The mechanistic action of lipopolysaccharides (LPS) at the crayfish neuromuscular junction (NMJ), with a focus on synaptic transmission

For the Bio350 honors section Spring 2026
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PURPOSE

To understand the properties of excitable membranes in relation to the ionic basis of the resting membrane potential and the relationship to the effects of LPS on membrane potential as well as synaptic transmission.

MODEL PREPARATION

The DEL1 and DEL2 abdominal muscle of the Northern Crayfish (*Faxonius virilis*). Crayfish obtained from Carolina Biological supply. These muscles are considered *Fast-type* or *Phasic* or as commonly called *White type* in mammals due to the type of myosin proteins they contain (Cooper et al., 1998; LaFramboise et al., 2000).

LEARNING OUTCOMES

1. To learn how a membrane potential occurs in cells.

2. To learn to explain how to obtain intracellular recording from a cell with glass microelectrodes.

3. To learn to be able to obtain recordings of membrane potential from DEL1 and DEL2 abdominal muscles of a crayfish.

4. To learn how to observe and record spontaneous quantal events on the DEL1 or DEL2 muscle fibers while obtaining a stable resting membrane potential.

5. To learn to switch the bathing media to different salines gentle while maintaining a resting membrane potential and recording spontaneous quantal events.

6. To investigate the effects of LPS on membrane potential and the frequency of occurrence in spontaneous quantal events.

7. Depending on the results, to predict the mechanisms of action of LPS in altering membrane potential , in the muscle fiber, and frequency of occurrence in spontaneous quantal events by actions on the muscle fiber as well as the presynaptic motor nerve terminals innervating the muscle fibers.

8. To learn to analyze and graph obtained data.

9. To learn to conduct reliable searches of the literature related to the subject.

10. To learn to composes a class report as a group working together.

ABSTRACT

In modifying a typical physiological laboratory protocol in measures of the resting membrane potential in relation to the concentration of extracellular potassium ions $[K^+]_o$, or other compounds, the effects of exposure to LPS on the membrane potential and frequency of occurrence in spontaneous quantal events are being addressed. The effects of LPS on the membrane potential are not commonly addressed in experimental physiology and neurophysiology student laboratory exercises. In terms of presenting an authentic application of the experimental design, various topics are highlighted. Experimentally, the skeletal muscle of a crayfish will serve as a model to obtain data. The crayfish model is robust for long term survival in minimal physiological saline, is easily obtainable, and allows for a relative ease in dissection. Graphing membrane potential in relation to exposure to LPS provides important distinctions and understanding of the relationship of LPS with the concepts of the Nernst and Goldman-Hodgkin-Katz (G-H-K) equations. Freely available online software is used in addressing the theoretical values one would expect. Discussion of other factors impacted by LPS in potentially altering the frequency of occurrence in spontaneous quantal events provides discussion and understanding of synaptic physiology.

1. Introduction

The description for this laboratory exercise is a mix of various previous protocols used in past years at the University of Kentucky in teaching and in research. Some text is copied from such protocols and research papers and will need to be modified and cited for any potential reports or publications. Sections of text is copied from Bio199, Bio350, Bio446 lab protocols provided by various people over the years (Baierlein et al., 2011; Naidugari et al., 2022; Dr. Danley's modifications of Bio350 lab protocols).

It is important that a report and any data obtained is not deposited online to such places as ChatGPT from OpenAI or Chegg postings as this can block the ability to publish novel research findings. ChatGPT will store any data and text to use AI if others search with similar prompts. For example, if you use ChatGTP to correct grammar or state the text in a new approach for a report , AI servers now have saved the text you posted, and it can be used by AI for other people. So, don't even use it to correct grammar on a report (use MS word standard grammar check which will not post text in AI storage servers).

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A common student physiology laboratory protocol for teaching content on the resting membrane potential is generally to vary the extracellular potassium ions $[K^+]_o$ in relation to the membrane potential.

If experimentally determining this relationship, participants would vary the $[K^+]_o$ while taking measures of the membrane potential with intracellular recordings. The experimentally obtained values are usually plotted in relation to the theoretical Nernst equation. The deviation in the curves would likely bring up the topic of the Goldman-Hodgkin-Katz (G-H-K) equation and why the G-H-K equation is a better fit for the experimentally obtained data (Atwood and Parnas, 1968; Baierlein et al., 2011; Johnson et al., 2014; Wyttenbach et al., 1999). With the same concept, adding a compound which alters membrane potential, would likely open or close leak channels or even effect voltage gated ion channels, receptors that are ion channels (ionotropic) or impact other channels indirectly (metabotropic) or even pumps or ion exchangers.

Common laboratory exercises become very standard and referred to as "cookie cutter" exercises. The exercise presented in this paper is designed to provide alternative tweaks to a standard protocol for novel investigative purposes. Hopefully this will engage more participation in learning the related content as well as enjoyment in conducting a laboratory exercise. We refer to this type of class exercise as an Authentic Undergraduate Research Experience (ACUREs) as these allow novel projects to be developed with a goal in publishing the findings and offering students an advanced scientific undertaking by publishing their results. In the past, this general lab in the resting membrane potential with varied extracellular concentration of potassium ions (i.e. $[K^+]_o$) was taught in the concept of muscle or neuronal injury and spillage of K+ adding to $[K^+]_o$. (Cooper et al., 2019; Thenappan et al., 2019).

To address a novel question and add to previous reported findings, this protocol address the effects of lipopolysaccharides (LPS) on the membrane potential and the occurrence in spontaneous quantal events on a phasic-like motor unit in a crayfish model.

By relating this integrative topic to real world applications, three general themes are presented for students to contemplate. Septicemia due to Gram-negative bacteria releases LPS and the following immune response causing the subsequent release of cytokines. This can lead to the breakdown of the BBB (blood brain barrier). Now LPS as well as cytokines have access to the central nervous system (CNS) and those

synapses. Initially, LPS already had access to the peripheral nervous system and synapses outside the BBB. When treating bacterial septicemia antibiotics would be used which can result in bacteria lysing and increasing the release of LPS causing a cytokine storm. It is not known what the direct action of LPS is on neurons and different types of synapses such as high- and low-output types in the CNS of animals or even at neuromuscular junctions (NMJs).

There are no prior reports on the direct acute action of LPS on synaptic transmission except for the Parnas et al., (1971) study and the more recent studies since 2019 with model crayfish and larval *Drosophila* preparations (McNabb et al., 2019). The differential effects of LPS on high output- and low-output synapses (phasic- and tonic-like) have not been fully addressed as to how evoked transmission is enhanced. Nor has the question of spontaneous quantal responses altered in their frequency of occurrence in the absence of evoked nerve stimulation been addressed for phasic- and tonic-like synapses.

