone will help it dry. Do not let the hydrogen peroxide touch the brain or the dura mater.
- During the drilling/perforation of holes in the skull, small pieces of bone (and even bone powder) must be cleaned. This will prevent inflammation. Use a cotton swab wet in saline to remove the powder. Alternatively, use vacuum to remove the debris.
- At the array implant window, clotting may occur. Remove all clots before lowering the electrodes.
- The dura mater is quite fibrous. Gentle repetitive movements of the thin needle (with tip bent at 90°C) will eventually catch the dura mater and allow you to rip it without damaging the underlying blood vessels. Once the bent tip is inserted under the dura mater, move the needle along a line to cut it as straight as possible.
- Support screws and ground screws can be placed frontally or caudally to the electrodes, according to the specific needs of the preparation.
- For grounding, it is recommended to implant two short-circuited ground screws in nearby regions.
- If there is dry blood over the bone at the end of the implantation, scrape it off with the scalpel tip, clean and dry the surface before applying dental cement.
- Build the helmet as small and low as possible. This will make the implant light and uncumbersome, decrease the odds of implant loss, and promote normal behavior. Avoid leaving any sharp surface that can hurt the animal. The use of a small paintbrush to apply the acrylic droplets greatly increases the ability to shape the helmet as desired, without having acrylic spilling to unintended places. The helmet anchorage must be firm. Add some screws distributed on both sides of the skull surface. To place the screws, use a drill bit with a smaller diameter than the screw (for example, if the screw has 1 mm diameter, use a 0.7 mm drill bit). Rotate each screw one and a half turn and test with a tweezer to ensure that they are firmly inserted. For each screw, add one drop of instant glue around the base to keep the screw steady. Remember to dry the screw before applying the glue. Sometimes, during rotation of the screws, blood drops may sprout. Use small pieces of filter paper cut at sharp angles to remove the blood. This will clear the access of the glue to the screw base. It is better to clean with filter paper instead of cotton because cotton leaves lint.
- The helmet surface must be smooth, without spicules. Tips may cause subcutaneous irritation. One or two surgical stitches will help in fixing the helmet. In the long term, the skin attaches to the acrylic cement.
- If the goal is to record well-isolated neurons over multiple recording sessions, use a microdrive. It will

increase your chances substantially. Remember to leave enough space to move the microdrive. Paraffin can be used to fill the space to be occupied by the drive.
- To obtain a clean signal, your system must be very well grounded. The ground must make contact with the cerebrospinal fluid ("wet ground"). The other screws should only touch the bone.
- It is very important to monitor each animal closely, weighing and handling them every day after the surgery. Pay attention to signs of suffering: Body posture, eating, movement, porphyrin surrounding the eyes and nose, temperature, fur, tonus of the tail.
- Avoid disturbing without intent the natural behaviors of the animals. Do not allow strange odors in the recording chamber, such as perfume, food smells, or organic solvents. Prevent loud and/or sudden sounds to interfering with the experiment.
- Keep a strict regime of circadian illumination inside the recording chamber, thus preserving the animal's circadian rhythm. The illumination may be reduced to as low as 80 lux, but the cycle should go on.
- Check temperature and humidity every day.

3.2 Rescuing Troubled Recordings

- When recordings are bad, check all the electrical connections.
- If your acquisition system behaves strangely, reboot the computer.
- If mechanical noise dominates the signal, the ground may be broken.
- The recording cable must be protected, otherwise the animals may bite it. Metal coil works well to protect the cables. A commutator is welcome to allow the animals to explore freely without tangling the recording cables. Be careful to ground the commutator very well to avoid adding noise to the signal.
- Sometimes the acquisition is jeopardized because the files become too large. Saving the data in smaller chunks is very advisable.
- Make two data backups immediately after recording, using, for instance, an external hard drive and an internet cloud.

