

Video Article

Membrane Potentials, Synaptic Responses, Neuronal Circuitry, Neuromodulation and Muscle Histology Using the Crayfish: Student Laboratory Exercises

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Abstract

The purpose of this report is to help develop an understanding of the effects caused by ion gradients across a biological membrane. Two aspects that influence a cell's membrane potential and which we address in these experiments are: (1) Ion concentration of K^+ on the outside of the membrane, and (2) the permeability of the membrane to specific ions. The crayfish abdominal extensor muscles are in groupings with some being tonic (slow) and others phasic (fast) in their biochemical and physiological phenotypes, as well as in their structure; the motor neurons that innervate these muscles are correspondingly different in functional characteristics. We use these muscles as well as the superficial, tonic abdominal flexor muscle to demonstrate properties in synaptic transmission. In addition, we introduce a sensory-CNS-motor neuron-muscle circuit to demonstrate the effect of cuticular sensory stimulation as well as the influence of neuromodulators on certain aspects of the circuit. With the techniques obtained in this exercise, one can begin to answer many questions remaining in other experimental preparations as well as in physiological applications related to medicine and health. We have demonstrated the usefulness of model invertebrate preparations to address fundamental questions pertinent to all animals.

Protocol

1. Introduction

The goals of these laboratory exercises are to understand the properties of excitable membranes, the ionic basis of the resting membrane potential, and methods to measure the membrane potential. In addition, staining and histology of muscle is presented, which can be used to teach muscle structure. Also, two different types of dissected preparations are used to demonstrate properties of synaptic transmission in various muscle groups. A complete sensory-central nervous system (CNS)-motor neuron-muscle circuit in the crayfish abdomen is also used to present a preparation to examine sensory stimulation and the influence of neuromodulators and neurotransmitters on aspects of a circuit.

The first part of this report presents the approaches used to measure resting membrane potential and the influence of extracellular K^+ on membrane potential. We will also introduce muscle structure. In the second part of this exercise, we present various means of measuring synaptic responses from different types of neuromuscular junctions (NMJs). The first exercise uses the crayfish abdominal extensor muscles and the second uses the abdominal superficial flexor muscles. In addition, we present a neural circuit (the ventral nerve cord of the crayfish with sensory inputs and motor outputs) that is easy to maintain, and which can be used for teaching as well as for research in various aspects of a sensory-CNS-motor neuron-muscle circuit. After completing the explanation of the initial exercises, we present the physiology of NMJs and CNS circuit.

The ion gradient across a biological membrane can result in a potential difference. For a cell at rest, this difference in electrical charge across the cell membrane is known as the cell's **resting membrane potential**. There are two main factors we will address that influence a cell's membrane potential. The first is the ion concentration on either side of the membrane. The second is the ionic permeability of the membrane. It is important to keep in mind that in a living cell there are a number of different ions with varying concentrations inside and outside the cell. The key ions we will address are sodium (Na^+), potassium (K^+) and chloride (Cl^-). The quantities and movement of these ions across a muscle membrane determines the membrane potential. From this foundation, we can address electrical potentials observed during electrical excitation and inhibition of a membrane from synaptic responses and examine the effects of pharmacological agents. We can also build biophysical models to represent these processes to experimental test concepts (Robinson *et al.*, 2010).

The use of glass capillary microelectrodes permits recording of membrane potentials. The electrode can be inserted through the cell membrane without damage, providing the tip is small enough and an accurate measure of the transmembrane potential can be obtained. The technique is particularly applicable to large cells, which are less likely to be damaged by the insertion of the intracellular electrode. This is one of the essential techniques in physiology.

The balance of Na^+ and K^+ across the membrane is maintained by the Na-K ATPase pump under physiological conditions. Under normal conditions the pump moves, on average, three Na^+ out of the cell and two K^+ into the cell. As a side note, a Nobel Prize in chemistry was awarded in 1997 for this discovery made back in the late 1950's. The fundamentals of the discovery were obtained from research using axons from a crab (Skou, 1965, 1998).

This pump is also considered electrogenic as it has a greater ability to pump when the membrane is depolarized (Skou, 1989a,b). In many cells, the pump speeds up when a cell is electrically activated by depolarization.

Potassium can also move through potassium "leak" channels while a cell is in a resting state. Due to these potassium leak channels, the cell membrane at rest is more permeable to potassium than to other ions. Thus, the cell's resting membrane potential is closer to the equilibrium potential for potassium than that for sodium. The resting membrane potential can then be examined to see if it depends upon the potassium equilibrium potential.

1) Muscle variability

Crustacean muscle fibers show greater variability of structural features, membrane electrical properties and contractile properties than do vertebrate muscle fibers. Phasic muscle fibers in crustaceans are modified for twitch-type contractions. They are characterized by short sarcomere lengths (2-4 microns), thin, straight Z-lines, a low ratio of thin to thick myofilaments, and well developed systems of T-tubules and sarcoplasmic reticulum. Phasic muscle fiber membranes may generate graded or all-or-none action potentials. Tonic muscle fibers, on the other hand, are modified for prolonged maintenance of tension. They often have sarcomere lengths of 10 to 15 microns, thick, wavy Z-lines, a high ratio of thin to thick myofilaments, and less well developed systems of T-tubules and sarcoplasmic reticulum. Tonic muscle fiber membranes are often electrically inexcitable, or they may produce graded electrical responses ("graded spikes"). A wide range of intermediate fiber types is found in crustacean muscles.

2) Equations

Equations that are commonly used to determine the equilibrium potential of an ion and resting membrane potential are the Nernst equation and the Goldman-Hodgkin-Katz (G-H-K) equation respectively. An important distinction between the two equations is that the Nernst equation is used only for one specific ion to determine the equilibrium potential for that ion, whereas the G-H-K equation is used to determine the resting potential by considering the permeability of multiple ions and their gradients across a cell membrane (Nernst, 1888, 1889; Goldman, 1943; Hodgkin and Huxley, 1952; Hodgkin *et al.*, 1952; Hodgkin and Katz, 1949; see Hille, 1992).

The Nernst equation is generally considered for ions across a membrane generating an electromotive force as commonly shown as:

$$V = (RT/zF) \ln([X]_{out}/[X]_{in})$$

X = ion of interest

V = equilibrium voltage for the X ion across the membrane

R = gas constant [8.314 J/(mol·K)]

T = absolute temperature [Kelvin]

Z = valence of the ion

F = Faraday's constant [9.649 x 10⁴ C/mol]

For the K⁺ ion at 20°C and transformation of Ln to Log₁₀ along with filling in the constants, one arrives at:

Potential = 58 log ([K_{in}]/[K_{out}]); expressed in mV

Let us assume that only K⁺ is permeant by diffusion. [K_{in}] is the K⁺ concentration on the inside of the cell and [K_{out}] is the K⁺ concentration on the outside of the cell.

As an exercise estimate [K_{in}]. _____

Assume for this calculation, membrane potential is only dependant on the K⁺ equilibrium potential.

Given the [K_{out}] = for the saline used is 5.4 mM. Also, assume membrane potential is -70mV.

Potential = 58 log ([K_{in}]/5.4).

In the experiment we will measure a cell's resting membrane potential and determine how it is influenced by altering [K_{out}]. The slope of the hypothetical line relating membrane potential and [K_{out}] is 58. After collecting data on the resting membrane potential at various [K_{out}] (range from 5.4 mM to 100 mM) we will plot the observed values to determine if there is a match with the hypothetical line. We will use the average resting membrane potential obtained at 5.4 mM [K_{out}] for initiating the hypothetical and observed lines for comparison.

Considering that a membrane can be permeable to more than one ion at rest, as well as at various depolarized states, one uses the G-H-K equation to take into account the permeability (P in the equation) for various ions. The G-H-K equation will reduce to the Nernst equation if a membrane is permeable to only one ion.

Here is a generalized G-H-K equation for Na⁺, K⁺, and Cl⁻ ions:

$$E_{m_{K,Na}} = \frac{RT}{F} \ln \frac{P_{Na^+}[Na^+]_{out} + P_{K^+}[K^+]_{out}}{P_{Na^+}[Na^+]_{in} + P_{K^+}[K^+]_{in}}$$

Since Cl⁻ has a negative charge, the concentration term is inverted in this equation for the inside and outside. This allows the Z (ion charge) to be left off.

3) Aims of this exercise

In this experiment we will measure the membrane potential of a crayfish muscle cell and apply the principles discussed above to address:

1. How to measure a cell membrane potential with appropriate instrumentation and technique.
2. Ion permeability of the muscle cell membrane and how it contributes to the membrane potential.
In addition, we will make a preliminary study of muscle structure:
3. Use stains to highlight the anatomy of the muscles in the dorsal aspect of the crayfish abdomen, which is used for conducting these electrophysiological experiments.
4. Examine the histology of different muscle fiber types.

In this laboratory exercise, we will use the crayfish abdominal extensor muscles. This preparation has been used in the past to teach these principles in physiology and anatomy (Atwood and Parnas, 1968). We have used many of the procedures from this source and modified others to accommodate current instrumentation and to complete the goals in a single 3 hour student laboratory period. These exercises are a foundation for other experiments used in the Animal Physiology course in the Department of Biology at the University of Kentucky (Instructor Dr. R.L. Cooper, 2010).