It is known that evoked transmission is increased more so at tonic-like NMJs than for phasic like NMJs (Saelinger et al., 2019). Thus, one could expect differences in mechanisms to account for the evoked differences. One possibility is that differences reflect release of internal Ca2+ stores triggered by LPS action and/or differences in the amount of Ca2+ influx into presynaptic terminals.

One mechanistic possibility is an increase in Ca2+ within the presynaptic motor nerve terminal. However, no increase in the frequency of quantal responses was observed in an earlier study of crayfish NMJs when exposed to LPS (Saelinger et al., 2019). Notably, these recordings were obtained at the tonic low-output opener neuromuscular junction. An early study that appears to have measured responses from a single preparation of an unidentified abdominal muscle of the crayfish showed an increase in the frequencies of quantal events with LPS (Parnas et al., 1971). In addition, the opener muscle study noted that some preparations increased the frequency of spontaneous quantal events while other preparations decreased the frequency of spontaneous quantal events. Thus, due to the variability there was no significant overall trend, but there were still effects—which is interesting. Why did some preparations increase in frequency while others decreased?

It is assumed that maybe the preparation used in the Parnas et al.1971 study was from an abdominal phasic NMJ of the crayfish, but the specific muscle used was not stated in

the publication. The abdomen has phasic and tonic muscles with various types of NMJs (Atwood and Cooper, 1996; Cooper et al., 1998; LaFramboise et al., 2000).

The high synaptic efficacy of phasic NMJs over a short period is due to a large number of synaptic vesicles docked within the presynaptic face which can be recruited quicky for fusion events. However, with high frequence evoked transmission they are depleted quickly as there is not enough time or possible energy to maintain the synaptic output. The phasic presynaptic terminals have fewer mitochondria than tonic-like presynaptic terminals.

To address if Ca2+ influx into presynaptic nerve terminals in increased by LPS one can reduce calcium in the extracellular saline, but the issue with that is the nerve will start to spontaneously fire. One might think that doesn't matter because then there'll be no calcium to come in the presynaptic nerve terminal to cause synaptic vesicles to fuse, and that could be the case; however, the sodium that comes in due to nerve depolarization could have an impact on the endoplasmic reticulum (ER) inside the nerve terminal and alter internal calcium release.

Another approach would be to leave calcium concentration the same in the saline, but block the voltage-gated calcium channels with something like cadmium Cd 2+. This would address the question of LPS resulting in the influx of Ca2+ through voltage gated Ca2+ channels. If any increase in frequency of spontaneous events occurred, it would be most likely due to internal release of calcium from the endoplasmic reticulum.

This could be addressed by pre-incubation of preparations with Thapsigargin (1 mM). This blocks the Ca2+ pump that pumps calcium into the endoplasmic reticulum, so there's no more calcium that can come out; however, to do this one has to pre-incubate for about 20 minutes with this drug so that the ER is depleted of calcium. This would tell one if, after LPS application, if there is no increase in minis or a change in mini frequency, but there was without the drug, then this suggests Ca2+ came from internal stores to account for LPS's action. This is, of course, contingent on observing an LPS-driven change in mini frequency in the first place.

If LPS causes an increase in quantal frequency even with Cd2+ and Thapsigargin, then an effect on the plasma membrane itself may be implicated. This could be due to LPS causing docked vesicles to fuse spontaneously. This would be unusual based on current understanding of synaptic vesicle fusion processes, but it would be a noteworthy result.

The lab is also designed to monitor any changes in the resting membrane potential of the muscle fibers. It was noted earlier that there is a slight hyperpolarization of the tonic muscle fibers with LPS exposure.

Proposed experimental sets (Bio350H-scale)

Experimentally, one would have:

1. Preparations exposed to LPS to determine if there's a change in minis (frequency) and membrane potential.
2. Preparations where LPS is added, but the saline includes Cd2+ to block voltage-gated calcium channels (Cd2+ + LPS).
3. Preparations incubated in saline with Cd2+ and Thapsigargin for 20 minutes and then exposed to LPS (Cd2+ + Thapsigargin → add LPS).

When one puts LPS on, one must use saline with the same mixture that the preparation was pre-exposed to (Cd2+ alone or Cd2+ + Thapsigargin) so one is not changing the contents of the saline before adding LPS. The only variable should be LPS.

There eight electrophysiological setups, so four could run LPS alone, and for other set ups with Cd2+ + Thapsigargin pre-incubation followed by LPS as an "extreme test" of the hypothesis that Ca2+ may be released from internal stores. Students in a research lab could follow up with additional experiments such as LPS + Thapsigargin alone (without Cd2+), which may take more time than Bio350 can accommodate.

Outside of Bio350, one additional validation is to examine Thapsigargin's effects on the preparation in the presence of cadmium and test whether serotonin (5HT), which is known to release calcium from internal stores, is blocked by the same incubation period with Thapsigargin. This would help document that Thapsigargin within the 20 minutes blocked release of calcium from internal stores due to depletion of ER calcium.

To begin this exercise the Nernst equation and Goldman-Hodgkins-Katz equation (GHK) need to be addressed. One can work on this prior to conducting the experiments.

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:

$$V = \frac{RT}{zF} \cdot ln \frac{[X]_{out}}{[X]_{in}}$$

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 × 104 C/mol]

For the K^+ ion at 20°C and transformation of ln to $\log_{10}$ along with filling in the constants, one arrives at:

$$Potential = 58\, log \frac{[K]_{out}}{[K]_{in}}$$

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 dependent on the K^+ equilibrium potential.

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

$$Potential = 58 \log \frac{5.4}{[K]_{in}}$$

-70/58=log 5.4/[K in]

-1.2069= log 5.4/[K in]

Antilog -1.2069= 5.4/[K in]

$10^{-1.2069}$= 5.4/[K in]

0.0621= 5.4/[K in]

[K in] =5.4/0.0621=86.95 mM

Double check

X= 58 Log (5.4/86.95)= -69.999 so close enough to -70 mV

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:

$$\mathrm{Em}_{K,Na,Cl} = \frac{\mathrm{RT}}{F} \ln \frac{\mathrm{P}_{\mathrm{Na}^+}[\mathrm{Na}^+]_{\mathrm{out}} + \mathrm{P}_{\mathrm{K}^+}[\mathrm{K}^+]_{\mathrm{out}} + PCl[Cl]in}{\mathrm{P}_{\mathrm{Na}^+}[\mathrm{Na}^+]_{\mathrm{in}} + \mathrm{P}_{\mathrm{K}^+}[\mathrm{K}^+]_{\mathrm{in}} + PCl[Cl]out}$$

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.

