Nucleated, outside-out, somatic, macropatch recordings in native neurons

Chapter xxx

Nucleated, outside-out, somatic, macropatch recordings in native neurons.

Francesco Tamagnini

Abstract

Patch-clamp recordings are a powerful tool for the live measurement of the plasma membrane biophysical properties, with the ability to discriminate fast events such as fast inactivating Na^+ currents (<1 ms c.a.). It can be used in virtually every cell-type, including cardiomyocytes, skeletal muscles, neurons, and even epithelial cells and fibroblasts. Voltage-clamp, patch-clamp recordings can be used to measure and characterize the pharmacological and biophysical profile of membrane conductances, including leak, voltage-gated and ligand-gated ion channels. This technique is particularly useful in studies carried out in cell-lines transfected with the gene expressing the conductance under investigation. However, voltage-clamp measures conducted on the soma of a native, adult neuron, for example in an acute brain slice or in the brain of a live individual, are subject to three major limitations: 1) the branching structure of the neuron causes space-clamp errors 2) ion channels are differentially expressed across different neuronal compartments (such as soma, dendrites and axons) and 3) the complex geometry of neurons makes it challenging to calculate current densities. While not preventing the experimenter to conduct patch-clamp, voltage-clamp recordings in native neurons, these limitations make the measures poorly standardized and hence often unusable for testing specific hypotheses.

To overcome the limitations outlined above, outside-out, patch-clamp recordings can be carried out instead (See chapter etc); however, the signal-to-noise ratio in outside-outs from native, adult neurons is usually too low for obtaining accurate measurements.

Here we describe how to carry out nucleated, outside-out, somatic, macropatch recordings (from now on abbreviated into “macropatch recordings”) to obtain accurate and standardised measures of the biophysical and pharmacological properties of somatic, neuronal membrane conductances.

1. Introduction

1.1 The use of patch-clamp, voltage-clamp to characterise voltage-gated channels biophysical properties.

The electrical properties of the plasma membrane are key factors driving the function of cells and tissues and they are central for the computational abilities of neurons and other excitable cells, such as muscle cells and glandular epithelia [1]. The pioneering and seminal work of Hodgkin and Huxley [2] demonstrated that neuronal plasma membranes work like equivalent electrical circuits, characterized by a charge separation between two poles, the

intracellular and the extracellular space, and a condenser (the phospholipid bilayer) in parallel with a resistor (the membrane leak conductances, such as non-gated K^+ and Cl^- channels) (Figure 1A). In addition, voltage- (and analogously ligand-gated) ion channels are responsible for the electrogenic, active membrane electrical properties of a cell (Figure 1B).

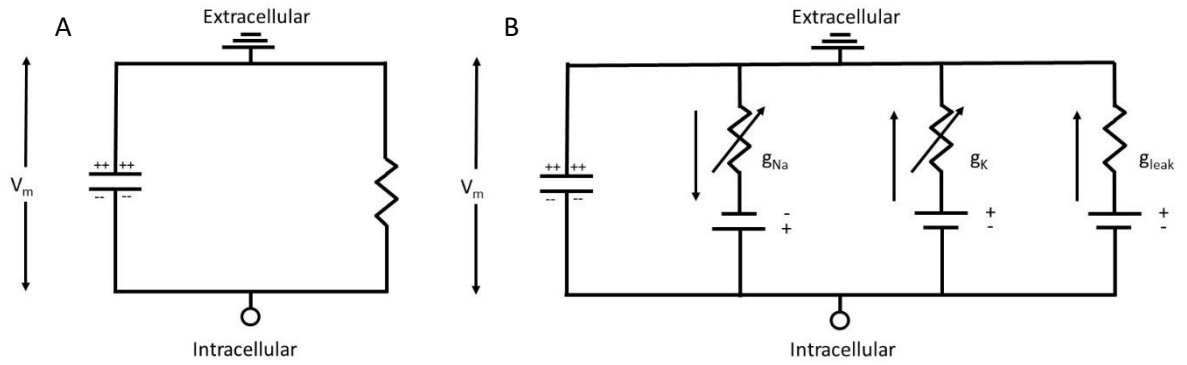

Figure 1. (A) Equivalent circuit representing the resistive and capacitive nature of the biological plasma membrane. (B) Excitable neurons possess voltage-gated conductances (such as g_{Na} and g_K) responsible for the generation of action potentials upon depolarization of the plasma membrane.

