SENSORY FIELD MAPS IN THE SKIN OF A LEECH FOR TOUCH, PRESUURE AND NOICEPTIVE NEURONS

By

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1. PURPOSE

The purpose is to learn how to map sensory fields for identifiable neurons using electrophysiological approaches.

2. PREPARATION

Leech (*Hirudo medicinalis*)

3. INTRODUCTION

Animals can perceive environmental cues through the use of sensory receptors. These receptors transform various types of stimuli of the environment into electrical signals that allow one to make use of the input to function. Sensory information is a partial assessment of the true environment (Mountcastle, 1975). The various types and intensity of stimuli that can be perceived is dependent on the sensitivity of the detectors (i.e. sensory receptors). In addition, the majority of the afferent information from sensory receptors to higher centers in the nervous system is transformed (integrated) before the body, as a whole, can utilize the information. In this experiment we shall see how a leech can detect mechanical stimuli on the skin surface from the outside world and how the acuity of the sensory system functions.

As you can test the sensitivity of your own sensory system on your own hand by slightly touching the back of your hand without looking at your hand and you can perceive very accurately where on your hand you are being touched. You can detect the slightest stimulus as well as a pinch of the skin on the back of your hand but other places on your body might not be as sensitive or be as accurate in differentiating between two stimuli given at the same time in relative close proximity to each other. This general phenomenon of differentiating two stimuli is referred to as 2-point discrimination. This is related to the overlap of receptor fields as well as the density of receptor innervation. In this exercise a receptive field of the leech skin is that area of skin that, when stimulated, will bring about excitation of a single mechanosensory neuron innervating that area.

The leech preparation has many advantages for investigating the function of identifiable primary neurons within the animal's central nervous system (CNS). There are only about 400 neurons within each ganglion; therefore electrophysiological and anatomical maps are not as complicated to obtain as compared to a mammalian

central nervous system. The astonishing nature of some neurons in the leech ganglion is that the shape of their action potentials and biophysical properties are characteristic for a cell type (ie., P, N, T, Rz). Even more amazing is that the neurons can be removed from the animal and maintained in a minimal saline. Also the cells can maintain the electrical characteristics for as long as 45 days (Fuchs et al., 1981; Ready and Nicholls, 1979).

The distinct advantage of the leech ganglion, in this species, for learning electrophysiology is that many of the cells are anatomically arranged in the ganglion in a particular pattern allowing identification of cell type within various ganglia as well as between animals. The unique location and morphology of neurons in leech ganglia were noted by Retzius as early as 1891 (Blackshaw, 1981). An identifiable neuron type is advantageous for investigating neural circuits and conducting repetitive experimentation with a given cell type. Due to the relative large somata of the neurons within a ganglion they are visible with a dissecting microscope and the somata can be selectively impaled with sharp microelectrodes for measuring their biophysical properties.

In addition, the segmental ventral nerve cord (or CNS) can be exposed with a patch of innervated skin. With the ability to keep a patch of skin and the neurons alive, sensory receptive fields can be probed by recording electrical signals from the cell body (i.e., soma) of sensory neurons within respective abdominal ganglion in the VNC. With this paradigm one can assess the sensitivity of the sensory endings by applying varying degrees of force on the skin and map receptive fields: light touch, pressure, and noxious (painful) stimuli (see Nicholls and Baylor, 1968; Yau, 1976).

An advantage of this leech ganglion-skin preparation is that one can draw anatomically the receptive map and trace the neuronal processes to the identified neuron once the electrical responses are being recorded. In addition, the synaptic connections to other neurons can be mapped to parallel electrical measures within neural circuits within ganglion.

4. METHODS

4.1 Materials

1. Scissors (1)
2. Forceps , one fine and 1 course (2)
3. One pair of fine iris scissors (1)
4. Silver Wire for ground wire (1)
5. Microscope (1)
6. Intracellular electrode Probe (1)
7. Sylgard coated large Petri dish for leech dissection (2)
8. Leech saline Solution (1)
9. Bleach (Small amount, Use for the tip of the silver wire to build Ag-Cl)
10. Glass Pipette (1), to remove and add solutions
11. Syringe 10 or 20 cc (1)
12. Amplifier/Acquisition System (1)
13. Faraday Cage (1)
14. Desktop/Laptop (1)

15. Fine dissection pins (10)
16. Large insect pins to stretch out the dissected leech skin
17. Leech (*Hirudo medicinalis*)
18. Fiber optic lamp
19. Mirror that can be tilted under a stand
18. Raised preparation stand with a glass bottom
19. Micromanipulators
20. Dissecting microscope with 100x magnification
21. Squirt bottle for leech saline (needed during dissection)
22. Dissection tools
23. Fire polished glass rods for touching the skin

4.2 Preparation/Dissection:

4.2.1Solution

Leech Ringer's solution:

Buffer Stock solution Tris 0.2M (24.2g) & Maleic acid (23.2g)

Final solution	mM
NaCl	115.3 (6.716 gram/1 L)
$CaCl_2$	1.8 (0.264 gram/1 L)
KCl	4.0 (0.298 gram/1 L)
Add Tris/Maleic acid	(50.0 ml) of stock for every 1000 ml

of saline to be made. Dissolve salts in 800 ml of H2O, pH to 7.4 with 5N NaOH, fill to 1 Liter.