A free software database allows one to put in different values for temperature and other variables in the GHK equation for a quick response in the expected membrane potential. The use of the rapid computer simulation helps to demonstrate the effect of the various parameters on the membrane potential as well as how slight changes in one parameter can have a large role in the outcome.

The main emphasis for this exercise is on how different pK values affects the membrane potential.

Given the values reported in the literature for crayfish muscle we use these as a reference. The concentration of K+ in the saline we start off with is 5.3 mM and the temperature is 21 C (Note: We will take the temperature of the saline in which we conduct these experiments in practice).

There are values estimated for neurons (Atwood 1982) which we could use as well for comparison in muscle fibers.

$[Na]_i$ = 17.4 mM (for neurons, Atwood 1982)

$[K]_i$ = 265 mM (for neurons, Atwood 1982)

$[Cl]_i$ = 12.7 (for neurons, Atwood 1982)

pK = 1 (for neurons, Atwood 1982)

pCl = 0.1 (for neurons, Atwood 1982)

pNa = 0.001 (for neurons, Atwood 1982)

For crayfish muscle:

"*Procambarus* and *Astacus* $[K]_i$ appears to be 171 and 167 mM, respectively." (Katz et al., 1972). We are using the values for *Procambarus.*

$[K]_i$ = 171 mM (determined for crayfish muscle)

$[K]_o$ = 5.3 mM (Saline)

$[Na]_i$ = 17.4 mM (assume for muscle)

$[Na]_o$ = 205 mM (Saline)

$[Cl]_i$ = 12.7 mM (assume for muscle)

$[Cl]_o$ = 232.15 mM (assume from saline; 205 mM NaCl; 5.3 mM KCl; 13.5 mM $CaCl_2 2H_2O$; 2.45 mM $MgCl_2\,6H_2O$)

pK = 1 (assume for muscle)

pNa = 0.001 (assume for muscle)

pCl = 0.01 (assume for muscle)

Use the on-line simulator from

Online https://www.physiologyweb.com/calculators/ghk_equation_calculator.html
(note: values of temperature are in K which is 273.15 + the # in centigrade)

Calculate an RP value (Vm) for the variables listed above from the online simulator (5.3 mM for $[K]_o$ and using 21°C). Now change pK to values by increasing or decreasing the values so you have an understanding how it alters the Vm

	pK	**Vm**
1st trail with the data given	1	?
2nd trial - Play		
3rd trial - Play		
4th trial - Play		

2. WET LAB PROTOCOL

A video of the following lab procedure is available online at:

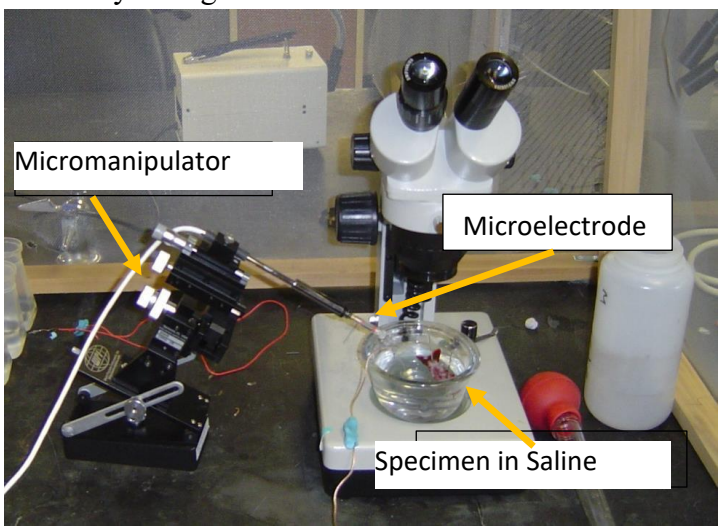
https://www.youtube.com/watch?v=24IvVf9e48s&feature=youtu.be

Some the equipment will be slightly different from what is shown in the movie and we will go over the details prior to conducting the experiments

2.1 Setting up the Specimen Dish and Micro-capillary Electrode

Your Lab instructor will demonstrate how to dissect the crayfish.

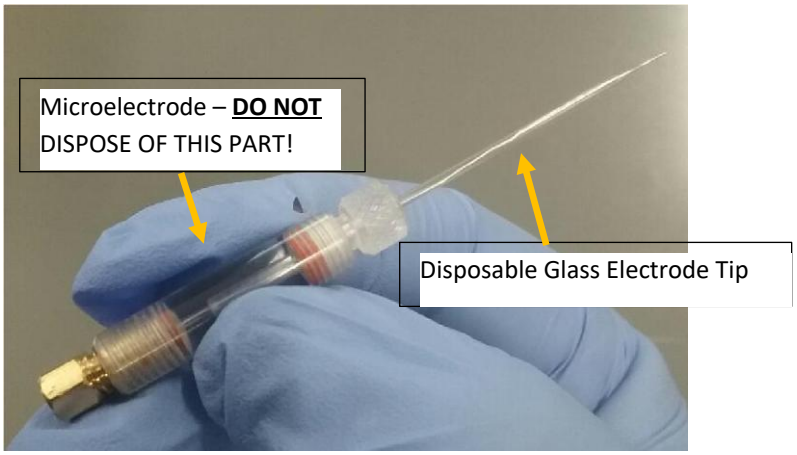
Have one member from your group dissect the crayfish, while the other members of your group get the software and equipment ready to begin.

