Whole-Cell Recording In Vivo

In vivo whole-cell patch-clamp recording provides a means for measuring membrane currents and potentials from individual cells in the intact animal. This allows for the study of intrinsic electrical properties of individual neurons, as well as network-related phenomena, such as post-synaptic responses to sensory stimuli, within the natural context of fully connected biological networks.

At a procedural level, in vivo whole-cell patch-clamp recording is very similar to the in vitro technique (see *UNITS 6.6, 6.7, & 6.10*), though there are important differences. Unlike the typical in vitro recording situation for mammalian preparations, one cannot utilize Differential Interference Contrast (DIC) optics to visualize neurons or the recording electrode in vivo, nor can the experimenter control the content of the "external solution" bathing the cells in the intact animal. Moreover, in the intact animal, electrode penetrations cause much more pronounced compression, or dimpling, of the tissue as compared with slice recording. However, the most significant differences are due to the motion that one must contend with in the intact animal, resulting primarily from cardiac and pulmonary pulsations. This unit focuses on strategies for overcoming the specific technical challenges posed by in vivo whole-cell recording.

The method described herein is designed to provide maximal recording time while minimizing the time and effort required for preparation. After preparing patch pipets and surgically exposing the cortex of the rat, patch electrodes are advanced through the cortex until an increase in electrical resistance indicates the presence of a neuron. Pipet pressure and voltage are then used to form a tight seal with the neural membrane and rupture the patch of membrane in the pipet tip, resulting in the whole-cell patch-clamp recording configuration.

Much of this protocol closely parallels that for blind whole-cell recording in brain slices (*UNIT 6.7*), and, with sufficient practice, the asymptotic data yield for this in vivo protocol can approach that of blind whole-cell recording in the slice. In addition, this procedure has been applied successfully to other rodent species and awake animals with minor modifications.

STRATEGIC PLANNING

There are several factors relating to the animal preparation that one should consider before starting an in vivo whole cell experiment: age, species, and anesthetic. Just as in slice recording, it is generally easier to obtain whole-cell recordings from the neurons of younger animals, but the data yield can be high for rats of any age. In addition to the factors that contribute to the ease of recording in brain slices taken from young animals, animals with smaller body sizes tend to exhibit smaller amplitude brain pulsations—so, if fully grown adult animals are needed for an in vivo experiment, adult mice, for example, might be easier to record from than much larger species. Mice have other advantages due to the vast amount of genetic work performed in that species.

When choosing an anesthetic, consider the action of the drug (if known) as it relates to the specific channels, currents, etc. to be studied, as well as the expected time-course of the effects of the drug. For example, make sure the frequency of injections necessary for sedation is compatible with the recording requirements.

This technique is sufficiently robust to animal motion that one option is to record without using any anesthetic at all. Young (post-natal day 20 to 30) rats and adult gerbils with

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no previous experience with head restraint can typically tolerate having their head fixed for ~4 hr. For awake recording, perform the surgery well in advance (i.e., ≥1 day), and allow the animal sufficient time to recover. During the surgery: do not perform a cisternal drain; attach a head-post to the skull; and attach a closable well over the craniotomy. It is also a good idea to surgically insert a Ag/AgCl ground wire under the skin at the back of the neck with a convenient electrical connector (e.g., SPC Technology gold pins and female connectors: CCD-206-1-SS and CCD-206-1-SP) that can quickly be plugged into the headstage at the start of a recording session.

Whole-cell recording provides a means for filling neurons with dyes or tracers that can be used for histological reconstruction of the recorded neuron(s) following the experiment. If histology is desired, then add a dye or tracer (e.g., 2% biocytin) to the internal solution (make sure to filter the solution after this is added) and take care to pull away from each neuron slowly after ~20 min of recording time, ideally while the neuron is still healthy. At the end of the recording session perfuse the animal with paraformaldehyde, extract the intact brain, and refrigerate for ~24 hr before slicing the brain and performing the required histological procedure (see *UNIT 1.1*).

If cell-attached recordings of spiking activity are all that is desired, the requirements of the seal are greatly relaxed—seals of greater than ~15 MΩ can provide sufficient signal-to-noise ratio to allow for essentially perfect identification of all spikes—and the same electrode may be reused to record from multiple neurons. In addition, histological reconstruction of neurons recorded in cell-attached mode can be accomplished with the use of juxtacellular labeling (Pinault, 1996).

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

BASIC PROTOCOL

IN VIVO WHOLE-CELL PATCH-CLAMP RECORDING

Materials

Physiological buffer (see recipe)
Normal saline: 0.9% (w/v) NaCl
Rats (male or female, post-natal day 17 to 30; Sprague-Dawley)
General anesthetic (e.g., 60 mg ketamine/0.5 mg medetomedine per kg)
Internal solution (potassium- or cesium-based, see recipes)
Agarose solution (1% to 2% agarose by weight in physiological buffer; Type III-A, A9793, Sigma), melted and kept up to 4 hr at ~40°C

Electrode puller (e.g., Narishige 2-stage vertical puller)
Electrode glass (e.g., filamented, fire-polished, thin-walled, borosilicate electrode glass 3 in. (75 mm) length, 1.5 mm o.d., World Precision Instruments)
Dissecting microscope
Patch pipet storage container with cover
Disposable 1-ml and 30-ml syringes (for anesthesia and pipet pressure control, respectively)
Disposable syringe needles: 25-G (for rats) or 27-G (for mice)
Temperature controller with heating pad and rectal thermometer
Stereotaxic frame (for rats) that allows access to desired cortical region
Cotton swabs
High speed pneumatic dental drill
Gel foam sponges
Dural hook
Recording chamber, electrically shielded

Computer-based data acquisition/analysis system, including A/D board and software (see *UNITS 6.1 & 6.6*)
Silver ground wire coated with AgCl at tip (e.g., model E201Ag-AgCl pellet; Axon Instruments)
Amplifier with headstage (e.g., Axopatch 200B from Axon Instruments)
Micromanipulater for headstage (e.g., MP-285 model, Sutter Instruments)
Small plastic alligator clip
Pipet holder with silver electrode wire coated with AgCl at tip
Tubing for pressure control (made of hard plastic, ~3 mm o.d.)
Three-way valve
Pressure gauge (e.g., DPM-1B model, Bio Tek Instruments)

Additional reagents and equipment for injection of rodents (*APPENDIX 4F*) and patch-clamp techniques (*UNITS 6.1, 6.3, 6.6, 6.7, 6.10, & 6.16*)

Prepare patch pipet electrodes

1. Immediately before beginning surgery, use the electrode puller to prepare between 10 and 50 patch pipet electrodes (e.g., see *UNIT 6.3*); this is a good point to measure their impedance in physiological buffer and visually inspect them with a dissecting microscope for defects, size, and shape (see Critical Parameters).