4.2.2. Dissection

The animal usually secretes slimy mucus from the skin. It is better to quickly deal with the animal and get it pinned dorsal side down with large pins placed in the head and tail regions as well as along the lateral aspects of the body wall (see Figure 1). If the animal is stretched longitudinally it will be easier to remove the ventral nerve cord.

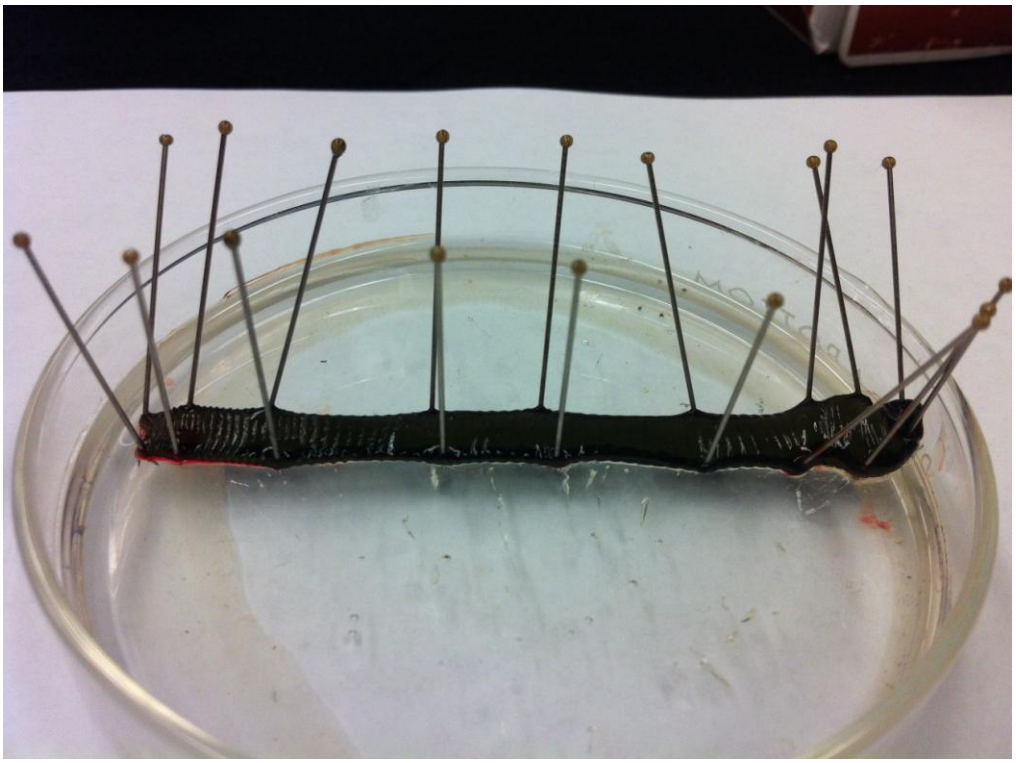

Figure 1: Leech pinned out along the sides and fully stretched.

Pin the skin to one side with roots on the other side of the severed ganglion or use an approach to expose a ganglion with the skin all around the ganglion (Figure 2).