4) Why this model animal

There are several good reasons for using the crayfish abdominal extensor muscles in this experiment:

1. Crayfish are commonly available and are relatively cheap and easy to maintain in laboratory conditions.
2. The dissection is relatively easy for students learning dissection techniques for live preparations.
3. The muscle is stable for several hours in a minimal normal saline which serves well for students learning electrophysiological techniques. The muscle preparation is fairly robust when external $[K^+]$ is altered for short time periods.
4. Various synaptic responses can be readily obtained by stimulating motor neurons.
5. The anatomical arrangement of the extensor muscles is easy to discern, and due to their large size it is relatively easy to obtain stable intracellular recordings.
6. The muscles and the innervation pattern can be observed easily with methylene blue staining. In addition, particular muscle fiber types can be readily processed for histology to observe the sarcomere structure.
7. No animal protocols are needed at this time for invertebrate animal preparations in laboratory experimentation in many institutions within the USA.

2. Methods

1) Materials

- Scissors (1)
- Forceps (1)
- Silver Wire for ground wire (1)
- Microscope (1)
- Electrode Probe (1)
- Petri Dish with Sylgard on the bottom (1)
- Saline Solution (1)
- Potassium Solutions: 5.4mM (normal saline), 10, 20, 40, 80, 100 mM
- Bleach (Small Amount, Use for the tip of the silver wire to build Ag-Cl)
- Glass Pipette (1), to remove and add solutions
- Syringe (1)
- Amplifier/Acquisition System (1)
- Faraday Cage (1)
- Desktop/Laptop (1)
- Dissection pins (4)
- Crayfish

2) Methods

2.1) Preparation/Dissection:

1. A crayfish approximately 6-10 cm in body length should be obtained (or a manageable size). Hold the crayfish at the back of the head or approximately a centimeter from the back of the eyes. Ensure that the claws of the crayfish or its mouth cannot reach the individual handling the crayfish. (The crayfish may be placed in crushed ice for 5 minutes to anesthetize it prior to cutting off the head.)
2. Use the large scissors to quickly remove the head. Make a clean and quick cut from behind the eyes of the crayfish. Dispose of the head and appendages.



Figure 1. Image shows placement of the cut to remove the head of the crayfish.

3. The legs and claws of the crayfish can be removed at this point to avoid injury. Stylets on males and swimmerets on both males and females can also be removed (Figure 2). Next, separate the abdomen from the thorax. Make a cut along the articulating membrane which joins the abdomen and thorax (Figure 3). Save the abdomen portion of the crayfish and dispose of the thorax.



Figure 2. The scissors are cutting the stylets. These can be removed from the crayfish.



Figure 3. Image shows the placement of the cut to remove the thorax from the abdomen.



Figure 4. Removal of the thorax from the abdomen. The cut should be made in circular fashion along the line in the joining of the segments.



Figure 5. The top image shows the abdomen with swimmeret appendages. Bottom image shows the abdomen without the swimmeret appendages.

4. With the abdomen, a cut should be made in the shell along the lower, lateral border of each side of the abdomen. Care should be taken not to cut too deeply into the crayfish. To help in the process of cutting the shell, the cut should be made with the scissors pointing slightly down towards the ventral side and at an angle. Follow the natural shell pattern of lines of the crayfish that run the length of each segment (Figure 6).

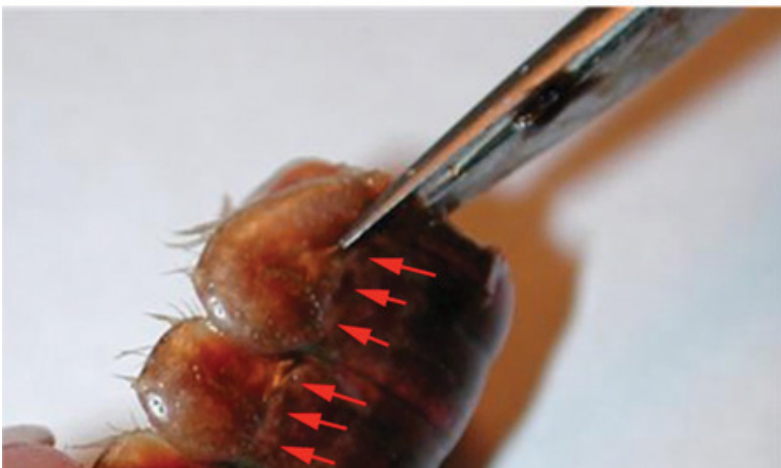


Figure 6. Scissors are placed at an angle and follow the natural alignment of the shell. Do not cut too deep and destroy the preparation. The arrow heads point to the natural line along each segment that should be followed for the cuts.

5. Remove the ventral portion of the shell. Take care not to destroy the abdominal muscles. Use forceps to remove the ventral portion. When the ventral portion of the shell is removed, a white mass of tissue can be seen on top of the deep flexor muscles. This tissue can be removed carefully with forceps.

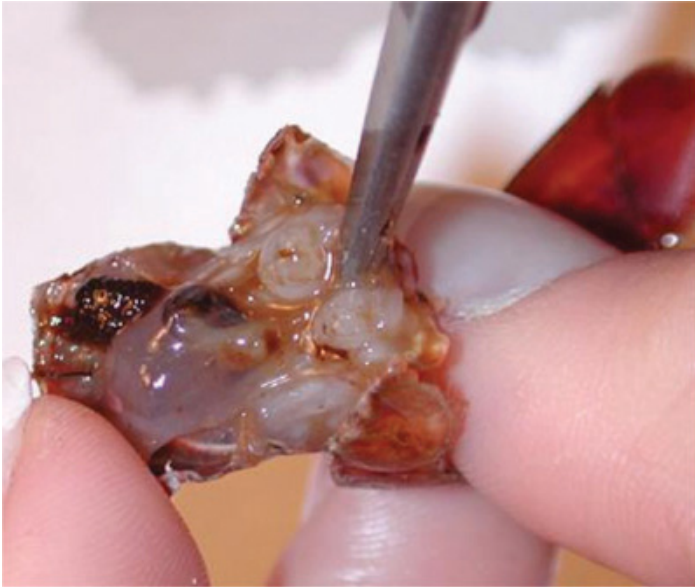


Figure 7. Removing the ventral portion of the shell with forceps. Pull up and back on the ventral portion to remove. Do not destroy muscles under the ventral shell.



Figure 8. Pulling back on the ventral portion of the shell which is to be discarded.



Figure 9. Cut the ventral portion of the preparation with scissors and discard.

6. The GI tract, a small tube running along the midline of the deep flexor muscles, can be removed from the crayfish. Pinch the top of the tract with the forceps and pull away from the abdomen. Cut the bottom of the tract - at the end of the tail. Rinse the dissection with saline to ensure the fecal waste does not interfere with the preparation.



Figure 10. Image shows the removal of the GI tract from the preparation.

7. Use dissection pins to secure the preparation to the Petri dish. The top and bottom corners of the preparation should be pinned down to the dish. Saline solution should be poured into the Petri dish and cover the preparation completely until intracellular recordings are performed. This dissection dish should have a Sylgard (Dow Corning) coating on the bottom (1cm thick) so that insect pins can be stuck into it. Dissected preparations are bathed in standard crayfish saline, modified from Van Harreveld's solution (1936), which is made with 205 NaCl; 5.3KCl; 13.5 CaCl₂; 2H₂O; 2.45 MgCl₂; 6H₂O; 5 HEPES and adjusted to pH 7.4 (in mM).

2.2) Intracellular Recording

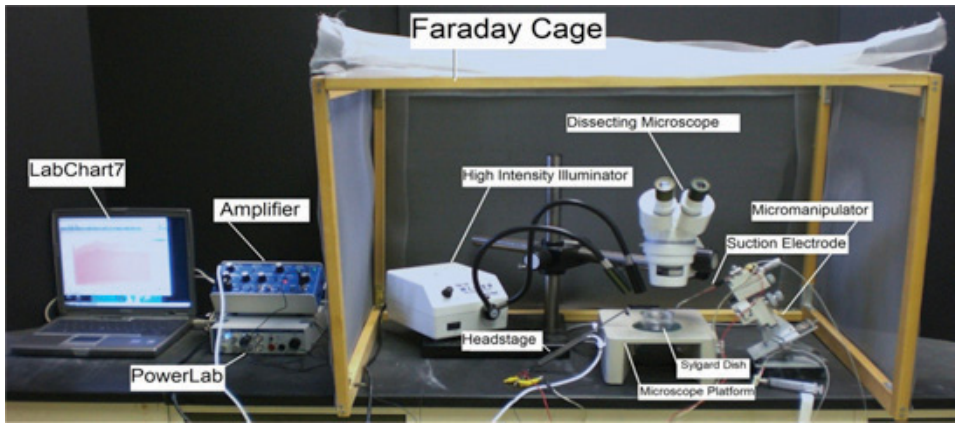


Figure 11. Overall setup of the recording equipment.

8. The Petri dish with preparation should be placed under the microscope and secured with wax at the bottom of the dish to prevent movement.