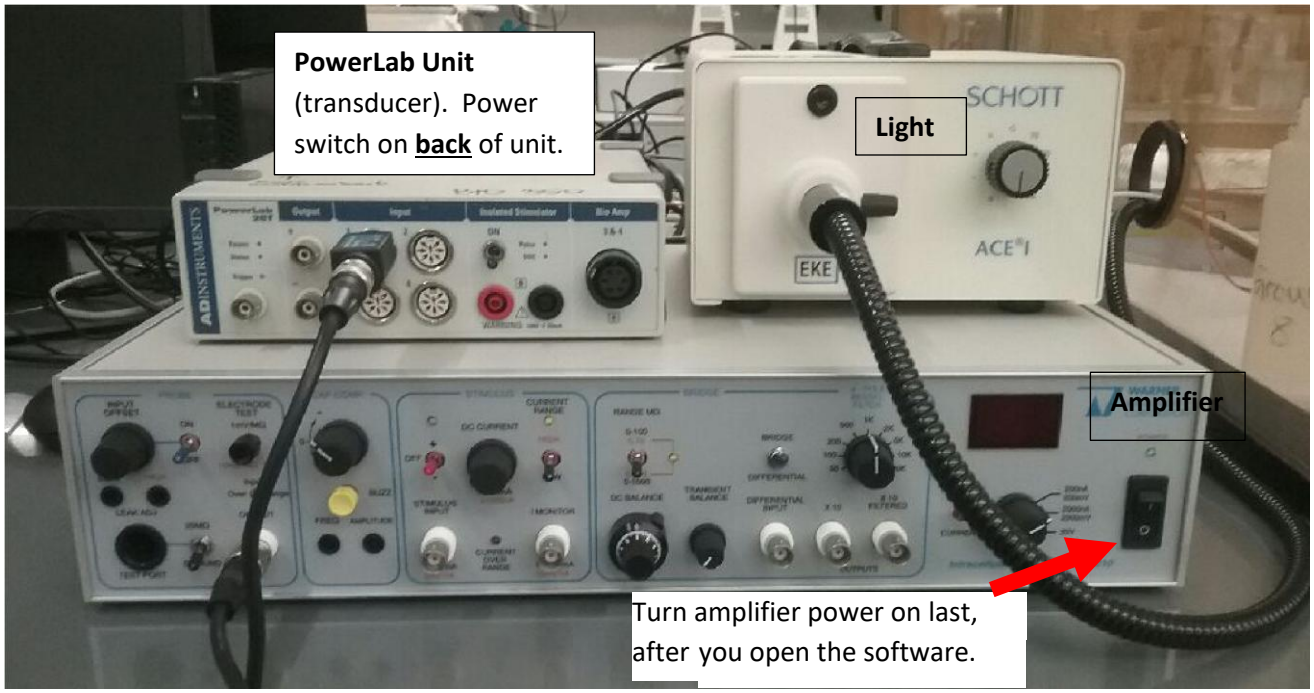


1. Use dissection pins to secure the crayfish preparation to the Petri dish. For ease if electrode insertion, role the crayfish preparation slightly onto its side (do not pin it down flat on its back, since this makes it difficult to angle the electrode tip in from the side and into the muscle). The top and bottom corners of the preparation should be pinned down to the dish and angled away from the prep so the electrode will not likely touch the pins and short out.
2. Chilled crayfish saline solution should be poured into the Petri dish and cover the preparation completely after the preparation has been pinned down.
3. The specimen dish with preparation should be on the platform of the microscope and secured with poster tack beneath the dish to prevent movement. DO NOT PLUG THE MICROSCROPE IN
4. Two wires each with a short length of silver wire attached to one end should be available in your Faraday cage. These are the ground wires. Carefully bend the wire and position them so that the silver tips are submerged in the saline bath. This should already be done.
5. A fine glass pipette should be obtained and carefully backfilled with 3 M KCl solution. There is are several syringes located on the side counter, each with long flexible fibers for this purpose. The tip of the pipette should be turned upwards (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. Turn the pipette upright when finished filling with potassium chloride solution. Check the pipette to ensure no air bubbles are present at the tip. The TA will demonstrate how to prepare your electrode.

6. Back at your station, there is a microcapillary electrode holder held in place on the black micromanipulator. A silver wire is hanging out of the end of the microcapillary holder. Carefully insert the silver wire into the pipette, sliding the microcapillary down into the barrel of the holder, then tighten the plastic screw cap of the holder to hold it in place.
7. Care should be made not to break the electrode tip.

CAUTION: DO NOT PUT YOUR HAND IN FRONT OF, OR AROUND THE GLASS OF THE ELECTRODE. THE ELECTRODE TIP IS EXTREMELY FRAGILE AND CAN PIERCE THROUGH FLESH VERY EASILY.





2.2 **Software Set-up**

1. Power up your equipment and software in the following order: Turn on your ADI PowerLab (power switch is on back side of the unit!). A small green light should be present on the front of the PowerLab unit if the equipment has been powered up correctly. Next, open the LabChart software. Do not turn on the amplifier, yet. Be sure the ADI PowerLab is turned on before opening the software!
2. Open the **LabChart software** by clicking on the icon on your desktop. Close the LabChart "Welcome center" window if it pops up. Adjust the chart to display only one channel by clicking "Setup", then "Channel settings." Under "Channel settings," change the number of channels (at bottom of the window) to one. Click "OK."
3. At the top of the chart, right hand corner, cycles per second should be 2K/s. Set volts (y- axis) to around 1V (underneath the 2K/s).
4. Click on "Channel 1" on the menu at the right side of the screen. From the drop-down menu, click "Input Amplifier" and check that the following settings are selected:

AC-Coupled	Unchecked
Anti-alias	Checked
Invert	Unchecked

5. Finally, turn on the amplifier by switching on the power switch located in the lower righthand corner on the front (see image). Check the PowerLab lights to ensure they are still green. Your equipment and software are now ready to use. Wait for your lab partner to bring the crayfish prep back to your station before continuing.

2.3 Preparation/Dissection:

To obtain the experimental data, we will measure in muscle fibers from a freshly dissected crayfish with sharp intracellular electrodes while changing the surrounding environment. Crayfish are used as the experimental organism as they survive for long periods of time with minimal saline composition.

https://www.youtube.com/watch?v=wveHR8jKTac **Only up to 9.26 TIME** (will not be stimulating the motor nerves.) This shows dissection and how electrodes are made.

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.

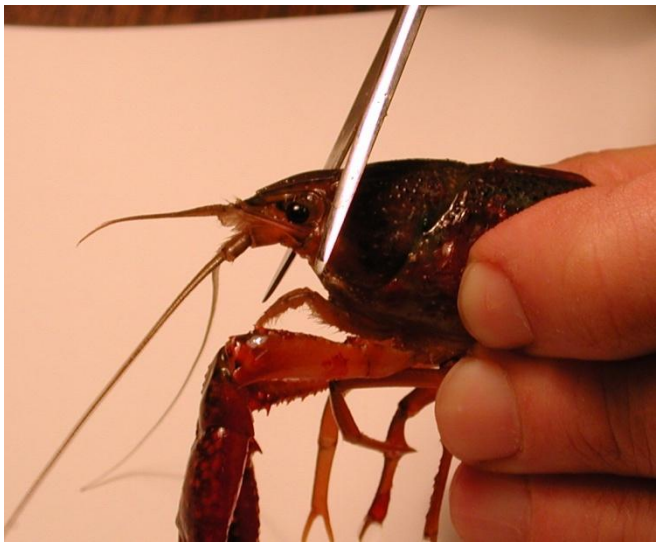


Figure 2: Image shows placement of the cut to remove the head of the crayfish.