For example, the action potential is generated via the activation of Na^+ and K^+ voltage-gated channels [3,4], while the release of neurotransmitter is triggered by the Ca^{2+} influx in the presynaptic terminal caused by the activation of voltage-gated Ca^{2+} channels [5,6]. To study the biophysical properties of these channels, it is necessary to measure the changes of conductance in presence of different levels of the stimulating factors (i.e. increasing depolarization steps for voltage-gated ion channels activated by depolarization). To this end, the voltage-clamp technique was developed (see Chapter xxx for details). Briefly, in voltage-clamp, a voltage recording electrode is placed in the intracellular space and is paired to a current generator: both the recorder and the generator are connected to an integration system, which controls the current generator by pumping charges intracellulary in order to keep the membrane voltage at the set value (Figure 2A), **known as holding potential (V_{hold})**, following Ohm's law (V=RI). Voltage-clamp, whole-cell patch-clamp works following the same principle, but the voltage recorder and the current generator co-exist in the same electrode and it is known as single electrode voltage-clamp (Figure 2B). The opening of ion channels will generate a current, in presence of an electrochemical driving force for that ion. To counteract the effects of that current on the membrane potential, according to the Ohm's law the system will have to generate a current which has the same shape but inverse direction to the one generated by the ion channels. The generated current, divided by the driving force, will correspond to the conductance. Plotting the conductance vs the voltage deflection, will result in the generation of data-points that can be fit by a Boltzmann sigmoid curve, describing the activation of voltage-gated channels upon voltage. The cell-to-cell Boltzmann fit can, in turn, be used to calculate the channel's half-activation potential ($V_{1/2}$) and its maximal conductance (G_{max}) (as in Figure 5).

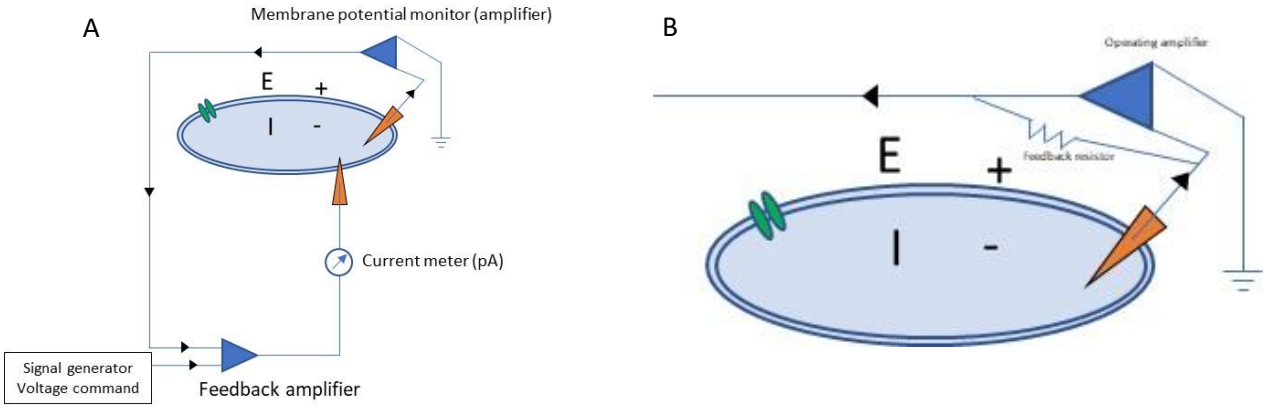

Figure 2. (A) Voltage-clamp can be reached with a two-electrode configuration, one to monitor voltage, one to measure/generate current, coordinated by a signal generator integrated with a voltage-command box. (B) However, neuronal membranes are usually too delicate to allow two electrode recording configurations. Single-electrode, voltage-clamp recordings can also be carried out: the same electrode is connected to an operating amplifier, to measure voltage, and a feedback resistor to generate the current needed for voltage-clamping.