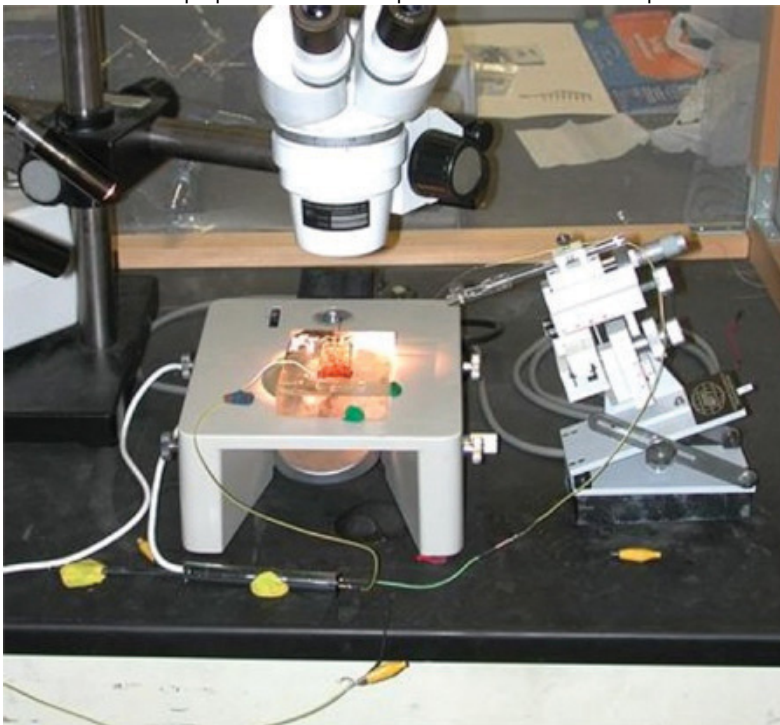


Figure 12. The placement of the preparation under the microscope. Use wax to secure the Petri dish and preparation.

9. Two wires each with a short length of silver wire attached to one end should be obtained. The silver wire should be dipped into a small amount of bleach for about 20 minutes to obtain a Ag-Cl coating. Wash the wire with water before using. A glass intracellular pipette should be obtained and carefully backfilled with a long needle attached to a syringe filled with a 3M KCl solution. The pipette should be turned down (with the opening facing the floor) and filled with solution. This will ensure that any excess KCl will drip out the back of the electrode. Be sure no KCl runs along the glass pipette that will enter the saline bath. Turn the pipette upright when finished filling with potassium chloride solution. The silver wire can then be placed into the pipette. The other end is connected to the +(positive) pole on the amplifier head stage. The pipette is then secured on the electrode probe. Care should be made not to break the electrode tip. A third wire attached to the Faraday cage should be placed into the green pole of the head stage. Lastly the Ag wire of the remaining lead should be placed in the bath and the other end attached to the - (negative) pole shown below. A wire should also be placed from the Faraday cage to the ground portion of the AD converter Powerlab. The head stage is connected to the 'input-probe' on acquisition/amplifier (Powerlab).

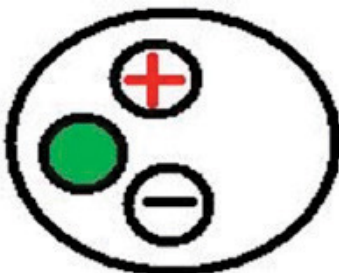


Figure 13. Head stage configuration. The wire connected to the green input of the head stage is grounded to the amplifier or Faraday cage. The wire connected to the red input is connected to the electrode wire. The black input is used to connect to the bathing solution.

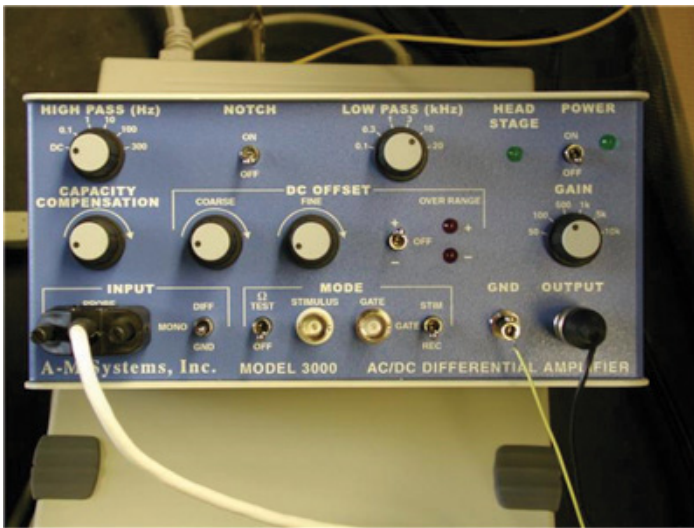


Figure 14. "Test toggle" is in the bottom row to test electrode resistance. The "coarse" knob is also found under DC offset which should be turned counter clock wise. Gain is set to 50, which amplifies signals by a factor of fifty. The ground wire from the head stage is placed in the "GND" pin jack opening.

10. The LabChart software should be opened on the desktop or laptop. Adjust the chart to display only one channel by clicking "Setup", then "Channel settings." Under "Channel settings," change number of channels to one. Click "OK." At the top of the chart, left hand corner, cycles per second should be 2K. Set volts (y-axis) to around 200mV to 500mV. Click on "Channel 1" on the right hand portion of the screen. Click "Input Amplifier." Make sure the Differential box is checked.

The amplifier output should be in channel one. The following settings should be used with the amplifier:

- High Pass- DC
- Notch Filter- OFF
- Low Pass- 20kHz
- Capacity Comp.- counterclockwise
- DC Offset Fine and Course knob- counterclockwise
- DC Offset (+OFF-) OFF
- Gain knob- 50
- Input (DIFF MONO GND)- DIFF
- MODE(STIM-GATE-REC)- REC
- QTEST- OFF

11. As a measure of the electrode resistance, the voltage should be divided by the current, which is 2.0 nA (ie., $R=V/I$, or Ohm's law). The resulting value is the resistance of the glass electrode. The resistance should be 20 to 60 MegaOhms. Lower (<20) and high resistance (>100) are not acceptable. Once the resistance has been determined, intracellular recordings can begin. Place the tip of the glass electrode into the saline bath. Make sure a ground wire is also in the saline bath.

To begin recording, press start at the bottom of the screen. Make sure the gain is set to 5 V/div. Use the course knob on the amplifier to move the line on the LabChart to zero before inserting the electrode. The toggle knob should be turned on and then off several times in order to test the electrode resistance. Next, the amplitude of the resulting values should be measured. Place one marker on the steady base line and then place the second at the peak to obtain the electrode resistance.

12. Use the electrode probe and microscope to insert the electrode into the longitudinal muscles (DEM or DEL1 or DEL2) of the preparation (see Figure 16). The electrode should barely be inserted into the muscle. Do not penetrate through the muscle. Use microscope and probe settings in order to find the longitudinal muscles and to insert the electrodes into the muscle. The high intensity illuminator should be adjusted to clearly see the muscle as the electrode is being inserted. When poking muscle fibres in this preparation one can commonly run into spaces and clefts within the muscle. This is the reason why the membrane potential can appear, then disappear, then reappear.

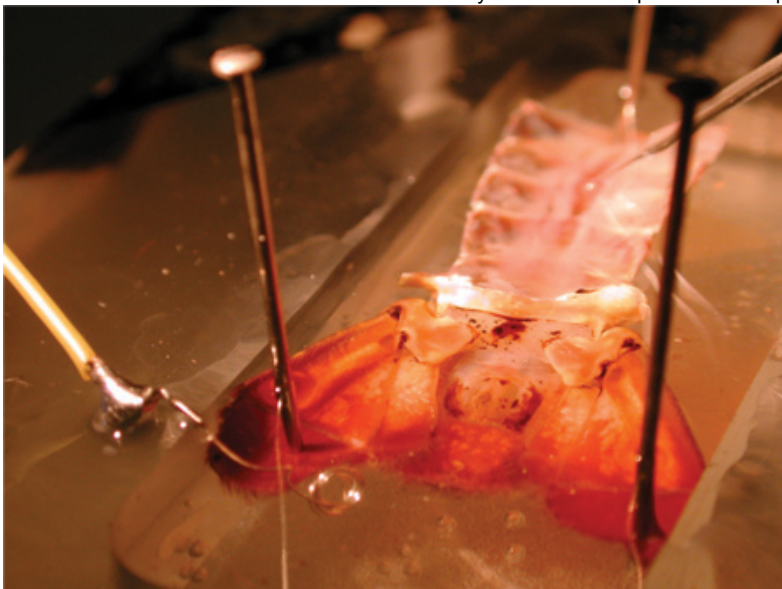


Figure 15. Insertion of electrode into the muscle.

13. To measure the membrane potential, use the coarse knob on the amplifier to move the line on the LabChart to zero before inserting the electrode. Poke a muscle fiber. Next, measure the amplitude of the resulting values. Place a marker on the steady base line and record the value.
The difference in the marker and the active cursor is displayed on the right side of the screen. The value is gives the voltage. The recorded voltage might need to be divided by the amount of amplification used on the amplifier (i.e. 10x or 100x amplification). The voltage should be converted from volts to millivolts (1 V = 1,000mV) if the values are reported on the software as volts.
14. Carefully use the microscope and manipulators to remove the electrode from the muscle. Poke another muscle fiber and note the resting membrane potential. One should take several recordings and be comfortable with measures as well as directing the intercellular electrode into the muscle fiber of interest.
The electrode probably will not stay in one muscle fiber during the changing of all the different altered $[K^+]_{out}$ solutions. It is best to withdraw the electrode, then change the solution, then penetrate again to avoid damaging the muscle. It is best to obtain 3 readings per solution from separate muscle fibers and use an average to avoid any spurious readings.
15. Use a syringe to remove and discard the saline solution from the Petri dish. The Petri dish should be filled with the next higher concentration of potassium chloride saline solution, covering the preparation completely. The same process should be repeated with each potassium solution and the changes in voltage/potential should be noted and recorded. The series of $[K^+]_{out}$ crayfish saline solutions we use are: 5.4, 20, 40, 60, 80, 100 mM.

