Topic Introduction

Patch-Clamping Fly Brain Neurons

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The membrane potential of excitable cells, such as neurons and muscle cells, experiences a rich repertoire of dynamic changes mediated by an array of ligand- and voltage-gated ion channels. Central neurons, in particular, are fantastic computators of information, sensing, and integrating multiple subthreshold currents mediated by synaptic inputs and translating them into action potential patterns. Electrophysiology comprises a group of techniques that allow the direct measurement of electrical signals. There are many different electrophysiological approaches, but, because *Drosophila* neurons are small, the whole-cell patch-clamp technique is the only applicable method for recording electrical signals from individual central neurons. Here, we provide background on patch-clamp electrophysiology in *Drosophila* and introduce protocols for dissecting larval and adult brains, as well as for achieving whole-cell patch-clamp recordings of identified neuronal types. Patch clamping is a labor-intensive technique that requires a great deal of practice to become an expert; therefore, a steep learning curve should be anticipated. However, the instant gratification of neuronal spiking is an experience that we wish to share and disseminate, as many more *Drosophila* patch clampers are needed to study the electrical features of so many fly neuronal types unknown to date.

BACKGROUND

Nowadays, scientists have many tools to explore neuronal activity. These techniques range from indirect approaches, such as the imaging of genetically encoded fluorescent calcium indicators or voltage sensors, to more direct approaches, such as electrophysiology. Historically, electrophysiology has relied on "giant" axons such as that of the squid (Hodgkin et al. 1952) or large somas such as those from *Aplysia*, another marine mollusk (Tauc 1955). This is mainly because initial methods relied on impaling electrodes in the cell of interest, which obviously had to be large to avoid bursting upon insertion. Muscle fibers are another tissue in which sharp electrodes can be conveniently inserted; and also considering their convenient accessibility, it is no surprise that the synapse between a motoneuron and its target muscle (known as the neuromuscular junction) has been extensively explored in many species.

The patch-clamp technique opened up the possibility of making electrophysiological recordings from small neuronal somas. As a matter of fact, this technique was not originally invented with this in mind; it was devised with the objective of recording the currents elicited by individual ion channels, thus demonstrating their existence and their working mechanisms (Neher and Sakmann 1976). For the patch-clamp technique, an electrode with a larger tip is used with the objective of removing a portion or "patch" of membrane from the cell in which individual ion channels can be isolated; this is

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referred to as excised patch techniques. When the patch of cell membrane is removed, but the electrode remains attached to the cell, the electrode solution is in contact with the cell's cytoplasm. This configuration is called "whole cell" because one can measure the resulting currents of (no surprise here) the whole cell. The reasons why an electrode with a larger tip permits electrophysiological recordings from smaller cells is explained in the next section.

WHAT DOES PATCH CLAMPING A NEURON REALLY MEAN?

The main difference between sharp electrophysiology and the patch-clamp technique is in how the electrode makes contact with the cell. The electrodes (sometimes also referred to as micropipettes or just pipettes in electrophysiology jargon) used for both of these techniques appear relatively similar; they are made out of pulled capillary glass that has a narrow ending (the side that contacts the neuron) and a wide ending (where it can be filled with the electrode solution). The electrode solution contains electrolytes that conduct electricity from the neuron to a wire that is inserted in the electrode and is connected to an amplifier, which is necessary to amplify the small currents and voltage differences elicited by biological membranes. Although sharp and patch-clamp pipettes may appear rather similar at first glance, they differ enormously in tip size, taper geometry, and the solutions that they must be filled with.