1.2 Limitations of voltage-clamp recordings in adult neurons.

The intensity of the currents recorded via voltage-clamp patch-clamp are directly related to the total number of current generating channels present on the plasma membrane. Such number depends on 2 factors: 1) the channel density and 2) the total extension of the plasma membrane. In whole-cell configuration, this can represent a problem. In fact, to avoid biases arising from cell-to-cell natural variability in cell size, the current values should be expressed as current densities (pA/µm^2). In practical terms, current densities are expressed in pA/pF: in fact, the capacitance value of a condenser is directly proportional to the surface extension of the armour

$$C = \frac{A\varepsilon_0}{d}$$

Where C=capacitance, A=area of the armour, d=distance between plates (in this case the thickness of the phospholipid bilayer) and ε_0 the dielectric constant. Assuming that d and ε_0 do not significantly change from cell to cell, the current density can be equally expressed as I/A (pA/µm^2) or I/C (pA/pF). Since in whole-cell patch clamp the C of a cell can be directly measured, current densities are usually expressed in pA/pF. However, one needs to be careful with this approach. In fact, the complex geometry of neurons may result in a reduction in the accuracy of the C reading. This is due to reduced space clamping in large, geometrically complex cells [7].

The current injected to maintain the V_{hold}, spreads radially from the point of injection and it exponentially decays with distance. This phenomenon is known as **space clamp**. In turn, this results in the lack of control of the membrane potential in those parts of the cell which are distant from the point of current injection [8].

Finally, ion channel subtypes are often differentially expressed across different parts of an adult, native neuron. Hence, the currents recorded in the point of patch (usually the soma), may be the result of a heterogeneous population of ion channels with different biophysical properties, reducing the relevance of the recording. For example, voltage-gated Na^+ $(Na)_v1.6$

channels are mostly expressed in the axonal initial segment and on the axon (especially in the nodes of Ranvier, in myelinated fibres), where they mediated the initiation and forward propagation of the action potential. On the other hand, $Na_v1.2$ channels are mostly expressed in the soma, where they mediate the back-propagation of the action potential [9].

1.3 Aim

To overcome the limitations outlined above, we will discuss here how to perform macropatch recordings. This method, while being technically challenging, can provide reliable and accurate data relative to the biophysical properties of both ligand- and voltage-gated channels on the neuronal somatic plasma membrane.

Materials

Brain slices

Brain slices can be obtained from rodents at different ages, using standard methods as the ones described in [10,11]. For the brain slicing process, you will need:

- Cutting solution (see below for details)
- Artificial cerebrospinal fluid (aCSF) (see below for details)
- Scalpel (large belly blade preferred)
- Surgical spring scissors
- Surgical tweezers
- Small bent spatula
- Larger bent spatula
- Modified Pasteur pipette for slices
- Ice bucket
- Carbogen mixture (95% O_2, 5% CO_2)
- Cutting matrix (optional)
- Water bath (37°C)

Solutions

- Cutting solution.

All cutting operations are performed at low temperatures (0-4°C), to help and reduce the damages arising from hypoxia, following decapitation and preceding the completion of slicing. Cutting solutions are used to help prevent the damages coming from both hypoxia and cold temperatures. For example, they can have low Ca^{2+} and high Mg^{2+}, to help preventing the excitotoxicity arising from NMDA receptor activation; sucrose or glycerol can be used, in substitution of the corresponding equivalents of NaCl, to reduce the nucleation of extracellular ice crystals which can damage the plasma membrane. Certain laboratories decide to use aCSF as cutting solution. As all these solutions are based on a $NaHCO_3$ buffer system, cutting solutions need to be constantly bubbled with a gas mixture containing 95% O_2 and 5% CO_2 (Carbogen). Use the cutting solution you prefer and keep it consistent across the whole project. Here, we report the composition of the cutting solution we use in our laboratory (Table 1A).

- Artificial cerebrospinal fluid (aCSF)

The composition of the cerebrospinal fluid slightly changes from one group to another. However, there are certain characteristics which are common. 1) the aCSF has a $NaHCO_3$-based buffer system and for this reason it needs to be constantly bubbled with Carbogen 2) It contains high concentrations of NaCl (usually >100 mM) and low of KCl (usually <5 mM) 3) the relative concentrations of Ca^{2+} and Mg^{2+} can change (2:1 is very common) and they are commonly 2 mM or less 4) Glucose levels can variate between 1 and 10 mM. Below, the aCSF formula we use in our laboratory (Table 1B). The pH of the aCSF must be 7.4 and the osmolarity = 300 mOsm/L c.a.

