AN ABSTRACT OF THE THESIS OF

Jay F. Howard for the degree of Master of Science in Physics presented on September 16, 2016.

Title: INTRACELLULAR MEASUREMENTS OF NEURONS USING THE SHARP ELECTRODE TECHNIQUE

Abstract approved:

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Ethan D. Minot

The Brain Research through Advanced Innovative Neurotechnologies (BRAIN) initiative seeks to understand how ensembles of neurons create neural networks. The initiative has spurred the pursuit of developing novel experimental tools for investigating how individual neurons propagate electrical signals (action potentials) to produce ensemble behavior. Classical techniques to measure the internal electrical dynamics of a neuron involve using a glass needle to either pierce through or dock to the membrane of the cell, drawbacks of which include damage to the cell and limits on the temporal resolution and number of simultaneous measurements. Biosensors made from graphene field-effect transistors (GFETs) are promising candidates for externally measuring the electrical activity of individual neurons, which avoids membrane damage. We aim to use the sharp electrode technique to correlate an internal measurement of an action potential with an external GFET measurement to confirm proof-of-concept. This thesis describes the progress made in establishing an experimental system in the Minot lab for making an internal measurement of a *Lymnaea stagnalis* action potential using the sharp electrode technique.

INTRACELLULAR MEASUREMENTS OF NEURONS USING THE SHARP ELECTRODE TECHNIQUE

by
Jay F. Howard

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APPROVED:

Major Professor, representing Physics

Chair of the Department of Physics

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Jay F. Howard, Author

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1 Introduction

Motivation for this project comes from the Brain Research through Advanced Innovative Neurotechnologies (BRAIN) initiative, which is led by the National Institutes for Health and National Science Foundation. The goal of the initiative is to seek an understanding of how individual neurons work together to form neural circuits - such as the human brain - which ultimately dictate behavior of an organism. The hope is that innovations in nanotechnology will play an important role in revealing the workings of neural networks, and that the results will help researchers determine how to better address medical issues in the human brain such as Alzheimer's and other neurodegenerative diseases [1].

To understand how an ensemble of neurons functions it is important to understand the electrical dynamics of an individual neuron. A classical method for doing this is the sharp electrode technique. In this technique a neuron is impaled by an electrolyte-containing microelectrode, which then becomes equipotential with the inside of the cell. The other end of this microelectrode connects to a recording device, and thus the sharp electrode technique allows for direct observation of electrical dynamics. However, the sharp electrode technique is difficult to implement in practice and harms the cell upon insertion. If the researcher is interested in the long-term dynamics of an unperturbed cell, this technique is not adequate.

Since field-effect transistors (FETs) effectively probe their electrical environment, biosensors built from FETs are a strong candidate for measuring neuronal activity. And since these biosensors sit on the surface of the cell rather than piercing the membrane, they should allow for longer-term experiments on relatively unperturbed cells. In addition, FET biosensors allow us to probe electrical dynamics with improved temporal resolutions and perform more simultaneous measurements compared to classical electrophysiology techniques [2]. Graphene acts as a field-effect transistor (GFET) and has been shown to be a promising candidate for this approach due to its low toxicity and high electrical mobility[34].

A technique called graphene kirigami, whereby a sheet of graphene is made more stretchable by plasma etching, is thought to be a good method for producing such a biosensor[5]. A good proof-of-concept experiment to show that a kirigami GFET will work as a biosensor is to make a simultaneous

intracellular measurement and extracellular GFET measurement and compare the two. The work discussed in this thesis is to form the groundwork for making the intracellular measurement.

This thesis explains how to understand the cell as a simple circuit and how this model can be used to understand the electrical recordings we obtain from a neuron from *Lymnaea stagnalis,* a pond snail. It then discusses the results of preliminary experiments and ends with suggestions for continuing the project.

2 Theory

2.1 Building a simplified model of the cell

The cell is a complicated system: to date, we understand the function of many types of cells, but many of the biochemical mechanisms involved are still a mystery. In the mid-20th century, biophysicists Alan Hodgkin and Andrew Huxley ignored biochemical complexity and used clever experiments to construct a simple circuit model of the cell - a set of voltage-gated resistors and capacitors in parallel. The Hodgkin-Huxley (HH) model correctly described the electrical behavior of the squid giant axon[6].

The experiments used to develop the HH model earned the researchers a Nobel Prize, and it is difficult to overstate the significance of their discovery. The HH model sits at the heart of electrophysiology research: researchers developing novel modeling techniques[2] test their new models versus HH to determine accuracy[7]. Details of applying the HH model are not discussed in detail in this thesis, but we follow their argument in spirit in order to derive a simplified model of our recording system.

2.1.1 Cell basics

All cells contain cytosol (an electrolyte solution). An insulating membrane (lipid bilayer) keeps the cytosol isolated from the extracellular environment, which is also an electrolyte solution (Fig 1). Transmembrane proteins span the lipid bilayer and behave as channels to allow selective passage of ions into or out of the cell. Most of these channels are voltage-gated. Cells regulate their internal chemistry such that they maintain a negative potential relative to their environment, and by convention we set the potential outside of the cell equal to zero, $V_{external}$ = 0. As a result, when we measure the internal potential of a cell, we are measuring the change in voltage across the membrane V_m:

$$V_m = V_{external} - V_{internal}$$
$$\Rightarrow V_m = 0 - V_{internal}$$
$$\Rightarrow V_m = -V_{internal}$$

2.1.2 Cell as a circuit

A physicist may look at this description and notice that the membrane acts as a capacitor (an insulating material separating two conductive media). Additionally, we can think of voltage-gated channels as variable resistors, and since they are all in parallel with each other we can simply define an effective variable resistance associated with all of them. This reduces the cell to an RC-circuit (inset, Fig 1), which is useful for understanding the general shape of an action potential and understanding the response of the cell to electrical stimulation.

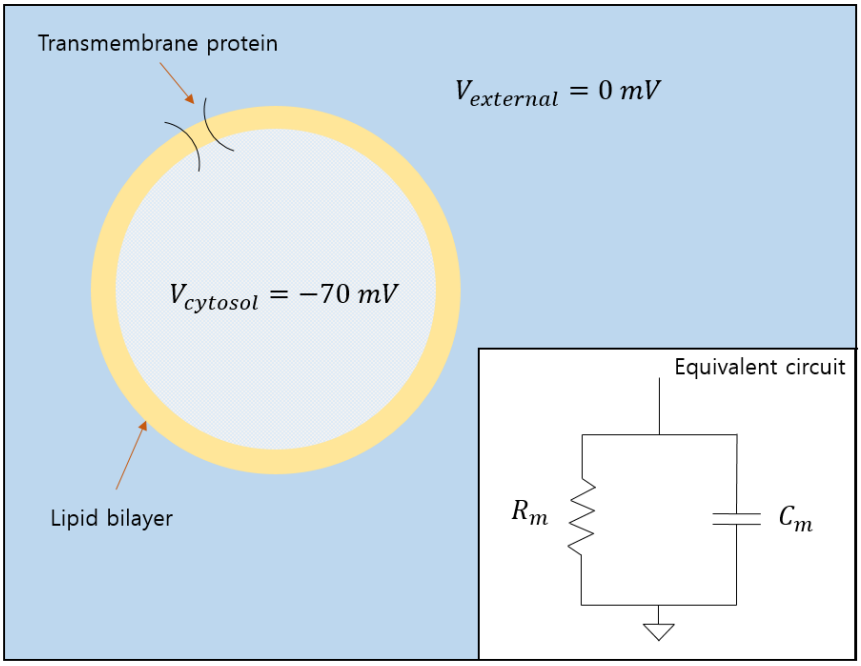

Figure 1: A cell is a complex machine, but can be approximated as a spherical insulating membrane of lipids which contains an electrolyte solution. A cell regulates its internal chemistry such that it maintains a negative potential relative to its environment. Since its external environment is also an electrolyte solution, the membrane acts as a capacitor. The membrane also contains transmembrane proteins which span the lipid bilayer and allow the selective passage of ions into and out of the cell. We can model these proteins as resistors, and can reduce the set of parallel resistors into one effective resistance. Thus, the cell may be modeled as an RC circuit.

2.1.3 Understanding the simplified model

In a typical electrophysiology experiment the researcher will perform either "voltage clamp" or "current clamp," holding constant either the membrane potential or the current through the membrane. In either case, an injection of current through the recording microelectrode is required. Recall that the current drawn on or off a capacitor is proportional to the derivative of the change in voltage with time across it:

$$I = C\,\frac{dV}{dt}$$

Finite current cannot cause instantaneous changes in the voltage across a capacitor. If there is an instantaneous change in current (such as the researcher injecting current into the cell), there will not be a corresponding instantaneous change in voltage to a new value, but rather one that approaches the new value exponentially with a time constant equal to $\tau = RC$ (figure 2). This is the origin of the smooth wavelike nature of action potentials.

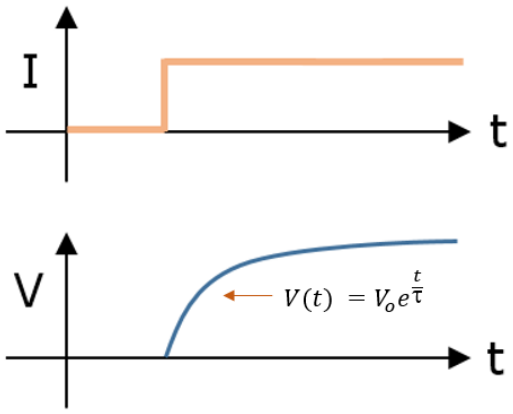

Figure 2 A plot of voltage versus time for a typical RC circuit. Note that the voltage approaches a steady value at an exponential rate governed by the rate constant $\tau = RC$

2.1.4 The measurement apparatus

Our recording device for measuring neurons is a glass microelectrode filled with a conductive solution of chlorine and potassium ions and a silver wire coated in a silver-chloride surface (Ag/AgCl). A diagram appears in figure 3.