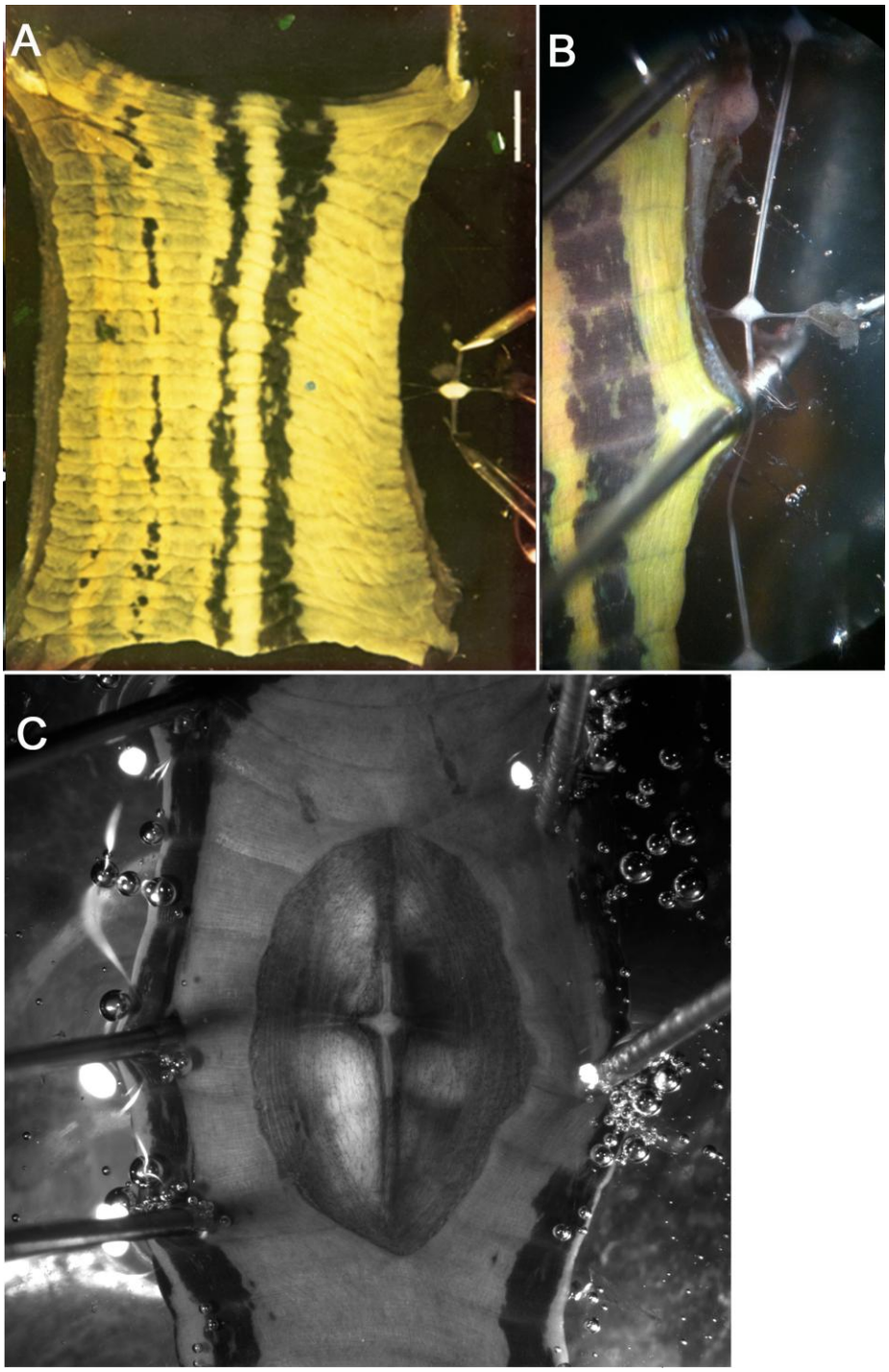

Figure 2: The leech ganglion-body wall preparation for mapping receptive fields. (A) One approach is to remove an entire section of the body wall along with 2-3 ganglia. Here the roots on one side have been cut and the ganglia have been pinned out to the side. (B) A higher magnification illustrates three ganglia next to the skin. (C) Another approach is to create a small window in the body wall and record from the intact ganglia while mapping a receptive field.

After the dorsal skin as been cut the full length of the animal, and some patches on the ventral side have been made, the skin needs to be pinned flat with a series of small pins. The fine iris scissors should be used to nick the black stocking (Figure 3). With one blade of the fine iris scissors slip it under the stocking and slight raise the blade to pull up on the stocking. Cut the stocking the length of the ganglion trying not to cut the nerve roots or the connectives between ganglia. Continue this process for each ganglion that is exposed where a ventral patch of skin removed. Note the ventral aspect of the ganglion is facing upwards so the observed can see the ventral aspect of the ganglion in order to identify neuron types based on their location in the ganglion.