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 3). Next, separate the abdomen from the thorax. Make a cut along the articulating membrane, which joins the abdomen and thorax (Figure 4). Save the abdomen portion of the crayfish and dispose of the thorax.

Figure 3: The scissors are cutting the stylets. These can be removed from the crayfish.

Figure 4: Image shows the placement of the cut to remove the thorax from the abdomen.

Figure 5: 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 6: 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 slighting 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 7).

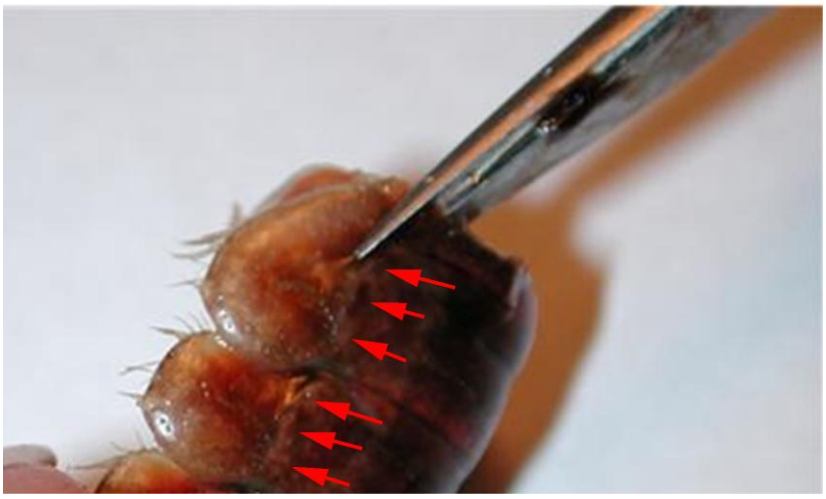


Figure 7: Scissors are placed at an angle and follow the natural alignment of the shell. Do not cut too deep and destroy the preparation. The arrowheads point to the natural line along each segment that should be followed for the cuts.

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 8: 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.

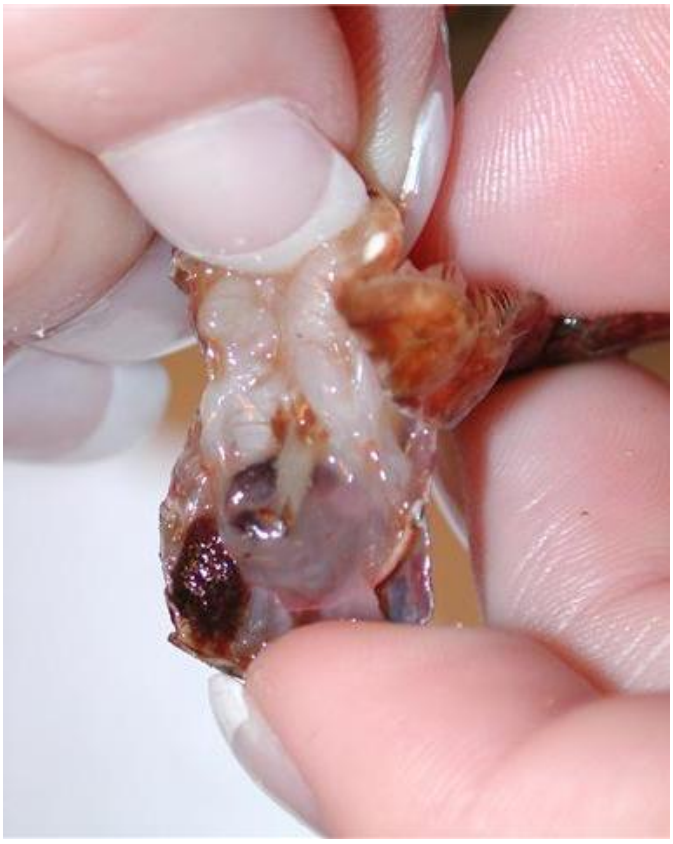


Figure 9: An image of the crayfish abdomen, after cut along each lateral line, allowing the abdominal flesh and some of the muscle groups to be peeled away. Pulling back on the ventral portion of the shell, which is to be discarded.

Figure 10: 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 11: 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 $CaCl_2$; $2H_2O$; 2.45 $MgCl_2$; $6H_2O$; 5 HEPES and adjusted to pH 7.4 (in mM).

2.4 Equipment Positions

1. When the crayfish tail/dish is ready, position it on top of the microscope base and push several small pieces of blue surgical wax around the base of the dish. This will keep it from moving while measurements are being made.
2. Next, position the micromanipulator so that the base is at the side of the dish. Use the knobs on the micromanipulator to adjust the angle and position of the glass electrode so that is can stab the muscle cells of the crayfish prep. Your TA will demonstrate this.