2.3) Anatomy

Now that the physiology is completed, we can examine the associated anatomy of the muscle fibers and innervation pattern. Transfer the preparation to the staining dish and add the methylene blue (1 gram of methylene blue mixed with 100 mL of crayfish saline) . Let the saline bathe the preparation for 5 minutes and then remove and add fresh crayfish saline without the stain. The anatomy of these muscles has been described in detail over the years (Huxley, 1880; Pilgrim and Wiersma, 1963). Only recently have some of the muscles been described anatomically, physiologically and biochemically (Cooper *et al.*, 1998; Griffis *et al.*, 2000; Sohn *et al.*, 2000).

The general anatomical layout of the muscles is depicted in Figure 16 (right side of figure for this purpose). Look for the main nerve that innervates primarily the muscles within a segment. Sketch the innervation pattern to the SEM, DEL2, DEL1 and DEM muscles in a segment. The abdomen needs to be stretched out fully by pinning the preparation in the dish firmly. Next remove the saline and add the fixative solution. The fix solution is a Bouin's solution (Prepared with saturated picric acid, formaldehyde and acetic acid; Sigma-Aldrich Co.).

CAUTION. Do not get this solution on your skin or in your eyes. Avoid the vapors of the solution by working under the fume hood. If your eyes start to burn wash your eyes out immediately at the eye wash station.

Let the Bouin's solution remain on the preparation for about 10 minutes and then use a pipette and exchange the solution for saline. Cut a thin piece of DEL1 or DEL2 muscle out and place on a glass slide. Label the slide. Repeat the procedure for the SEM muscle. View the sarcomere banding pattern in both tissue preparations. You can use the compound microscope and adjust the objectives accordingly to see the banding patterns. If possible take a digital photo through the eye-piece of the microscope (note: some cell phone cameras work well for this procedure).

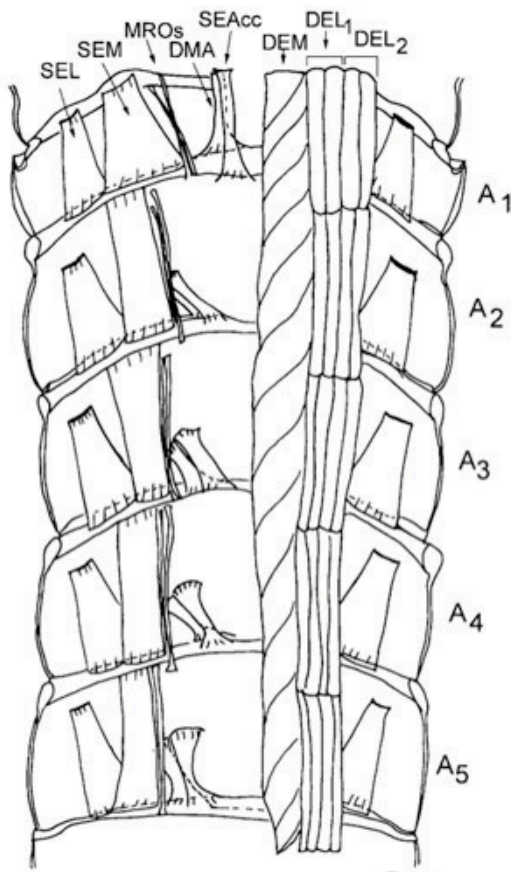


Figure 16. Schematic drawing from a ventral view of the dorsal part of the crayfish abdomen showing the extensor musculature of each segment. The dorsal membrane abdomen muscle (DMA) and the superficial extensor accessory muscle head (SEAcc) occur in segments 1 through 5 of the abdomen with a different orientation for each segment. With the exception of segment 1, these muscles have their attachment sites at their

anterior end to the calcified tergite and at the posterior end in the articular membrane. In segment 1, the homologous muscles have their anterior attachment sites to the articular membrane located between the thorax and abdomen. The illustration was based upon photographic montages of methylene blue stained preparations. On the left side of the figure all the deep extensor muscles have been removed to show the dorsal superficial extensor muscles. Scale = 2.35 mm. (Taken from Sohn *et al.* 2000).

3. Results

The following questions and data processing illustrate the main principles and objectives for this laboratory procedure.

1. Plot the measures obtained for the resting membrane potentials at each $[K^+]_{out}$ used. See if the observed and hypothetical lines are matched in their slope. To plot the values use a semi-log plot with the x-axis of varied $[K^+]_{out}$ as a log and the y-axis of the membrane potentials (as shown below; Figure 17). (Download free graph paper if needed <http://incompetech.com/graphpaper/logarithmic/>)

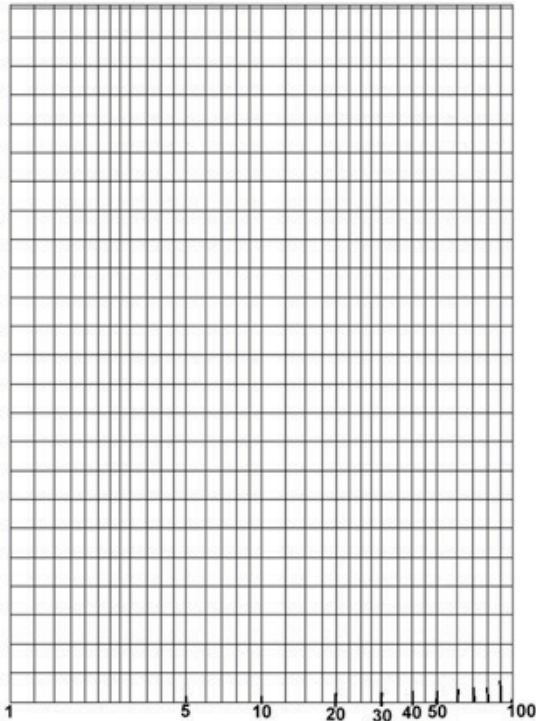


Figure 17. Graph paper

Use the average resting membrane potential obtained at 5.4 mM $[K^+]_{out}$ for initiating the hypothetical and observed lines for comparison. If the lines do not match discuss why this might be.

2. If you altered the external level of Na^+ ions, would you expect the same type of alterations as observed for changing the K^+ concentration ?
3. How well did methylene blue stain the muscles as compared to the nerves? Why might there be differences ? Is methylene blue used today for identification of tissue or contrast in live human cells ? What relationship is there with Sigmund Freud and stains used in crayfish ?
4. Note any differences in the sarcomere patterns between the DEL and SEM muscles. If so, what might be the reason? Do all muscles have the same resting sarcomere distances ? Draw the muscle banding pattern you observed with the microscope and label as much of the figure as possible (in relation to known sarcomere muscle anatomy).

4. Measuring Synaptic Responses

1) INTRODUCTION

The abdominal extensor muscle preparation used to demonstrate the resting membrane potential is also ideal for demonstrating induction of synaptic responses at the NMJs from the various muscles. Some muscles in crustaceans are selectively innervated by either a phasic or a tonic motor neuron, although some single fibers can be innervated by both phasic and tonic excitatory motor neurons, such as for extensor muscle in the crayfish walking legs (Atwood, 2008; see JOVE production id#2319-Wu and Cooper, 2010) and most other limb muscles (Wiersma, 1961a). By selectively stimulating phasic and tonic motor neurons, physiological differences in the EPSPs may be measured. Phasic motor neurons produce rapid twitching of muscle fibers and evoke EPSPs on the order of 10-40 mV. The phasic response can depress rapidly with 5-10-Hz trains of stimulation. The tonic motor neurons give rise to smaller EPSPs that can be facilitated in the presence of a higher frequency (10-50 Hz) of stimulation. Structurally, the presynaptic phasic and tonic terminals at the NMJs are different (Atwood and Cooper, 1996; Bradacs *et al.*, 1997; Cooper *et al.*, 1998).

Surprisingly the phenotype of the phasic physiological responses can undergo a transformation to a tonic-like state by electrically conditioning phasic neurons for a few hours daily over 7 days (Cooper *et al.*, 1998; Mercier and Atwood, 1989). Also the sensitivity to neuromodulation of the transformed NMJs is prime for investigating the regulation of receptor expression (Griffis *et al.*, 2000).

In this relatively robust preparation (crayfish abdominal muscles), both tonic and phasic responses are easily recorded and examined for facilitation and/or depression of the synaptic responses with varied stimulation paradigms. With these preparations, students will be able to recognize generalities of the phasic and tonic synaptic responses by stimulating a nerve bundle.

An additional NMJ preparation presented is used for monitoring intrinsic motor activity and sensory stimulus induced motor activity from the CNS. This is the superficial flexor muscle on the ventral side of the crayfish abdomen. This preparation will also be used to monitor the sensory-CNS-motor-muscle circuit and the effects of neuromodulators (Strawn *et al.*, 2000).