4. FUTURE PERSPECTIVES

Since the development of vacuum-tube amplifiers and cathode ray oscilloscopes more than 80 years ago (Garson, 2015), electrophysiological recordings have been an extensively used technique to study the functions of excitable cells. They allowed the first descriptions of the action potential itself and greatly enhanced our understanding of neurophysiology—how muscle fibers generate movement, how sensory cells encode stimulus attributes, how memories are encoded (Mountcastle, 1998). By directly probing the activity of individual neurons and neuronal ensembles, neurophysiologists were able to demonstrate that brain activity is in an entirely different processing mode during sleep in comparison with waking. Slow oscillations, spindles, K complex, theta oscillation, sharp waves, and ripples are conceptual entities emerged from LFP patterns recorded using electrophysiological setups. Nevertheless, we still lack a general theory or governing principles capable of explaining the functions and physiology of sleep. With the development of new techniques based on functional magnetic resonance imaging (fMRI) and light emission molecular sensors (calcium imaging and voltage-sensitive dyes), one may question what will be the place of conventional electrophysiology in future studies on sleep physiology. Will electrophysiological techniques be able to decipher how complex information is encoded in neural circuits, assemblies, and networks? How will electrophysiology applied to sleep research look like in the (near) future? We will next discuss the challenges associated with (1) reducing tissue damage associated with electrode positioning, (2) identifying neuronal phenotypes *in vivo*, (3) following unit activity over time in long-term recording sessions, (4) making sense of a large amount of data, and (5) probing ensemble activity of distant neuronal populations connected by long-range projections.

4.1 The Persistent Relevance of Electrophysiology

Electrophysiology can be considered one of the primary sources of any model of brain function. The rationale of recording action potentials during the sleep–wake cycle arises from the idea that discharges encode information, either from previous waking experiences (Pavlides and Winson, 1989; Wilson and McNaughton, 1994; Nadasdy et al., 1999; Ribeiro et al., 2007; van de Ven et al., 2016) or simulations of future events (Dragoi and Tonegawa, 2011; Ólafsdóttir et al., 2015; Guo et al., 2017). Therefore, the determination of specific firing patterns allows the experimentalist to get insights on the role of a particular structure in the sleep–wake cycle or in the memory process associated with it.

4.2 Toward Inert Recordings

An important aspect to be resolved in the future of *in vivo* electrophysiology is the damage produced by electrode positioning. As technology evolves and the miniaturization of amplifiers increases, the number of electrodes that can be implanted in the brain of an

experimental animal is reaching a limit. Therefore, one can expect nanotechnology to provide new types of electrodes and thinner and stiffer supporting materials, as it happened with the Utah and Michigan probes (Wise et al., 2008). Multifunctional organic devices already allow for spike and LFP recordings without damaging the brain surface—"NeuroGrid" (Khodagholy et al., 2015)—and for the combination of electrical recordings and chemical stimulation on the same site—"Neural Pixel" (Jonsson et al., 2016). Another possibility is to consider the use of genetically modified cells, specifically designed to produce and release conducting nanoparticles that can grow inside the tissue, leaving a low resistance track that can be used to conduct electrical signals from multiple sites and to reduce tissue mechanical damaging related to electrode positioning. Also, the merge of 3-D printer technology with the advances in design and synthesis of nanomaterials will enhance our ability to customize electrode arrays allowing the measurement of the activity of hundreds or thousands of cells simultaneously (Gonzales et al., 2017).