   Electrodes should all be as close to the same length as possible to facilitate depth estimation during multiple penetrations through the agarose and cortex.

Surgically prepare animal for recording

Make sure the animal is properly hydrated with periodic subcutaneous injections of saline, in which case it should urinate periodically throughout the experiment.

2. Fill a 1-ml syringe with the general anesthetic and, using a 25-G needle (for rats) or a 27-G needle (for mice), inject the animal intraperitoneally (*APPENDIX 4F*), place on heating pad, and mount on a stereotaxic frame.

3. Perform a cisternal drain as follows:

   a. First make a horizontal incision in the skin over the occipital bone at the base of the skull. Retract the skin, and use forceps to remove neck muscles from the base of the skull and occipital bone with a downward peeling motion until the allanto-occipital membrane is exposed; it should appear as a white membrane.

   b. Hold a cotton swab horizontally and place the side of the cotton tip on the retracted muscles just below the exposed membrane. While pushing down and rolling the swab down and away from the skull to pull the membrane taut and away from the spinal cord, use a scalpel to poke a small hole in the membrane with your other hand, taking care not to cut the spinal cord.

   Clear cerebrospinal fluid often appears outside the membrane when it is successfully cut.

   This step is not crucial, but a cisternal drain can reduce cardiac- and pulmonary-induced pulsations of the cortex, and it can reduce the risk of damaging the cortex during the craniotomy and durotomy.

4. Expose the cortex as follows:

   a. Make an incision and remove sufficient skin from the scalp to expose the skull over the cortical region to be recorded from. Remove fascia from the exposed skull and, using the scalpel or drill, remove a 2-mm × 2-mm area of bone over the desired cortical region, taking care not to damage the dura or cortex.

   b. Use gel foam sponges soaked in physiological buffer and cotton swabs to extract blood from any bleeding parts of the skull or dura.

c. Using the dural hook or a disposable syringe needle, cut and retract the dura to expose the cortex, taking care to avoid touching blood vessels and the brain with any of the surgical instruments.

If a drill is used for the craniotomy, irrigate the bone with cortex buffer and take frequent pauses to avoid overheating the cortex.

Position pipet above the exposed cortex

5. Place stereotaxic frame with rat and heating pad in a recording chamber.

6. Attach silver ground wire from the headstage to the rat by wrapping the moist underside of a flap of skin from the edge of the cisternal drain around the AgCl pellet at the end of the ground wire and clamping the fold of skin together with a small plastic alligator clip.

If injectable anesthesia is used, it is best to administer whatever supplemental dose of anesthesia is required to ensure sedation for the desired duration of the recording session before advancing the electrode into the cortex, rather than to wait and have to administer a shot while the recording is taking place.

7. Partially fill a patch-pipet with internal solution and tap the side of the pipet with a finger or forceps to dislodge any air bubbles near the tip and place it in the pipet holder. Make certain that the recording electrode wire reaches the fluid in the pipet.

Potassium-based internal solution is best for current clamp recordings and cesium-based internal solution is often better for voltage clamp applications.

8. Apply positive pressure to the back of the pipet via a piece of tubing attached to the side port of the pipet holder, either by mouth or with a 30-ml syringe. Maintain pressure by closing a valve or three-way trap. Monitor the pressure with a pressure gauge.

About 75 mmHg works well for a 3.5 MΩ electrode. A good rule of thumb is to set the pressure in proportion to the electrode impedance as measured in a bath of physiological buffer; i.e., for a 4.0 MΩ electrode, set the pressure to about 85 mmHg.

9. Lower the pipet tip to just above cortical surface, well away from the edge of the craniotomy and any visible blood vessels. Advance the pipet axially until it just touches the cortical surface, quickly note the reading of the position of the micromanipulator, and withdraw the electrode axially by $\geq$2 cm.

10. Take up ~0.2 ml of melted agarose into a disposable 1 ml syringe (with no needle). Periodically test the temperature of the cooling agarose in the syringe (e.g., on wrist) until it reaches approximate body temperature and then apply melted agarose to the cortex until there is between 2 and 5 mm of agar built up.

The agarose covering the cortex can remain soft enough to be penetrable by new patch pipets for well over an hour if it is kept moist. If it becomes hard or opaque, it should be removed and replaced.

Hunt for cells in voltage clamp mode

11. Increase pressure to ~200 mmHg and advance electrode until it penetrates the agarose. Monitor the electrode resistance on an oscilloscope or computer screen by applying small (~10 mV), brief (~10 msec) steps in voltage at least 5 times per second and applying Ohm's law (see *UNITS 6.6 & 6.7*). Quickly note the resistance of the electrode in the agarose, and then swiftly (at a rate of ~25 μm/sec) advance the electrode into the cortex.

If the lowest layer of the dura was not entirely removed during the durotomy, then entering the cortex is usually accompanied by a brief but large jump in the DC current level indicated by the oscilloscope trace before and after the current pulse.

Because of brain dimpling, the electrode might not actually enter the cortex until the patch pipet is 100 µm or more below the value recorded before agarose was applied. If the upper layer of the dura was not completely removed, the tip of the patch pipet is likely to break as it enters the brain.

By Ohm's law, the electrode resistance is given by the test pulse command voltage divided by the height of the current response on the oscilloscope (See Fig. 6.6.2).