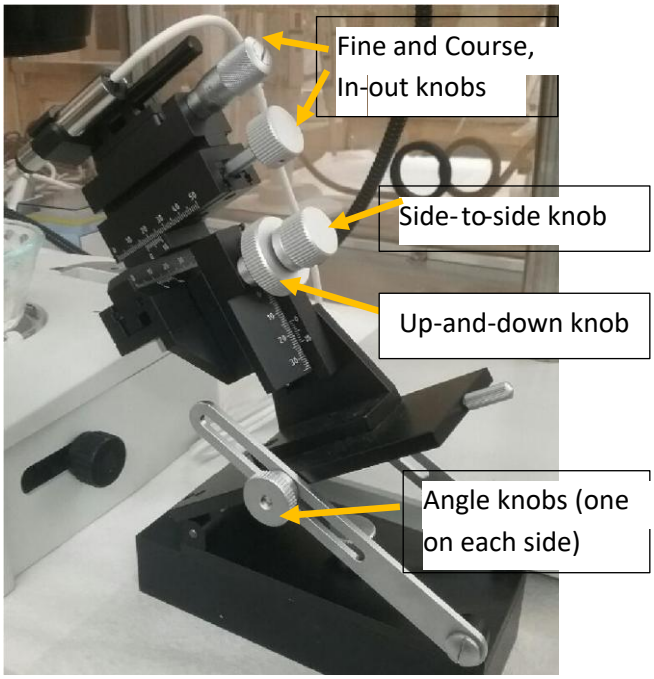
Amplifier and Electrode Tip Checks

1. With the tip of the electrode in the crayfish saline, turn the amplifier on (see image above). Note – if the tip is not in the saline, the amplifier will sound an alarm.
2. Zero the digital read-out on the amplifier by turning the "Input Offset" knob on the front-left side.
3. Next, check that your tip is the right diameter (not too large or broken, and not too small or plugged) by running a resistance check. To measure the resistance of your electrode tip (how difficult it is to send current through the tip), place the tip of the glass electrode into the saline bath. Make sure a ground wire is also in the saline bath.
4. Press and hold down the "Electrode Test" button in the front-left side of front). The resistance of your electrode tip should be between 10 and 40 MegaOhms.
    a. If resistance is too high, the tip may be clogged or sealed closed. Is so, remove the glass tip and replace it with a new one. Do not throw out the electode itself!
    b. If resistance is too low, it indicates the tip of your probe is probably broken. If so, remove the glass tip and replace it with a new one. Do not throw out the electode itself!
    c. Each time the tip is replaced, or as your measurements progress, check the resistance again. You will want to continue to check the resistance of the tip throughout today's lab. If you observe a substantial decrease in the resistance, then change the tip out with a new one, and dispose of the broken tip in a broken glass box. It is normal for the tip to chip away after repeated uses.

Additional Resistance Check Troubleshooting Tips:

1. *Check the electrode to ensure there is adequate saline within the shaft of the electrode tip and no large air bubbles are present between the inner wire and the tip.*
2. *Check your prep to ensure both the electrode is properly positioned, and the abdominal prep is still submerged in the saline.*
3. *Check to ensure the electrode tip is completely immersed in the saline (not in air).*
4. *Check that the end of the ground wire is in the saline bath.*
5. *Check to ensure no bare electrode wires are touching any metal surfaces.*
6. *Check to ensure the glass tip was filled with 3 M KCl (not crayfish saline which is much more dilute)*
7. *If you are still having trouble, ask the lab instructor to help you*

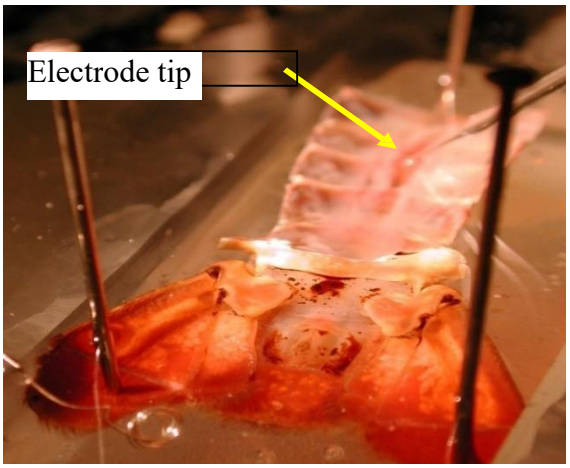
5. Once the electrode resistance has been verified, SET YOUR EXTRACELLULAR VOLTAGE TO ZERO. Begin recording by pressing "start" at the bottom of the screen. Use the DC offset knob on the amplifier to adjust the recording trace to zero (or close to it) before inserting the electrode into the tissue. This sets your extracellular voltage to zero. If it is off a little bit, that's ok. You will be taking the difference in voltage changes for your measurements.



6. Use the micromanipulator and dissecting scope to insert the microelectrode tip into the longitudinal muscles of the preparation as indicated by your lab instructor. **Do not insert the electrotype into the muscle physically moving the micro manipulator instead** position
the tip close to the muscle, then use the knobs on the micromanipulator to pierce the tissues (in-out, and side-to-side knobs). The electrode should barely be inserted into the muscle. You will likely see the muscle dimple as the electrode penetrates. Do not penetrate completely ***through*** the muscle since this can push the tip against the shell (exoskeleton) and break the tip. The high intensity illuminator (light) should be adjusted to clearly see the muscle as the electrode is being inserted. Turn the light down (not off) when you are not needing it, so it will not kill the preparation.

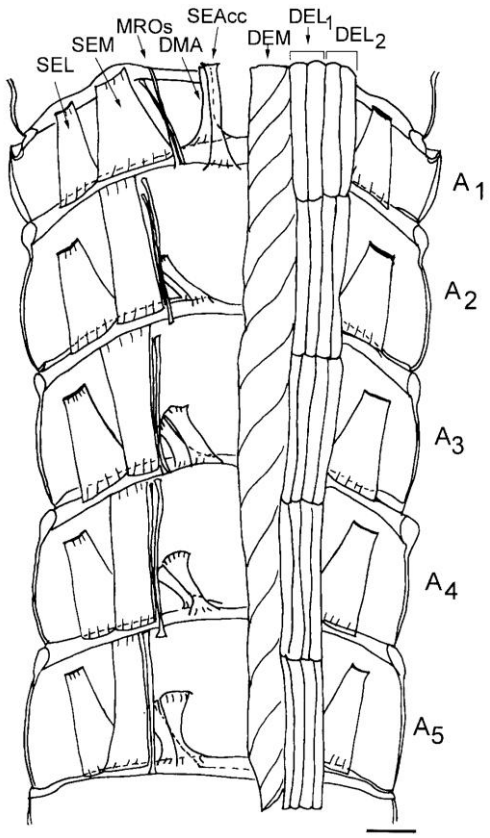
The specimen dish with preparation should be placed under the microscope and secured with poster tack beneath the dish to prevent movement.

7. Troubleshooting: When poking muscle fibers in this preparation one can commonly run into spaces and clefts within the muscle. This is one reason why the membrane potential can appear, then disappear, and then reappear.



Insertion of electrode into the muscle.

Use the micromanipulator and dissecting scope to insert the microelectrode tip into the longitudinal muscles (DEM or DEL1 or DEL2) of the preparation (see Figure below). The electrode should barely be inserted into the muscle. You will likely see the muscle dimple as the electrode penetrates. Do not penetrate completely ***through*** the muscle. The high intensity illuminator should be adjusted to clearly see the muscle as the electrode is being inserted.



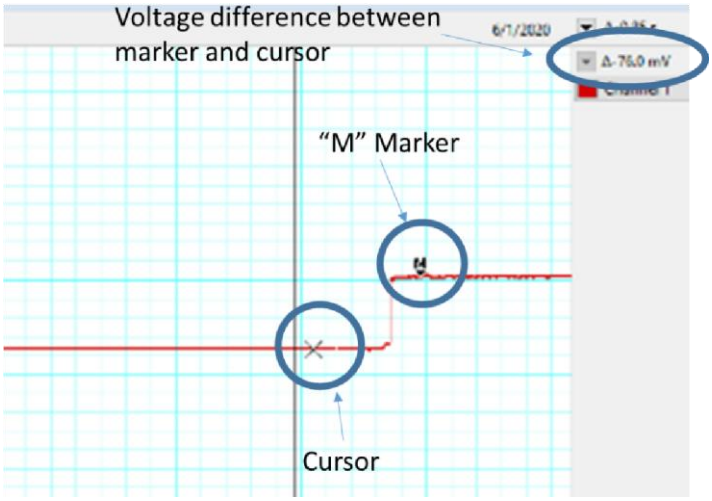
Schematic drawing from a ventral view of the dorsal part of the crayfish abdomen showing the extensor musculature of each segment. The dorsal membrane abdomen muscle (DMA) and the superficial extensor accessory muscle head (SEAcc) occur in segments 1 through 5 of the abdomen with a different orientation 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).