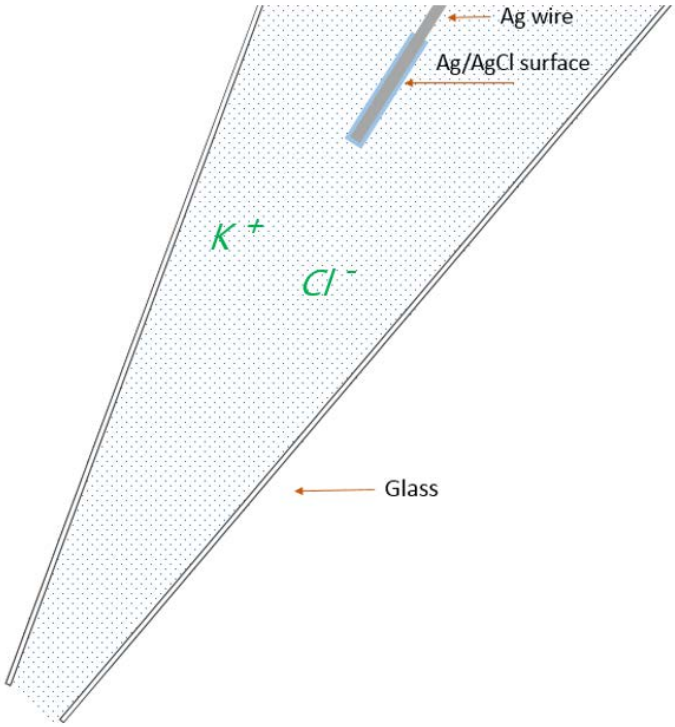

Figure 3 The microelectrode we use for experiments is glass capillary tube with an inner diameter of 0.5 mm at one end that has been pulled to a fine point at the other end, with a diameter on the order of hundreds of nanometers. The glass is filled with an electrolyte solution containing potassium and chlorine ions, the latter of which allows redox reactions to occur at the Ag/AgCl surface. Thus, current may flow from solution to the Ag/AgCl wire or from the wire to the pipette solution.

In an experiment, a cell is impaled by this glass microelectrode as seen in Figure 4a. Reduction-oxidation (redox) reactions take place at the Ag/AgCl surface as described by the following reaction:

$$AgCl(s) + e^{-} \rightleftharpoons Ag(s) + Cl^{-}$$

One can see that on either side of the equation there is a net negative charge; this allows the redox electrode to act as a bridge between current in solution and current in the silver wire. The silver wire connects to a preamplifier called a headstage, which sends a signal to an instrument called an electrophysiology rig. The rig is the central instrument for electrophysiology experiments and is discussed in more detail in the Methods section.

We use the microelectrode as a source or sink for current to pass into or out of the cell. Current is injected into the cell by the following mechanism: an electron in the silver wire reacts with AgCl to produce solid Ag (neutral charge, stays bound to the surface of the electrode) and a negatively charged chlorine ion enters the bulk filling solution. Thus, the electron is "converted" into a negatively charged chlorine ion, and current flows to the cell. Assuming the researcher adequately chlorides the silver wire (discussed in Methods section) this is a reversible reaction and thus Ag/AgCl makes a great microelectrode.

Impaling a cell with a microelectrode introduces two new circuit elements to our model, electrode resistance R_e and a parasitic capacitance C_p.

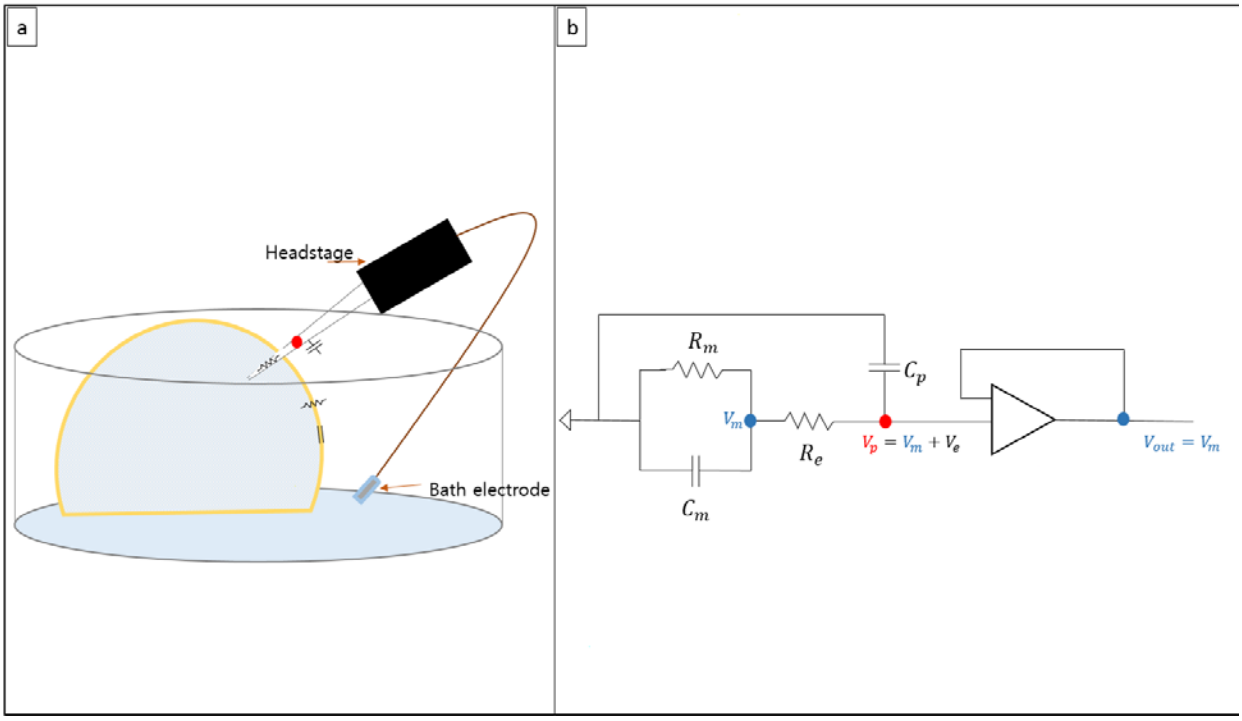

Figure 4 a) A sharp electrode experiment requires the researcher to impale the cell with a microelectrode, which allows recording of the internal cell potential. This introduces new circuit elements to our model.
b) The equivalent circuit of the cell plus microelectrode. Note that there are two resistive terms: a bath resistance for current flowing from the cell to the ground and a resistive term from the microelectrode. Since these resistances are in series we may define a new series resistance, which is the sum of the other two. We keep the label Re because electrode resistance is by far the dominant term in this sum. The additional elements C_p and R_e are electronically subtracted from recordings using an op-amp, represented by the triangle

2.1.4.1 Electrode resistance and bridge balance

Figure 5 shows the voltage measured from a microelectrode, $V_p(t)$, when there is a sudden change in current from zero to I. The presence of R_e adds an additional voltage change to the beginning of the voltage versus time plot equal to $V_e = IR_e$, as seen in figure 5. We can account for and subtract V_e from our recordings using the bridge balance knob on our electrophysiology rig, which activates an op-amp to sample and subtract current before the signal is sent to the electrophysiology rig.

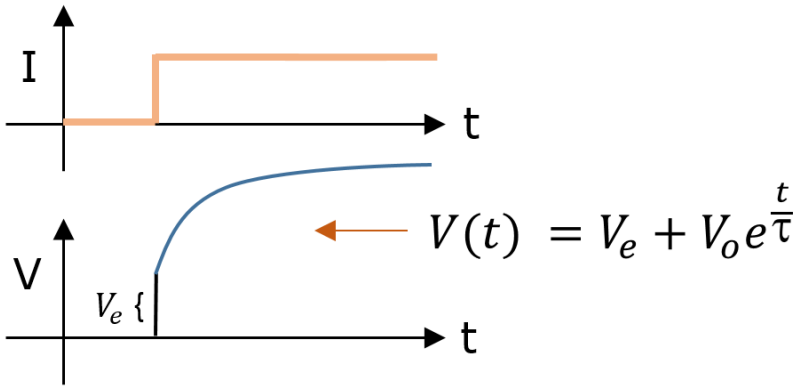

Figure 5 The presence of R_e in series with our RC circuit causes the recording to have an additional voltage "bump" at the beginning of a plot of voltage versus time. We can account for and subtract this from our recordings using bridge balance on the electrophysiology rig.

In practice, bridge balancing not only gives us a "correct" recording but also tells us R_e. Note that R_e lumps together resistance due to the injection microelectrode and resistance through the saline bath into one term. Since these resistances are in series, we sum them and obtain a series resistance, which remains labelled R_e. The main contribution to R_e is the resistance of the narrow tube of KCl solution at the tip of the microelectrode. The resistance of a cylinder of 3 M KCl is given by

$$R = \rho_{KCl} \frac{L}{\pi \left(\frac{D}{2}\right)^2}$$

where D is the diameter of the cylinder and $\rho_{KCl} = 5 * 10^{-2}\ \Omega \cdot m$ is the resistivity of 3 M KCl at room temperature[8]. The relationship between R_e and D is used to estimate the diameter of microelectrode tips. We can use this relationship, along with an empirical rule of thumb that $1\ M\Omega$ of resistance corresponds to a $1\ \mu m$ diameter tip, to derive a general relationship between tip diameter and resistance:

$$D \sim \left(2.5 * 10^{-4}\, m \cdot \Omega^{1/2}\right) R^{-\frac{1}{2}}$$

Which is a convenient form for estimating diameter from measured tip resistance. The presence of R_e and the procedure to correct for it does not affect the behavior of the cell, it only affects our recording. The term "bridge balance" is an historic term stemming from the fact that this technique was originally done using a Wheatstone bridge. Today, the functionality of the Wheatstone bridge is achieved with an op-amp.

2.1.4.2 Parasitic capacitance

Parasitic capacitance C_p, on the other hand, introduces artifacts into our recording and also perturbs the behavior of the cell. This term is a combination of effects from (i) the capacitive coupling between the liquids inside the microelectrode and the bath and (ii) capacitances in the external circuit. Since a capacitor only affects a circuit when it is charging or discharging, to remove its effect on our system we need to keep C_p fully-charged. Electrophysiology rigs have a "capacitance neutralization" knob that is used to achieve this, using an op-amp for positive feedback. This method can reduce the effect of C_p, but it cannot eliminate its presence entirely: C_p will be variable in time, but the capacitance neutralization knob only allows us to account for a static capacitance value. Further methods for reducing noise from parasitic capacitance (such as coating the glass microelectrode in metal or Sylgard) can be found in chapter 12 of the Axon Guide[9].

The researcher needs to balance desire for noise reduction with the risk of killing the cell with positive feedback.

2.2 The Model Cell

An informative way to understand the circuit model for the neuron/microelectrode circuit (Fig 4) is to walk through the calibration procedure for our electrophysiology rig, which begins on page 13 of the Axoclamp-2B Manual. This is done by attaching a "model cell" to our headstage (we used a "CLAMP-1U MODEL CELL"). The model cell mimics the experiment shown in figure 6 and has the ability to toggle between "bath" and "cell" modes. We begin in "bath" mode for our calibration.