In each of the abdominal segment (except the last) there are three functional groups of muscles: (1) those controlling pleopod (swimmerets) movement, (2) three extensor muscles and (3) three flexor muscles. The flexors and extensors are antagonistic groups of muscles which bring about either abdominal flexion or extension by causing rotation about the intersegmental hinges. The phasic musculature occupies most of the volume of the abdomen, while the tonic muscles comprise thin sheets of fibers that span the dorsal (extensors) and ventral (flexors) aspect of each abdominal segment.

In crayfish, the tonic abdominal flexor muscles of crayfish are innervated in each half segment by five motoneurons and by a peripheral inhibitory neuron. The excitatory motoneurons use glutamate as a neurotransmitter. Glutamate depolarizes the muscle fibers by causing an increase in permeability primarily to sodium ions. The inhibitory neurons release gamma-amino butyric acid (GABA), which usually hyperpolarizes the muscle fibers by causing an increase in permeability to chloride ions. In some crustacean muscles (mainly in limbs), the peripheral inhibitory neurons make synaptic contacts with motor neuron terminals as well as with the muscle fibers, and reduce the amount of transmitter released by the motor neuron (presynaptic inhibition) (Dudel and Kuffler, 1961). This phenomenon is not present in the tonic flexor muscles of crayfish.

The ventral nerve cord of crayfish is a bilaterally symmetrical structure running the length of the animal. There is one ganglion per body segment. In the abdomen (6 segments), each ganglion contains several hundred neurons, and each of the two connectives consists of a few thousand axons. The nerve cell bodies form a layer several cell bodies thick on the ventral surface of each ganglion. Immediately above the cell body layer is a fine meshwork of neuronal processes, the neuropile. All synaptic interactions occur here; the cell bodies are devoid of synapses.

Each abdominal ganglion (except the last) has three roots on each side. The first root contains axons of neurons innervating the pleopod musculature and sensory axons; the second root contains axons innervating phasic and tonic extensor musculature and sensory axons; and the third root, which leaves the nerve cord several millimeters caudal to the ganglion, contains axons innervating phasic and tonic flexor musculature. There are two branches of the third root. The deep branch (IIIa) innervates only phasic flexor muscles. The superficial branch of the third root (IIIb) in each half-segment contains six axons, which innervate the tonic flexor muscles.

The neurons innervating the tonic flexor are spontaneously active, unlike the phasic efferent neurons, and in a good preparation, they will continue to fire for many hours after the abdomen has been removed from the animal. For a review of the historical nature of the discoveries made in these abdominal preparations see Atwood (2008). The cell bodies of four of the motor neurons and of the peripheral inhibitory neuron innervating the tonic flexor muscle in any half segment are located in the ganglion of that segment. The cell body of the remaining motor neuron is located in the next caudal ganglion. These neurons may be reliably distinguished from each other on the basis of extracellularly recorded spike amplitudes. If the tonic flexor muscle from one half segment is removed along with the two ganglia containing the neurons innervating this muscle, five neurons usually show some degree of spontaneous activity. These neurons are numbered on the basis of relative extracellular spike amplitude, in ascending order. f1 to f4 are motoneurons and f5, the largest spontaneously active neuron, is the peripheral flexor inhibitor. f6, the largest motor neuron, is an excitatory motor neuron which is seldom spontaneously active.

The spontaneous nature of tonic motor neuron activity can be modulated by exogenous application of compounds or by providing a sensory stimulus to the cuticle within the same segment that is being monitored for motor nerve activity.

2) Dissection

To obtain the abdominal extensor preparation the same procedure as described above for examining the resting membrane potentials in relation to extracellular potassium. The difference is to take care of the segmental nerve bundle that runs along the side of the carapace. This nerve will be pulled into a suction electrode which will serve as the stimulating electrode. Stimulate at 1 Hz for monitoring phasic responses. Stimulate with short bursts of pulses 10Hz for 10 to 20 stimuli while monitoring the tonic responses.

The experimental procedures for carrying out experiments on the crayfish tonic flexor muscles are different and one needs to leave the ventral nerve cord intact. A preparation consisting of several abdominal segments is made. This is obtained as follows:

1. A crayfish approximately 6-10 cm in body length should be obtained (or a manageable size). Obtain the crayfish by holding it from the back of the head or approximately 2 or 3 centimeters from the back of the eyes. Ensure that the claws of the crayfish or mouth cannot reach the experimenter when handling the crayfish. Dispose of the head and appendages after removing them.
2. Use the scissors to quickly remove the head. Make a clean and quick cut from behind the eyes of the crayfish.



Figure 18. Image shows placement of the cut to remove the head of the crayfish.

The legs and claws of the crayfish can be removed at this point to avoid injury. Stylets on males and swimmerets on both males and females can also be removed (Figure 19 and 20). Next, separate the abdomen from the thorax. Make a cut along the articulating membrane which joins the abdomen and thorax (Figure 20).

3. Save the abdomen portion of the crayfish and dispose of the thorax.



Figure 19. Image shows the placement of the stylets that can be removed from the crayfish.



Figure 20. Image shows the placement of the cut to remove the thorax from the abdomen.



Figure 21. Removal of the thorax from the abdomen. The cut should be made in circular fashion along the line of the joining of the segments.



Figure 22. The top image shows the abdomen with appendages. Bottom image shows the removal of the abdominal appendages.

4. Place the isolated tail preparation in saline solution in a large Petri dish. Pin down the tail and upper portion of the preparation to the dish. Make sure the preparation is secure. Use a scalpel to remove a square portion of the ventral side of the preparation between the ribs.

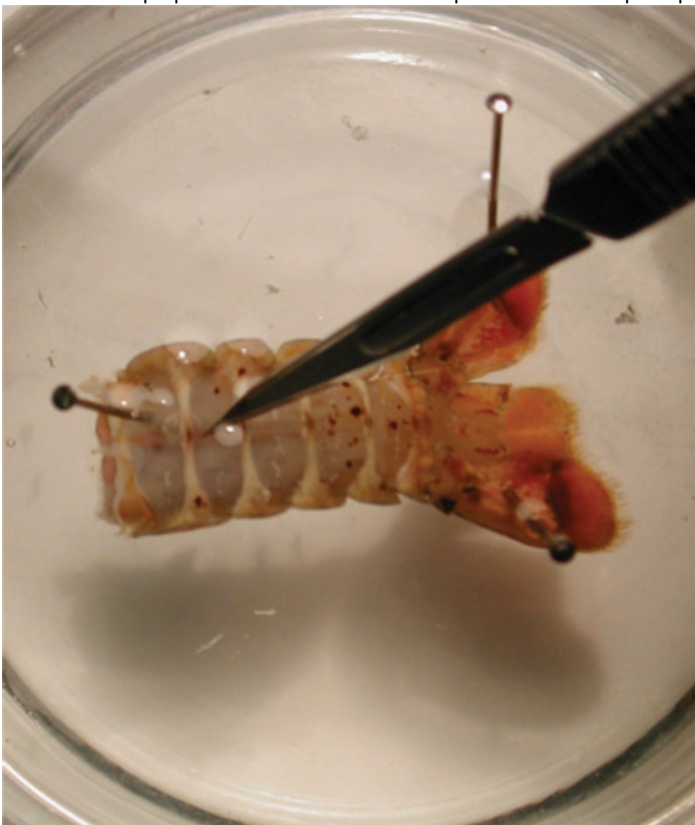


Figure 23. Shows where the cut should be made to remove the ventral portion of the preparation.

5. A small cut should be made (can also be done with scissors). A flap should be cut and lifted upward. The flap can then be removed with scissors, exposing the deep flexor muscles. The microscope should be used during this process to ensure precision in removing the ventral portion of the preparation.

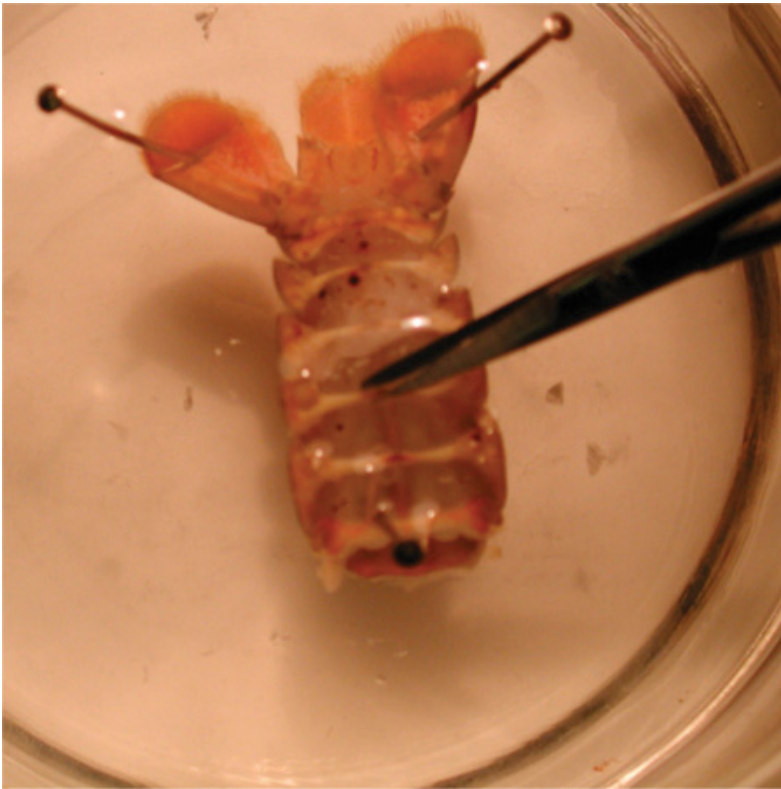


Figure 24. Cutting preparation with scissors to expose muscles.