12. Using a 30-ml syringe, very briefly flush the electrode with a sharp pulse of high positive pressure and then lower the pipet pressure to the level used during step 8. Advance electrode at about 1 to 3 µm/sec, paying close attention to changes in the height of the test pulse responses.

    Periodically check the depth of the electrode relative to the cortex surface as indicated by the manipulator to avoid driving the electrode too deep into the brain, and thus bypassing the cortical cell bodies; the thickness of cortex in the young rat is ∼1.5 mm.

13. When a sudden decrease in test pulse height is noted, indicating an increase in electrode resistance, which can signal that the electrode is approaching a cell, continue to advance until the maximum increase in resistance is roughly 50% of the previous maximum height, and then simultaneously release the pipet pressure and switch the DC voltage command to −70 mV.

    Due to the motion of the heart and lungs, a cell in front of the electrode will tend to rhythmically affect the height of the test pulse responses.

    Due to the amount of motion and the extent of brain dimpling, the electrode resistance can first increase due to brief contact with a neuron long before the electrode has advanced enough to form a seal.

Form seal

14. Continue to monitor the responses to the test pulses and apply very light suction by mouth until a high-resistance seal (>1 GΩ) is formed.

    Ideally, part of the cell membrane will immediately enter the open tip of the patch pipet, as indicated by an abrupt increase in resistance as soon as the pressure is released, and a gigaohm seal will form over a period of tens of seconds, with little applied suction (-10 mmHg or less) and no advancement of the electrode. If, however, resistance does not immediately increase when the positive pressure is released, a seal can sometimes be established by steadily increasing suction by mouth and/or slowly advancing the electrode. If the electrode resistance never increases beyond a factor of two of its original value, apply large positive pressure (∼150 mmHg) and advance the electrode until the resistance drops to the original value, and return to step 12. If the electrode resistance rises by more than 50% during the attempted seal formation, retract the electrode from the brain, replace the patch pipet, moisten the surface of the agarose with a few drops of physiological buffer, and return to step 11.

    If a gigaohm seal does not form within 1 to 2 min following the release of positive pressure, it becomes increasingly less likely with time to form at all. However, seals can sometimes be achieved by employing more extreme manipulations, such as applying strong positive or negative pressure, switching the holding potential to +70 mV, advancing and withdrawing the electrode, etc.; it is worth occasionally trying such manipulations if only to gain intuition about the environment of the electrode, such as better judging distances to cells in front of the electrode.

15. Once a gigaohm seal is formed, increase the amplifier gain (≥20 mV/pA), using the fast capacitance compensation controls on the patch-clamp amplifier (see *UNITS 6.6 & 6.7*). Recordings can now be performed in cell-attached mode.

    Different patch pipets pulled on the same day tend to have similar properties, such that it is often unnecessary to readjust the capacitance compensation settings on the amplifier once they have been set for the first electrode. Unfortunately, it is not always possible to perfectly remove the capacitive charging transients for any setting of the compensation

controls. This can obscure the increase in the transient that occurs during break-in, and it can even hinder the formation of a gigaohm seal in some cases.

16. Gently apply brief (1 to 3 sec) mild suction (approximately −4 mmHg) to the pipet by mouth and observe whether the DC current, indicated by the level of the oscilloscope trace before and after the test pulse, changes in response to suction. If not, slowly (∼2 μm/sec) retract the electrode by ∼15 μm, and again apply mild suction and check for movement in the DC current level. Continue to withdraw the electrode until mild suction results in perceptible movement of the DC current level, or until an abrupt decrease in pipet resistance occurs—indicating either that the seal has been compromised, in which case the test pulse response will still appear as a square pulse, or that the cell membrane has been ruptured and a whole-cell recording configuration has been achieved, as indicated by prominent capacitance transients on the oscilloscope trace (see Fig. 6.6.2).

    This method requires that the vertical scale of the test pulse response be fixed, and magnified enough to resolve changes in the DC level that are comparable in magnitude to the height of the test pulse response.

    Empirically, it is the observation of the authors that the susceptibility of the DC current to fluctuations in pipet pressure is an indicator of the degree to which the electrode is pushed into the cell. It is sometimes necessary to retract the electrode by over 100 μm before pipet suction results in a noticeable deflection of the DC current, but it is important to remember that due to the relaxation of brain dimpling around the shaft of the patch pipet during electrode withdrawal, it is unlikely that the pipet tip is moving very far relative to the neuron even in these extreme cases.

    Occasionally, the capacitance compensation settings may need readjustment following withdrawal of the pipet.

Break in

17. Rupture the small patch of membrane at the tip of the pipet by applying mild, punctate pulses of suction by mouth; if break-in does not occur immediately, continue to administer brief pulses, applying increasingly stronger suction on subsequent attempts.

    One can also break into the cell by applying steady, slowly increasing suction by mouth; if this method is employed, it is important to stop applying suction immediately after breaking into the cell. If these methods fail, use a brief pulse of current to rupture the patch membrane (e.g., by pressing the "zap" button on the Axopatch 200B). Start with the lowest setting (i.e., on the Axopatch 200B, rotate the dial to the left of the zap button, which controls injected current duration, as far counter-clockwise as possible without clicking it into the "manual" setting), and continue to administer stronger pulses until either a whole-cell configuration is achieved, or the seal is compromised.

18. Once the whole-cell recording configuration has been achieved, compute the series resistance (sometimes called "access resistance") by applying Ohm's law to the peak value of the oscilloscope trace at the initiation of the current response to the test pulse. Next, compute the sum of the series and cell input resistances by applying Ohm's law to the asymptotic value of the exponentially falling current response, which should be well approximated by the height of the trace towards the end of the test pulse.

    If desired, it is now possible to null the whole-cell transients (UNIT 6.6), and/or record in voltage or current clamp mode.

    By imagining a simple, one-compartment model for a neuron, the series resistance can be thought of as the resistance experienced by current flowing from the recording electrode wire to a point just inside the cell; and the cell input resistance can be thought of as the resistance of the cell membrane (see Fig. 6.6.4). Note that some investigators refer to the sum of these two resistances as the "input resistance."