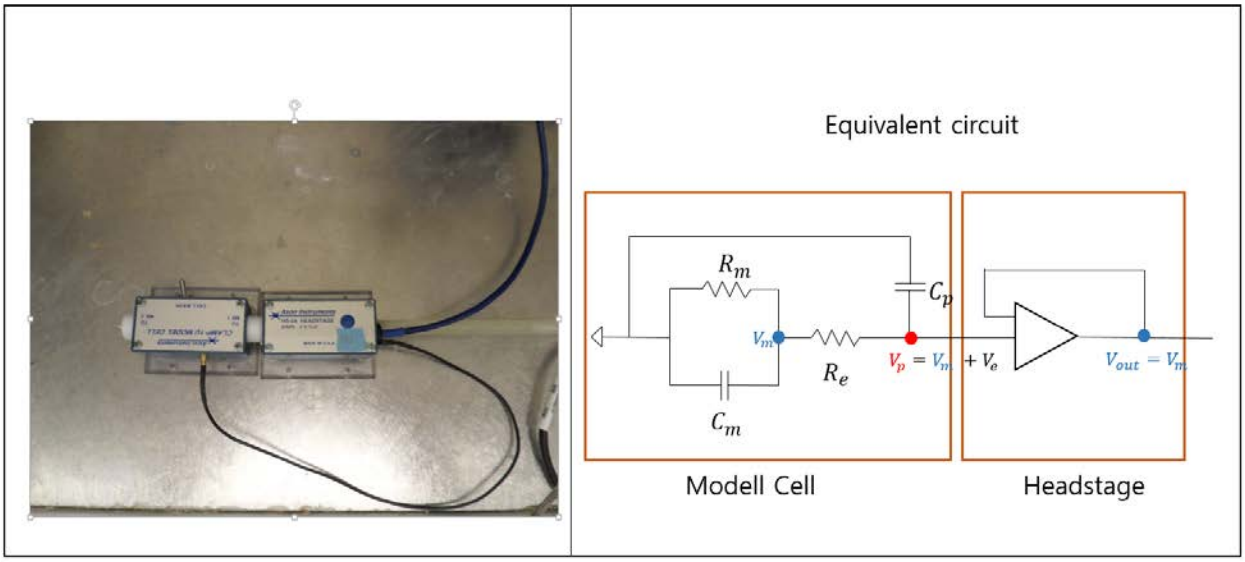

Figure 6 An image of our experimental setup when using the model cell in "cell" mode. Note that the model cell and microelectrode both have a plastic base to keep them off of the surface of the faraday cage. The equivalent circuit mimics the circuit of the sharp electrode technique.

A function generator is attached to the rig and square wave current pulses are sent through the rig to the model cell (see the Methods section for practical details). An oscilloscope attached to the output of the rig monitors the membrane potential as it responds to these current pulses.

Since our circuit has a resistive term R_e in series with the capacitor, when we send in a current pulse we see an initial jump in voltage followed by an exponential charging of the membrane

capacitance. A plot of a resistor in series with an RC circuit appears in Figure 5. It is this initial bump that the bridge balance corrects.

Prior to bridge balancing, the waveform on the oscilloscope will appear similar to Figure 7-a. Balancing the bridge reduces the quick voltage steps seen at the beginning and end of the waveform, and when the bridge is correctly balanced the waveform should look like Figure 7-b. An overbalanced bridge looks like Figure 7-c on the oscilloscope.

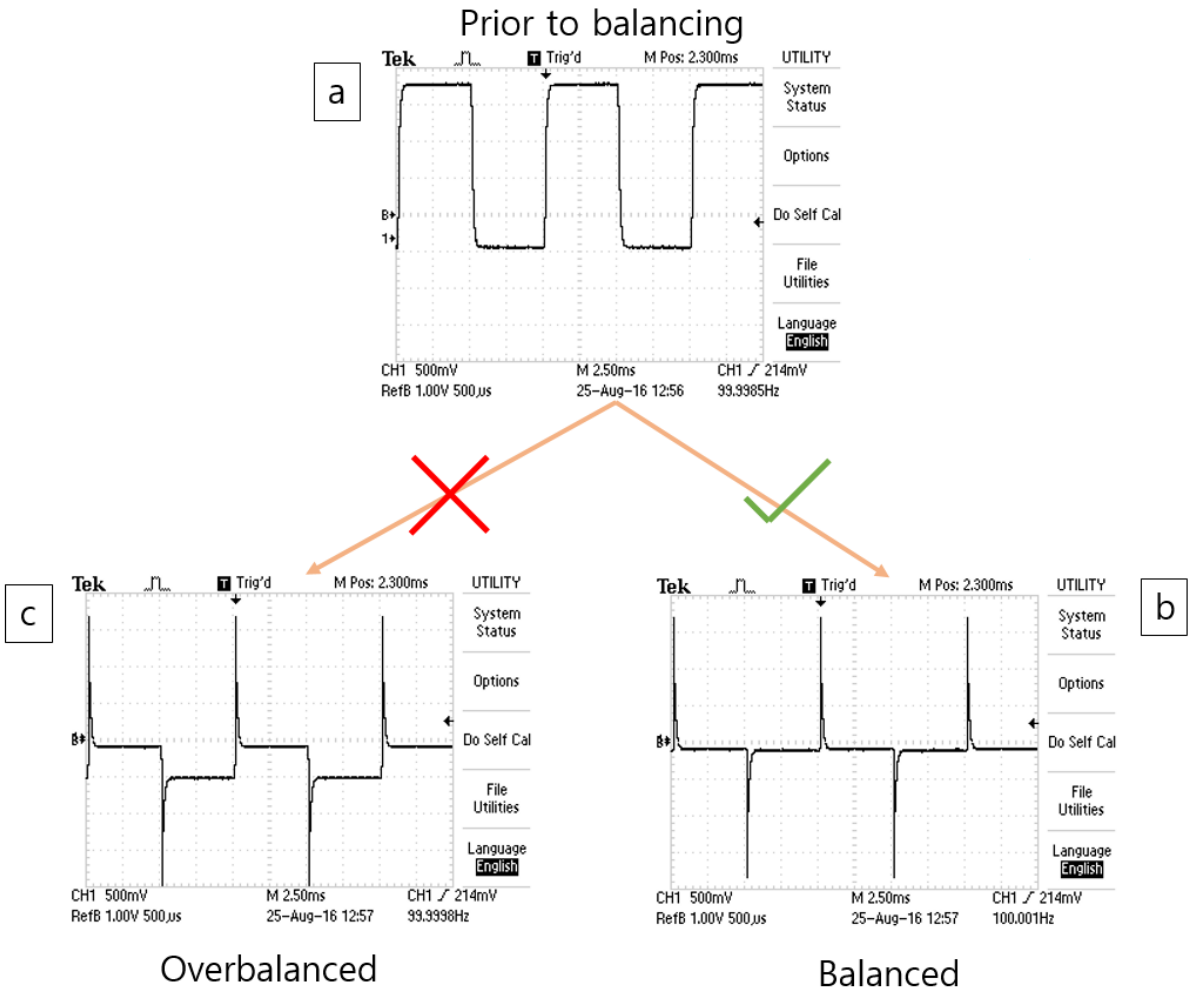

Figure 7 a) Prior to bridge balancing, the waveform on the oscilloscope should look like this while square wave current pulses are applied.. b) After bridge balancing, the waveform on the oscilloscope should have a sharper peak. Note that the system returns to a steady potential before and after the peak. c) an overbalanced bridge has an unequal potential before and after the peak.

After bridge balancing, the user can now read off the electrode resistance by examining the bridge knob: multiply the number of turns by 10 and divide by the gain (0.1 for our headstage). This should come out to 50 MΩ for the model cell.

To obtain clean recordings of cell dynamics we still need to account for C_p. After the bridge is balanced, toggle the model cell to "cell" to mimic impaling the cell with a sharp electrode. Adjust the capacitance neutralization knob until transients seen at the beginning and end of the current pulse are eliminated. It is important to be careful with this step of the procedure: if capacitance neutralization is adjusted past a certain point, positive feedback will rapidly inject too much current and destroy a cell. When properly adjusted, the waveform should appear as in figure 8-b; additionally, the end of the waveform should be as smooth a decay possible without additional oscillations (figure 8-c). In practice, after a researcher finds what seems like a "good" amount of capacitance neutralization, they reduce the amount of compensation to err on the side of caution.

We have now calibrated our system to properly display electrical dynamics of the cell we are monitoring. We have also obtained our series resistance, which is important for comparing results to those in the literature.