The difference in the computer recorded values might need to be adjusted to account for any amplification used on the amplifier (i.e. 10X amplification). The voltage should be converted to millivolts if the values are reported on the software as volts (1 V = 1,000mV).

2.4 Experiment: What is the effect of changing extracellular saline with LPS or the compound you are using on the resting membrane potentials of crayfish skeletal muscle cells?

1. You are now ready to begin collecting data for your experiment. Assign one group member to manipulate the electrode with the micromanipulator. Assign a second group member to operate the software and observe the voltage responses (recording) being detected by the electrode.
2. To measure the membrane potential, ensure the digital read-out on the amplifier is still at zero. If not, adjust the "input offset" knob on the amplifier to zero before inserting the electrode.
3. Place the normal crayfish saline in the petri dish first and fully cover your preparation. Completely fill the bowl to keep the tissues alive and help buffer the saline temperature.
4. To poke a muscle fiber, slowly use the dials move the tip of the electrode against a muscle cell membrane at about a 90^{o} angle. You will see the surface of the muscle begin to dimple. At this point, STOP! If one continues to force the electrode further into the muscle, it is likely to slice straight through, hit the exoskeleton of the crayfish on the dorsal side, and break the tip off.
5. Once the dimple is visible on the surface of the muscle where the tip is, the group member manning the computer should be simultaneously watching the recording for the voltage to change.
    a. *A decrease in voltage to a new, lower but stable voltage usually indicates the electrode tip has punctured the cell membrane.*
    b. *A brief decrease in voltage that is unstable or returns to the previous resting level indicates the electrode tip may be only partially through the membrane, or may have slipped back out. If this is the case, have the group member manning the manipulator <u>gently</u> tap the top knob of the micromanipulator. Often, this gentle movement will be enough to drive the electrode the rest of the way through the cell's membrane.*
    c. *If no decrease in voltage is observed while the electrode is inserted into the muscle, check the resistance of your electrode to ensure the electrode tip is still intact.*
        i. *If no resistance is detected, the tip has likely been broken off and the electrode should be changed. This is the most common problem.*
        ii. *If the resistance is unusually high, it may indicate tissue is clogging the tip. If a clogged tip is suspected, withdraw the electrode tip and try the insertion again. If the resistance is still high, change out the electrode tip.*

2.5 Measuring the responses on the recordings



Find the portion of the recording where the electrode was removed from the muscle cell after a successful insertion. If you are doing this as you go through the in-person portion of the lab, you can measure the difference after each successful insertion event. If you are doing this lab online using a complete recording, start at the beginning of the recording with the control saline (5.3 mM) treatment conditions, and work your way through the recording treatment tested.

1. To determine the membrane potential associated with each insertion event, you will use the marker and cursor to measure the difference on the recording when the electrode was outside the cell versus inside the cell.
2. First, click and drag the marker to the extracellular region of the recording immediately after removing the electrode from the muscle. Release the marker onto the recording.

3. Next, use move the computer mouse so that the cursor (not the marker) follows the recording and trace backwards to the point where the electrode was still in the muscle.

4. Measure the amplitude (difference) of the resulting voltage (mV) between the Marker (extracellular reading) and cursor (intracellular reading). The difference indicated by the marker and the active cursor is displayed on the upper-right side of the screen. This value is the voltage. Note, if the observed voltage decreased when the electrode tip was inserted (compared to resting/outside voltage), the voltage difference should be reported as a negative value.

5. Record the value into Table 1. DATA!

Obtain an RP value (Vm) for the results you obtained from intracellular recording in saline. To reach the empirical value obtained prior to exposure of LPS and modify the pK values again to obtain as close as possible to the values you obtained with the exposure of LPS if your membrane potential showed and alteration.

6. VERY IMPORTANT RECORD A STABLE BASELINE FOR at least 10 minutes. Hit the stop record and make a note on the Chart software file.

7. To change the bathing solution, use a syringe or pipette to remove and discard the saline solution from the Petri dish. The Petri dish should be filled with the next treatment saline, covering the preparation completely. The same process should be repeated with each solution and the changes in voltage/potential should be noted and recorded.

AFTER carefully changing the bath VERY IMPORTANT RECORD for at least 10 minutes. Hit the stop record and make a note on the Chart software file.
SAVE YOUR FILE TO YOUR DESKTOP WITH YOUR INTIALS (Yours and partners)

The series of crayfish saline solutions used this semester will be noted and data worksheet.

Obtain an RP value (Vm) for the results you obtained from intracellular recording in saline. To reach the empirical value obtained prior to exposure of LPS and modify the pK values again to obtain as close as possible to the values you obtained with the exposure of LPS if your membrane potential showed and alteration.

	mV	**estimated pK**
RP in initial saline		
RP during exposure to LPS What saline mixture did you use ? (write it here)		
Differences		

2.6 Analysis in the occurrence of quantal events

https://www.youtube.com/watch?v=zvvvoCcclI0 **General idea of synaptic transmission with quantal responses**

https://www.youtube.com/watch?v=PTdzlhM4dx4 **(only watch 5:27 to 5:47)**

to understand how to use lab chart.

Here is a sample in how to analyze a data set. https://youtu.be/3j55G20_rcw

This protocol is to record the traces over time for spontaneous quantal events within each 30 seconds. So, bins of 30 seconds.

Fill in the table:

10 minutes of baseline, 10 minutes after changing the bathing saline. Use 30 sec bins.

Table: # of minis every 30 seconds

Time		# of minis	Time	# of minis	CHANGE Saline	Time	# of minis	Time	# of minis
30s			5.5			30s		5.5	
1 min			6			1 min		6	
1.5			6.5			1.5		6.5	
2 min			7			2 min		7	
2.5			7.5			2.5		7.5	
3 min			8			3 min		8	
3.5			8.5			3.5		8.5	
4			9			4		9	
4.5			9.5			4.5		9.5	
5			10			5		10	

You can make a graph Y axis -the number of events and X-axis over time (each 30 sec bin).