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; *for suppliers, see* **SUPPLIERS APPENDIX**.

Internal solution, potassium-based

140 mM potassium gluconate
10 mM HEPES
2 mM $MgCl_2$
0.05 mM $CaCl_2$
4 mM MgATP
0.4 mM Na_2GTP,
10 mM Na_2-phosphocreatine
10 mM BAPTA (Sigma)
Adjust pH to ∼7.25 with KOH
Dilute to ∼290 mOsm
Filter with 0.2-μm filter
Store in 0.5-ml aliquots up to 1 year at −20°C.

Internal solution, cesium-based

115 mM cesium methanesulfonate ($CsMeSO_3$; Sigma-Aldrich)
20 mM CsCl
10 mM HEPES
2.5 mM $MgCl_2$,
0.4 mM Na_2ATP
0.4 mM NaGTP
10 mM Na-phosphocreatine
10 mM EGTA
Adjust pH to ∼7.25 with CsOH
Dilute to ∼290 mOsm
Filter with 0.2-μm filter
Store in 0.5-ml aliquots up to 1 year at −20°C

Physiological buffer (example)

127 mM NaCl
25 mM Na_2CO_3
1.25 mM NaH_2PO_4
2.5 mM KCl
1 mM $MgCl_2$
25 mM glucose
Filter with 0.4-μm filter
Store up to 1 month at 4°C

COMMENTARY

Background Information

In the decades since the inception of the patch-clamp technique (Neher and Sakmann, 1976; Hamill et al., 1981), patch- clamp methods in general, and whole-cell recording in particular, have largely been developed in vitro (e.g., Blanton et al., 1989), with rat brain slices among the most common of the in vitro preparations. This body of work has contributed enormously to the understanding of many important phenomena in excitable cells. However, in recent years, an increasing number of groups have also applied whole-cell recording techniques in the intact animal (e.g., Ferster and Jagadeesh, 1992; Borg-Graham et al., 1998; Hirsch et al., 1998; Chung et al., 2002; Larkum and Zhu, 2002; Wehr and Zador, 2003; Bureau et al., 2004; DeWeese and Zador, 2004), including unanesthetized preparations (Covey et al., 1996; Aksay et al., 2001;

Margrie et al., 2002). In vivo studies, such as these, offer the tantalizing opportunity to uncover the underlying principles and mechanisms of neural interactions within the natural context of fully intact networks.

Sharp electrode recording (often referred to as simply "intracellular recording") is another method for recording membrane currents and potentials within individual cells that has been successfully employed in vivo (Gray and McCormick, 1996; Svoboda et al., 1997; Stern et al., 1998; Las et al., 2005), and even in awake animals (Fee, 2000; Aksay et al., 2001; Steriade et al., 2001). The whole-cell technique has several advantages over sharp recording, including greater recording stability in the face of motion (however, see Fee, 2000), and better electrical and chemical control of the interior of the cell. In patch recording, various constituents of the internal milieu of the neuron are diluted and extracted as the interior of the cell is dialyzed by the internal solution in the patch pipet. This effect can be applied to an advantage in some experimental paradigms, but may be a drawback in others. In general, dialysis proceeds slower during whole-cell recording with higher series resistance. Perforated patch recording (Falke et al., 1989; Spruston and Johnston, 1992), in which the internal solution is augmented with agents (such as antibiotics) that introduce pores in the neural membrane while the pipet is in cell-attached mode, offers electrical control and mechanical stability similar to whole-cell recording, while preventing the indiscriminant dialysis of the interior of the neuron. This method has found many in vitro applications (*UNIT 6.23*), but it may be challenging to implement in vivo due to the potential for widespread neural damage resulting from the release of these pore-inducing agents as the electrode is advanced through the brain with positive pipet pressure. One disadvantage of all of these techniques is that they typically require the animal to be at least partially restrained, which limits the range of behaviors that can be studied in the awake animal (however, see Fee, 2000; Lee et al., 2006).

If spike records are sufficient, a variety of extracellular recording methods can be used, such as single tungsten wire recording, tetrode recording (see *UNIT 6.16*), and cell-attached recording using a whole-cell patch pipet (see Basic Protocol step 15). All of these methods can produce a high yield of well-isolated neural spiking records, although cell-attached recording provides near-perfect isolation with minimal effort during the data analysis phase, even when applied in vivo (e.g., DeWeese et al., 2003). Tetrodes can provide simultaneous extracellular records from multiple neurons in freely behaving animals. Most single-wire tungsten recordings are performed with the head fixed, but these can also be employed in unrestrained animals. The cell-attached method has the disadvantage that it requires head restraint, but it is unique among these approaches in providing a means for histological reconstruction of recorded neurons via juxtacellular labeling (i.e., using pulses of current to induce small pores in the cell membrane and inject the neuron with a labeling agent contained in the pipet solution; Pinault, 1996). Another important difference between these methods is selection bias: neurons with high spontaneous or stimulus-evoked firing rates are more easily detected and isolated using tungsten or tetrode recording methods, whereas cell-attached recording is biased towards neurons that are more easily patched onto, regardless of their spiking activity.

Critical Parameters

Two of the most critical components of whole-cell recording are the patch pipet electrodes (*UNIT 6.3*) and internal solution (see Reagents and Solutions, and *UNITS 6.6 & 6.7*). It is not unusual to have to replace the patch pipet 30 or more times during the course of an in vivo experiment. After gaining experience and when everything is working perfectly, a stable recording can be obtained on nearly every penetration, but even experts experience periods of low yield. Determining the best parameter settings for the electrode puller on any given day and actually pulling 30 or more electrodes takes some time (see Time Considerations), so it is helpful to prepare a batch of electrodes before beginning the surgery to maximize the available recording time once the preparation is ready. However, electrodes can be adversely affected by dust and condensation from the air, so it is best not to prepare them too long before their use; and it helps to keep them in a covered patch pipet storage container, which also prevents the delicate electrode tips from being broken. If there is any question whether the electrode tip has come in contact with a hand, the counter top, or any other object, it should be discarded.