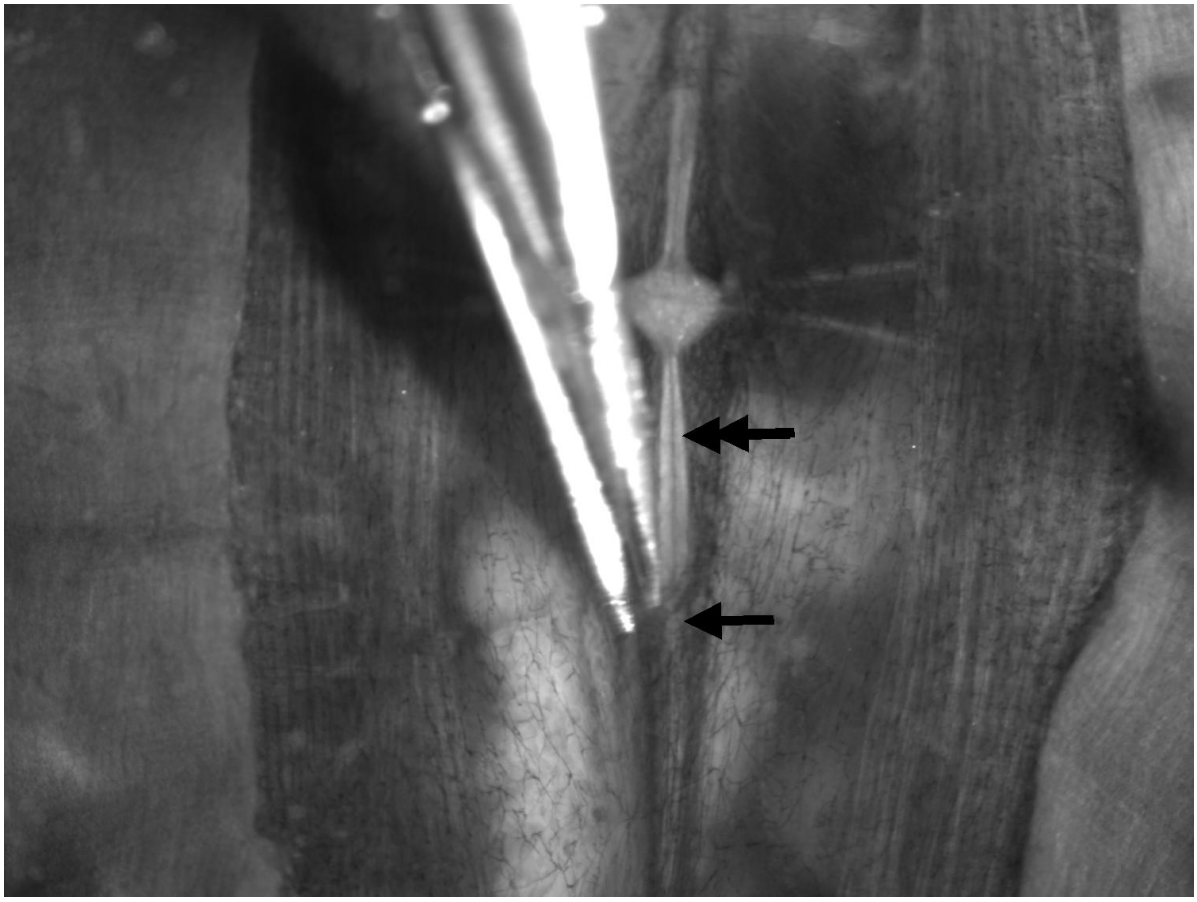

Figure 3. Dissection of the black sinus. The blood vessel (arrow) should be carefully cut on one side to expose the ventral nerve cord (double arrow). Leave segments of the blood vessel intact around the segmental roots for use as a handle for manipulating the ventral nerve cord when transferring between dissection and recording dishes.

4.2.3 Cell identification anatomically

See that the arrangement of neurons matches the typical arrangement as shown in Figure 4. If it does not match then potentially the preparation is pinned dorsal side up. The two large Retzius cells in the middle of the ganglion should be observed.

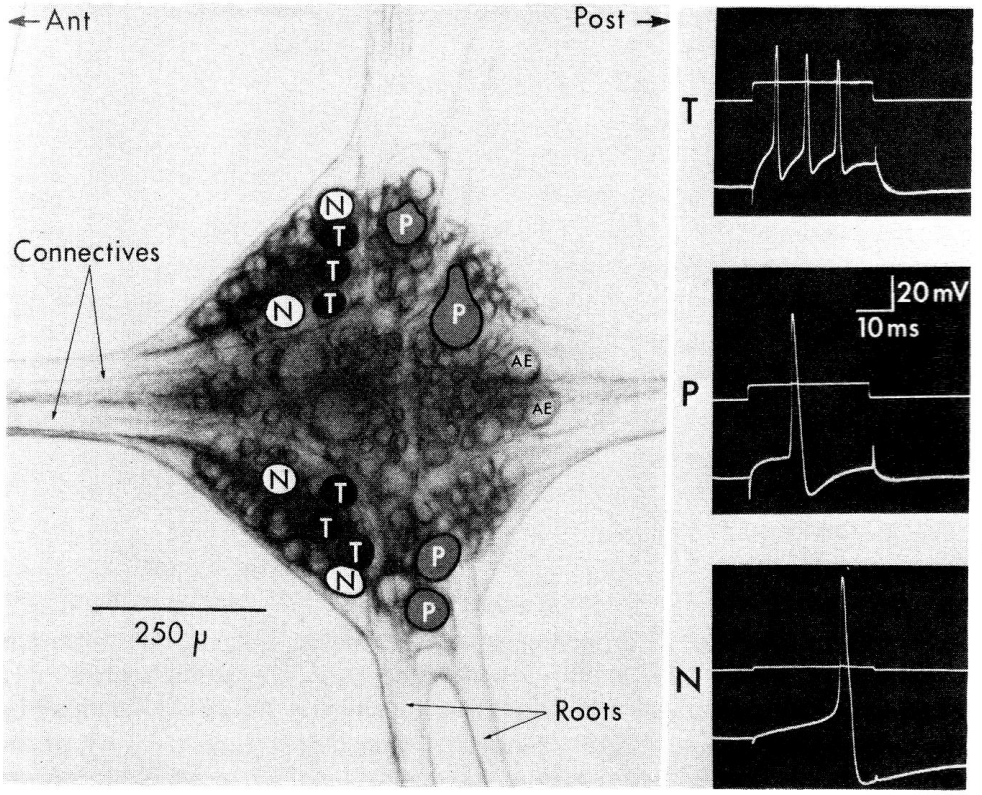

Figure 4 Cellular anatomy of the leech ganglion. Ideally the Retzius cells (R) and other large cell bodies (P- pressure, T- touch, N-nocieptive, AE- annulus erector) will be clearly visible if the microscope and light condenser are set up properly (from Blackshaw, 1981 modified from Nicholls and Baylor, 1968).

4.2.4 Intracellular identification of neuronal types

From a ventral view, one is now to obtain an intracellular recording of the Rz neurons. Afterwards, then impale the known sensory T, P and N neurons and measure the RP and electrical signals. In order to perform this, one may have to change the saline level and adjust the mirror to obtain the best light intensity so that the cell bodies of the neurons to be recorded from are clear (Figure 5).