- Intracellular solutions

Intracellular solutions can vary, depending on the kind of current that must be measured. For example, to record K^+, outward currents, starting from a hyperpolarised V_{hold}=-100 mV, a high K^+, low Cl^- internal solution is recommended. In our laboratory, we normally use K-gluconate internal solutions. However, other counter-ions for K^+ can be used, such as methansulphonate ($KMeSO_4$). The pH should be 7.4. The osmolarity of the internal solution needs to be 5 % lower than the extracellular solution, to avoid cell swelling and weakening/loss of the seal.

A

Molecule	[Final] mM	Mr
Sucrose	189	342.30
Glucose	10	180.16
$NaHCO_3$	26	84.01
KCl	3	74.55
$MgSO_4.7H_2O$	5	246.48
$CaCl_2$	0.1	
NaH_2PO_4	1.25	137.99

B

Molecule	[Final] mM	Mr
NaCl	124	58.44
KCl	3	74.55
$NaHCO_3$	24	84.01
NaH_2PO_4	1.25	137.99
$MgSO_4.7H_2O$	1	246.48
$CaCl_2$	2	110.98
Glucose	10	180.16

Table 1. An example of cryoprotective, cutting solution (A) and artificial cerebrospinal fluid (B). Note that in the cutting solution, in comparison with aCSF, the sucrose replaces NaCl (to prevent the formation of ice crystals), the [Mg^{2+}] is higher and the [Ca^{2+}] is lower (to reduce NMDA-R dependent excitotoxicity).

So, for an aCSF with an osmolarity of 300 mOsm/L, the intracellular solution should have an osmolarity comprised between 280 and 290 mOsm/L. If an intracellular marker needs to be added to the internal solution (i.e. biocytin for post-hoc reconstruction) the levels of the K^+ salt should be decreased accordingly, to keep the osmolarity constant. Below, an example of internal solution used in our laboratory, with and without biocytin (Table 2).

Molecule	[Final] mM	Mr
KGluconate	145	234.2
NaCl	5	58.44
HEPES free acid	10	238.3
EGTA	0.2	380
GTP- Na salt	0.3	523
ATP- Mg Salt	4	507

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HEPES free acid	10	238.3
EGTA	0.2	380
GTP- Na salt	0.3	523
ATP- Mg Salt	4	507
Biocytin	13.4	372

Table 2. Example of KGluconate based internal solutions with (A) and without (B) biocytin. Note that, to make space to the intracellular marker biocytin and keep the osmolarity constant, the concentration of KGluconate has been decreased in (B), in comparison to (A).

Recording setup

The recording setup to perform macropatches in acute slices needs to be built as follows.

1. Microscope. For visualising cells in brain slices, you will need a fixed-stage, upright microscope, mounted on a translation table. To better visualise the cells within the brain parenchyma, we recommend using infrared, differential interference contrast (DIC). Epifluorescence or confocal (1- or 2-photon based) microscopy can also be implemented, to visualise specific subpopulations of neurons or patched neurons filled with a fluorescent probe, but this part is optional.
2. Hydraulics. During the recordings, the slices will have to be constantly perfused with carbogen-bubbled, heated (35°C), aCSF. To this end, they will be placed in a submersion-style recording chamber, while the aCSF is pumped in and out the chamber by a pump system or let in and out via gravity.
3. Micromanipulator. A motorized or hydraulic or piezoelectric micromanipulator, with low drift and sub-micron resolution. This will be used to mount the amplifiers headstage and direct the micropipette to the cell.
4. Isolation table. The whole setup should be mounted on an antivibration table. This increases the stability of the patch and decreases the probability to lose the seal. The anti-vibration table can be an active control air table or a passive control one (i.e. a heavy metallic plate placed onto inflated tyres/inner tubes).
5. Amplifier. We recommend using an amplifier designed for patch-clamp (Molecular Devices, HEKA etc). The amplifier is connected to a headstage, which is mounted directly on the micromanipulator. Patch-clamp amplifier headstages are designed to be connected to both an AgCl electrode via a micropipette holder and to an Ag/AgCl reference electrode which is placed in the recording chamber. The pipette holder allows to firmly place a micropipette, filled with internal solution, over the recording electrode: the Ag/AgCl will work as a bridge between the metallic circuitry within the amplifier (where electrons are the charge-bearing particles) and the solutions (where the ions are the charge bearing particles).
6. Analog/Digital (A/D) interface. This is needed to convert the amplified analogic signal into a digital signal which can be visualised with a dedicated data acquisition software, such as pClamp (Molecular Devices), PatchMaster (HEKA), Signal