Another consequence of having to replace the patch pipet so often is that one recording session can cause substantial wear and tear on the electrode wire, which can shorten its useful lifetime, and can result in small bits

of AgCl breaking off and clogging the patch pipet tip. An easy way to alleviate this is to use fire-polished electrode glass (i.e., fire-polished on the end *opposite* the tip). Fire-polishing is commonly performed on the patch pipet tips as well (see *UNIT 6.3*), but this can have the detrimental effect of causing fragile gigaohm seals that form and dissipate immediately. This is presumably because the fire-polished glass is sufficiently smooth and/or blunted to permit seals to form between the very tip of the pipet and a locally flat patch of the membrane of the cell, rather than allowing the membrane to deform and enter the pipet tip so that a more mechanically stable seal can form between a larger patch of membrane and the inner surface of the pipet.

In the hands of the authors, the best range for patch electrode sizes is between 3.0 and 5.0 MΩ, measured while the electrode tip is immersed in a bath of physiological buffer with the electrode filled with a potassium-based internal solution; the range is slightly less (∼2.6 to 4.5 MΩ) for a cesium-based internal solution (see Reagents and Solutions). Ideal electrodes are ∼3.5 MΩ in the bath, which corresponds to a tip diameter of a little over 3 µm. It is advisable to visually inspect the tip of each electrode under a microscope after it has been pulled in order to ensure that it is the right size and shape and free of obvious defects, such as a protruding filament, jagged edge at the tip, or debris inside the electrode. With experience, visual inspection will be sufficient to judge the impedance of the electrode, obviating the need to directly measure impedance in buffer prior to surgery. Some electrode pullers heat up with use, so it might be necessary to periodically lower the temperature settings of the puller when preparing a large batch of electrodes. In addition to size, electrode shape also matters (see *UNIT 6.3*).

There are a great many considerations for determining the composition of the internal solution, but perhaps the most critical for establishing gigaohm seals are the osmolarity and pH (see Reagents and Solutions). It is also crucial to filter (0.2 µm) the internal solution. A good rule of thumb is that patch pipets and internal solutions that perform well for whole-cell recording in brain slices typically also work well in vivo. Specifically, when things are not working, the best way to rule out the possibility that a particular batch of internal solution—or a particular set of electrode puller parameters—is suitable for in vivo whole-cell recording is to try it in a slice. As a control, one can test a batch of internal solution (stored in the freezer) that has worked in the past, either in vivo or in vitro; for this reason, it is extremely useful to always maintain at least one batch of internal solution that has been proven to work.

Troubleshooting

Success at blind whole-cell recording is extremely sensitive to many intangible factors concerning the fine manipulation of electrode position and pressure, as well as the ability to infer the current state of the electrode's environment based solely on the response to brief voltage pulses. Below are some practical suggestions for overcoming problems commonly experienced with this technique. There are two categories of experimental problems covered in this section—*basic technical issues*, which are problems that have to do with the proper functioning of the equipment and maintenance of the preparation, and *failure modes*, which are commonly experienced manifestations of these underlying technical issues as well as more subtle procedural errors—followed by some *general advice* on how best to approach these types of experiments.

Basic technical issues

Equipment not working properly

As with any other type of experiment, always check that all electrical equipment is properly connected, plugged into power, and turned on.

Electrical noise

Using a voltmeter, make sure that all metal objects in the recording chamber, including the microscope, manipulator, isolation table, and the Faraday cage itself, are grounded. Ungrounded metal objects can act as antennae for electromagnetic radiation emanating from the building wiring. Also, minimize the number of "ground loops" in the setup by checking that each metal object or piece of electrical equipment has only one path to ground; keep in mind that the power cord of many devices has a ground connector that is electrically continuous with the chassis.

A ground loop in the apparatus, particularly if it involves the recording electrode and ground wire, can add noise in two ways. First, changes in the magnetic flux through a ground loop will induce a current in the loop. In this way, electromagnetic radiation from the alternating currents in the wiring in the wall can inductively couple the loop to wiring in the wall. Second, a device that is connected to the ground via more than one path can experience different, time-varying voltages at its

contact point to each of these paths depending on the relative amounts of current passing through each path, and the relative impedances of each path. Thus, the device can experience different, time-dependent voltages at points that were designed to expect the same value.

The usual culprit for noise in an ostensibly working setup that contains neither ungrounded metal objects nor ground loops is a bad electrical contact between the animal and the ground wire. Merely disconnecting the ground wire from the animal and reattaching it often solves the problem. A reliable configuration is to wrap the underside of a cut section the animal's skin around the AgCl end of the ground wire and hold the fold of skin closed with a small plastic alligator clip. Connecting to skin rather than muscle lessens the risk of picking up electrical impulses from muscular contractions. Choose a location far from the heart, and not too far from the recording site to minimize motion artifacts. AgCl pellets (e.g., model E201Ag-AgCl pellet; Axon Instruments) provide stable contact between the animal and a fixed amount of surface area of AgCl.

If noise persists, check that the recording electrode wire is making good contact inside the electrode holder. Clean all electrical contacts on the headstage with alcohol. Re-chloride or replace the ground wire and the recording electrode wire. There are several ways to coat the end of the electrode wire with AgCl. Immersing the end of the electrode wire in bleach for at least ~20 min is a popular method, but it can result in electrode drift as well as crumbling of the AgCl coating (see Electrode drift and Sudden increase in resistance prior to establishing contact with cell below). A better method that tends to alleviate these problems is to coat the silver electrode wires with molten AgCl, which can be done with a Bunsen burner and a Pyrex petri dish.

There are myriad sources of intermittent electrical noise (ceiling fans, refrigerators, etc.). In addition to 60-Hz hum, it is common to pick up voltage spikes occurring at 120 Hz that result from the abrupt change in polarity that occur every half-cycle in an electric motor being driven by 60 Hz alternating current.