For sharp electrophysiology, the glass capillary must be pulled to produce a very narrow taper with a small tip, with the aim of minimizing damage when it is impaled into a large neuron or muscle. Because the tip is so small, the area of exchange between the cell and the electrode solution is also small; therefore, the electrode solution needs to have a high concentration of electrolytes to allow current to flow properly from the cell to the wire and vice versa. On the contrary, the glass used to produce electrodes for patch-clamp electrophysiology has thick walls, and it should be pulled to produce a pipette with a relatively larger tip and a wide taper. Before the patch can be opened, the glass should attach firmly to the cell, achieving a high-resistance seal (which reaches the order of gigaohms and is therefore sometimes called a "giga-seal"). To improve the contact between the membrane and the electrode, the electrode's tip can be polished with fire to smooth the broken glass ending; and, as a matter of fact, this step is mandatory when recording from *Drosophila* neurons. (For instructions on how to pull and polish pipettes, see Protocol: **Preparation of Pipettes and Pipette-Filling Devices for Patch-Clamping *Drosophila* Neurons** [Fernandez-Chiappe and Muraro 2022a]). The composition of the electrode solution for patch clamping should mimic that of the cell's cytoplasm because, once the patch breaks open, there will be continuity between the interior of the pipette and the cell contents. In this case, a high concentration of electrolytes is not only unnecessary (because of the larger ending of the pipette, which favors current flow) but would also be detrimental, as it would change the neuron's internal ionic concentrations and affect its physiology, eventually killing it. For this reason, weighing the components when preparing the internal solution for patch clamping should be done conscientiously (the final concentration of osmolytes could be confirmed with an osmometer, if available, to avoid swelling or shrinkage of cells). Sharp and patch-clamp electrophysiology are different approaches; neither technique is better than the other. Deciding which approach to use in any given experiment will depend primarily on the size of the cell from which one wishes to record. Figure 1 summarizes the characteristics of these two intracellular electrophysiological approaches. Extracellular electrophysiology, in which electrodes are placed in a tissue and record electrical information from the multiple neurons that surround it, is not discussed here.

Although any technique in neurobiology requires time and practice to be performed correctly and efficiently, this is particularly true for patch-clamp electrophysiology, which is famously a "difficult" method to learn. The reason for this is that a patch clamper needs to perform a series of very precise movements that involve manipulations with the hands and looking through the microscope and at the computer screen almost simultaneously. Until this motor pattern is internalized and these movements flow in sequence almost without intention, many pipettes will be broken and many preparations will

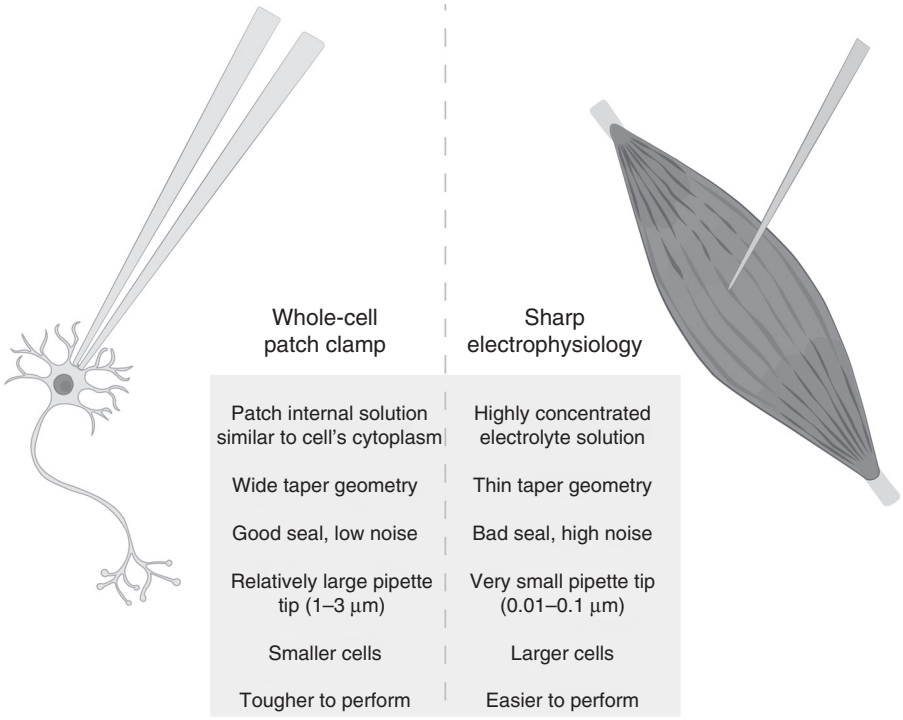

FIGURE 1. Summary of the characteristics of sharp and patch-clamp approaches to intracellular electrophysiology.

be damaged; thus, high tolerance of frustration is the most important trait in anyone who wishes to learn this technique. To master patch clamping as quickly as possible, the trainee should devote a solid period of time to practice (maybe combined with some fly pushing but not much more), such that their whole day in the laboratory is spent in a continuous loop of preparing a brain and attempting to patch, over and over, for several weeks. Unfortunately, patch clamping cannot be learned during spare time while performing other experiments. Patch clamping is not rocket science; anyone wishing to invest time and effort can become a good patch clamper. There is also a large psychological component to patch clamping. If nothing is working, either when learning the technique or even when the method has already been mastered, stop for the day or the morning, take a break, and have a cup of tea. If you are sad or worried, or your mind is somewhere else, that is probably not the day for patch clamping.

