Experimental approach for studying structural changes in axonal membrane upon nerve excitation

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Tiivistelmä – Referat – Abstract

The Hodgkin and Huxley (HH) model of action potential has become a central paradigm of neuroscience. Despite its ability to predict action potentials with remarkable accuracy, it fails to explain several biophysical findings related to the initiation and propagation of the nerve impulse. The isentropic heat release and optical phenomena demonstrated by various experiments suggest that action potential is accompanied by a transient phase change in the axonal membrane.

In this study a method was developed for preparing a giant axon from the crayfish abdominal cord for studying the molecular mechanisms of action potential simultaneously by electrophysiological and optical methods. Also an alternative setup using a single-cell culture of an Aplysia sensory neuron is presented. In addition to the description of the method, the preliminary results on the effect of phloretin, a dipole potential lowering compound, on the excitability of a crayfish giant axon are presented.

(146 words)

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1 Introduction

1.1 The Hodgkin and Huxley model of action potential

The Hodgkin and Huxley (HH) model of the action potential (1-4) has become a central paradigm of neuroscience. The model presents the axonal membrane as an equivalent electrical circuit and deciphers its function by voltage-clamp measurements on the squid giant axon. It explains the formation of action potential through variable sodium and potassium conductances of the axonal membrane. The model proposes that the conductances are controlled through specific voltage-sensitive gating particles and describes their function through a set of empirically derived equations fitted to the voltage-clamp data. The action potentials calculated by the model fit the recorded action potential with remarkable accuracy. The variable conductances were attributed to specific proteinaceous sodium and potassium channels; the first evidence for their existence was the discovery of specific blockers of sodium and potassium conductance (5-6). More recently, single-channel recording techniques have in turn allowed further studies on the gating and conductance properties of ionic channels described in the HH model (7-9).

To summarize, the electrical behavior of the action potential can be excellently explained by the HH model. This approach has failed to explain several findings on the biophysical grounds accompanying the action potential. A transient heat release followed by equal heat absorption coincides with the nerve impulse (10-12). This contradicts the expected entropic increase caused by ionic fluxes in the HH model, as the heat is fully reabsorbed after the impulse and no net heat is released (3). 8-anilinonaphtalene-1-sulfonate (ANS) fluorescence, light scattering and birefringence studies on a nerve demonstrate a transient change in the observed optical signal during the course of the action potential (13). Evidence acquired from fluorescence experiments performed with pyrene probes suggests that the fluidity of the axonal membrane lipids decreases during excitation (14). The biophysical changes recorded in phospholipid model membranes during liquid crystalline → crystalline (cooling) transition have a striking resemblance to the changes recorded in a nerve membrane during excitation (15).

1.2 Giant nonmyelinated nerve fibers in studies of action potential

When studying the role of axonal phospholipid membrane in action potential initiation and propagation, the nonmyelinated nerve fibers of simple invertebrates have important advantages over vertebrate myelinated nerve fibers. Nonmyelinated fibers lack the myelin sheath and nodes of Ranvier, and thus nerve conduction along them can be considered uniform and continuous (16). The axonal diameter of nonmyelinated nerve fibers is generally larger than of myelinated fibers, as slow nerve impulse conduction due to the lack of a myelin sheath is partially compensated for by increasing the diameter of the axon. The large size of the neuron provides an advantage when optical studies of the axonal membrane are performed.

A classical giant nerve fiber used in electrophysiological research is the squid (*Loligo spp.*) giant axon that may reach a diameter of 1 mm. The immense size of the squid fiber enables an intracellular longitudinal electrode to be inserted inside the axoplasm, a technique used in the development of the HH model (17-18). The lobster (*Homarus americanus*) (19-20) and crayfish (*Procambarus clarkii*) (21) have also been used as a source for giant axons. The giant axons of these species are smaller than the squid axon, but since these animals are more readily available their axons have become widely utilized in the study of action potential.

A special case of model animals in invertebrate neurophysiology is the California sea slug (*Aplysia californica*) which has provided an excellent source of large neurons for studies of the mechanisms of memory storage (22). Extensive work has been done to identify and characterize the electrophysiological properties of Aplysia neurons (23-24). When set on a culture dish, certain neurons are able to form synapses between each other (22), providing a method for building custom neuronal circuits for experimentation.