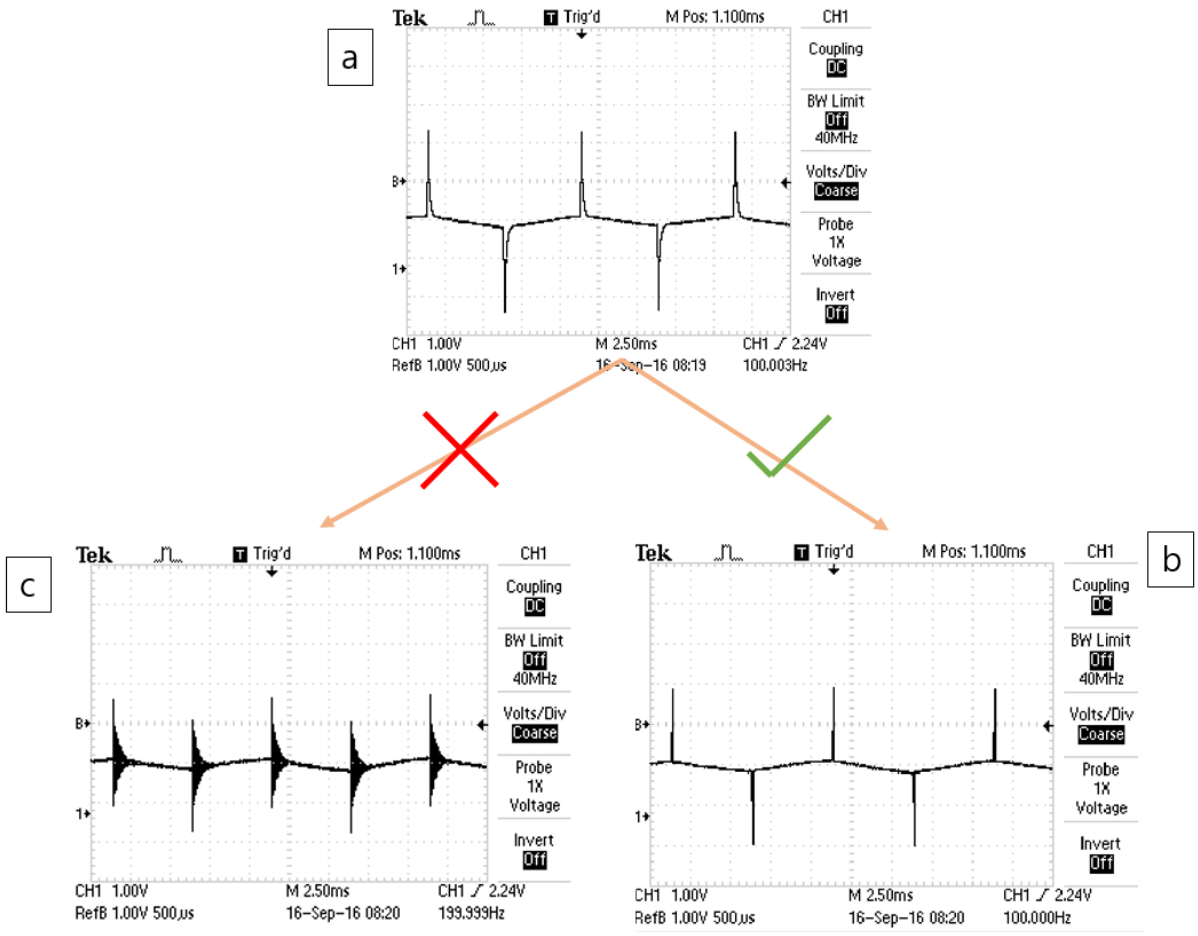

Figure 8 Overdoing it on the capacitance neutralization knob will kill the cell, so it is important to understand what an over-neutralized waveform looks like. a) Prior to neutralization the waveform should look like this. The waveform does not return immediately to a flat value between pulses as it did during bride balance because we are now using the model cell to mimic the whole cell circuit, which includes the exponentially-charging and -discharging cell membrane. b) After neutralization, the width of the peak should be narrower than it was. c) An over-neutralized circuit will have additional oscillations. The waveform pictured here is drastically over-neutralized for illustrative purposes. An over-neutralized circuit may not appear as drastic as this, so we recommend zooming in on the oscilloscope and looking for oscillations in the potential that overshoot the base value. If these appear, turn the neutralization down until the waveform no longer overshoots the base value.

2.3 Advantages and Disadvantages of simple model

This model of the neuron/microelectrode circuit is helpful for a few key reasons: it gives us a simple model with which to understand the waveform we record and it helps us understand the purpose of bridge balance and capacitance neutralization, two essential procedures for measuring neurons. In addition, having a circuit model of our cell helps us identify sources of noise: Johnson noise arises from thermal fluctuations in resistors, so identifying the resistance in various elements is important. Thus our simple model helps us become familiar with our measurement apparatus and recordings.

But like many simple models, ours is not sufficient for describing some of the more complex behavior of neurons. For example, we know that sodium and potassium channels have different gating voltages, but we ignore this and lump them into a single resistive term. Additionally, most neurons have more structure than the roughly-spherical insulated ball we have assumed, and thus require additional compartments to model correctly; think of our current model as the trunk of a tree and the additional compartments as branches. These new compartments appear as additional RC circuits in parallel with our first circuit, and would need to be accounted for if the researcher plans to use their results to model electrical dynamics using the HH model[6]. We do not intend to use the HH model in the near future, so considering a single-compartment model with one variable resistor is adequate for the purpose of this thesis.

2.4 Electrophysiology rig principles of operation

We used an Axon Instruments Axoclamp-2B electrophysiology rig for the experiments described in this thesis (Axon Instruments is now owned by Molecular Devices). When a researcher does electrophysiology experiments they have control over a number of experimental parameters. What appears below is the reasoning for our set of parameters. A detailed walkthrough of how to set up the rig itself is in Methods section.

2.4.1 Filling solution

In our experiments we want to record the internal potential of the neuron V_m as a function of time. To achieve this we impale a cell with a glass microelectrode filled with an electrolyte solution ("filling solution"); the filling solution then becomes equipotential with V_m. In the other end of the glass microelectrode is an Ag/AgCl wire that connects back to a preamplifier, which then connects to the electrophysiology rig.

In order for redox reactions to take place at the Ag/AgCl surface and allow current to flow, we need chlorine ions in our filling solution. 3M KCl is a good candidate because it contains chlorine ions and has a relatively low resistivity, so contributions to thermal noise are minimal. Saturated KCl is around 4.5M, but having a saturated solution poses a problem: if liquid begins to evaporate from the glass pipette, salt crystals will form, which will likely interfere with measurements. Additionally, a higher concentration filling solution will undergo faster ion exchange with the cytosol. We want to perturb the internal chemistry of the cell as little as possible, so a saturated solution puts a limit on the longevity of experiments.

2.4.2 Glass

We use 1.0 mm outer diameter (OD) and 0.5 mm inner diameter (ID) filamented borosilicate glass (ITEM-#: BF100-50-10) We use this glass because the Sutter Cookbook suggested this was a good diameter glass for making electrodes with tip diameters on the order of 100 nm to 1 µm. Using thicker-walled glass may reduce the parasitic capacitance from the microelectrode, but will make it harder to obtain fine tips[10]. To make the sharp glass tips we put the glass tubing into an instrument called a pipette puller. For our experiments we used a P-80/pc flaming/brown micropipette puller. Details for operating the puller and obtaining appropriate tip diameters may be found in the Methods section.

Using filamented glass is important because it allows our filling solution to fill the small-diameter tip by a process called back-filling. The wide end of the pipette is placed in a salt solution as indicated in figure 9, and the filament acts as a wick to pull the filling solution to the tip. The rest of the pipette is filled by direct injection of filling solution into the wider end of the glass pipette. It is important to backfill first because otherwise an air bubble may form near the fine tip, which will interfere with measurements.

Figure 9 We backfill pipettes in our lab by putting the wide base in a 1.5 mL Eppendorf tube that's been filled with about 1 mL of 3 M KCl. This is an important step for ensuring the electrolyte fills the entire interior of the glass. If this step is skipped, there will be air in the tip and we cannot make recordings. After the glass is backfilled, the researcher can directly fill the rest of the glass (details in the "Methods" section)

3 Results and Discussion

3.1 Experimental results

The results presented here use the experimental geometry depicted in figure 4. For these measurements we used neurons harvested from *Lymnaea stagnalis,* a pond snail. Detailed methods for dissection of *lymnaea* and isolating the neurons we used for these measurements may be found in the methods section.

Cells maintain a negative internal resting potential relative to their environment. We set the potential of our recording electrode to zero when the electrode is in the bath, and thus we expect to see a sharp drop in potential as the microelectrode impales the cell. For most neurons this potential drop should be on the order of -40 to -70 mV. In our measurements we saw a potential drop of ~-20 mV (figure 10).

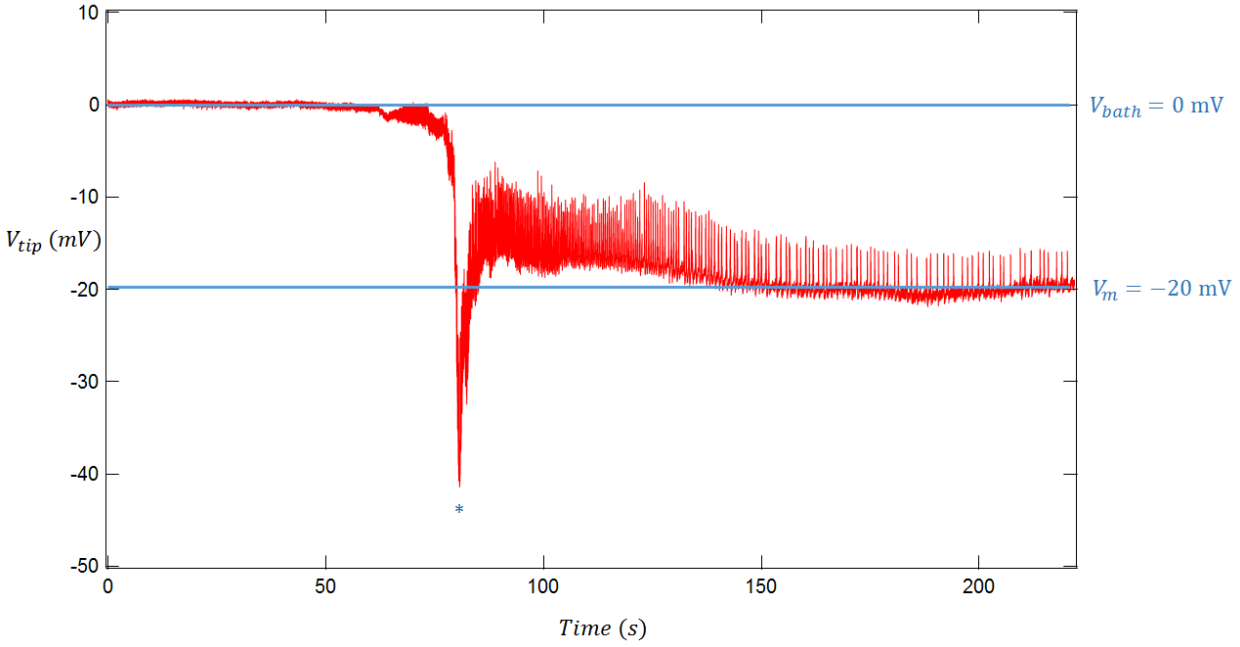

Figure 10 Cells maintain a negative potential relative to their environment, so we expect to see a large decrease in potential as our pipette tip enters the cell. While the tip is still in the bath we zero the potential, then approach the cell. The star marks where we entered the cell, as evidenced by the ~ -40 mV change in recorded voltage. We believe the subsequent increase to a new semi-steady value is due to membrane rippage due to a having a large-diameter pipette tip. The rapid spikes that begin at around 100 seconds and continue for the rest of the record are action potentials.

The wavelike shape of an action potential comes from the charging and discharging of membrane capacitance from the relative influx and outflux of sodium and potassium ions[6] [7].Typical action potentials have a 1 ms duration and change of about 100 mV from internal resting potential to the top of the action potential. Our measurements show a smaller change of about 5 mV (figure 11).