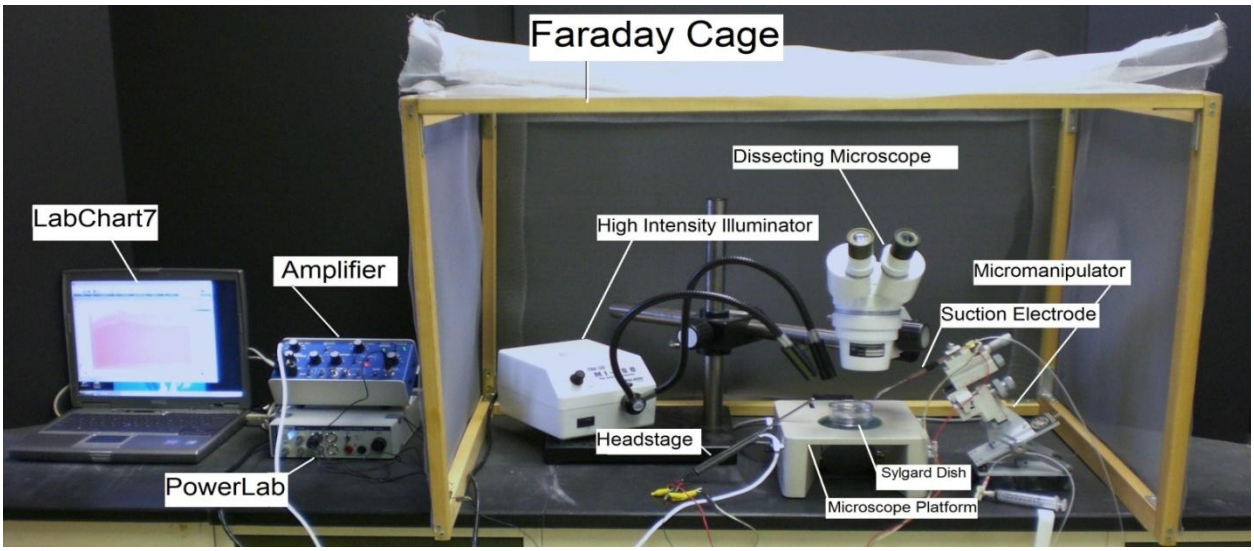

Figure 5: Overall setup of the recording equipment.

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

The specimen dish with preparation should be placed under the microscope and secured with wax or clay on the sides of the dish to prevent movement. One might find using a tilt mirror of use under the preparation to shine light up through the preparation (**Figure 5 and 6**).

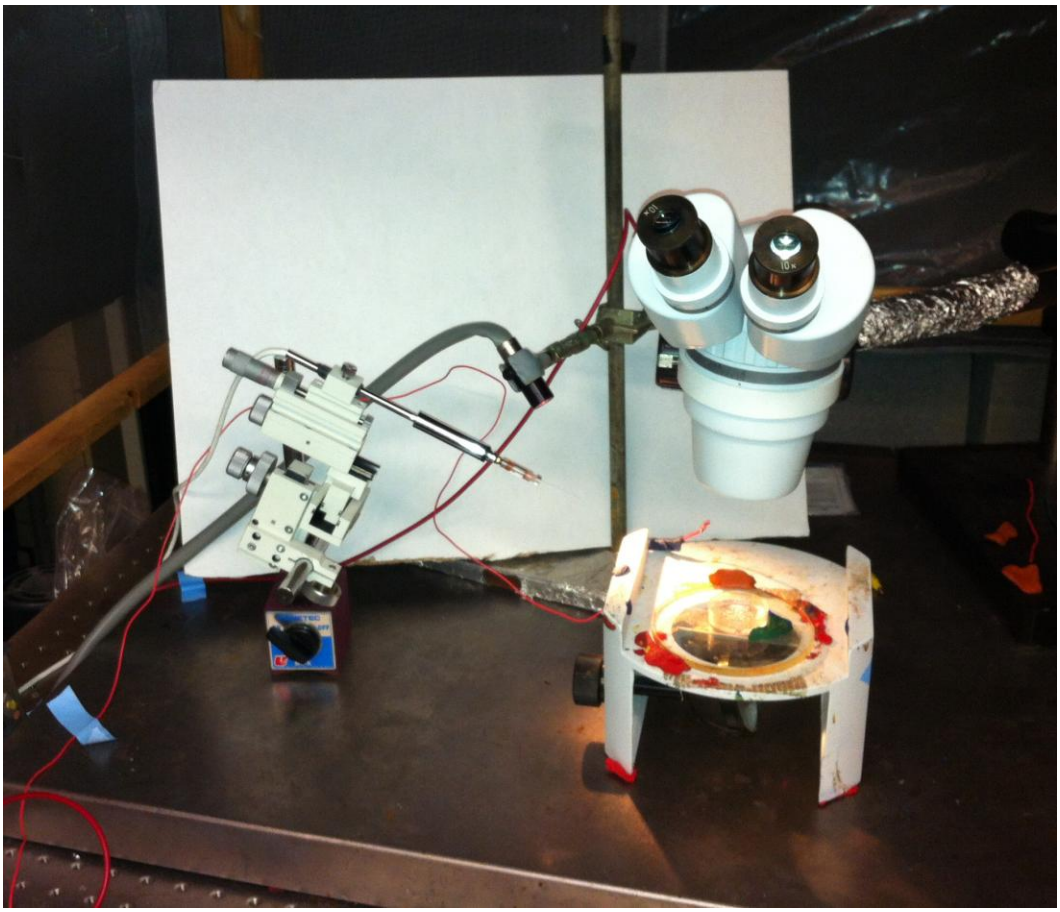

Figure 7: Placement of the preparation under the microscope with the intracellular head stage mounted on a micromanipulator

