

# Examining Motor and Sensory Units as an Educational Model for Understanding the Impact of Localized Tissue Injury on Healthy Cells

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This series of laboratory modules uses common neurophysiological teaching exercises which are modified to provide a focus on a medical related physiological topic. The scenario is theoretically related to what could be encountered with traumatic skeletal muscle injury. The testable hypothesis is that skeletal muscle injury raises extracellular K<sup>+</sup> concentration, and other constituents due to the lysing of the injured cells, which impacts healthy muscle, sensory and motor neurons in the vicinity. The primary sensory neurons of the crayfish muscle receptor organ (MRO) and neuromuscular preparations of the crayfish are used as models to address this topic. The change in the neural activity of the MRO, the resting membrane potential of skeletal muscle and evoked synaptic transmission at the neuromuscular junction are used as indices to assess the effect of raised extracellular K<sup>+</sup> and exposure to homogenized muscle tissue.

**Keywords:** Electrophysiology, neuromuscular junction, physiology, neurobiology, cell injury, CURE

## Introduction

The theme in the effects related to deep tissue injuries (DTIs) of skeletal muscle was divided into three modules in the ABLE workshop. Each of these modules is presented herein as independent laboratory exercises. One module relates to the effect on primary sensory neurons of the crayfish muscle receptor organ (MRO) in the abdomen which is responsible for proprioception. The second module is related to the effects on the resting membrane potential of cells due to a surrounding injury and the third module is on the effect of synaptic transmission at the neuromuscular junction (NMJs) around a skeletal muscle injury site. The invertebrate crayfish is used for all three modules as it is a common organism to use for college level courses teaching of neurophysiological principles as well

as there are fewer regulations in animal use protocols since they are invertebrates (Johnson *et al.* 2014).

The modules are left open ended in several ways, so instructors and students can modify them to tackle new questions. The testable hypothesis is that skeletal muscle injury raises extracellular K<sup>+</sup> concentration, due to the lysing of the injured cells, which impacts healthy muscle as well as the healthy sensory and motor neurons in the vicinity. The primary sensory neurons of the crayfish muscle receptor organ (MRO) and the skeletal muscle of the crayfish are used as models to address this topic. The change in the neural activity of the MRO while displacing the joint or holding the joint in a static position is used as an index to assess the effect of raised K<sup>+</sup> in the bath as well as exposure to homogenized muscle tissue. The resting membrane potential of skeletal muscle and evoked synaptic transmission at the neuromuscular junction is also

used for indexing the same conditions as the MRO. The sensory activity, resting membrane potential as well as synaptic responses are dampened by raised  $K^+$  in the bath and when exposed to muscle homogenate. These educational modules can also help establish another animal model to explore the potential effects of deep tissue injury of skeletal muscle as well as other tissues, such as brain (Hartings *et al.* 2017), in urgent care centers for mammals.

The theme of a potential medical scenario or a topic in which a student can relate to, as a possible situation they could find themselves facing, provides a practical tie in conducting the laboratory exercises. And by allowing the students to develop novel modifications to the experimental design allows the students to be independent thinkers. Having the hands-on experiences and to have a role in the designing, implementing and re-designing protocols helps students develop and apply scientific practices (Coll *et al.* 2005). This course project is part of a new trend in teaching science to undergraduates (Linn *et al.* 2015). Course-based undergraduate research experiences (CUREs) are relatively new and is an approach being adopted by science educators in high schools and colleges (Bakshi *et al.* 2016). We refer to these CUREs as Authentic Undergraduate Research Experience (ACUREs) as these allow novel projects to be developed with a goal in publishing the findings.