1.3 Membrane dipole potential and the action of phloretin

The hydrophobic interactions that drive the assembly of a phospholipid membrane also force the dipoles of membrane lipids and water molecules into a preferred orientation

with respect to the membrane normal. As a consequence, planes of positive and negative charges form within the membrane. The potential difference between these layers is called the dipole potential (25).

The dipole potential in phosphatidylcholine bilayers has been estimated to be on the order of 100-300 mV (26-27) positive towards the membrane interior, and due to the limited size of the membrane region where the potential difference manifests, the electric field generated by the dipole potential is approximately ten times the magnitude usually created by transmembrane potential (25). Dipole potential is considered to affect the folding and conformation of transmembrane proteins (26), and modifying the dipole potential has been shown to influence the rate of a redox reaction driven by a membrane fluorescent probe (28) and the activity of the antimicrobial lipopeptides syringomycin (29-30) and surfactin (31).

Phloretin is a flavonoid naturally present in apples (32-33) and one of the most potent attenuators of dipole potential (25, 34). The major dipole potential lowering mechanisms of phloretin are thought to be its penetration to the phospholipid membrane and the alignment of the phloretin dipoles in opposite direction to the lipid ones, and the disorientation of the water dipoles at the lipid-water interface (35).

1.4 Aims of this study

Data reported in literature implies that a transient phase change of membrane phospholipids accompanies the propagation of the action potential. The purpose of this study was to design and construct an experimental setup enabling the recording of action potentials from a single axon simultaneously by electrophysiological and fluorescence techniques.

The combining of electrophysiological and fluorescence methods set specific requirements on the axonal preparation and measurement setup. The axonal preparation must be optically accessible to a fluorescence spectrophotometer and free of interfering connective tissue debris. The axon must also be of adequate size in order to provide a large enough membrane area for emitting a sufficient optical signal when stained with

fluorescent membrane probes; this is particularly important since the fast propagation of action potentials necessitates fluorescence measurements of a high temporal resolution.

In this study two approaches to overcome these challenges are presented. A dissection method was developed for extracting a giant axon from the abdominal cord of the signal crayfish (*Pacifastacus leniusculus*). Also, a method for preparing a single-cell culture of a sensory neuron from the pleural ganglion of Californian sea slug (*Aplysia californica*) is described.

During the development of the crayfish dissection methods and testing of their applicability on our electrophysiological measurement setup, an experiment studying the effects of membrane dipole potential modification on the excitability of the crayfish giant axon was performed. The preliminary results of this experiment are presented.

2 Methods

2.1 Dissection of the giant axon from the abdominal cord of signal crayfish (*Pacifastacus leniusculus*)

The protocols presented were developed by the author during 2007-2010. Signal crayfish were ordered from Länsi-Suomen Rapu, Säkylä, Finland. The crayfish were kept in an oxygenated bath filled with tap water (+6 °C). An estimated 100 crayfish were used during the process. The dissection of the abdominal nerve cord was performed in a crayfish buffer solution of the following composition: 207 mM NaCl, 5.4 mM KCl, 13.6 mM $CaCl_2$, 2.6 mM $MgCl_2$, 5 mM Hepes. The pH of the buffer was adjusted to 7.7. Images of the fine dissection of the crayfish abdominal cord were taken with a digital camera attached to an Olympus SZX10 stereo microscope.

The crayfish are anesthetized prior to dissection by incubating them for 10-15 minutes in a container filled with iced water (+0 °C). The crayfish are decapitated (Image 1) and the chelipeds and walking legs are cut off to facilitate the dissection procedure. The cephalothoracic carpace and the underlying gills and viscera may also be removed (Image 2).

The abnominal nerve cord is located in the midline on the ventral side of the abdomen, dorsally adjacent to the abdominal sternites of the exoskeleton. A convenient way to expose the nerve cord for removal is to cut the lateral ends of the ventral sternites with scissors on one side of the animal (Image 3), and similarly cut the lateral ends of the dorsal tegrites on the other side (Image 4). The exoskeletal rings surrounding the abdomen are thus broken in two parts, and the breaking points are located diagonally on the opposite lateral sides of the animal. The abdominal exoskeleton is opened like a book by separating the tegrites from the sternites on the side where the sternites were cut. The lateral abdominal muscle bundles are cut and the ventral nerve cord is exposed (Image 5). The nerve cord is separated from the ventral sternites by gliding tweezers under the nerve cord and carefully breaking its lateral roots (Image 6). The proximal and distal ends are cut and the nerve cord is lifted on a Sylgard-coated Petri dish filled with buffer.