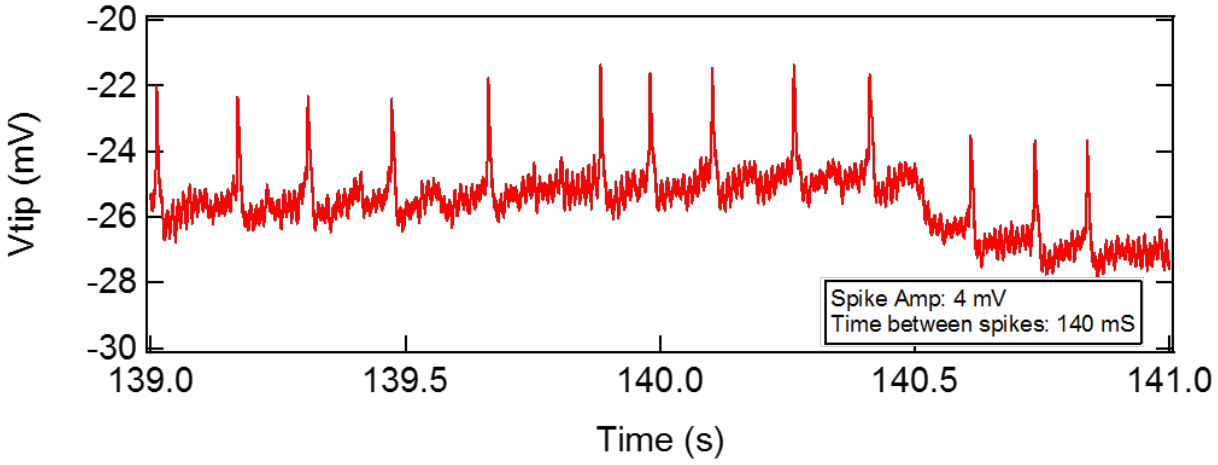

Figure 11 action potentials recorded from a Lymnaea stagnalis neuron. We expect magnitudes of spikes to be on the order of 70 mV. Although the magnitudes of the action potentials is much smaller than expected, we can say with certainty that these are action potentials (Dr. Pat Chappell, Veterinary Dept. Oregon State University, private communication). The smaller magnitude is due to damaging the cell membrane during the insertion process.

Our measured electrode resistance for these results was $R_e \sim 1\ M\Omega$, which means our pipette tip diameter was on the order of 1 µm. For these experiments we did not soften the neuron membrane prior to pipette insertion (see "Methods" section for more detail on membrane softening), and we believe this resulted in damage to the cell membrane during the insertion process.

Structural damage to the cell due to using a large-diameter pipette tip would explain the lowered magnitudes present in both figures. A large diameter pipette may result in structural damage to the cell membrane, which would be consistent with lowered magnitudes of action potentials and a measured intracellular potential higher than that predicted (Private Communication, Dr. Bruce Johnson, Neurobiology dept., Cornell University).

3.2 Johnson noise

To understand sources of noise in our system, we can calculate the frequency-independent thermal noise (Johnson noise) using the relationship

$$S_v = 4\,kTR$$

Where S_v is the voltage power spectral density (measured in units of Volts^2/Hz), T is the temperature in Kelvin, k is Boltzmann's constant, and R is resistance in Ohms. For the measurements presented here, $R \sim 1\ M\Omega$ and $T = 298\ K$, and we obtain:

$$S_v \sim 1.65 * 10^{-14} \qquad V^2/Hz$$

We can compare this value to a power spectral density (PSD) of our V(t) measurement data to determine if there are other sources of noise in our system other than Johnson noise.

3.3 Power spectral density

The plot in figure 11 shows a voltage versus time trace for electrodes sitting in the bath. Figure 12 shows the PSD of this data. Doing a PSD on our V(t) data tells us the contribution to noise from various frequencies, from which we can infer sources of noise. For example we can easily identify 60 Hz AC wall noise, harmonics of which are indicated by arrows in figure 12.

Johnson noise is a frequency-independent (white) noise - at least up until the sampling frequency, when aliasing begins to affect recordings. Thus, we expect Johnson noise to contribute the same amount of noise at any given frequency, and a horizontal line drawn through the floor of a PSD of V(t) data represents an approximation for the magnitude of Johnson noise. Doing this, we obtain an approximate value of

$$S_v \sim 2 * 10^{-13} \qquad V^2/Hz$$

This is within an order of magnitude of the expected value.

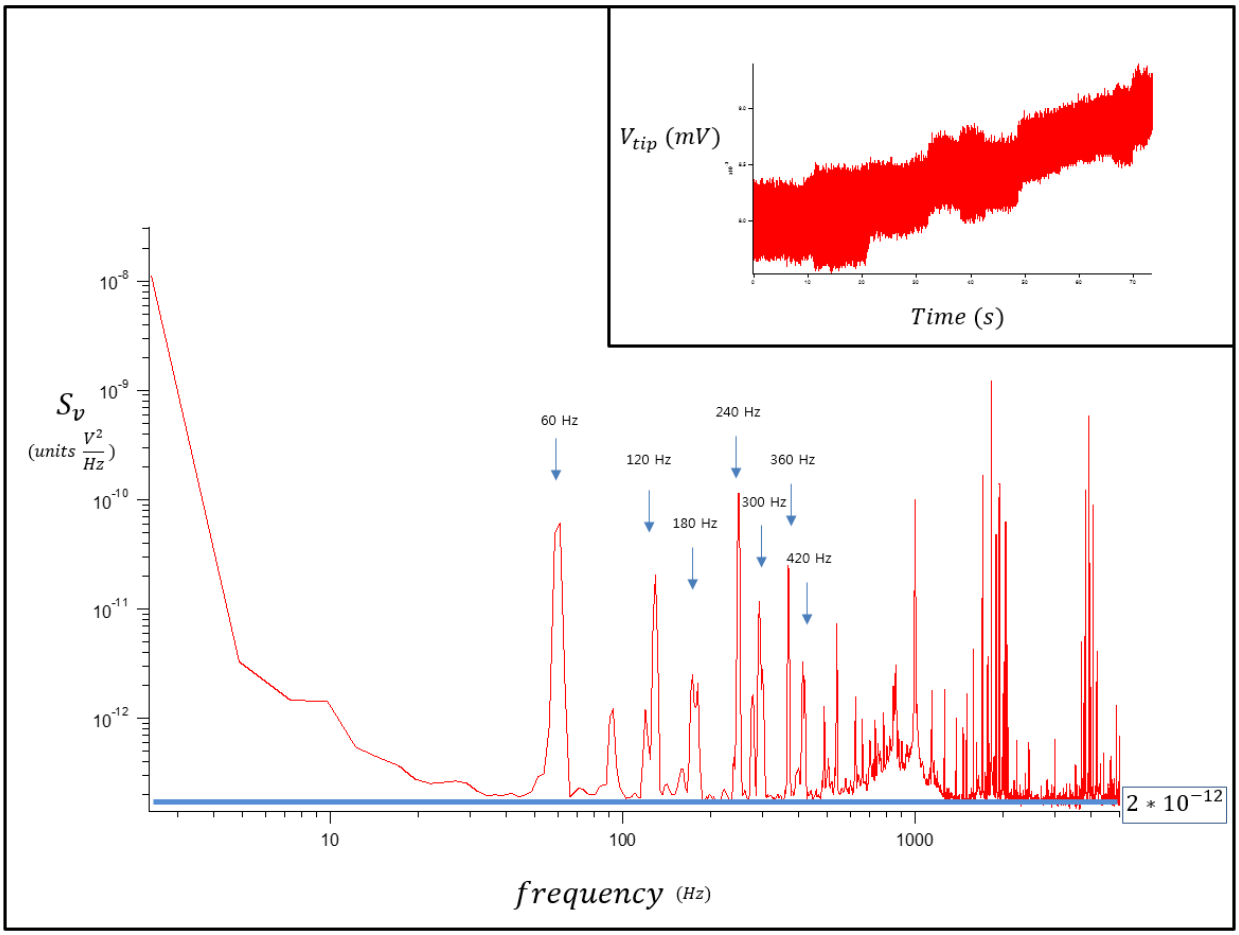

Figure 12 Power spectral density (PSD) of voltage-versus-time data of electrodes sitting in the bath (inset). This plot tells us about sources of noise in our system. We can easily identify 60 Hz AC wall noise, and its subsequent harmonics, which are labelled with arrows. Johnson noise is white noise (independent of frequency), so we expect it to have an equal contribution to noise at all frequencies. We plot a horizontal line across the base of our spectrum to obtain an estimate of Johnson noise. This estimate is greater than the predicted value derived from statistical fluctuations of thermal energy in resistors. However, it differs by less than an order of magnitude, indicating that Johnson noise is a primary source of noise in our system.

PSDs were performed using IGOR Pro. Information about using IGOR and performing a PSD can be found on the Minot lab wiki and in IGOR's built-in Fourier Transform documentation.

3.4 Junction potential

A junction potential is a constant voltage arising from a difference in redox potentials at the two electrodes (bath and cell)[9]. Redox potentials reflect the probability that a given redox reaction will go in the forward or reverse direction. A difference in redox potential from two Ag/AgCl electrodes will be due to the relative ease with which a chlorine ion is able to interact with the Ag/AgCl surface.

At the beginning of an experiment we expect to see a junction potential due to different chlorine concentrations at the two electrodes, but this can be subtracted electronically using the input offset knob on the rig and poses no practical problem. It is possible for a difference in redox potential to arise over the course of an experiment if, for example, one of the electrodes becomes unchlorided due to too much current passed in one direction. However, there are chlorine ions available in both the bath and filling solutions and our experiments use a large surface area of Ag/AgCl and short timescales for experiments, so we do not expect to see an electrode become unchlorided. Thus, we do not worry about junction potential as a source of noise or drift in our measurements.

4 Conclusion

Our results demonstrate that our current equipment is adequate for making a measurement of the intracellular potential of a neuron using the sharp electrode technique. A PSD of our measurements reveals the presence of noise beyond just Johnson noise, so if we are interested in making Johnson noise-limited measurements we must identify and eliminate other sources of noise. We also must do a better job in the future of making measurements without ripping the cell membrane, so some work will have to be done to determine the best protocol for proteasing the membrane.

We can make buffers, prepare dissection samples, and make intracellular measurements on-site. We are poised for the next phase of the research project, which is to incorporate a kirigami GFET device with the recording system described in this thesis, and to make a simultaneous intracellular and extracellular potential measurement. There are many hurdles between the results described here and interfacing our electrophysiology system with the GFET system, and explaining them in detail is beyond the scope of this thesis.

5 Methods

5.1 Buffer and reagent recipes

Snail Buffer

Mix the following proportions, fill to 1L with DI water, then bring to pH 7.3 by titrating NaOH after all ingredients are mixed. We titrated with 1M NaOH.

Ingredient	Mass	Concentration
KCl	127 mg	1.7 mM
NaCl	3 g	51.3 mM
CaCl2*2H20	603 mg	4.1 mM
MgCl2*6H20	305 mg	1.5 mM
HEPES buffer	1.19 g	5 mM

Filling solution: 3M KCl

Dissolve 45.3 g KCl into 200 mL DI water. It takes some time for this to happen. We recommend using a stir bar and plate to help mix. The solution can also be heated to quicken the process, but care should be taken as some water will evaporate and change the concentration. KCl should be stored, covered and labelled, in the fridge. If the researcher wishes to improve contrast of the pipette tip under the microscope, dissolving some ink from a sharpie or highlighter into the filling solution should help, although we did not find this necessary.