Then let's think how we might gather everyone's data and uses statistics. We can discuss this topic, but you should think about it.

Some groups might just have had preparations exposed to LPS to determine if there's a change in minis (frequency) and membrane potential. While others might have had preparations where LPS is added, but the saline includes Cd2+ to block voltage-gated calcium channels (Cd2+ + LPS). The third group had preparations incubated in saline with Cd2+ and Thapsigargin for 20 minutes and then exposed to LPS (Cd2+ + Thapsigargin → add LPS).

DISCUSSION AND THOUGHT PROBLEMS

We will have to wait and see the results before speculating if there were any changes in the membrane potential as well as the number of occurrences in the spontaneous quantal events.

Please provide some description of the issues you had in conducting measure of the membrane potential and obtaining spontaneous quantal events. Also, please provide your insightful thought on the analysis and on the accuracy of the analysis.

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: 5.3mM (normal saline)
- 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

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Glossary: common biological terms and definitions (used in this proposal)

These terms appear throughout the proposal. Definitions are written for quick reference and BIO 350 readiness.

Immune / clinical context

- **Gram-negative bacteria**: A group of bacteria with an outer membrane containing LPS; infections can trigger strong inflammatory responses.
- **Septicemia (sepsis)**: A severe, body-wide response to infection that can involve inflammation, altered organ function, and changes in barrier tissues.
- **Lipopolysaccharide (LPS)**: A large molecule in the outer membrane of Gram-negative bacteria; can trigger immune responses and, in some models, rapid effects on excitable tissues.
- **Cytokines**: Small signaling proteins released by immune cells that coordinate inflammation and immune responses.
- **Blood–brain barrier (BBB)**: A selective barrier that limits what substances can enter the CNS from the bloodstream.

Nervous system organization

- **Central nervous system (CNS)**: Brain and spinal cord; protected by the BBB.
- **Peripheral nervous system (PNS)**: Nerves outside the brain/spinal cord; peripheral synapses may be exposed to circulating factors earlier than CNS synapses.
- **Hemolymph**: The circulating fluid in many invertebrates (functionally similar to blood) that can contain signaling molecules.

Synapses and NMJ basics

- **Synapse**: A junction where one cell communicates with another (often neuron → neuron or neuron → muscle).
- **Neuromuscular junction (NMJ)**: A synapse between a motor neuron and a muscle fiber.
- **Motor neuron**: A neuron that controls muscle contraction by releasing neurotransmitter onto muscle.
- **Glutamatergic synapse**: A synapse that uses glutamate as its main neurotransmitter (common at arthropod NMJs).
- **Neurotransmitter**: A chemical released from the presynaptic terminal that binds receptors on the postsynaptic cell to change membrane potential.

Pre- vs. post-synaptic terms

- **Presynaptic**: The "sending" side of a synapse (motor nerve terminal).
- **Postsynaptic**: The "receiving" side (muscle fiber membrane).
- **Synaptic terminal / nerve terminal**: The end region of an axon that contains synaptic vesicles and releases transmitter.
- **Varicosity**: A swollen region along a terminal where release machinery is concentrated.

Electrical signals at synapses

- **Evoked synaptic transmission**: Transmitter release triggered by intentionally stimulating the nerve.
- **Spontaneous synaptic transmission**: Transmitter release that occurs without intentional stimulation.
- **Quantal event / quantal response**: The postsynaptic response produced by release from a single synaptic vesicle (a "unit" of release).
- **Miniature EPSP (mEPSP) / "mini"**: A small spontaneous EPSP produced by one vesicle release event.
- **EPSP (excitatory postsynaptic potential)**: A depolarization in the postsynaptic cell that increases excitation.
- **Mini frequency**: How often spontaneous quantal events occur (events per unit time); used here as a readout of presynaptic release mechanisms.
- **Mini amplitude**: The size of each spontaneous event; often reflects postsynaptic receptor sensitivity and/or vesicle content.

Phasic vs. tonic synapses (core concept here)

- **Phasic NMJ / high-output terminal**: Produces large responses at low stimulation frequencies; often fires brief bursts.
- **Tonic NMJ / low-output terminal**: Produces small responses at low frequencies but shows strong facilitation as frequency increases; often more active over time.

- **Facilitation**: A short-term increase in synaptic strength during repeated stimulation, often due to residual presynaptic Ca2+.
- **Mixed phenotype preparation**: A preparation where the same muscle fiber is innervated by both phasic- and tonic-like motor neurons, enabling comparisons.

Ions and calcium signaling

- **Ion**: A charged atom/molecule (e.g., Na+, K+, Ca2+, Cl−) that carries electrical current across membranes.
- **Depolarization**: Membrane potential becomes less negative (more positive).
- **Hyperpolarization**: Membrane potential becomes more negative.
- **Voltage-gated calcium channels**: Channels that open with depolarization to allow Ca2+ influx—often a key trigger for vesicle fusion.
- **Calcium influx**: Entry of Ca2+ from the extracellular fluid into the presynaptic terminal.
- **Intracellular Ca2+ stores**: Calcium stored inside organelles (especially the ER) that can be released to influence cellular processes.
- **Endoplasmic reticulum (ER)**: Internal membrane network that stores Ca2+ and participates in calcium signaling.
- **Ca2+ pump into ER (SERCA)**: The pump that loads Ca2+ into the ER; blocking it depletes internal stores over time.

Pharmacological tools mentioned

- **Cadmium (Cd2+)**: Commonly used to block voltage-gated calcium channels, reducing Ca2+-dependent transmitter release.
- **Thapsigargin**: Inhibits the ER Ca2+ pump (SERCA); with incubation it depletes ER Ca2+ stores and reduces store-based Ca2+ release.

Experimental design and rigor terms

- **Preparation (“prep”)**: The dissected, maintained tissue used for recording (e.g., a specific crayfish muscle with intact innervation).
- **Incubation / pre-incubation**: Exposing a preparation to a drug/condition for a set time before testing the main manipulation.
- **Physiological saline**: A salt solution designed to mimic extracellular conditions and keep tissues functioning during experiments.
- **Control**: A comparison condition used to isolate the effect of the variable being tested.
- **Variable**: The factor intentionally changed (here, LPS presence/absence and blocker conditions).
- **Standardization**: Keeping protocols identical across student groups to reduce variability and improve publishability.
- **Sample size (n)**: Number of independent preps/animals/recordings used per condition.
- **Reproducibility**: Obtaining similar outcomes when the same experiment is repeated across days/groups/setups.