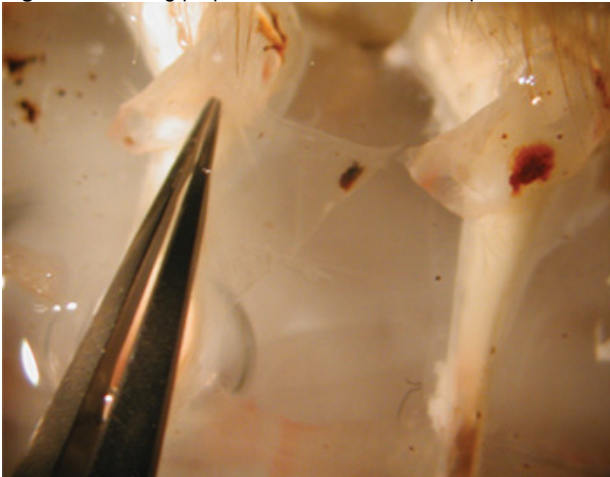


Figure 25. Top image shows the grasping of the flap with forceps. Bottom image shows the removal of the flap from the preparation using the microscope.

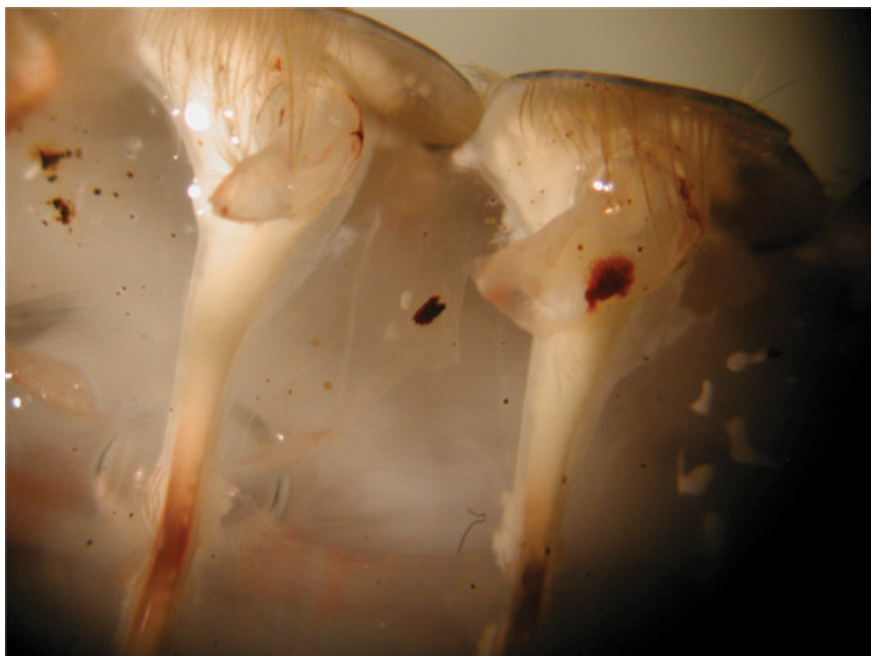


Figure 26. Exposure of the superficial flexor muscles.

3) Intracellular Recording:

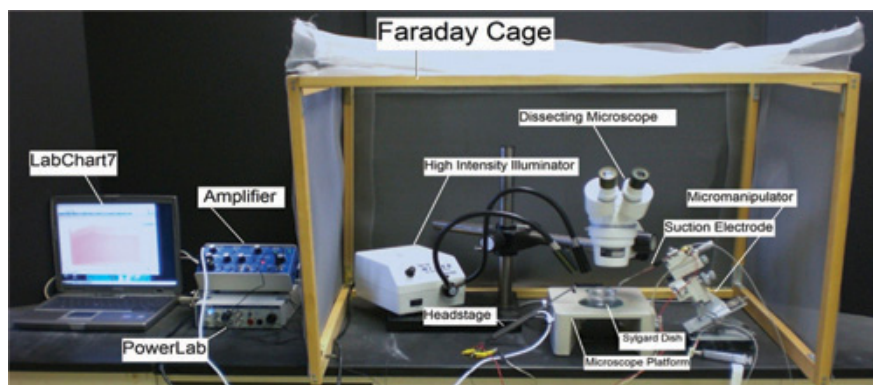


Figure 27. Overall setup of the recording equipment.

1. The Petri dish with preparation should be placed under the microscope and secured with wax at the bottom of the dish to prevent movement.

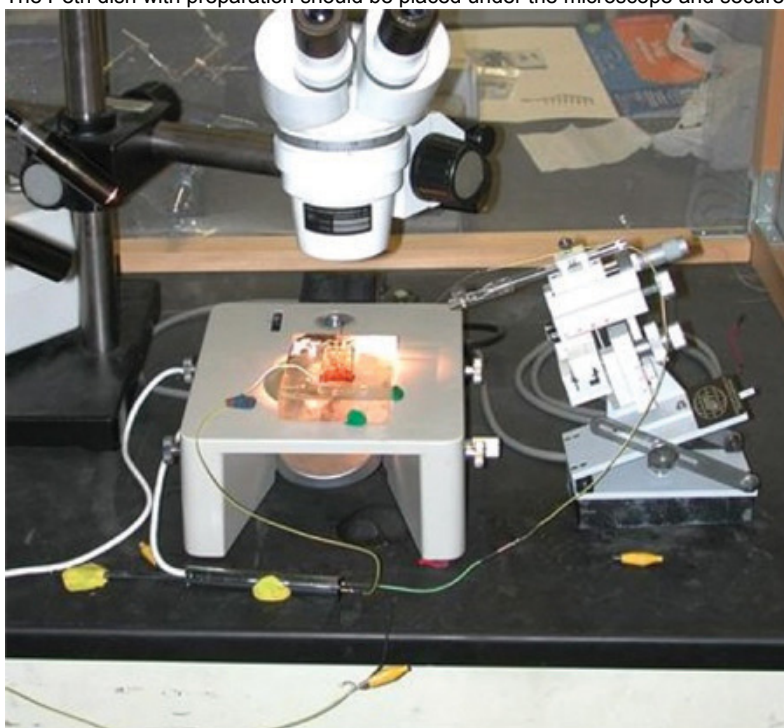


Figure 28. Shows the placement of the preparation under the microscope. Use wax to secure the Petri dish and preparation.

- Two wires with short length of silver wire attached to one end should be obtained. The silver wire should be dipped into a small amount of bleach for about 20 minutes to obtain a Ag-Cl coating. Wash the wire with water before using. A glass intracellular pipette should be obtained and carefully filled with a KCl (3 M) solution. The pipette should be turned down (with the opening facing the floor) and filled with solution. The latter will ensure that any excess KCl will drip out the back of the electrode. Be sure no KCl runs along the glass pipette that will enter into the saline bath. Turn the pipette upright when finished filling with potassium chloride solution. The silver wire can then be placed into the pipette. The other end is connected to the +(positive) pole on the head stage. The pipette is then secured on the electrode probe. Care should be made not to break the electrode pipette. A third wire attached to the Faraday cage should be placed into the green pole of the head stage. Lastly the Ag wire of the remaining lead should be placed in the bath and the other end attached to the - (negative) pole shown below. A wire should also be placed from the Faraday cage to the ground portion of the AD converter Powerlab. The head stage is connected to the "input-probe" on acquisition/amplifier (Powerlab).

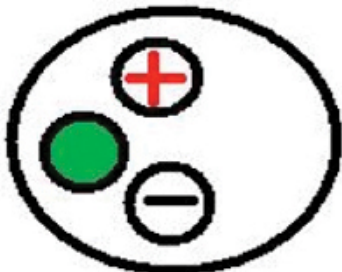


Figure 29. Head stage configuration. The wire connected to the green portion of the head stage is grounded to the amplifier or Faraday cage. The wire connected to the red portion is connected to the electrode wire. The black portion is used to connect to the bathing solution.



Figure 30. "Test toggle" is in the bottom row to test electrode resistance. The "coarse" knob is also found under DC offset which should be turned counter clock wise. Gain is set to 50, which amplifies signals by a factor of fifty. The ground wire from the head stage is placed in the "GND" pin jack opening.

- The LabChart software should be opened on the desktop or laptop. Adjust the chart to display only one channel by click "Setup", then "Channel settings." Under "Channel settings," change number of channels to one. Click "OK." At the top of the chart, left hand corner, cycles per second should be 2K. Set volts (y-axis) to around 200mV to 500mV. Click on "Channel 1" on the right hand portion of the screen. Click "Input Amplifier." Make sure the Differential box is checked.