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 an Ag-Cl_2 coating. Wash the wire with distilled 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 or 3M K-acetate solution (**Figure 7**). 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 or potassium acetate solution. The silver wire can then be placed into the pipette (**Figure 8**). Care should be made not to break the electrode tip. Another wire is attached to the Faraday cage or into ground directly on the intracellular amplifier. 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).

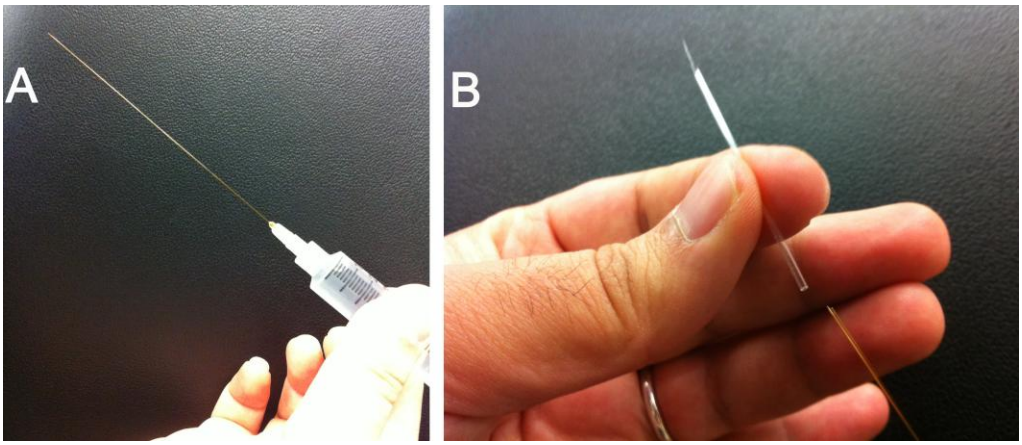

Figure 8: Filling microelectrode

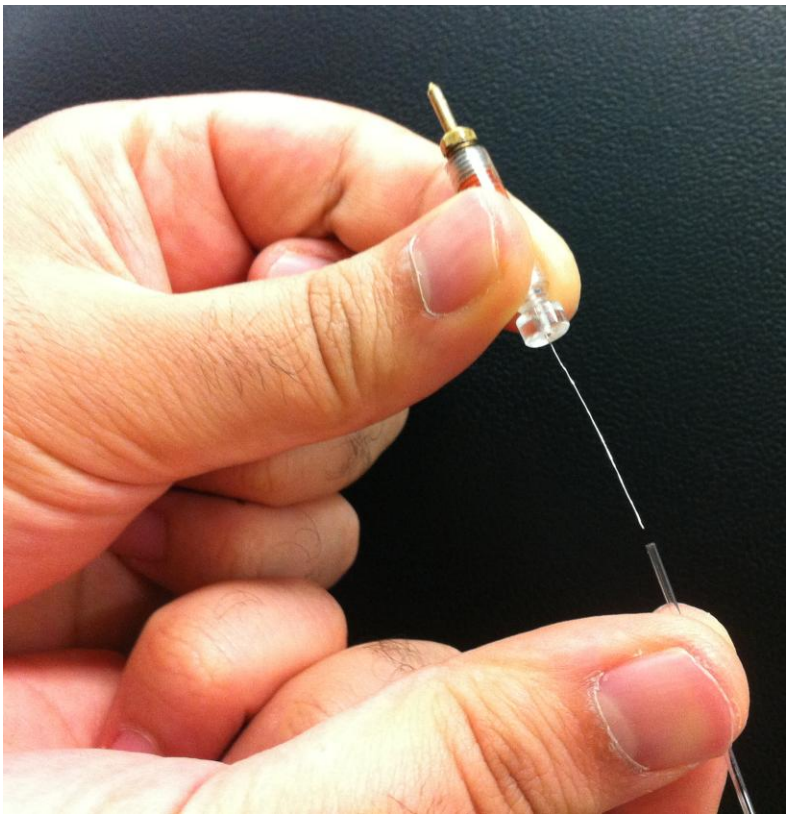
Figure 9: Microelectrode and holder

Figure 10: Front face of the intracellular amplifier used during the intracellular membrane potential recordings. The "Ω TEST" switch used to test electrode resistance is center left. The DC offset knob is in the upper right corner, and should be turned counter clockwise to start. The ground wire is placed in the "GND" pin jack opening. The amplifier in this set-up amplifies the signal by 10X.