Dissection dish:

Make a 1:10 dilution of SiH to Sylgard and mix it in a plastic cup. Pour this solution into a glass petri dish. Put this dish under suction for an hour or until all air bubbles are gone. Then put the dish into an incubator at 37 degrees Celsius for at least an hour to solidify.

Protease:

Mix at a proportion of 5 mg of protease to 1 ml of snail buffer.

5.2 Microelectrodes

It is important to able to reproducibly pull the same geometry glass microelectrode. The thickness of the microelectrode governs a portion of the parasitic capacitance: since capacitance is inversely proportional to distance between parallel plates, thicker glass corresponds to less capacitance. Since parasitic capacitance is impossible to eliminate entirely, the best we can do is minimize the difference in error between experiments by having consistent microelectrode geometry and thus wall thickness.

In principle, to create a glass microelectrode all one needs to do is heat a small hollow tube of glass and pull one end; the glass will naturally stretch and taper to a point, and the researcher can break the point to create a small-diameter tip. But this is barbaric and does not give tips of a reproducible size.

Modern micropipette pullers use a set of programmable parameters to tune how much the glass should be heated, how hard it should be pulled, and how the glass is cooled. These parameters ultimately dictate the geometry of the microelectrode. Each piece of glass placed in the puller ostensibly results in two symmetric electrodes, although in our experiments we found about 10-20% disagreement of measured resistances between each of these. This may be fixed by repositioning the filament over the gas as detailed in the Sutter Cookbook[10].

Anyone using a pipette puller should have a copy of the Cookbook on hand. The Cookbook answers most questions a researcher will have about pulling pipettes, and the author (Adair) is easy to contact and an incredible resource for anything not answered in the document.

5.2.1 Pulling the ideal diameter microelectrode

The puller used for experiments in this thesis was a Sutter P80/PC Flaming/Brown Micropipette Puller. We used a 2.5 mm x 2.5 mm box filament with pressure (out-going regulator) set to 50 psi. The vertical micrometer dial behind the box filament sets the airflow; we set this to 1.27.

The P80/PC is an older model of puller, so guides that update frequently (such as the Sutter Cookbook) require translation. These guides do include instructions on how to translate to an older model puller, but here I present our discoveries with our instrument to save the reader some time. Principles we discovered for modifying pipette geometry are listed below. Images are taken from the Sutter Cookbook.

Measuring pipette resistances is as simple as doing a bridge balance and reading it off of the knob. Multiply the number of turns on the knob by 10 and divide by the gain (0.1 for our headstage). This will give a value for resistance as measured in mega ohms.

5.2.1.1 Parameter Modification

There are four parameters we have freedom to manipulate: Heat, Time, Velocity, and Pull.

Heat: The heat value is determined by running the RAMP test and subtracting 10 from the value. Details for performing the ramp test can be found on page 14 in the P80/PC Manual.

Time: Time indicates the amount of delay between a pull and when cooling step (gas flow) begins. We found that increasing time made our tapers more wispy, while decreasing time made the taper stubbier.

Velocity: Relates to the speed at which the two carriages move. Increasing velocity should result in smaller diameter tips and increasing in larger diameter

Pull: Pull dictates the amount of strength behind the pull. Higher pull values should result in smaller tips with longer taper, while lowering the value should result in larger tips with shorter tapers.

Finally, it should be noted that pipette pulling is somewhat of an art. For example, some program parameters (Pull and Time) change the same geometric parameters (taper and tip diameter). Thus, one should expect to have lots of bad pipettes pulled (we had upwards of 50) before arriving at a good set of values.

The values we used to obtain pipettes that were consistently measured to have resistances around 1 MΩ were: Heat=737, Time=250, Vel=50, Pull=150. This is currently saved as Program 4 in our puller.

5.2.1.2 Instrument Modification

The instrument itself can be modified to achieve different shaped tips. If tips are too whispy, a smaller box filament can be installed (details for installation found in the Cookbook). We did not need to replace the filament for our experiments so I can offer no further advice.

5.3 Snail dissection

For our experiments with *Lymnaea stagnalis*, neurons we want to study are on the order of 100 microns and are contained in tissue called ganglia. Conveniently, neurons in *lymnaea* have a natural optical contrast to their surrounding tissue. Their relatively large size and ease of identification makes them ideal candidates for electrical measurements.

We wish to remove and isolate these neuron-containing sections from the rest of the snail so we can impale them with a microelectrode. There are a few different nerve clusters the researcher can study in a snail, depending what they are looking for. Some other dissection protocols focus on isolating the buccal mass, but we found the buccal ring easier to isolate and pin down than the buccal mass. The following section describes our method for dissecting snails and removing the buccal ring, which contains the neurons we used for measurements. There are many online resources, some free, that

discuss *lymnaea* dissection in detail, many of which show a dissection as seen through a microscope. We recommend becoming familiar with one of these videos before attempting a dissection. The purpose of the following protocol and images is to complement such a video, and hopefully answer any remaining questions.

Materials required: Dissection pins (at least 5, we recommend having at least 6 on hand); microscissors (referred to as "scissors" here for brevity); microtweezers (referred to as "tweezers" here for brevity).

Begin by cutting off the shell. Cut along the spiral of the shell, being careful that the tip of the scissors is between the snail and the shell – we do not want to cut the snail at this point, just remove the shell. Keep cutting around the spiral until you get to a point where you can fully remove the shell.

Pin the snail down. The first pin should go straight through the back (Figure 13).

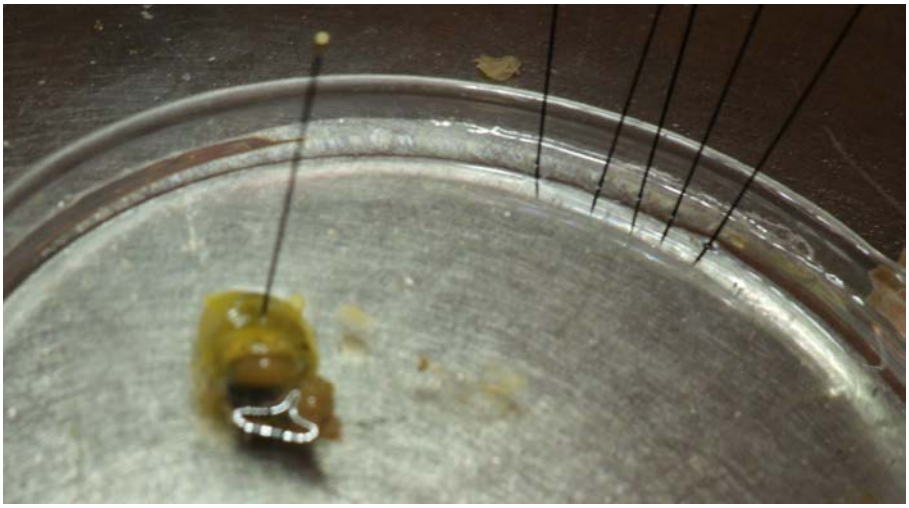

Figure 13 The first pin should go straight through the back of the snail (after the shell as been removed) to pin it to the dissection dish.

The next two pins should go to the right and left side of the head: be careful to avoid the red buccal mass in the center. We recommend pinning the ears/horns.

Then pull back the mantle (a flap of tissue on the "back" of the snail, which is currently facing up) and pin it back behind the snail, using two pins to hold it in place. After these four pins are in place, remove the center pin (Figure 14).

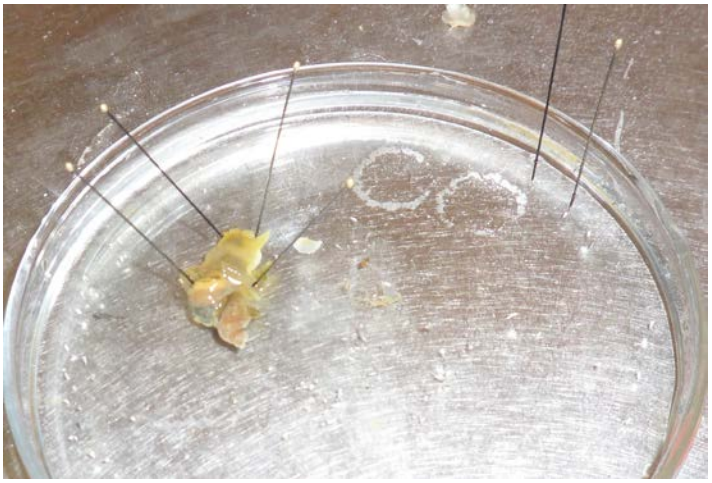

Figure 14 Pin the corners of the snail down to make the back taught. This makes the next cut, which opens up the snail through the mantle, much easier.

Use scissors to cut from the back (where the mantle is currently peeled to) up to the head, being careful to keep a shallow insertion – the bottom edge of the scissors should be very close to the top surface of the snail. You have now exposed the buccal mass (reddish lump), which is attached to the esophagus (gray/brown tube) that runs down to the main part of the body. The buccal ring is wrapped around the esophagus.

To have some more room for making the next incisions, “open up” the snail. While the four pins are still in, grab a new pin and use it to peel and pin the skin away from the main body of the snail. Do this by peeling skin from a position further toward the interior of the snail than the original pin is sitting, and stab this new pin in further out from the snail than the original pin, then remove the original pin. Do this for each of the four pins (Figure 15).

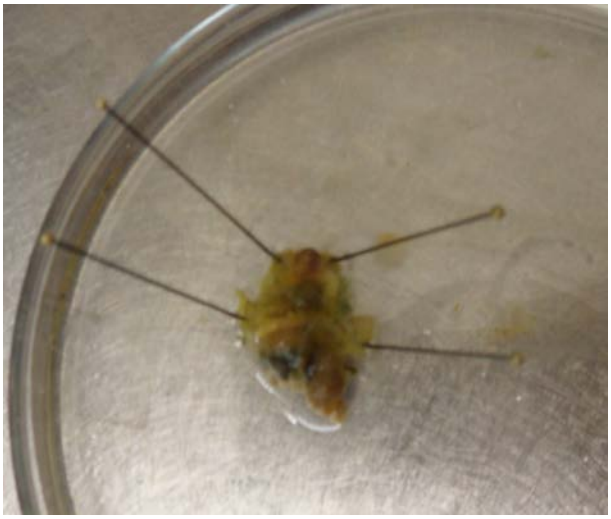

Figure 15 The pins are re-placed so that it is easier to remove internal organs.