The amplifier output should be in channel one. The following settings should be used with the amplifier:

- High Pass- DC
 - Notch Filter- OFF
 - Low Pass- 20kHz
 - Capacity Comp.- counterclockwise
 - DC Offset Fine and Course knob- counterclockwise
 - DC Offset (+OFF-)- OFF
 - Gain knob- 50
 - Input (DIFF MONO GND)- DIFF
 - MODE(STIM-GATE-REC)- REC
 - Ω TEST- OFF
- To measure the electrode resistance, the voltage should be divided by the current, which is 2.0 nA. The resulting value is the resistance of the glass electrode. The resistance should be 20 to 60 MegaOhms. Once the resistance has been determined, intracellular recordings can begin. Place the tip of the glass electrode into the saline bath. Make sure a ground wire is also in the saline bath. To begin recording, press "start" at the bottom of the screen. Make sure the gain is set to 5 V/div. Use the course knob on the amplifier to move the line on the LabChart to zero before inserting the electrode. The toggle knob should be turned on and then off several times in order to test the electrode resistance. Next, the amplitude of the resulting values should be measured. Place one marker the steady base line and then place the second at the peak to obtain the electrode resistance.

5. Use the electrode probe and microscope to insert the electrode into the muscle. Do not penetrate through the muscle. Use microscope and probe settings in order to find the thin layer of muscle fiber and to insert the electrodes into the fibers. The high intensity illuminator can be used as a light source when penetrating the muscle.



Figure 31. Insertion of electrode into the muscle.

6. Care must be taken to avoid damaging the nerve roots to the superficial muscles. It is advisable to keep the saline bathing the preparations cool (10-15 degrees Celsius) and well oxygenated while carrying out the experimental procedures. If cooling units are not available replace the saline with fresh, cooled saline regularly. Oxygen gas, or at least air, should be bubbled through the saline.
7. Record the spontaneous activity of the EPSPs. Note the different sizes of the EPSPs and if IPSPs are present.
8. Very carefully take a small paint brush and by hand stimulate along the cuticle edge within the same segment that one is monitoring the spontaneous activity. Note a change in frequency of the responses and if different size EPSPs appear that were not there prior to stimulating the cuticle.

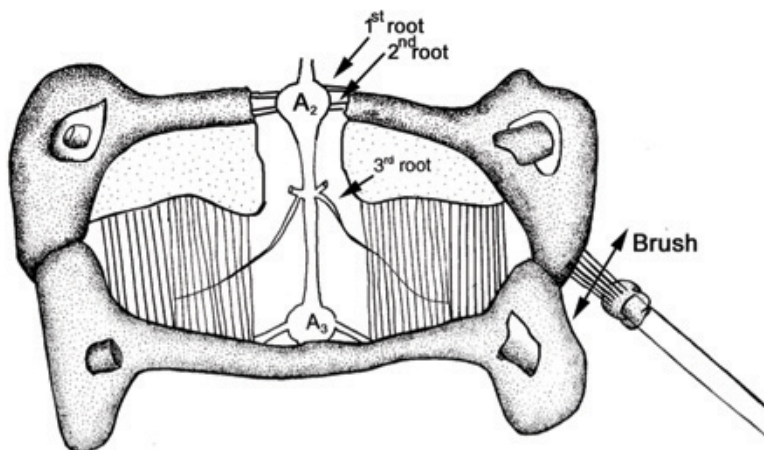


Figure 32. Preparation with stimulating brush and nerve roots. (modified from Strawn *et al.*, 2000)

9. The stimulation can be repeated after carefully exchanging the saline bath with one containing a neuromodulator such as serotonin (1 microM) or saline bubbled with CO₂. Note the effect on the activity profile for a given stimulus. Also note if exchanging the saline back to fresh saline returns the activity to its initial condition.
10. Next, one can monitor neural activity within the sensory-CNS-Motor neuron circuit in various ways. We can use a suction electrode instead of an intracellular electrode (Figure 33) to monitor motor neuron activity. At the tip of the glass suction electrode, plastic tubing is placed which has an opening of the correct size to pull the nerve into the tip. The opening should not be too large, as the nerve would fall out; or too small, because the nerve would be damaged by the pressure of the electrode. The plastic tubing is pulled over a flame and trimmed back to the size needed.

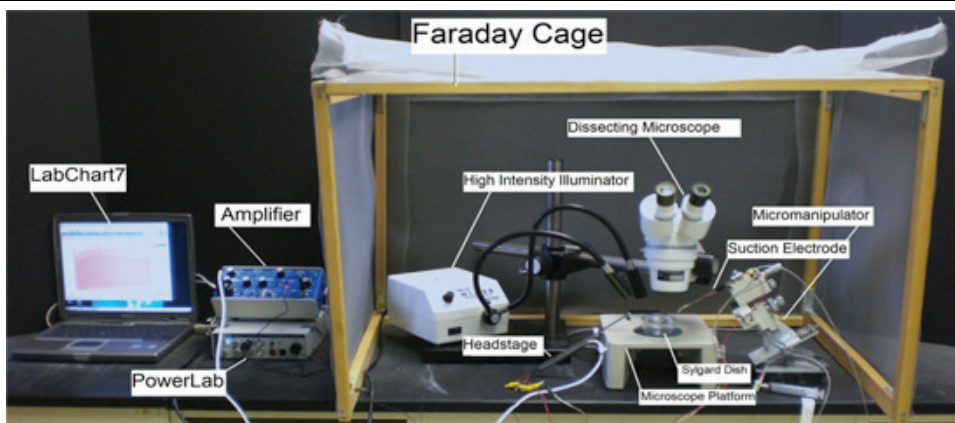


Figure 33. Set up with suction electrode recording arrangement.

Position the micromanipulator in a position where the suction electrode has easy access to the saline bath. Suction up saline until it is in contact with the silver wire inside the suction electrode. Arrange the other wire on the cut-side of suction electrode close to the tip of electrode, so both wires will be in contact with the saline bath.

As for the electrical monitoring connect the AC/ DC Differential Amplifier (amplifier) to the Power Lab 26T. Do this by connecting the proper cord from Input 1 on the PowerLab 26T to the output on the amplifier.

- The amplifier instrument controls should be set to the following settings:
 - High Pass- DC
 - Notch Filter- OFF
 - Low Pass- 20kHz
 - Capacity Comp.- counterclockwise
 - DC Offset Fine and Course knob- counterclockwise
 - DC Offset (+OFF-)- OFF
 - Gain knob- 50
 - Input (DIFF MONO GND)- DIFF
 - MODE(STIM-GATE-REC)- REC
 - Ω TEST- OFF

Connect the head stage to the 'input- probe' on the amplifier.

Connect the electrical wires from the suction electrode to the head stage. The wires should be connected with the red (positive) at the top left, green (ground) in the middle, black (negative at the bottom). This is indicated in Figure 34. The ground wire can just be put in the saline bath.

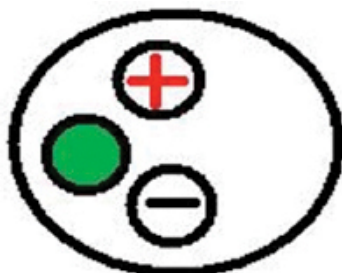


Figure 34. Head stage configuration

11. Now connect the USB cord from the PowerLab 26T to the laptop. Ensure that both the amplifier and PowerLab26T are plugged in and turned on before opening LabChart7 on the computer.
12. Open LabChart7.
 - The LabChart Welcome Center box will pop open. Close it.
 - Click on Setup
 - Click on channel settings. Change the number of channels to 1 (bottom left of box) push OK.
 - At the top left of the chart set the cycles per second to about 2k. Set the volts (y-axis) to about 500 or 200mv.
 - Click on Channel 1 on the right of the chart. Click on Input Amplifier. Ensure that the settings: single-ended, ac coupled, and invert (inverts the signal if needed), and anti-alias, are checked.
 - To begin recording press start.

We can record from the branch of the 3rd root that innervates the superficial flexor muscle (branch IIIb) to monitor size of the action potentials with extracellular recording. The extracellular nerve impulses are referred to as 'spikes'. Recall that there are five excitatory motor neurons and one inhibitor motor neuron in this root (Kennedy and Takeda, 1965; Velez and Wyman, 1978). Stimulation of the cuticle with a brush or the exposure of neuromodulators can be utilized (Figure 35). The paintbrush could be used by hand or for consistent stimulation it could be mounted on a micromanipulator to control the amount of pressure and movement.

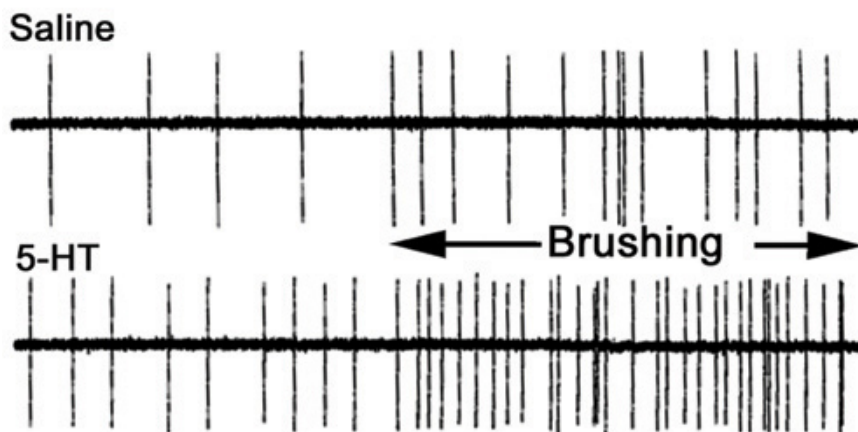


Figure 35. Activity of the 3rd root before and during cuticular stimulation in saline (top) and in 100 nM 5-HT (bottom). The time during cuticle stimulation is indicated by the bar. Note the enhanced activity before and after stimulation when the preparation is bathed in 5-HT (modified from Strawn *et al.*, 2000).