The three modules are extracts of various other laboratory exercises but combined here in the theme of a DTI and the associated effects on neighboring healthy sensory neurons, motor neurons and skeletal muscle. The effects of raised  $K^+$  in the bath on the activity profile of primary sensory neurons for proprioceptors in the walking legs of a crab as well as the MRO of crayfish was previously described (Malloy *et al.* 2017). The effect of raised  $K^+$  on the resting membrane potential is related to the concepts to determine the equilibrium potential of an ion and the mechanism driving the resting membrane potential. The fundamentals are used to explore how the Nernst equation and the Goldman-Hodgkin-Katz (G-H-K) equation can be used to explain the biological phenomenon. The concept of raised  $K^+$  affecting the resting membrane potential is covered in an exercise developed for college level students taking a physiology course using the same crayfish model as presented herein (Baierlein *et al.* 2011). The consequences of raised extracellular  $K^+$  ions from a DTI in altering synaptic responses at the NMJs of the crayfish model has recently been detailed in Thenappan *et al.* (2018). Combining the main content and shortened protocols of the previous exercises to cover the same salient features in the learning outcomes is the goal of these modules which were presented in the ABLE workshop.

The dissection and extracellular recording of electrical activity from the MRO is relatively easy to accomplish and has been obtained for comparative physiology courses in many university settings in the USA as well as other countries. If the dissection is done for the

students, then the electrical recordings with each saline bath change of the various solutions is rapid. If the data can be stored and analyzed later this will save wet lab experimental time. If the data is to be obtained and analyzed in one laboratory setting this module will take about 2 hours.

If intracellular recordings are already being utilized and training on the instrumentation is not necessary, the protocol of measuring resting membrane potential with exchanging the bathing media for saline with raised  $K^+$  concentration or with muscle homogenate is relatively quick. Recording the resting membrane potential at each experimental condition of switching the bath and repeating the measure of the potential takes about 5 minutes. Thus, collecting the data, graphing the data and having some discussion on the topic takes about 1 and 1/2 hours.

The time and difficulty of the third module in measuring synaptic responses depends on which preparation is used and if the instructor provides the dissected preparations for the students. The phasic abdomen muscles (DEL1 and DEL2) are quick to expose (10 minutes); however, the tonic SEL muscle is prone to damage if one is not careful in the dissection of removing the ventral aspect of the abdomen. If the intracellular instrumentation and general procedures are already mastered by the students, then setting up a stimulating suction electrode for the motor nerve is quick and responses will be able to be obtained within 20 minutes if the tissue preparations are provided. The stimulating electrodes are easily put into place to stimulate the motor nerve but sometimes obtaining an intracellular recording and managing not to lose the placement of the electrode with a twitching muscle fiber can be a challenge. The muscles to be used need to be stretched to avoid muscle movements during the stimulation. The tips of the microelectrodes also will break if impaled in a muscle which undergoes a large twitch contraction. Thus, the 3 M KCl within the microelectrode can spill out and damage the tissue for the recordings. In past experiences, this lab requires about 2-3 hours for students who have already completed the previous two modules in which they learned to use suction electrodes for recording the extracellular activity and mastered intracellular recording for obtaining the membrane potentials. Once the students have obtained a synaptic recording changing the bathing medium to the various ones of varying  $K^+$  concentrations or with the muscle homogenate needs very careful attention as not to displace the recording or one can remove the electrode and impale the fiber again after changing the bathing medium.

*Learning Objectives:*

(i) Module One: MRO of the Crayfish

1. Students will learn the associated anatomy and physiology of the MRO.

2. Students will learn how to monitor neuronal activity with extracellular recordings and to use common electrophysiological equipment.

3. Students will learn the effect of raised extracellular  $K^+$  and muscle homogenate on the activity of primary sensory neurons in relation to DTIs.

4. Students will learn to graph and interpret the data obtained based on the sensory stimulation provided. The sensory stimulation will range from static positions as well as dynamic movements of the segment being monitored.

(ii) Module Two: Resting Membrane Potential

5. Students will learn about physiological conditions of membrane potential.

6. Students will learn terminology and meaning of the terms related to membrane potential (depolarization,

hyperpolarization, resting membrane potential, voltage gated channels, passive leak channels, permeability)

7. Students will learn the meaning and how to use the Nernst Equation and Goldman Hodgkin Katz equation.

(iii) Module Three: Synaptic Transmission

8. Students will learn to record synaptic responses at NMJs of a crayfish motor unit preparation.

9. Students will learn how the resting membrane potential influences the amplitude of the evoked excitatory postsynaptic potentials (EPSPs).

10. Student will learn the differences in the effects of a muscle homogenate as compared to raised extracellular  $K^+$  ions on the amplitude of the EPSPs.