(Cambridge Electronic Design). A/D boards specifically designed for electrophysiology recordings are Digidata 1550 (and previous versions), from Molecular Devices and several adaptable boards from National Instrument.

7. Glass micropipettes. These pipettes can be purchased already pulled and polished. However, most electrophysiology laboratories pull them from borosilicate capillaries with the use of a horizontal/vertical puller. Narishige is one of the most common producers of these pullers. A Narishige microforge can be purchased separately to round up the sharp edges of the pulled pipette. The pipette diameter, after pulling and polishing, should be 1 µm c.a., corresponding to a pipette access resistance of 2-7 MΩ.
8. Faraday cage. A Faraday cage should be placed around the recording system and every device directly connected to AC current should be left out of it. Reducing the noise via adequate shielding, cleanliness and removing contacts with the space outside the cage (i.e. by introducing a dripper in the inlet perfusion if the reservoir is placed outside the cage) is always better and advisable, compared to filtering and active noise control. In fact, the latter instances can lead to data loss or, in certain cases, even add noise.
9. Temperature control. Temperature control within the recording chamber is of paramount importance, as the temperature has a dramatic effect on the electrical properties of the plasma membrane. An active temperature control system based on constant feedback (i.e. Scientifica Temp Controller) is advisable. However, a constant current system is also adequate, if the room T conditions remain stable.

Methods

Overview

Manual patch-clamp techniques are known to be challenging. The experimental setup comprises many separate components and much can happen which can make the recordings unusable or the experiments inconclusive. A good rule of thumb is to get into the recordings as prepared as possible. For example, before starting an important experiment, make sure your technique is good by exercising on less important samples, such as the leftovers from someone else's experiment. In addition, make sure that your dissection technique is accurate and fast: the dissection time is a key factor in slice electrophysiology. In addition, plan your experiment well in advance: make sure you have a clear hypothesis in mind and the experimental protocol you are using is the most adequate to test it. Finally, make sure that both the rig and the laboratory are clean and tidy: things tend to work out better in a well-ordered lab and, in case a problem occurs, it is usually easier to find a solution.

Slice preparation

The animal needs to be decapitated, following cervical dislocation. Subsequently, the brain is rapidly removed and placed into an ice-cold (0-4°C), cryoprotective, cutting solution (see above for details), which is constantly bubbled with Carbogen. Depending on the brain area of interest certain parts can be removed with single scalpel cuts. For example, if the

prefrontal cortex is required, the cerebellum and the caudal part of the telencephalon can be removed with single scalpel cuts. To improve the accuracy and the precision of these cuts, a cutting matrix can be used. Depending on the anatomical plane required, the brain can be glued to a vibratome stage in different ways. For example, to cut horizontal slices containing striatum, hippocampus and temporal cortices, you should remove with single scalpel cuts the cerebellum, the prefrontal lobes and the dorsal part of the cortex; subsequently, the brain should be glued on the dorsal side (ventral side facing up). The sample should be quickly placed into a vibratome tray, covered with ice-cold, carbogen bubbled, cutting solution and sections should be cut. The thickness of the slice can be comprised between 250 µm and 500 µm. While 400 µm is the thickness preferred for field potential or blind patch-clamp recordings, for visualised patch, using infrared DIC microscopy, we recommend to not exceed a thickness of 300 µm. Each slice should be immediately placed in Carbogen-bubbled, aCSF to recover. The recovery period should be carried out for 30 minutes at 37°C, followed by >60 minutes at room temperature, prior to the beginning of the recordings.