Begin removing unnecessary organs. The neurons we want are either in the buccal ganglia (the reddish mass), which is located in the head of the snail, or the buccal ring, which is wrapped around the esophagus. Use a pair of scissors to carefully remove the back (spiral) part and toss it. Next, remove the grey-green glob that is present at the apex of the part of the snail that is still pinned to the dish. Then remove the sex organ, which is a white glob to the left of the buccal mass. Then snip the esophagus from the posterior end of the buccal ring. Grab the esophagus anterior to the buccal ring and pull it through the buccal ring such that the buccal ring is left exposed.

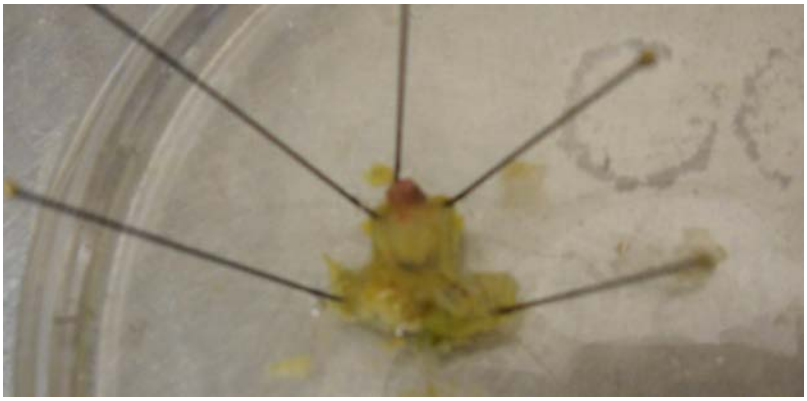

Figure 16 The internal organs have been removed. The buccal mass is prominent at the top of the snail. Below this was the esophagus, which has now been removed. Around the esophagus is the buccal ring, which is not pictured here.

Grab onto the esophagus and use it to pull the buccal mass out of the way of the rest of the body. Then pin the buccal mass down outside of the body, thus exposing the buccal ring (Figure 16). Once the buccal ring is exposed, use scissors and cut the tissue connecting the body to the buccal ring. Try to leave some of this tissue still connected to the buccal ring as it helps with pinning down the ring.

Once the tissue is cut, carefully grab the buccal ring by the connecting tissue with tweezers and remove it from the rest of the body. The buccal mass is the red mass still connected to the body. On top of this are the buccal ganglia, which hold many neurons. The buccal mass is physically difficult to separate, as it requires you to cut tissue that is between the snail and the dissection dish. The buccal ring has adequately large neurons for a sharp electrode measurement, so we used these.

Now that the ring is removed from the rest of the body, we can discard the rest of the snail body: remove it from your dissection dish. Cut open the commissure, which is a piece of connecting tissue that holds the buccal ring in a ring formation. This allows you to pull open the ring into a straight line and pin it down. You have now isolated the buccal ring, which contains neurons for electrophysiology experiments (Figure 17).

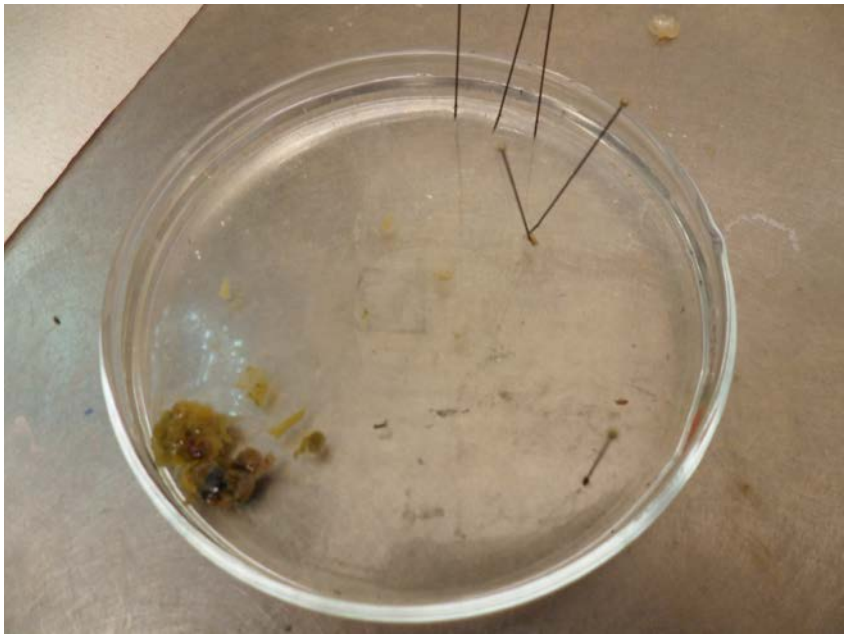

Figure 17 The buccal ring is pinned to the dish by the two pins in the top of this picture, while the discarded portion of the snail is pictured in the bottom left. The pins are placed such that they hold the ring pinned under tension, which makes the microelectrode insertion process easier.

5.3.1 Soften the membrane

As a final step for preparing the neuron for measurements, protease may be used to help soften the neuronal membrane and make it easier to penetrate. Proteases are enzymes that break down proteins, so a researcher handling protease should be very careful not to spill any on themselves. For pond snails, use 5mg/ml of Sigma type XIV at a concentration of 5mg/ml in snail buffer (described as "Snail Buffer" in the methods).

The protocol for "proteasing" a membrane is as follows: add protease to the bath, let it incubate for a set amount of time, then rinse off the neuron with snail buffer and replace the bath with fresh snail buffer. The goal is for no protease to be present in the system after incubation. The amount of time necessary to soften the membrane without dissolving it completely varies with neuron size. For *lymnaea* neurons, one should begin with a one-minute incubation and increase or decrease time depending on results.

5.4 Snail care

Lymnaea stagnalis want to be between 18C and 24C. A 10 gallon tank has been plenty of room for our population, which began as 20 snails. We purchased our snails from Androscoggin Scientific.

Equipment and Supplies: 10 gallon water tank, Aqueon brand water filter, air stone, air pump, aquarium chiller (optional, depends on ambient temperature of room), Instant Ocean brand water treatment powder, crushed oyster shells; sinking frog pellets (any brand, we use Aqueon Shrimp Pellets) and dark-leafed lettuce (food).

Tank setup:

- Place air stone (connected to air pump) in bottom of tank.
- Add treated water to desired water level. To prepare water: add 1g/2.5L of Instant Ocean to DI water.

- Add crushed oyster shells to the tank. Sprinkle enough shells to make a sparse layer over the bottom of the tank. Oyster shells are an important source of calcium for shell growth.
- Install filter on side of tank.
- Add about 15 sinking pellets to the tank. They are important as a source of food for the babies. Make sure to remove any pellets that look like they have a white fuzz on them – it is a sign they are rotting, which typically happens after a few days.
- Add lettuce. We use a fork tied to a dowel as our feeder, which allows for easy addition of more lettuce without having to reach to the bottom of the tank. It also weighs the lettuce down, which we have found convenient: floating lettuce ends up floating to the filter and clogging it up, eventually causing it to stop running

Tank Maintenance:

We typically add an additional ~3L of water per week to the tank to offset water loss to due evaporation.

Take the top off the filter a few times a week and check how the water is flowing. Change the filter once water is running over the plastic lip instead of around it (the filter itself will be green/brown and noticeably thicker). Before installing the new filter, rinse it in tap water for ~10 seconds. Do not rinse the blue plastic pieces – the slime on it is a biological filter and is necessary for the health of the snails.

Make sure the intake to the filter is kept clear at all times. If the plastic slits get clogged up, water will cease to flow into the filter and it will stop working.

Food:

Make sure there are a few leaves of lettuce in there all the time. Drop a few pellets into there every other day or so – scatter them around so any babies can reach them. If they aren't consumed after a few days, remove the remains or else they will begin to rot.

Further information can be found in the "SNAIL REF" folder in the group drive.

5.5 Making the measurements

If the reader is using this as an operational guide, **read the entirety of this section before setting up.**

The following instructions assume the researcher has prepared the following materials:

Buffers: 3 mL of 3 M KCl, 70 mL Snail Buffer, 10 mL Protease. See “Buffer recipes” for details.

- Exposed neuron (See “Snail dissection” for instructions on how to isolate and pin down tissue that contains the neurons). Do not yet protease the membrane.
- 3x BNC cables and a BNC splitter
- 2x alligator clips or similar; 1 jumper wire (bananclip ends).
- Headstage: HS-2A, Gain: x 0.1 LU (comes with the Axoclamp-2B rig)
- Microelectrode holder – we used a <Find model number>
- Silver for bath electrode. We found 5 feet of silver wire to be a convenient source, from which we can make a new electrode with ease by clipping off a small portion (~2 inches at a time)
- 1 mL syringe
- Flexible needle. We used the following: MICROFIL 28 GAUGE/67MM LONG, World Precision Instruments, Inc., Item number MF29G67-5
- Pre-pulled glass pipette
- Micromanipulator. We used a Burleigh TS-5000-150 (MIS-5000 Series). We did not need to connect this to a piezo-electric controller for our experiments, but the user can do so if they wish.
- Function generator. We used a “Tektronix AFG 3021B single channel arbitrary/function generator”
- Oscilloscope. We used a “Tektronix TDS 1001B Two channel digital storage oscilloscope”
- Dissection microscope (at least 40x zoom between objective and eyepiece)
- Secondary light source – we found applying light at a shallow angle rather than from straight above helped us resolve our experiment. We used an AmScope HL150-AY.
- P1000 micropipettor.

Setting up an experiment is straightforward. The protocol described here assumes the user is working at the “undergrad probe station” faraday cage in the Minot lab in Weniger 306.

Overall setup:

Frontpanel

Begin with the rig off. Make sure the following dials and switches on the front panel of the rig are in the following positions:

STEP COMMAND: Toggle set to EXT; bridge balance 0 (fully counterclockwise).

DESTINATION set to ME1 (xH nA). The parenthetical indicates that, for example, when STEP COMMAND is toggled to CONT., a continuous DC current will be injected equal to the reading on the STEP COMAND thumbwheel multiplied by the gain (H), in nanoamps. For example, a reading of 50 on the thumbwheel will inject a DC current of 5 nA. When toggled to EXT., current pulses will be delivered of the magnitude indicated, as controlled by an external device (our function generator). We recommend using negative current pulses: either polarity will work, but negative pulses tend to work better in biological systems.

MODE: BRIDGE mode; we do not use other modes.

VOLTAGE CLAMP: These settings will not affect our measurements.

I DISPLAY SELECT: Set to I_m; the I (nA) display will now show the amount of current being injected by the user. This should read 0 (to plus or minus 0.1 nA).

H1 knob: set to the gain of the headstage (x.01 for ours).