## Student Outline

The overall theme of these three modules is to aid in understanding how the spread of injury can damage healthy cells in the vicinity of the injury and elsewhere. The general outcomes can be applied to the central nervous system as well as skeletal muscle. For the ease in experimentation in student laboratories the focus is to use the neuromuscular junction and associated sensory neurons of the crayfish as physiological models.

Throughout conducting these experiments think of the following scenario. Imagine if one was injured in which the large skeletal muscles in one's thigh are smashed against the bone (i.e. femur) but the skin is not broken. With this mind set, imagine how the cells of the surrounding tissues which were not initially injured will respond over time. There are many variables which can be addressed such as ischemia, ischemic reperfusion injury, impaired lymph drainage, alteration in interstitial fluid flow, alteration in capillary wall permeability, edema, and inflammatory changes (Honaker *et al.* 2013; Thenappan *et al.* 2018). Also, consider the impact of the intracellular constituents of the damaged muscle cells spreading to the localized healthy tissue and the rest of the body. The higher concentration of free  $K^+$  ions, amino acids and proteins increasing rapidly in the extracellular fluid can have a multitude of consequences. As part of this exercise, list a series of potential impacts and how one might test the consequences with experimental procedures.

Likely, you came up with effects on skeletal muscle, sensory neurons associated with skeletal muscle such as proprioceptors in the muscle spindles as well as motor neurons and the potential impact on synaptic transmission. So now, you need to think how the effects can be monitored in the tissue of interest and what variables to examine. If one considers the ionic differences between extracellular and intracellular fluids, then the rise of  $K^+$  ions from the damaged cells might be a factor to consider. So various concentrations of  $K^+$  could be examined for the physiological effects on the tissues of interest. In addition, the rise in amino acids and proteins as well as other intracellular constituents one might want to examine. Instead of trying to expose the tissue to the many individual intracellular components possible, an approach might be to examine the effect of various dilutions of skeletal muscle homogenate. The results in the two approaches of varying the  $K^+$  concentration and muscle homogenate can be compared.

As early as the 1930's it has been known that damaged skin tissue causes sensory neurons to stop responding and this was speculated to be due to the raised  $K^+$  concentration (Feng, 1933). Since  $K^+$  ions can also move through potassium "leak" channels while a cell is in its resting state, cells are usually close to the equilibrium potential for potassium rather than for other ions. The resting membrane potential depends upon the potassium equilibrium potential so when extracellular  $K^+$  rises so will the resting membrane potential. It was shown in the leech nervous system that a rise in extracellular  $K^+$  ions from activity of cells (neurons) could depolarize the resting membrane potential in neighboring cells that were not initially electrically active (Baylor and Nicholls, 1969; Frankenhaeuser and Hodgkin, 1956; Orkand 1966). The depolarization in the surrounding cells in these cases is due to small changes in the extracellular  $K^+$  levels.

The crayfish abdominal extensor muscle preparation is used to demonstrate effects on resting membrane potential with ion substitution in saline and is a good preparation for demonstrating synaptic responses for different types of motor units. The topic of muscle phenotype can be examined as well as investigating if different types of motor units are affected differently by cytoplasm spillage of a DTI. 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. With the techniques obtained in these three modules one can proceed to answer many questions remaining in other experimental preparations as well as in physiological applications related to medicine and health. The modules presented will demonstrate the usefulness of invertebrate model preparations to address fundamental questions pertinent to all animals.

### *Background: MRO*

The consequence of a rise in extracellular  $K^+$  or exposure to a muscle homogenate on the function of sensory neurons associated with the skeletal muscle can be examined by measuring the activity of proprioceptors during various stimulations paradigms. Abnormal activity of proprioceptors in animals can result in the animal not knowing where body parts are or how they are moving in respect to the body. In humans, this could result in fall risks if leg proprioceptors are malfunctioning. Proprioceptors monitor not only the rate of movement but also static positions of limbs. The muscle receptor organ (MRO) is analogous to the mammalian muscle spindle and is found within the crayfish abdomen (Kuffler, 1954; Rydqvist *et al.* 2007). We utilized the crayfish MRO to examine both the effects of raised extracellular  $K^+$  ions ( $[K^+]_o$ ) and a saline mixed with a homogenate of crayfish skeletal muscle on the function of the MRO. The sensory endings of the MRO are embedded within the thick skeletal muscle fibres, which are also neurally innervated by motor neurons. This exercise is detailed in Malloy *et al.* (2017).