4.3 *In Vivo* Identification of Neuronal Phenotypes

Beyond the exquisite but quite delicate methods for juxtacellular staining (Pinault, 1996; Klausberger et al., 2003; Houweling and Brecht, 2008; Tang et al., 2014; Katona et al., 2016; Ebbesen et al., 2017), the precise characterization of the neuronal types recorded was improved in recent years by the development of genetically encoded tools as the adeno-associated virus delivery of target genes (Daya and Berns, 2008) and the transcription factor–dependent expression of fluorescent proteins and opsins (He, 2016). These protocols promise to boost studies of activity-dependent processes within specific neuronal populations during adaptive and maladaptive behaviors (Wallace et al., 2008; Sorensen et al., 2016; Wang, 2017; DeNardo and Luo, 2017). Also, combining the genetic approach with other sorts of physiological monitoring, as miniaturized chemical sensors used for cyclic voltammetry (Wei et al., 2015), will contribute to the creation of a "taxonomy" of neuronal phenotypes (Migliori and Shepherd, 2005) in which causality between neuronal activity and specific behaviors will be inferred.

4.4 Computer-Based Algorithms for Classification of Neuronal Subtypes

An indirect but convincing manner to determine neuronal phenotypes, and allow data mining on a large scale, is to use computational tools to putatively classify neuronal responses according to known profiles of activity, such as ISI and relation to neuronal oscillations (McBain and Fisahn, 2001; Li et al., 2015; Senzai and Buzsaki, 2017).

4.5 Long-Term Recordings

Individual recording sessions usually last minutes to hours, but certain questions in the sleep and memory field require recording for several days in a row (Ribeiro et al., 2004; Hengen et al., 2016). Stability in long-term recordings is of primary importance when testing the role of sleep in modulating neural plasticity (Tononi and Cirelli, 2014). In this type of studies, it is required to demonstrate the changes in the activation pattern of individual neurons or population of neurons over multiple time points. Usually, spike sorting can be used to fill the gaps in intermittent recording sessions (Hengen et al., 2016) by comparing the spike waveforms between sessions. However, one cannot be entirely sure that similar waveforms indeed represent the same neuron across successive sessions. Technical challenges toward gap-free recording sessions must consider automating waveform tracking (Dhawale, 2017) and electrode drift associated with brain movements (Liu, 2015) and chronic immune responses (Luan et al., 2017). Examples of such achievement have been recently demonstrated in mice, where the stability of single-neuron responses to electric stimulation lasted for up to 8 months (Fu et al., 2016).

4.6 Big Data

Managing and making sense of large amounts of data is one of the most critical challenges facing neurophysiology (Landhuis, 2017), as the recording capability expands to hundred and even thousands of neurons simultaneously (Sejnowski et al., 2014). Most spike acquisition systems sample something between 20,000 and 40,000 points of voltage information per second. A minimum of 20 kHz sampling rate is necessary for any acceptable approach of spike sorting. Thus, considering you have 32 channels and that your system stores that information as a single precision value (i.e., 4 bytes per sample), 1 h of recording will need 2.3 GB of memory space (at the minimum sampling rate) to store that information. Therefore, most commercial setups and some open-source systems will not store raw, wide-band, signals but instead, will filter, detect, and "cut" spike waveforms before storing them into memory. Although this approach can dramatically reduce memory space, it does not allow offline modifications of the detection threshold for unit activity. Other computational techniques have been developed to further compress, encrypt, and store large datasets (Brinkmann et al., 2009), which can deal with the combined unit and LFP

data (Worrell et al., 2012). Also, extracting meaningful information from dozens of units and their relation with LFP can be a daunting task. Therefore, mathematical tools for the identification of higher-order multineuronal entities, such as cell assemblies (Lopes-dos-Santos et al., 2011, 2013) and assembly (phase) sequences (Almeida-Filho et al., 2014), are growing increasingly popular (Gulati et al., 2014; Bower et al., 2015; Trouche et al., 2016; Chenkov et al., 2017).

4.7 Combining Recordings With RNA or Protein Labeling Techniques

There is much to be learned from the combination of spike and LFP recordings with methods to assess protein and mRNA levels during particular sleep–wake states of interest (Ribeiro et al., 2007), including the levels of phosphorylated proteins (Blanco et al., 2015). The envelope to be pushed is to perform such studies using large-scale proteomic or transcriptomic methods.

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