Patching

1. Place the slice in the recording chamber and make sure it is not moving. Using a harp to hold it down can help (Figure 3a).
2. Use the 4x objective to choose the brain area of choice.
3. Once the iris has been closed on the area of interest, switch to the high magnification, salt-water dipping, long working distance objective (usually 40x or 60x).
4. Use the focus to select a cell which looks healthy. A healthy cell, in adult slices, usually looks plump and with regular borders.
5. Fill the pipette half-way, mount it on the pipette-holder and place it just above the surface of the slice. Lock positive pressure inside the mounted pipette. Keep an eye on the pipette electrical resistance by constantly applying pulses of voltage difference between the recording and the reference electrode (1-5 mV). These will generate a current with an intensity inversely correlated to the access resistance of the electrode (Ohm's law: V=RI). The amplifier, in this stage, should be in voltage-clamp mode; whole-cell capacitance and series resistance compensation should be switched off and the pipette offset should be kept around 0 mV.
6. Slowly lower the pipette in the brain parenchyma: you will see the internal solution being pushed out by positive pressure and moving debris away. The borders of the cell should now appear clearer.
7. Using an approach angle on the micromanipulator and the focus, approach the surface of the cell with the pipette. Once close enough to the plasma membrane, the access resistance to the electrode should increase and a dimple should appear on the surface of the plasma membrane. Immediately release the positive pressure.
8. Quickly switch on the voltage-clamping and set a V_{hold} = -70 mV. Run a protocol enabling the transient passage of V_{hold} from -70 mV to -65 mV. The overall injected current and the current step between V_{hold} = -70 mV and V_{hold} = -65 mV will reveal the tightness of the seal.

9. Once the Gigaohm seal has been obtained, apply short pulses of negative pressure (i.e. you can apply brief and quick suctions to the pipeline connected to the pipette holder) paired to low intensity and short (25-100 µs) electrical shocks ("Zap" function on most amplifiers) to reach the whole-cell configuration (Figure 3b).
10. Leave the cell to adjust for 1 or 2 minutes: follow up the current injected to keep the V_{hold} (I_m) and make sure it is stable.

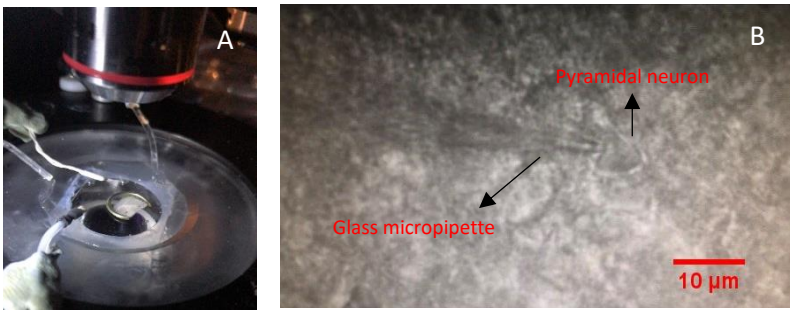

Figure 3. (A) Example of a brain slice placed in a submersion-style chamber and held down by a harp. (B) Example of a pyramidal neuron in whole-cell patch-clamp configuration in a brain slice, visualised with DIC, infrared microscopy.

Pulling the macropatch

1. Apply a light, negative pressure and lock it in the pipette. This will allow the nucleus of the cell to move towards the pipette opening.
2. After 10-15 seconds, start to very slowly manoeuvre the micromanipulator and pull the pipette away from the cell, following an approach angle. The speed of pulling should not exceed 1 µm/s.
3. Monitor the I_m while pulling: once the macropatch is pulled, the access resistance will be in the order of GΩ.
4. Once the macropatch has been excised, keep on pulling until the macropatch has been completely removed from the slice. This will help the quality of the recording: in fact, the slice's parenchyma is an environment which is less standardized, in comparison with the rest of the recording chamber. The excised macropatch, once pulled outside of the slice, should look like the ones in Figure 4: you can see the nucleus enclosed within the excised macropatch. The size of a macropatch is usually comprised between 2 and 5 µm,
5. Compensate the whole cell's capacitance and series resistance. Macropatches have usually a capacitance comprised between 1 and 5 pF. The series resistance of the macropatch should be under 20 MΩ.
6. Record the signals. For example, if the biophysical, activation properties of voltage-gated, outward channels are being recorded, start from a V_{hold}=-75 mV and apply short (i.e. 30 ms) V_{hold} depolarization steps (i.e. 10 mV, for 12 iterations). For each V step record multiple sweeps (at least 3) and remember to perform leak subtraction (see chapter…).
7. The current densities generated upon depolarization can be measured and IV curves can be generated for the calculation of the conductance activation properties ($V_{1/2}$ and G_{max}). Macropatches can be used to measure several currents,