### *Methods: MRO*

The dissection as well as the recording procedures are shown in detail via video format (Leksrisawat *et al.* 2010). In brief, the dissected crayfish abdomen was placed in a Sylgard-lined dish filled with crayfish saline. The MRO was moved using

a wooden dowel from a relaxed position to a stretched position. An insect dissecting pin was used to mark the displacement range, and each displacement marked on the computer recorded file. The displacement rates can be varied by pushing on the joint to the end of the displacements range. We used a 1 second displacement rate. The displaced position can be held for different lengths of time. We used 10 seconds in a static position followed by withdrawing the wooden dowel for the joint to pull back into place (Dayaram *et al.* 2017). The crayfish saline to use is a modified Van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 CaCl<sub>2</sub>·2H<sub>2</sub>O, 2.45 MgCl<sub>2</sub>·6H<sub>2</sub>O, and 5 HEPES adjusted to pH 7.4).

Suction electrodes made from glass pipettes fitted with plastic tips were used to record extracellular signals from the cut nerves (details of making the suction electrodes is provided in Baierlein *et al.* 2011). A P-15 amplifier (Grass Instruments, Astro-Med West Warwick, Rhode Island, USA) in conjunction with a PowerLab/4s A/D converter and Lab Chart 7 software (ADI Instruments, Colorado Springs, CO, USA) was used to obtain the signals on a computer at a 10 or 20 kHz sampling rate. For this set of experiments, the frequency of extracellular spikes is used to assess the MRO function in the varied conditions. A plot of the activity before and after changing the conditions along with use of a Sign test is used to quantify if any significant effects are induced.

### *Background: Resting Membrane Potential*

The conceptual details for this this module are explained in Baierlein *et al.* (2011) and parts are reproduced herein. 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 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 ion to address for this module is potassium (K<sup>+</sup>). The quantity and movement of this ion across a muscle membrane influences the membrane potential. From this foundation, we can address electrical potentials observed during electrical excitation. One 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, 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<sup>+</sup> and K<sup>+</sup> across the membrane is maintained by the Na-K ATPase pump under physiological conditions. Under normal conditions the pump moves, on average, three Na<sup>+</sup> out of the cell and two K<sup>+</sup> 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 for sodium. The resting membrane potential can then be examined to see if it depends upon the potassium equilibrium potential and how skeletal muscle homogenate alters the potential.

### *Methods: Resting Membrane Potential*

The crayfish abdominal extensor muscle preparation in this protocol is used to demonstrate effects on resting membrane potential with ion substitution in saline. The dissection as well as the recording procedures are shown in detail via video format (Baierlein *et al.* 2011).

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 (Goldman, 1943; see Hille, 1992; Hodgkin and Huxley, 1952; Hodgkin *et al.* 1952; Hodgkin and Katz, 1949; Nernst, 1888, 1889).

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 [ $9.649 \times 10^4$  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, assume membrane potential is negative 70mV.

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

In the experiment one 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) one will plot the observed values to determine if there is a match with the hypothetical line. One should 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:

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

Plot the measures obtained for the resting membrane potentials at each  $[K^+]_{out}$  used using Excel. 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. (Download free graph paper if needed <http://incompetech.com/graphpaper/logarithmic/>)

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.

In new preparations repeat the procedures but this time with various dilutions of the muscle homogenate solution. If the muscle has an estimated 120 mM  $K^+$  does a 50% dilution with crayfish saline (made without adding KCl) of the muscle homogenate solution mimic the 60 mM  $K^+$  saline examined previously?

### *Background: Synaptic transmission*

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 to examine the impact of raised extracellular  $K^+$  or the effect of skeletal muscle homogenate. Some muscles in crustaceans are selectively innervated by either a phasic or a tonic motor neuron. 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. 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 and ion substitutions or effects of the muscle homogenate. The background content of this module is detailed at a greater depth in Thenappan *et al.* (2019).