Electrode drift; not operating within the range of the amplifier

A steadily increasing (or decreasing) DC current level while in voltage clamp mode is usually a sign of a changing junction potential occurring at either the interface between the ground wire and the experimental animal, or the recording electrode wire and the internal solution in the patch pipet. This can happen if the amount of surface area in contact between the ground wire and the skin of the animal changes with time, or if this contact becomes drier or wetter with time. Detaching and reattaching the ground wire should fix this problem. Drift can also result if there is either insufficient AgCl coating on one of the two wires, or if the AgCl is actively crumbling or flaking away. Re-chloriding (preferably with molten AgCl, rather than dipping in bleach) or replacing one or both of the wires should solve the problem of insufficient AgCl, but if there are cracks in the AgCl, then it is best to replace the wire altogether. If the junction potentials at the electrode and ground wires are sufficiently different, the DC signal applied to the input of the amplifier will be so large that it cannot be offset and the output will be "railed" or "pinned" to one extreme or the other. A ground wire that works well for a particular ionic bath solution used to test the setup may not work when connected to an animal.

Microphonics

Under some conditions electronics in the headstage or amplifier can act as a microphone, converting acoustic vibrations into electrical signals that can corrupt the output data stream; this is a particular nuisance for studies in auditory physiology where a sound signal is applied as part of the experiment. If this occurs, make sure that the headstage being used corresponds to the amplifier, since some manufacturers adjust each individual amplifier to compensate for the specific microphonic (and other) properties of its corresponding headstage. If the problem persists even with a model cell (electrical circuits that simulate the passive electrical properties of a biological cell and are often supplied by the amplifier manufacturer) in place, then the headstage may require servicing.

Photovoltaics

Under some conditions, incident light falling on the AgCl coating on the recording electrode wire or the ground wire can induce a measurable voltage drop. If replacing both wires does not solve this problem, the simplest solution is to maintain constant lighting conditions during the experiment, or both wires can be shielded with an opaque covering.

Unhealthy tissue

The overall health of the experimental animal can affect the health of neurons in its

cortex. Check that the animal appears to be healthy—breathing should be regular and relaxed, the skin should not be too gray, etc. Check for bleeding from injection sites. Make sure the animal is properly hydrated with periodic subcutaneous injections of saline, in which case it should urinate periodically throughout the experiment. The urine should not be too dark or contain blood. Avoid exposing the cortex to toxins (e.g., Krazy Glue) and deionized water (as opposed to physiological buffer). Obviously, one should avoid damage to the cortex during surgery, but the visual appearance of the surgical field does not always reflect the viability of the underlying cortex. When making multiple electrode penetrations, it is good practice to continually move to fresh cortical tissue, away from previous penetrations. A large craniotomy can help in this regard, but it can also lead to increased cortical pulsations.

If the animal appears gray or blue, it may not be adequately oxygenated, which can result from too deep anesthesia (particularly with barbiturates), excessive pressure from the stereotaxic frame on the nasal passages, or a buildup of bronchial secretions, which can be prevented by administering atropine (1.6 mg/kg) 0 to 30 min before surgery. Low blood oxygen (which can be conveniently monitored with a with a pulse oximeter) can be easily remedied by flowing pure oxygen in front of the nose of the animal.

Even if the blood oxygen level is normal, cells can become unhealthy if the blood vessels supplying them are damaged, or if they are directly exposed to blood due to damage within or near the durotomy. Hypoxic neurons can be more difficult to patch successfully, and any blood on the pipet tip can prevent it from forming a seal, even with healthy neurons.

Avoid overheating the cortex. When applying melted agarose, it is helpful to check the heat level by applying a drop on your wrist, and to bathe the cortical surface under a shallow pool of cool physiological buffer before applying the agarose. If a dental drill is used during surgery, irrigate the skull regularly with physiological buffer and pause frequently to prevent excessive heating.

In addition to the issues already discussed, there are many other reasons that an experimental animal may die unexpectedly. For example, topical anesthetic (e.g., lidocaine) used on the scalp or neck can come in contact with the spinal cord if it is not flushed away before the cisternal drain is performed. In addition, since hypothermia or hyperthermia can decrease the viability of an animal, it is important that the heating pad or heat lamp be properly adjusted.

Reducing motion

It is not uncommon for the exposed surface of the cortex to move by hundreds of microns as it pulses with the movement of the heart or lungs, even in a small animal such as a young rat. Performing a cisternal drain and applying agarose to the cortical surface before advancing electrodes into the brain can significantly reduce the amount of motion. Small craniotomies (∼100 µm to ∼1 mm diameter) can also reduce cortical motion, but this must be weighed against the greater access to potential recording sites and the ability to avoid blood vessels provided by a large craniotomy, as well as the higher probability of exposing cortical region(s) of interest despite animal-to-animal anatomical differences.

The posture of the animal can also affect the level of motion. In particular, if the head is raised too high relative to the heart, heart movement can increase in magnitude resulting in strong motion artifacts due to the increased difference between the systolic and diastolic pressure measured at the cortex. Conversely, if the head is not high enough relative to the heart, the average blood pressure in the cortex can be high enough to cause excessive bleeding. In addition, changing the angle of the head can sometimes lessen the mechanical coupling between the body and the cortex. For example, if the head is turned in such as way as to increase the tension of the muscles on one side of the neck, those muscles can tug on the head as the body moves due to heard or lung motions.

Another technique for reducing motion is to press against the preparation with a "pressure foot," as some groups have done for in vivo sharp electrode intracellular recording (e.g., Phillips, 1956). Similarly, the shape of the patch electrode itself can have an influence on tissue motion in the vicinity of the electrode. Many available electrode pullers allow for four or more separate pulls of the electrode glass, so that patch pipets with long slender necks can be produced. Such electrodes can have a tip shape that is excellent for patch recording and at the same time can lessen the amount of damage to the preparation; this also makes deeper recordings possible. Two stage pullers tend to produce electrodes with wide "shoulders," which can, in effect, act as a pressure foot. This is especially convenient, given that the most crucial time to minimize motion is

during seal formation, which is when the electrode provides maximal pressure on the cortical surface—once a seal is established, the electrode is withdrawn to facilitate break-in, and the pressure is relieved.