including voltage-gated Na^+ and K^+ currents, which are involved in action potential generation (Figure 5).

8. The rate of success of pulling macropatches is relatively low. Exercising, persistence and passion are key. Just keep on trying. A particular important point is to be very slow but constant while pulling away. Also, it is useful to respond to changes in I_m while pulling, to adjust the rate of pulling accordingly (slowing down or accelerating, depending on how wobbly the signal is).

Notes

1. *Brain areas and cell subtypes.*

Our recordings have been carried out in hippocampal and cortical pyramidal neurons and interneurons, at both room temperature and 34˚ C. Excising macropatches from interneurons has proved to be more challenging than from pyramidal neurons. This is probably due to the smaller size of interneuronal somas. For these reasons, we infer that macropatches are a viable technique for different cell-types in virtually every brain area.

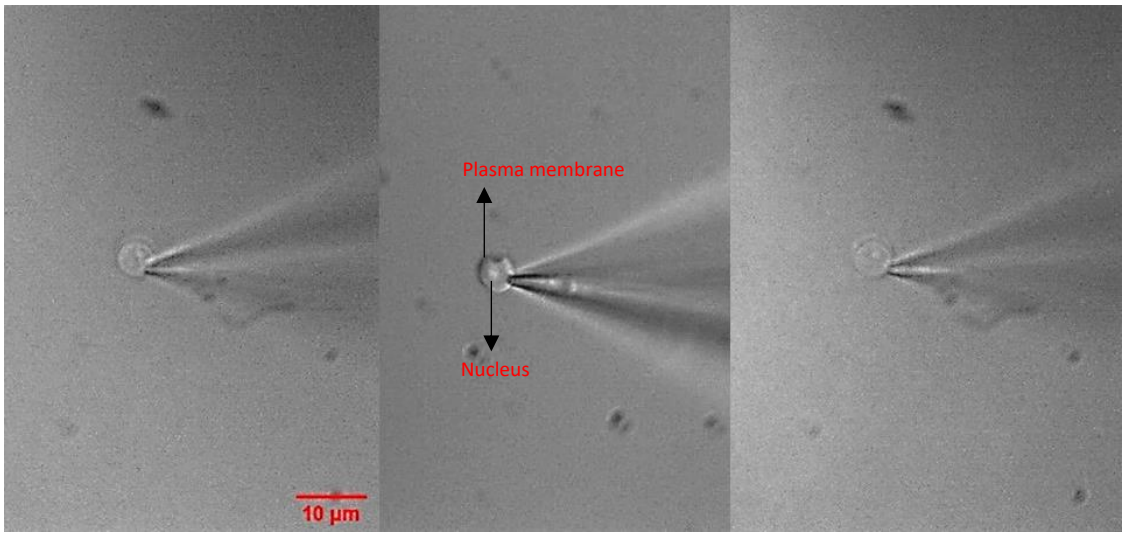

Figure 4. Three examples of somatic, nucleated, outside-out macropatches pulled from an adult neuron which was in a brain slice. In each image, the nucleus and the somatic membrane can be visualised.