*Methods: Synaptic Transmission*

These are the same muscles that were used in an earlier exercise to examine the effects of altering extracellular  $K^+$  on the resting membrane potential. Please refer to the previous protocols and references utilized in the earlier module. Details of the dissection in video format are provided in Baierlein *et al.* (2011) and in text format in Thenappan *et al.* (2019), both of which are open resource.

The procedure for exposing the deep abdominal preparation is described in several studies and in protocols (Baierlein *et al.* 2011; Johnson *et al.* 2014; Parfitt, 2002; Sohn *et al.* 2000; Wyttenbach *et al.* 1999). There are two main types of muscles and motor unit groups to examine. The deep extensor lateral (DEL1) or the more lateral deep extensor muscle bundles (DEL2) for phasic muscle and the superficial extensor lateral (SEL) for a tonic muscle phenotype and their associated motor nerve innervation (Pilgrim and Wiersma, 1963; Sohn *et al.* 2000). To record the EPSPs of the L1 or L2 or the SEL fibers, we found it best to split the abdomen down the midline and pin the preparation in a stretched position. Now two halves can be used. One of the halves is placed in another dish for a lab partner to use or as an extra preparation.

We stimulated the segmental nerve with a standard suction electrode set up and a SD9 ASTRO-MED GRASS stimulator (ASTRO-MED Grass Natus neurology square pulse stimulator, model SD9, Pleasanton, CA). The segmental nerve is readily seen just passing over the SEL and then along the lateral side of the segment where it was transected when cutting off the ventral aspect of the abdomen. See previous reports on methods (in a video Baierlein *et al.* 2011; Johnson *et al.* 2014; Sohn *et al.* 2000; Wyttenbach *et al.* 1999;).

Part of the exercise focuses on determining the  $[K^+]_i$  which would likely spill out of a damaged muscle or what would be the extracellular  $[K^+]_o$  in the crayfish hemolymph or for a mammal with a massive DTI injury for a given amount of muscle tissue.

## Materials

### MRO:

1. Faraday Cage
2. Micromanipulator
3. Suction Electrode
4. Dissecting Microscope
5. High Intensity Illuminator (light source)
6. Microscope Platform
7. AC/DC Differential Amplifier (A-M Systems Inc. Model 3000)
8. PowerLab 26T (AD Instruments)
9. Head stage
10. LabChart 7 (ADI Instruments, Colorado Springs, CO, USA)
11. Crayfish Saline (mM: 205 NaCl; 5.3 KCl; 13.5 CaCl<sub>2</sub>·2H<sub>2</sub>O; 2.45 MgCl<sub>2</sub>·6H<sub>2</sub>O; 5 HEPES adjusted to pH 7.4)
12. Potassium Solutions: 5.4mM (normal saline), 10, 20, 40, 80, 100 mM
13. Sylgard coated dishes (Dow Corning, SYLGARD® 184 Silicone elastomer kit; Dow Corning Corporation, Midland, MI. USA)
14. Dissecting tools
15. Insect pins
16. Crayfish

### Resting Membrane Potential:

1. Scissors
2. Forceps
3. Silver wire for ground wire
4. Dissecting microscope
5. Electrode probe
6. Petri dish with sylgard on the bottom
7. Saline Solution
8. Potassium Solutions: 5.4mM (normal saline), 10, 20, 40, 80, 100 mM
9. Bleach (Small Amount, Use for the tip of the silver wire to build Ag-Cl)
10. Glass Pipette to remove and add solutions
11. Syringe
12. Amplifier/Acquisition System
13. Faraday Cage
14. Desktop/Laptop
15. Dissection pins
16. Crayfish

### Synaptic Transmission:

1. Each item per set up. 2 to 3 students per setup is ideal
2. Scissors (1 fine for muscle and nerves, 1 course for cutting cuticle)
3. Forceps (#5)
4. Silver Wire for ground wire
5. Dissecting microscope (final magnification at least 30X)
6. Electrode Probe for intracellular recordings
7. Stimulator to use with the suction electrodes