Yet another approach is to actively compensate for motion in an automated fashion by measuring fluctuations in the position of the surface of the cortex and moving the recording electrode with the brain in real time while recording. In the right hands, this elegant approach has been rewarded with spectacular success even in an awake, *running* animal (Fee, 2000). In contrast, the approach outlined here relies primarily on the tight seal formed between the cellular membrane and the glass surface just inside the relatively broad tip (~3.5 μm diameter) of the whole-cell patch pipets we favor to achieve stable recordings in the face of motion. Amazingly, at least one group has succeeded in obtaining whole-cell records from freely behaving rats by mounting the patch pipets directly to the animal's skull (Lee, 2006).

Cardiac and pulmonary pulsations pose more challenging problems in larger animals because, although the amplitude of these motions scales with animal size, the physical scale of the neurons and patch electrodes are essentially the same as for smaller species. When working with a larger animal, it is therefore common practice to suspend the body of the animal in a sling to dampen movements, and to use paralytics and artificial respiration to eliminate pulmonary motion (e.g., Ferster and Jagadeesh, 1992).

Failure modes

Once the basic technical issues listed above have been attended to, there is a progression of failure modes that novice electrophysiologists typically encounter as they learn to perform blind, whole-cell patch-clamp recording in vivo. Even those with a great deal of experience with this technique tend to progress through these same stages, albeit on a much faster timescale, before obtaining useful recordings when they return to their experiments after a prolonged period away. The basic stages can be summarized as: 1) not encountering any cells while advancing electrode; 2) observing sudden increases in resistance while advancing electrode, but the apparent neurons do not "jump on" to the electrode when pipet pressure is released; 3) forming only low impedance seals; 4) forming gigaohm seals, but losing them quickly, or not being able to break into cells; and finally, one successfully breaks into a cell and obtains a stable recording. Each of these four failure modes are discussed below, along with some others.

Abrupt increase in resistance that does not decrease when the electrode is withdrawn

This usually indicates that the tip of the pipet has been clogged, and should be replaced. This can also reflect AgCl residue settling into the pipet tip. With the dissecting microscope, look for small black particles inside the pipet, and check that the AgCl coating on the recording electrode wire is not crumbling. In either case, replace the electrode wire. This can be avoided by using molten AgCl, rather than a bleach solution, to coat the electrode wire with AgCl.

Abrupt decreases in electrode resistance

This is usually due to a broken pipet tip, but small decreases in resistance can also result from entering a region with a different local geometry or different ionic composition as the electrode is advanced. In the latter case, the resistance should return to the original value as the electrode is withdrawn. Otherwise, replace the pipet.

Not encountering any cells

This is the worst failure mode because the experimenter gets little or no feedback about what went wrong. Possible causes include: patch pipets that are the wrong size or shape at the tip; bad internal solution; too much pressure while advancing electrode; too much time spent between entering the agarose and attempting to approach a cell; too much electrical noise, or too many motion artifacts, to assess subtle changes in electrode resistance; unhealthy tissue; and a bad angle of approach with respect to the three-dimensional orientation of the cells in the area. The following procedures may produce desirable results: lower the pipet pressure while looking for cells; move quickly from the moment of initial penetration of the agarose to the cell-hunting stage of the protocol; move to a fresh area of cortex; go deeper or less deep than previous attempts before looking for cells; and change the angle at which the patch pipet enters the cortex. However, if one has previously had success with similar patch pipets and internal solution from the same batch, then it is best to not change the protocol too much even after several unsuccessful recording sessions.

Observing sudden increases in resistance, but the putative cells do not "jump" onto the patch pipet when pressure is released

This can result from most of the same causes listed for the first failure mode, but in this case there is some useful feedback in the form of the time dependence of the pipet resistance fluctuations. If the fluctuations in pipet resistance are extremely regular, like a sinusoid, or if the resistance rises very slowly as the electrode is advanced, or if the DC current level of the oscilloscope trace rises appreciably as the putative neuron is approached, it may indicate that the obstruction before the patch pipet is either a cell in a poor orientation for obtaining a seal, or not a neuron at all (e.g., a blood vessel). Large, highly stochastic fluctuations in the electrode resistance, and a very sudden increase in resistance as the electrode is advanced are auspicious signs.

It may also be that the electrode has not been advanced close enough to the cell before the pressure was released. Try approaching the next few neuron(s) *too closely* before releasing the positive pressure. This may result in passing some cells by, but it is better to err in different ways on subsequent attempts, rather than to always make the same mistake. Other measures to try include: use higher pipet pressure while hunting for cells; attempt to time the release of pipet pressure with the peak of the pulsations in electrode resistance; and switch the voltage command to +70 mV, rather than −70 mV (but once a seal is formed remember to switch back to −70 mV before breaking in). It is a good policy to be gentle when applying suction or positive pressure while trying to form a seal, but some coaxing of the neuron during seal formation is usually helpful for obtaining a stable seal.

"Lancing" the cell with the patch pipet before forming a seal

This may result from too much motion, unhealthy tissue, or patch pipets that are too narrow at the tip. Another hallmark of small-tipped pipets is a tendency towards quickly formed, but highly transient, gigaohm seals. This can also result from fire-polishing the pipet tips.

Achieving high impedance seals, but not being able to break in

There are several schools of thought on how best to break into a neuron—one can apply suction that is mild or strong, and pulsed or sustained. When having difficulty breaking in, try them all. It is generally easier to break in soon after a gigaohm seal is formed, than to wait longer than 1 or 2 min. The electrode may be jammed up against the neuron; try backing out the electrode. Try injecting brief, strong current pulses ("zap" button on the Axopatch 200B).

Losing cells soon after break-in

This can be a sign that the cells are unhealthy; check other indicators of cell fitness such as resting potential, which should be roughly −60 mV or lower for most healthy cortical neurons (corrected for junction potential). Note that using series resistance compensation (*UNIT 6.6*) in the presence of strong, motion-induced fluctuations in access resistance can result in fatally large current injections into the neuron.