We can record from the 1st or 2nd roots by making an en passant recording of the nerve; or we can transect the root away from the VNC and record pure sensory input arising from the periphery which would be sending signals into the VNC. Thus, you would record from the transected root leading to the periphery for sensory activity.

The 2nd root contains very large primary afferent axons from the muscle receptor organs (MRO) and smaller axons of efferents to extensor motor neurons (Fields and Kennedy, 1965). There are many sensory axons in the 1st and 2nd roots.

The mechanosensory neurons have direct connections, by electrical synapses with the lateral giant axons (LG) (Krasne 1969; Zucker 1972). Also, mechanosensory neurons are known to excite interneurons via chemical synapses.

To examine how sensory input can influence motor neuron activity, through a sensory-CNS-motor neuron circuit, we can record the synaptic responses in a muscle. Various aspects of the circuit we will use can be examined. For example, we can record from the sensory nerve root alone or the motor root with or without intact sensory input into the VNC. To analyze the spike frequency recordings, one can count over a period of time in different conditions. The measures can be made prior to brush stimulation and during the brush stimulation for a given amount of time (Figure 35). One can repeat the conditions 5 times and obtain the average percent change in frequency as a measure to make comparisons.

One can also apply exogenous compounds such as serotonin (Strawn *et al.*, 2000) or acetylcholine (ACh), nicotine or glutamate. Various behavioral actions have been described for nicotine in invertebrates. This would suggest the presence of nicotinic receptors (Tsunoyama and Gojohori, 1998). Glutamate is a major excitatory neurotransmitter in most invertebrates at the NMJ and ACh is the major excitatory neurotransmitter within the CNS (Monaghan *et al.*, 1989; Watkins, *et al.*, 1990).

One can try heptanol or CO₂ bubbled saline since it will uncouple the crayfish septate (or gap) junctions within the circuit as Dr. Sonya M. Bierbower (University of Kentucky) has shown in her dissertation research. This action may account for altered whole animal behavior when exposed to high CO₂ in the environment (Bierbower and Cooper, 2010). When you stimulate the cuticle with a brush and drive sensory input and record a response in the motor neurons, note if there is a difference in the activity before and during heptanol or CO₂ exposure. This may or may not suggest gap junctions to have a role in the sensory-CNS-motor neuron circuit.

Disclosures

No conflicts of interest declared.

Discussion

Membrane Potential

As early as 1902, Bernstein was dealing with the issues of a resting potential in the axon of a squid. It is intriguing to consider how these early ideas and observations of Bernstein (1902) and Nernst (1888) later influenced research in membrane physiology. (See review by Malmivuo and Plonsey, 1995; also available on the www <http://www.bem.fi/book/>). There are still, to this day, breakthroughs being made about ion channel function and properties of biological membranes that are very significant in understanding the cellular physiology which relates to the function of tissues, organs and systems.

The comparison of the experimental and theoretically derived effects of external [K⁺] on the resting membrane potential indicates the influence of ions on the membrane potential. Additional experiments using this same preparation remain to be performed to address fundamental physiological questions. Some were highlighted back in 1968 by Atwood and Parnas and have yet to be fully tackled. With the techniques obtained in this exercise, one can proceed to answer many questions remaining in other experimental preparations as well as in physiological applications related to medicine and health. We have demonstrated the usefulness of a model invertebrate preparation to address fundamental questions pertinent to all animals.

With the knowledge gained on the electrochemical gradients of ions in this above exercise, you can now advance to the excitability of membranes by examining synaptic transmission at neuromuscular preparations in the crayfish.

Measuring Synaptic Responses

The details provided for the first part of this laboratory, and the associated movie, have provided key steps for recording membrane potentials and investigating muscle structure. In the second part of this laboratory, the demonstration of dissection and recording synaptic transmission at the NMJs of phasic and tonic motor units provided an exposure to fundamental concepts in physiology. The exposure to a neural circuit, which can in

part can be used to explain associated behaviors, in the intact animal has potential not only for students to investigate various open ended questions within their laboratory exercise but also for future research on neuronal circuits in a well established invertebrate preparation (Kennedy *et al.*, 1969; Antonsen and Edwards, 2003)

These preparations can also be used to investigate synaptic facilitation, depression and long-term plasticity (not investigated in this laboratory study). Even within some species of crayfish, neuronal plasticity depends on the experimental stimulation conditions (Mercier and Atwood, 1989; Cooper *et al.*, 1998) as well as their natural environment. To what extent the ability to alter synaptic efficacy and muscle dynamics serves the animal remains to be investigated. Since crayfish do alter their behavior in relation to seasonal variation and the molt cycle, there are relatively long-term activity differences in their neuromuscular systems. It has been shown that the phasic motor nerve terminals of claw closer muscles exhibit the classic phasic morphology during the winter, but swell and become more varicose along the length of the terminal during the summer months (Lnenicka 1993; Lnenicka and Zhao, 1991).

Some early studies conducted in crayfish lateral giant (LG) interneurons within the ventral nerve cord demonstrated the presence of gap junctions (Johnson, 1924; Watanabe and Grundfest, 1961). It is well known that CO₂ has an effect on electrical communication by uncoupling gap junctions (Arellano *et al.*, 1990). It was recently shown that the nerve cord and communication within the sensory-CNS-motor-muscle circuit, as described in this report, is also sensitive to CO₂ exposure, indicating the presence of gap junctions (Bierbower, 2010; Bierbower and Cooper, 2010)

The spontaneous activity of the 3rd motor root has been a topic since the 1960's when Eckert (1961) examined if the tonic firing static muscle receptor organ (MRO) within the same or neighboring segment could account for the spontaneous motor drive. In these earlier studies it became apparent that the activity was driven within the ventral nerve cord (VNC) possibly from higher centers (Eckert, 1961; Kennedy and Takeda, 1965a,b; Strawn *et al.*, 2000). Since the presence of CO₂ stopped the spontaneous activity, one can assume somewhere in the drive to the motor neurons there might be gap junctions or glutamatergic excitatory drive. The NMJs are blocked or exhibit decreased sensitivity to glutamate in the presence of CO₂, and they may be blocked as well within the CNS (Bierbower, 2010; Bierbower and Cooper, 2010; see also Badre *et al.*, 2005).

The action of various neuromodulators is also readily studied at the various types of NMJs (Cooper and Cooper, 2009; Griffis *et al.*, 2000; Southard *et al.*, 2000; Strawn *et al.*, 2000). In addition, various influences are exerted by neuromodulators on the CNS circuitry. It has been suggested that the 5-HT and octopaminergic neurons may function as 'gain-setters' in altering the output of neuronal circuits (Ma *et al.*, 1992; Schneider *et al.*, 1996; Hörner *et al.*, 1997; Edwards *et al.*, 2002). Much work remains to be done before we can fully understand the effects of neuromodulators on individual target cells. Given that different neuromodulators may work in concert with one another, analysis of their mixed action is an area for future research (Djokaj *et al.*, 2001). In addition, few studies, particularly in the vertebrates, address the effects of neuromodulators on entire pathways which can regulate a specific behavior. In this sensory-CNS-motor unit preparation one can examine the influence of both sensory input and neuromodulators on the activity of the motor neurons (Kennedy *et al.*, 1969).

Since it has been postulated that 5-HT plays a role in regulating the behavioral state of crayfish, lobsters, and crabs (Livingstone *et al.*, 1980; Sneddon *et al.*, 2000), several attempts have been made to determine its concentration in the VNC, the hemolymph, and in isolated ganglia of lobsters (Livingstone *et al.*, 1980; Harris-Warrick and Kravitz 1984; Fadoo *et al.*, 1988). However, there has been considerable variation in the recorded measurements, precluding specific dose-response relationships which could account for behavioral actions.

A crayfish with the claws held in a raised position and with the tail tucked under its abdomen has been thought to exhibit a dominate posture (Livingstone *et al.*, 1980). The state of abdominal flexion in crayfish does not appear to be the posture that dominant crayfish, within a pair, exhibit during the social interactions or while maintaining a dominant hierarchical status (Listerman *et al.*, 2000). Submissive crayfish will even tuck their abdomens under themselves as they retreat from an opponent. Such tail tucking is also seen as a defense posture (Listerman *et al.*, 2000). These behaviors have been readily observed in the field and in laboratory settings (Bovbjerg, 1953, 1956; Bruski and Dunham, 1987; Li *et al.*, 2000; Listerman *et al.*, 2000). Interestingly, the behavioral postures noted in lobsters (Livingstone *et al.*, 1980) are reversed for 5-HT and octopamine injections in the Australian crayfish, *Cherax destructor* (McRae, 1996). Possibly, entirely different responses would be observed in the superficial flexor preparation in the Australian crayfish. In addition, since dominance is generally size related among crayfish, one would expect a very plastic response system for rapidly altered social conditions (Strawn *et al.*, 2000). The plasticity in responsiveness to neuromodulators in invertebrates is an open area of investigation.

Wytenbach, Johnson, and Hoy (1999) have produced digital media and a laboratory manual for various crayfish experimentations involving the same muscular presented in this report in addition to other crayfish preparations. This is an excellent resource for student exercises.

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