8. Petri Dish with Sylgard on the bottom
9. Normal saline crayfish saline.
10. Potassium Solutions: 5.4mM (normal saline), 10, 20, 40, 80, 100 mM
11. Bleach (Small Amount, Use for the tip of the silver wire to build Ag-Cl)
12. Glass Pipette to remove and add solutions
13. Syringe
14. Amplifier/Acquisition System
15. Faraday Cage
16. Desktop/Laptop
17. Four strong dissection pins
18. Crayfish

## Notes for the Instructor

As discussed in Thenappan *et al.* (2019), these laboratory modules are designed on commonly used laboratory protocols to measure RP with varying  $[K^+]_o$  and measures of EPSPs. Generally, with any physiological lab one needs to ensure the preparations are healthy when starting and that the animals have been maintained in good conditions. Sometimes right after receiving a shipment of crayfish a number will die in 1 or 2 days which is likely from the stress of the transportation. It is a good idea to house the crayfish for a few days prior to using to insure robust physiological preparations.

It is important to see that the students have removed the clumps of damaged muscle attached to the cuticle after the main dissection is completed and to rinse the preparation with fresh normal saline before starting. We used room temperature to maintain the freshly made saline and muscle homogenate. If the saline is stored cold and then applied the RP as well as the amplitude of the EPSPs will be altered.

A common mishap with intercellular recordings and glass electrodes is breakage of the tip in the dish and the spilling out the KCl. If the student notices the break and can quickly change out the solution there may not be too much concern, but this would depend on the situation.

A stimulating suction electrode tip which is too large in diameter and, in which the nerve is not snug, results in having to pass larger voltage to activate the nerve. In such a case the stimulus artifact is sometime large and obscures the EPSPs. This can be remedied by placing a petroleum jelly plug around the nerve and the tip of the suction electrode. One has to be sure not to use scented petroleum jelly as we found this can kill the nerve. The same issue can arise when recording from the MRO nerve such that the signals are too small if the electrode tip opening is too large. Petroleum jelly is used to seal the nerve around the opening of the suction electrode.

A topic in which an instructor may wish to engage after such experiments are completed is the neurotransmitter used at these neuromuscular junctions. A unique aspect in crustaceans and insects neuromuscular

junctions is that the neurotransmitter is glutamate (while in humans it is acetylcholine). The receptors on the muscle fibers in insects and crustaceans will desensitize with exposure to glutamate. Glutamate receptors on the hippocampal neurons of vertebrates decay with a time constant of 1.2 msec, which is primarily due to the desensitization of the receptors (Clements *et al.* 1992). Though muscle fibers likely contain 120 mM  $K^+$ , even if it is diluted to an estimated 30 mM  $K^+$ , it did not depolarize the muscle to the same level as saline made to 30 mM  $K^+$ ; this is likely due to  $K^+$  still being bound to some the constituents within the homogenized muscle. In addition, the synaptic responses are still able to be measured with a saline containing 30 mM  $K^+$ , but with the estimated muscle homogenate dilution to 30 mM  $K^+$  synaptic responses are not able to be obtained. This is most likely due to the amino acids and glutamate, which come from the homogenized muscle (Abdel-Salam, 2014; Camien *et al.* 1951; Simpson *et al.* 1959). Thus, as a follow up experiment to the exercises presented, various concentrations of amino acids can be added to the known saline and investigate the effects on resting membrane potential and on synaptic transmission (Robbins 1959; Thieffry, 1984).

This module on synaptic transmission is narrated on line via YOUTUBE by Ashwatha Thenappan for visualization of the dissection and recording procedures. Additional material is also presented on line related to effects of raised extracellular  $K^+$  on sensory neurons which is not covered in the exercises highlighted below. Please see:

Part 1: Resting membrane potentials with varied  $K^+$  and muscle homogenate.

<https://www.youtube.com/watch?v=f7YySz7Rz9Q>

Parts 2 & 3: Sensory stimulation and motor nerve recording; Synaptic responses at NMJ.

<https://www.youtube.com/watch?v=mrRFVcrwJhA>

After obtaining an average RP in normal saline, the electrode should be removed from the muscle fiber and the bathing solution changed to the next saline, and new values of membrane potential should be obtained. In describing the results, the Nernst and Goldman Hodgkin Katz equations can be discussed to explain the measures that are obtained in this part of the exercise.