Software Set-up

Be sure your amplifier and PowerLab units are on before opening the software.

Open the LabChart software. 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 1V.

Click on "Channel 1" on the right hand portion of the screen. Click "Input Amplifier" and that the following settings are selected:

Single ended	OFF
Differential	Checked
AC-Coupled	OFF
Anti-alias	Checked
Invert	OFF

Amplifier Set-up

The amplifier output cable should be plugged into channel one. The following settings should be used with the intracellular amplifier (see **Figure 10** for reference):

Current Comp.(3 knobs)	counter clockwise
Capacity Comp.	counter clockwise, OFF
Capacity Amp (ΔA)	counter clockwise
DC Offset knob	Varies (see part 16 below)
Low Pass knob	50 kHz
Notch	OFF
Current Injection	0 µA
ΩTEST	OFF

CHECK THE RESISTANCE OF YOUR ELECTRODE.

To measure the resistance, place the tip of the glass electrode into the saline bath. Make sure a ground wire is also in the saline bath. While recording, the Ω TEST switch should be turned on and then off several times (**Figure 11**).

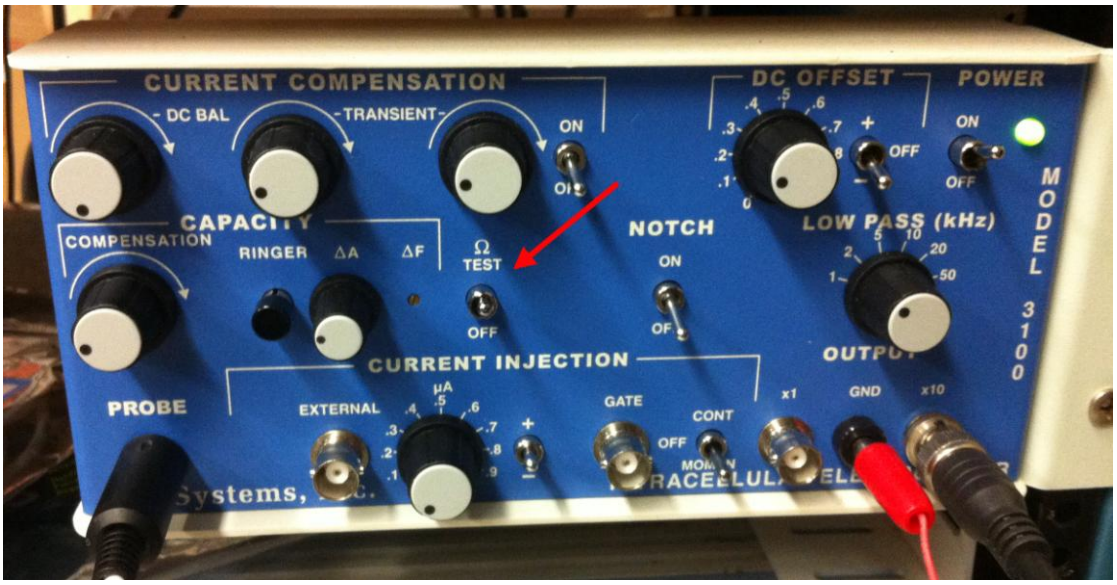

Figure 11: Front face of the intracellular amplifier with the "Ω TEST" switch in the on position.

The amplitude (mV) of the resulting changes should be measured. To measure the amplitude changes in the trace, place the marker on the steady base line and then move the cursor to the peak amplitude. The trace might be condensed so use the "zoom function" under the "window" menu. Then move the "M" at the bottom left to

base line and the cursor over the peak response (**Figure 12**). Then the delta value will display at the top in mV. In the figure it is 222.7 mV.