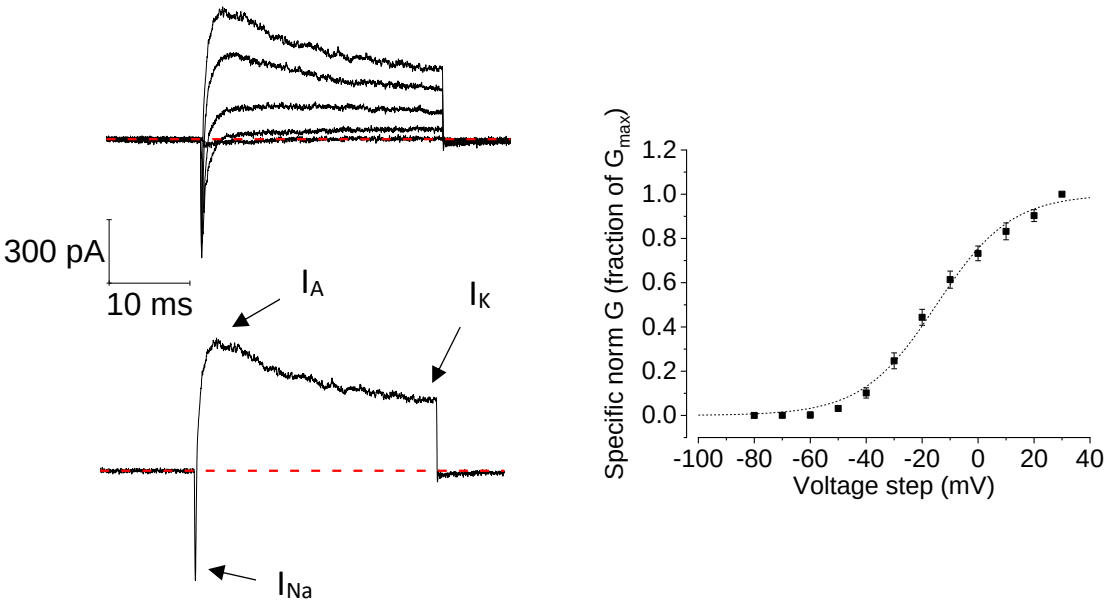

Figure 5. (A) Example of inward Na^+ and outward K^+ currents. Amongst the outward currents, a fast-inactivating (I_A) and a non-inactivating (I_K) component, can be observed. These currents were evoked by progressive, 10 mV/30 ms, voltage steps, starting from an initial V_{hold}=-90 mV. (B) Currents evoked by a 30 mc voltage step from -90 mV to +10 mV. (C) IV, Boltzmann curve generated by plotting the current amplitudes vs the applied change in V_{hold}. This curve can be used to calculate cell-to-cell (or in this case, macropatch-to-macropatch) values of the half-activation voltages and maximal conductance of the voltage-gated channel under investigation. Modified from [12].

2. *Isolating individual currents in macropatches.*
   Native neuronal membranes contain a variety of different voltage-gated channels. For this reason, the currents recorded from macropatches may result from the activity of different channels. Teasing out the contribution of each one to measure their respective biophysical properties can be challenging. A viable approach can be using pharmacological tools to isolate each component: for example, using TTX and 4-AP to block I_{Na} and I_A, respectively, can be used to isolate I_K currents. However, caution should be used in interpreting the results coming from these recordings, as the signal, as pharmacologically or biophysically isolated as it can be, may still arise from different channel subtypes.
3. *Slices recovery time.*
   Slice electrophysiology majorly relies on slice viability. Many factors affect it, including dissection time, the use of cryoprotective, cutting solution and the temperature of the cutting solution. Each group tends to have its set of rules, which are equally valuable as long as they fit with an effective experimental plan. We recommend moving the slices directly from the ice-cold, cutting solution to 37˚C aCSF, for 30 minutes. Subsequently, the slice can be left to recover for additional 60 minutes in aCSF at room temperature. The first temperature boost, in our

experience, allows higher cell viability and, consequently, higher probability to excise macropatches.

4. *Macropatches for the study of ligand-gated ion channels.*
   The focus of this chapter was on the use of macropatches for the characterization of voltage-gated channels biophysical properties. However, the same technique can be used for detecting currents generated by ligand-gated ion channels, such as GABA-A or AMPA-R dependent currents. This can be reached by pairing the patch-clamp equipment to a pico-/femto-spritzer, to apply localised chemical stimuli directly on the macropatch. This approach would be preferred to bath application, to avoid biases due to the inactivation of these conductances associated to the persistent presence of the transmitter in the extracellular space[13].

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