In order to make dilutions of homogenized skeletal muscle, a volume of supernatant of the homogenized crayfish muscle was measured and diluted as determined by the students for testing their predictions. For these experiments, a small dish was used so only small volumes of the homogenized crayfish muscle solution were needed for each preparation. The crayfish muscle is homogenized after measuring the volume in a graded Eppendorf tube. The tissue was homogenized with a small mortar and pestle. The homogenized tissue and solution were added back to an Eppendorf tube to be spun slightly at 1000 RPM for 1 min. Earlier attempts with needle

aspiration back and forth were problematic, because the needles would clog, so this method was not preferred.

The students quickly learned that phasic muscle fibers will twitch dislodging the intracellular electrode and that movement of the electrode tip in the contracting fiber may also cause artifacts in a recording.

To avoid these problems it is best to stretch the muscle but not to overstretch as this can damage the fibers. In addition, the nerve to DEM muscle can be transected to avoid this muscle from twitching while EPSP measures are made on L1 and L2. The DEM muscle may be used if recordings of the RP in various bathing solutions are to be obtained, but it is difficult in this muscle to keep an intracellular electrode impaled while measuring EPSPs.

To record the EPSPs of the L1 or L2 or the SEL fibers, we found it best to split the abdomen down the midline and pin the preparation in a stretched position. Now two halves can be used. One of the halves is placed in another dish for a lab partner to use or as an extra preparation.

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## Acknowledgments

We thank Thomas Schwarcz, MD for the clinically relevant matters related to this topic. Funded by the Department of Biology, University of Kentucky for the development of this laboratory exercise

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Dr. Robin L. Cooper obtained a dual BS Chemistry and Zoology from Texas Tech Univ. in 1983. He obtained a Ph.D. in 1989 in Physiology from the School of Medicine, Texas Tech Univ. He then went on for postdoctoral training (1989-1992) at the University of Basel, Sch. of Medicine, Basel, Switzerland and a second postdoctoral stint (1992-1996) in the Department of Physiology at the Univ. Toronto, Sch. of Medicine, Toronto, Canada. He then joined the Dept. of Biology at the University of Kentucky in 1996 and is now professor and director of the undergraduate neuroscience program at the University of Kentucky. He also obtained a BSN in nursing in 2012 and practiced nursing as an RN from 2011 to 2017. He has received several teaching awards: Kentucky Academy of Sciences Excellence in high education teaching, Arts and Sciences Excellence in undergraduate mentoring and, Provost Award for excellence in teaching.

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Dr. Esther E. Dupont-Versteegden graduated from State University Limburg at Maastricht, the Netherlands, with a Bachelor in Science degree in Movement Sciences. In 1995 she received a Doctor of Philosophy degree in Physiology from the University of Texas Health Science Center at San Antonio, Texas. Esther Dupont-Versteegden completed a postdoctoral study in molecular physiology with Dr. Charlotte Peterson at the University of Arkansas for Medical Sciences. In 1999 she became Assistant Professor of Geriatrics at the University

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## Appendix A

Dissection tools: Fine #5 tweezers, fine scissors, knife blade holder, #26002-20 insect pins (all obtained from Fine Science Tools (USA), Inc., 373-G Vintage Park Drive, Foster City, CA 94404-1139).

Sylgard-bottomed glass dish (instructor should pour the Sylgard about 1 to 2 inches deep in a glass dish prior to heating for harden the sylgard).

A PowerLab 26T (ADInstruments, Colorado Springs, CO, USA) was interfaced to a computer.

The standard software from ADInstruments named Chart or Scope was used to collect data and to perform the data analysis.

A model 3000 AC/DC amplifier for intracellular as well as extracellular recordings can be used. For intracellular recordings we use glass capillary tubing (catalogue # 30-31-0 from FHC, Brunswick, ME, 04011, USA).

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### Citing This Article

Cooper RL, Thenappan A, and Dupont-Versteegden EE. 2019. Examining motor and sensory units as an educational model for understanding the impact of localized tissue injury on healthy cells. Article 6 In: McMahon K, editor. *Tested studies for laboratory teaching*. Volume 40. Proceedings of the 39th Conference of the Association for Biology Laboratory Education (ABLE). <http://www.ableweb.org/volumes/vol-40/?art=6>

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