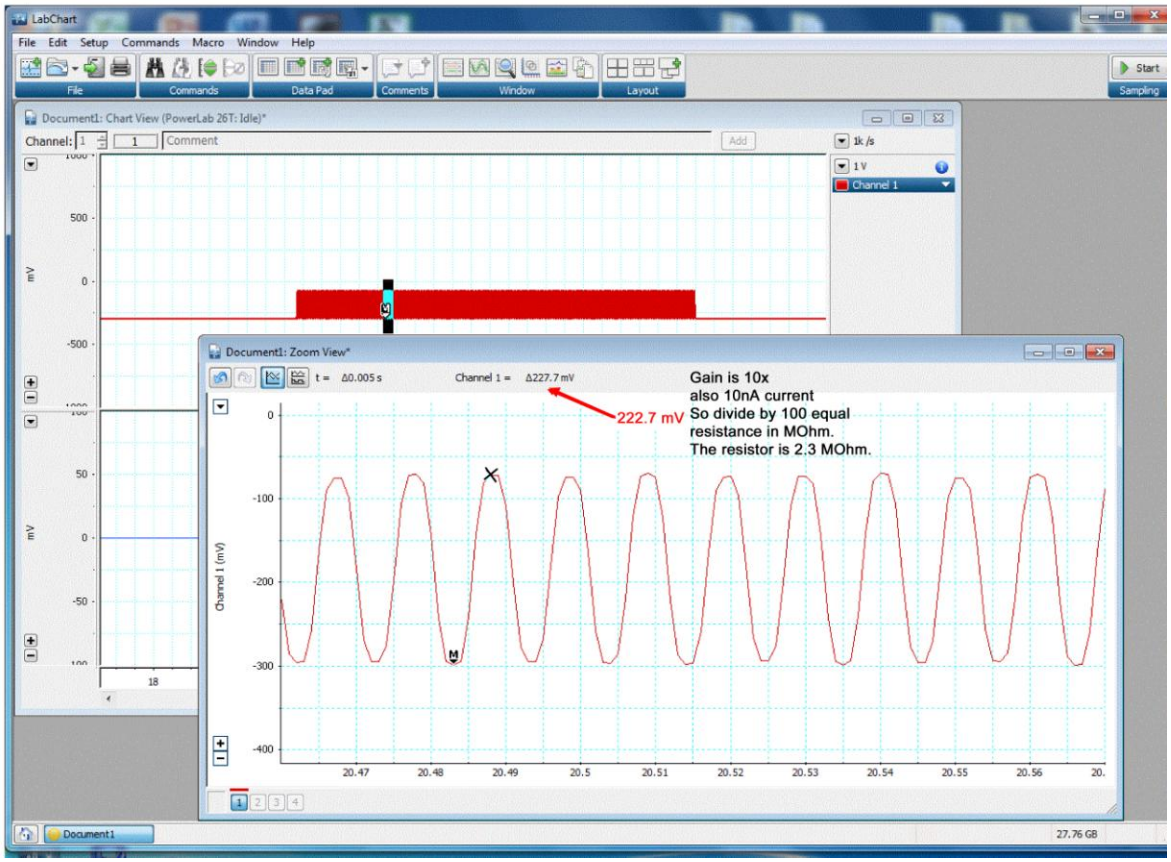

Figure 12: Traces of the responses for measuring electrode resistance.

As a measure of the electrode resistance, the voltage should be divided by the current, which is 10 nA (ie., R=V/I, or Ohm's law). The resulting value is the resistance of your glass electrode. Recall the BNC output of the intracellular amplifier is connected to a 10X output. Thus, divide by 100 to obtain the correct electrode resistance.

Electrode Resistance (MΩ) = _____

The resistance should be within 20 to 60 MegaOhms. Low (<20) and high resistance (>100) are not acceptable. Troubleshoot as necessary to bring your electrode's resistance within the acceptable range.

Set the gain in your software to 1 or 5 V/div. 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 before inserting the electrode into the tissue. This sets your extracellular voltage to zero. This is the difference from the glass microelectrode to the ground wire which should be both in the saline.

Record potentials

Carefully place the tip of the electrode over the cell of interest and gently lower the tip until a dimple is seen in the glial sheath. One might very carefully tap the electrode holder or manipulator so that the electrode tip “pops” into the cell of interest. Since there are at least two of the same cell types (Rz, T, P, N) try to obtain the resting membrane potential from the same cell type.

4.2.4 Mapping of sensory fields on the skin while recording with an intracellular electrode in the sensory cell body

After obtaining a stable intracellular recording in ones of the sensory cells (T, P, N) use the small glass rod that has been fire polished to lightly touch the skin in the same segment. If the neuron being recorded from is an N cell then a more forceful pressure will need to be applied to the skin. In most cases, the N cell will only respond if the skin is pinched with tweezers. For this reason these cells should be tried last as one might damage the skin but also one might displace the intracellular recoding with disturbing the preparation in such a manner.

One should be able to quickly sketch the skin and ganglion with details sufficient enough that a map of where one touched can be used to determine the receptive filed for a particular neuron. After recording from as many as possible T and P cells on both sides of a ganglion and try the N cells.

In order to examine if the sensory neurons have process that travel through the connectives in a rostral or caudal manner and then out the roots in the adjoining segment to the skin, the connectives between ganglia can be cut and the receptive fields reexamined in regards to the spread of detection. Basically one is examining if there has been any loose in the receptive felid.

5. RESULTS

For the laboratory write up report your findings in the receptive fields and provided the sensory maps that you drew. Report on the findings from cutting some of the connectives and the changes in your sensory map the skin.

6. DISCUSSION

These sets of experiments were to teach the fundamental principles of intracellular recordings from identifiable neurons and to recognize the characteristic shapes of

electrical signals that can arise from these particular neuronal types. In addition, one will have learned how to map sensory filed and to know what is meant by a sensory field for an identifiable neuron. One might be asking such questions: (1) how the fields form? (2) Do receptive fields overlap with the same cells type in the adjacent ganglion? (3) Do receptive fields overlap within the same segment for the same neuronal type? (4) How is that some sensory endings are more sensitive than others to light touch? Then think how one might go about experimentally to address such questions.

There is a rich history of investigations using the leech model for neurophysiological investigations and still an active area of research is ongoing in addressing questions of the development in the neural circuitry and gene regulation during development as well as during synaptic repair and regeneration (Friesen et al., 2011; Jellies and Kueh, 2012; Lamb and Calabrese,. 2011; Meriaux et al., 2011; Mladinic et al., 2009; Mullins and Friesen 2012; Ngu et al., 2007; Urazaev et al., 2007; Weisblat and Kuo, 2009).

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