WHICH NEURONS CAN BE PATCHED?

In theory, the patch-clamp technique can be applied to any neuron of interest (or any cell; remember, all cells have a membrane potential) in larvae or adult flies as long as it is physically accessible. Accessibility is therefore the main concern when the electrophysiological approach is to be applied, and the damage that could be caused to the surrounding tissue in reaching the neuron of choice should be assessed wisely. It is not surprising that many patch-clamp electrophysiological recordings performed thus far on flies relate to sensory systems and the neurons that correspond to the first steps of sensory information processing, as they are located in more external parts of the brain. Examples of these are the antennae and olfaction glomeruli (Kazama and Wilson 2009) or the retina and underlying visual circuits (Tuthill et al. 2014). Additionally, some neuronal types located in the central brain have also been accessed and recorded using the patch-clamp technique. Some examples of these are embryonic and larval motoneurons (Baines and Bate 1998), and adult mushroom body (Gu and

O'Dowd 2006), ellipsoid body (Liu et al. 2016), and fan-shaped body (Pimentel et al. 2016) neurons, to mention a few.

In the accompanying protocols, we provide instructions for accessing and recording from two neuronal types. Protocol: **Dissection of *Drosophila* Wandering Larval Brains for Patch-Clamping Neurons** (Fernandez-Chiappe and Muraro 2022b) explains how to dissect a wandering third-instar brain and glue it down to later record from the fairly well-characterized aCC or RP2 motoneuron somas (highlighted by the *RN2*-Gal4 driver, also referred to as *eve*-Gal4), which are located fairly superficially in the ventral nerve cord (VNC). Smaller larvae can be filleted to produce a preparation that contains both the motoneuron somas and their innervating muscles. These are complicated dissections that require more practice; the methods to produce these can be found elsewhere (see Protocol: **Dissection of First- and Second-Instar *Drosophila* Larvae for Electrophysiological Recording from Neurons: The Flat (or Fillet) Preparation** [Marley and Baines 2011]). In the case of larger larvae, such as third instars, a filleted preparation can be obtained, but, without any pharmacological intervention, the muscle contractions produce waves of muscle movement that preclude the recording of the motoneuron somas. A simple preparation in which the brain is pulled off from the body is described here, in which superficial VNC motoneuron somas can be accessed and recorded without these problems.

Protocol: **Dissection of *Drosophila* Adult Brains for Patch-Clamping Neurons** (Fernandez-Chiappe and Muraro 2022c) provides detailed steps for the dissection of the *Drosophila* adult brain with the aim of recording from large lateral ventral neurons (lLNvs). lLNvs are clock neurons (Helfrich-Forster 1995) with a role in arousal (Sheeba et al. 2008a) and, because of their relatively large soma size and their fairly good accessibility, comprise the most recorded *Drosophila* clock neurons to date. There are four to five lLNvs per hemibrain with their somas localized in the accessory medulla, the tissue that lies at the intersection of the optic lobes and the central brain. To identify and record from lLNvs, a fluorophore should be genetically expressed using a lateral ventral neuron (LNv)-specific promoter such as the one for the pigment-dispersing factor (Pdf) neuropeptide. Pdf expression in the central brain is restricted to the 16 to 18 LNvs, highlighting not only the eight to 10 lLNvs but also the eight small LNvs (sLNvs), but their difference in soma size allows the identification of each LNv type univocally. The fluorophore expressed to label the lLNvs should not be membrane-bound green fluorescent protein (GFP), as this may alter neuronal physiology; instead, a cytoplasmic fluorescent protein should be used. In this sense, the construct that provides the most flexibility for experimentation is from a direct *Pdf*-red fluorescent protein (RFP) line (Ruben et al. 2012) (available on the second and third chromosomes) that can be genetically combined with driver and effector lines to manipulate genes independently of the labeling of lLNvs. The classical *Pdf*-Gal4 driver (Park et al. 2000) could also be used to drive an upstream activating sequence (UAS)-fluorophore element.