High series resistance during recording

There are several ways to lower the series resistance after breaking into a cell. Applying positive pipet pressure after breaking in often helps—try smoothly ramping the pressure up to 30 mmHg for a second or two, and then lock in a low (≤4 mmHg) level for the duration of the recording. Locking in more than about 4 mmHg can result in blowing the cell off of the pipet.

Withdrawing the electrode ∼10 μm after breaking in can also help. Other steps that can minimize series resistance include not waiting too long after a seal is formed before breaking in, and breaking in as gently as possible, applying mild suction at the moment the membrane is ruptured. One can also try switching to an internal solution with higher osmolarity, which has a similar effect as raising the pipet pressure.

General advice

Success at this technique is a function of many hidden parameters that cannot be directly measured or assessed, and straying too far from a working protocol can easily result in not even encountering cells, much less recording from them, throughout any number of electrode penetrations. Therefore, when optimizing the protocol, it is good practice not to change more than one aspect of the experiment at a time, and to assess the effect of this change over several recording sessions. Experience is crucial—if one can manage to remain in a parameter regime that allows one to encounter neurons and get feedback, the yield and quality of in vivo whole-cell data typically improves steadily with time.

Anticipated Results

With sufficient practice, the amount of useful data produced with this technique can approach that of blind whole-cell recording in the slice. A typical 6 hr experimental session may yield one high quality ∼30 min recording, along with one or two mediocre recordings. On good days, one can obtain excellent recordings on every penetration, though this is more common in slice preparations. Recording quality can be very similar to that of the best in vitro recordings, but the affects of motion on the in vivo recordings will typically be more pronounced. Series resistances range from >10 MΩ to >100 MΩ, with values typically in the low tens of megaohms.

Figure 6.22.1 shows an 8-sec trace from a stable in vivo whole-cell recording in current-clamp from the auditory cortex of an anesthetized rat. Note the healthy resting potential level (approximately −60 mV, uncorrected for junction potential) and the presence of clear post-synaptic potentials occurring both spontaneously and in response to brief tone pips (hash marks below trace); several of the tones elicit spikes.

Time Considerations

Electrode fabrication (*UNIT 6.3*) can require between 10 and 40 min depending on the desired number of patch pipets. Anesthetizing the rat takes ∼5 to 10 min. The surgery tends to require ∼25 min; when all goes well, the surgery can take as little as 5 min. Replacing the patch pipet and positioning the electrode takes ∼1 min, and each electrode penetration takes between 1 and 10 min. Recording times vary from 1 min to several hours, with an average duration of roughly 20 min of stable recording from a healthy neuron. Anesthetized rats can routinely be maintained for over 8 hr for acute experiments. Even with no previous exposure to the recording chamber or stereotaxic frame, awake gerbils and young rats will usually tolerate up to ∼4 hr of head restraint.

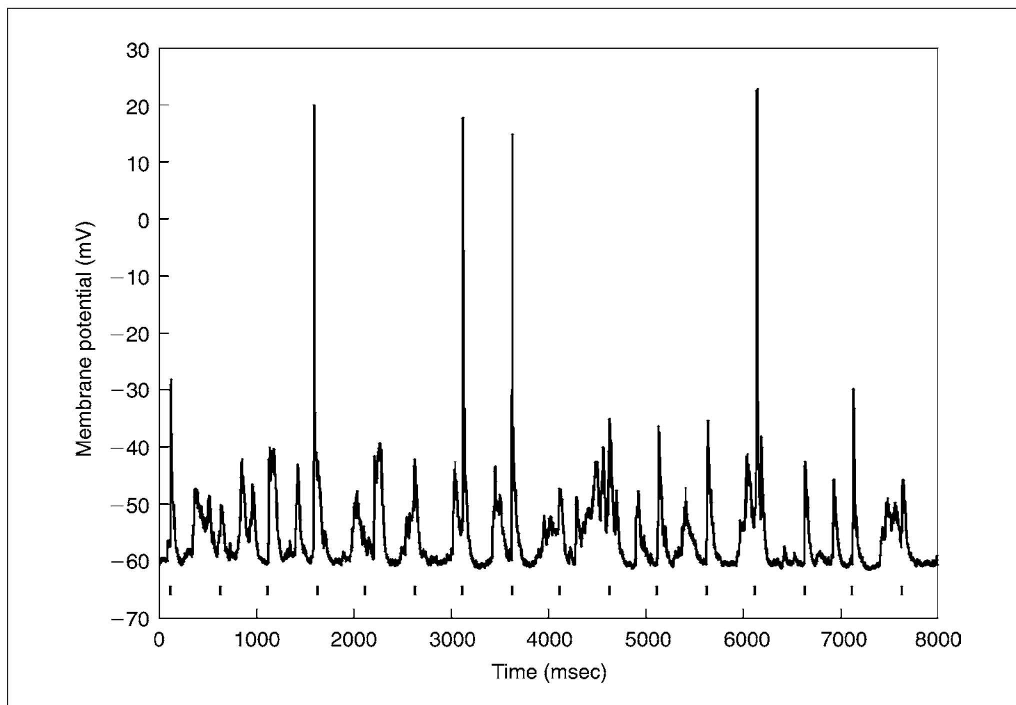

Figure 6.22.1 An example trace from a current-clamp whole-cell recording from rat auditory cortex. The black hash marks below the trace indicate 16 brief (25-msec duration) acoustic tones of varying frequency and intensity that were presented to the rat during the recording. Three of the tones evoked single spikes and one tone (presented at 6100 msec) evoked a spike doublet; most of the 12 remaining tones elicited substantial post-synaptic potentials (PSPs), but no spike. Note the occurrence of spontaneous PSPs that do not immediately follow auditory stimulation (e.g., 1400 msec). For this recording, series resistance was ∼25 MΩ and input resistance was ∼60 MΩ. The amplifier gain was set to 20 mV/pA, and 3 mmHg of positive pipet pressure was maintained for the duration of the experiment. The subject was a post-natal day 18, Sprague-Dawley rat anesthetized with ketamine and xylazine. The recording remained stable for ∼30 min.

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