CAPACITANCE NEUTRALIZATION: Turn all the way counterclockwise

INPUT OFFSET: Set around the middle of the dial (5), which is an offset of about zero. This will be varied at the beginning of the experiment so don’t worry about it too much, just get it near 5.

OUTPUTS: Set the 10 V_m, I_m OUTPUT BANDWIDTH to 30 kHz; we do not want to prefilter our signal.

MICROELECTRODE 2: Ignore this section of the front panel

Wiring

Plug power cords into the wall.

Make sure the back of the rig is grounded to the cage by connecting the "Signal ground" port to the faraday cage – to make a good contact, we did this by plugging a jumper cable into the port then stripping the other end and wrapping the exposed wire around a screw in the cage, using electrical tape to hold it in place. Then plug a function generator into the back port labelled STEP ACTIVATE. Use the TTL (logic) output of the function generator and set the function generator to deliver 2ms logic pulses. Plug the microelectrode into the ME1 port ("ME1 PROBE") and screw in the set screws so it is secure. Run the microelectrode through the hole in the side of the faraday cage and set it down. The microelectrode should sit on a plastic base to help electrically isolate it from the floor of the probe station, which is now our system ground.

Connect a BNC splitter to the $10V_m$ output on the front panel. Connect one output of the junction to an oscilloscope and the other to the DAQ. Note that this will output a signal equal to 10 times the measured membrane voltage, so be sure to account for that in recording software (in our lab's LabView software, this is reflected by setting the "Factor" to 0.1). The oscilloscope will be used to watch response to injected current pulses, which we will use to calibrate the system.

Micromanipulator

Our headstage is attached to a plastic rod, which is how we attach it to our micromanipulator. Slide the rod into the appropriate spot on the micromanipulator (it is obvious where to attach it), and angle it downward at roughly a 45-degree angle. This angle may be adjusted later depending on what the researcher identifies as the easiest descent direction for impaling the cell: this is part of the art of electrophysiology, and different references will advise different angles. For measurements presented in this thesis, we had a very shallow descent angle, coming in as parallel to the table as the lip of our dissection dish allowed.

We found operating a micromanipulator via handscrews was adequate for the measurements presented in this thesis. However, we did notice that even small bumps to the micromanipulator showed up in our recordings, so for more precise recordings we advise connecting this to a piezo-

electric controller. The piezo-electric will also help keep cells alive, as small vibrations at the micromanipulator will propagate down the lever arm of the plastic rod and likely move the pipette tip enough to damage the cell membrane.

Beginning an experiment:

Chloride the electrodes**.** Both the silver wire in the holder and the bath electrode need to be chlorided. We did this by filling a small container with 5 mL of bleach (Clorox brand, concentrated), then bending silver and hanging it over the edge of the container into the bleach. We chloride for 15 minutes each experiment to be safe, but some resources will recommend chloriding for only 2 minutes[11]. When a wire is properly chlorided, it should appear a dark purple or grey color[11]. For the bath electrode, make sure that a portion of the wire remains unchlorided for electrical attachment. Chloride at the point of attachment to the rest of the circuit will add noise to the system.

Dissect a snail according to the instructions in "Snail dissection." **Do not yet protease the snail.** This sample should last up to a couple of hours as long as it is in Snail Buffer, so the researcher has freedom to backfill a pipette before or after a dissection. It should be noted that some samples will last longer than others, depending on the amount of damage during dissection. We recommend all experiments take place as soon after dissection as possible.

Backfill a pipette by placing the wide end into a 1.5 mL epindorff tube that has been filled with ~ 1 mL of 3M KCl (exact volume doesn't matter). After a minute or two the fine tip of the pipette should be filled. Verify this by holding it up to the light: you should be able to see liquid from around the "shoulder" of the pipette (where the tapered section meets the wider diameter part) and upward.

To fill the rest of the pipette, follow this procedure: fill a 1 mL syringe with 3M KCl, using a microFil flexible needle. Insert this needle into the wide end of the glass until it has gone as far into the glass as possible. Begin pushing down on the plunger to inject solution into the glass. As you are injecting solution, begin pulling out the needle in a fluid motion. The goal is that liquid is injected into the pipette as the needle is pulled out, which (i) ensures the entire pipette is filled without air bubbles and (ii) ensures that pressure from injected liquid does not build up and break the fine tip of the glass.

If air bubbles are present, tip the pipette so that the wide end is face up and begin tapping on the glass. The bubble should eventually rise to the end of the glass and leave, but may not. We have had variable success trying to re-fill a pipette that has a trapped air bubble in it and find it saves time and frustration to just toss the pipette and fill a new one.

Attach the pipette to the holder by doing the following: Lightly loosen the threaded end of the holder by unscrewing it. Thread the (now-chlorided) silver wire that is in the holder into the wide end of the pipette and slide the wide end of the pipette into the holder. The wide end of the pipette will pass through a rubber o-ring and emerge on the other side. When you see the wide end emerge, tighten the end of the holder by screwing it back the other direction, which will close the o-ring and hold the glass pipette in place. The silver wire should be as far forward toward the fine tip of the glass pipette as possible. Attach the holder to the headstage by screwing it into the white plastic port. Use alligator clips to connect the gold grounding cable coming out of the back of the headstage to the bath electrode.

Make the insertion process easier by weakening the membrane with protease. Follow the instructions in section 5.3.1.

Clip the bath electrode to the side of the dissection dish, with the chloride section of the electrode submerged in the bath solution. Be careful to keep some room between the bath electrode and the bottom of the dissection dish – we have noticed lots of noise and a brown discoloration of the PDMS base over time when these were in contact.

Angle the headstage as desired using the micromanipulator and put the tip of the electrode into the bath (far from the dissected sample, if possible – we notice more noise the closer the tip is to any biology). *Turn off the dissection microscope.* This step is imperative for removing noise from the recording system. Use the secondary light source to apply low-angle light to the dissection dish. Follow the calibration procedures to bridge balance and neutralize parasitic capacitance.

Find the tip of your glass pipette. We recommend the following: drop the tip into solution, estimate where the tip is, then use the micromanipulator to move it into the field of view of the microscope. Then, raise the tip out of solution using the vertical axis (“z-axis”) of the micromanipulator. Begin watching the oscilloscope, and reverse direction in the z-axis. There is a clear signal (change in resting potential and onset of noise) when the tip of the pipette enters solution. Look back at the microscope and oscillate the z-axis up and down. You may be able to infer presence of the tip by

watching surface tension. Iterate this process until you can think you have found the tip. Keep in mind that the end of the tip may be on the order of a hundred nanometers, so you likely cannot resolve it. The actual tip is likely further from the headstage than you have estimated, but this is okay, as it is primarily important to at just have an estimate of location.

Identify a neuron by looking at the buccal ring through the dissection microscope and looking for small yellow/white dots. An image of what these should look like under the dissection microscope appears in Figure 18. Adjust the micromanipulator until the pipette tip is angled toward the neuron.

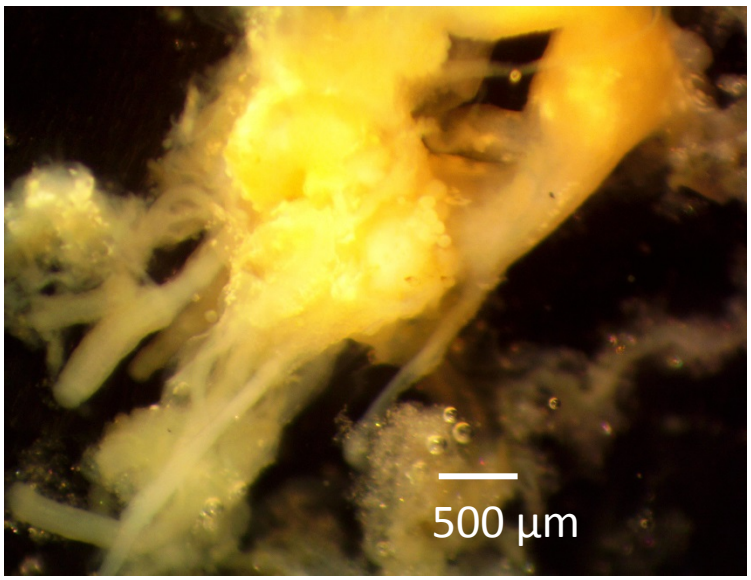

Figure 18 Closeup of snail neurons

Begin recording using the DAQ and move the tip toward the neuron very slowly. You will likely be too far up in the z-direction on your first approach, but this will help you determine if your initial estimate of the tip location was accurate, as sometimes the biology underneath the tip provides enough contrast to help resolve it. If you are too far in the z-direction – which can be determined either by a lack of change in signal or, hopefully, by being able to resolve the glass directly – use the micromanipulator to reverse direction, lower the z-axis by a small amount, then approach the cell again. We typically repeat this process about a dozen times before finding the correct z-axis. It is important to be patient with this step procedure because if you overdo it you may run the tip into the PDMS at the bottom of the dish, which will break the pipette tip. Eventually, you will be able to impale the cell, and you should see a sharp decrease in the recorded potential (figure 10). If the cell is not currently producing action

potentials, begin injecting positive current, starting with 0.1 nA and slowly ramping up. You may trigger potentials, but it's possible this neuron is damaged. If you see no response after you have ramped up a few nA of current, pull the tip out, find a new neuron, and repeat.

6 Bibliography

1. Bargmann, C. I. & Newsome, W. T. The Brain Research Through Advancing Innovative Neurotechnologies (BRAIN) initiative and neurology. *JAMA Neurol.* **71,** 675–6 (2014).
2. Scha, R. & Fromherz, P. Neuron – silicon junction with voltage-gated ionic currents. **10,** 1956–1962 (1998).
3. Bolotin, K. I. *et al.* Ultrahigh electron mobility in suspended graphene. *Solid State Commun.* **146,** 351–355 (2008).
4. Hess, L. H. *et al.* Graphene transistor arrays for recording action potentials from electrogenic cells. *Adv. Mater.* **23,** 5045–5049 (2011).
5. Blees, M. K. *et al.* Graphene kirigami. *Nature* **524,** 204–207 (2015).
6. Hille, B. *Ionic Channels of Excitable Membranes*. (Sinauer Associates Inc, 1992).
7. Bean, B. P. The action potential in mammalian central neurons. *Nat. Rev. Neurosci.* **8,** 451–465 (2007).
8. Franklin Breschneider, J. R. de W. *Introduction to Electrophysiological Methods and Instrumentation*. (Academic Press, 2006).
9. Molecular Devices. *The Axon Guide*. (2012).
10. Oesterle, A. *Pipette Cookbook 2015*. (Sutter Instruments, 2015).
11. Corporation, W. I. Techniques for Chloriding Silver Wires. 99–99 (1999).