Something that should be taken into account when recording electrophysiologically from lLNvs is that their firing varies with the time of day, a feature typical of clock neurons. During the day, lLNvs fire spontaneous action potentials, organized in bursts of spikes, at a higher rate; however, at night, their firing frequency is lower, and a higher proportion of tonic-firing neurons are present (Sheeba et al. 2008b; Muraro and Ceriani 2015). Although some researchers have reported a proportion of lLNvs in a silent state in this type of ex vivo preparation (Cao and Nitabach 2008; Sheeba et al. 2008b), our experience is that all lLNvs are spontaneously active if they are recorded quickly after dissection.

An important feature of lLNv ex vivo preparations that should also be kept in mind is that the dissection itself has an effect on the firing of these neurons. This is because important visual inputs that support their high bursting frequency are lost during dissection; therefore, their bursting frequency decays as a function of the time after dissection (Muraro and Ceriani 2015). For this reason, recording the starting time of the dissection is crucial for later analysis of electrophysiological recordings, because all observations must be considered at the same time after dissection if results from different cells are to be pooled and groups of cells are to be compared.

sLNvs can also be recorded using this preparation. But they are, as their name indicates, smaller in size, making the recordings more challenging. To avoid frustration, only after mastering recording from lLNvs should an attempt to record from sLNvs be made. sLNvs are also bursting neurons during

the day, and their bursting frequency also decays with time after dissection (Fernandez-Chiappe et al. 2021); therefore, the same considerations regarding timing mentioned for lLNvs should be taken into account when using this preparation for sLNv recordings.

The patch-clamping method explained in detail in Protocol: **Patch-Clamping *Drosophila* Brain Neurons** (Fernandez-Chiappe and Muraro 2022d) covers preparation to whole-cell patch clamping; once you have mastered this demanding technique, an entire universe of recording possibilities will open up. Getting to this point will require time and practice; therefore, the initial objective should only be to consistently obtain recordings of healthy cells with a good seal, negligible leaking, and a good resting membrane potential and that fire action potentials upon current injection (or even spontaneously, depending on the neuronal type). After this milestone is achieved, the proper experiment should be planned in advance according to the hypotheses to be tested, so that the experimental settings (such as modified internal and external solutions) and the electrophysiological procedures once you reach whole-cell configuration are adequate. For instance, if the hypothesis is that a particular ion current is affected by a specific manipulation, a step procedure in voltage-clamp mode should be used. In this type of procedure, the membrane is held at different voltages and the elicited currents are recorded. This is extremely useful for characterizing ionic conductances by building *I–V* (current–voltage) curves. In this case, use different combinations of internal and external solutions to block the currents that you do not wish to measure. For example, if you wish to isolate voltage-gated sodium currents, the voltage-gated potassium and calcium currents need to be blocked; otherwise, inward and outward voltage-sensitive currents will open all at the same time and the resulting currents may not be as informative.

Alternatively, if you are interested in assessing neuronal excitability, you should design a step procedure in current-clamp mode. In this type of procedure, pulses of increasing amounts of current are injected sequentially, and the reaction of the cell to these pulses is recorded. The number of action potentials elicited by each step will reflect the excitability of the neuron (more excitable neurons will fire more action potentials in a given current step and will start firing action potentials with less current injection than less excitable neurons).

Many modern techniques in neurobiology such as whole-brain imaging of fluorescent reporters, single-cell RNA sequencing of whole tissues or organisms, and connectomics, to mention a few, involve obtaining large quantities of data about different characteristics of brain cells. Electrophysiology is a low-throughput, labor-intensive technique; so, is it worth investing time and effort in discovering the electrical characteristics of a few neurons in the "whole-brain" era? There are many reasons why the answer to this question is a resounding yes. First, not every electrical change in a neuron would be reflected by a difference in calcium levels detectable with fluorescent calcium sensors, and fluorescent voltage indicators have yet to reach a signal-to-noise ratio such that they are widely adopted. Moreover, those techniques usually measure changes in fluorescence, not absolute values, and the richness of information gained simply by the accurate measurement of resting membrane potential is something that only electrophysiological methods can provide. The ability to detect subthreshold currents, to dissect ionic conductances, and to apply pharmacological treatments only to the intracellular medium (by adding chemicals to the patch pipette) are possibilities that patch-clamp techniques are particularly convenient for. Finally, at the end of the day, electricity is the language of neurons; let us talk and listen to them in their own language. So, our proposal to you is: Let's have it all! The "high-throughput," "omics," and "whole-brain" approaches, the unbeatable genetic tools available for our favorite fly, and patch-clamp electrophysiology.

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