Image 1 - Decapitation of the crayfish

Image 2 – Removal of the cephalothoracic carpace

Image 3 – Cutting the ventral sternites

Image 4 - Cutting the dorsal tegrites

Image 5 - Exposing the ventral nerve cord

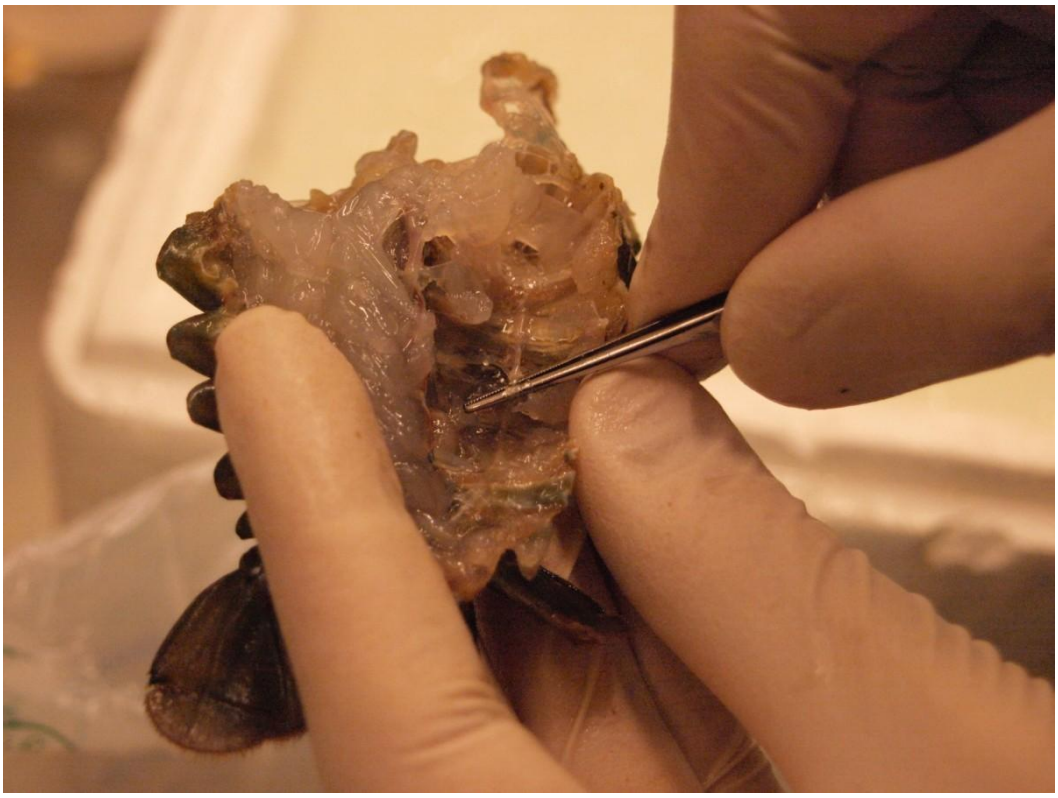

Image 6 - Detaching the ventral nerve cord

The abdominal nerve cord is attached to the Sylgard dish by microneedles that are pinned through the lateral roots. The giant axons are located on the dorsal surface, just beneath a layer of connective tissue that surrounds the nerve cord.

If only electrophysiological measurements are performed, as was done in the phloretin experiment described in sections 2.2 and 3.3, it is not necessary to separate a giant axon from its surroundings, and only a small section of the connective tissue on the dorsal surface of the nerve cord needs to be removed to expose the giant axons. This can be done by making a cut on the lateral edge of the connective tissue sheath surrounding an abdominal ganglion, and carefully separating a small patch of connective tissue from the axons.

If optical studies of a specific giant axon of the abdominal cord are planned, a more thorough dissection procedure might be needed, depending on the optical detection system used. A potential method is described for extracting an interganglia section of a giant axon that is relatively clean of connective tissue debris that would interfere with the optical measurements. It should be noted that the giant axons are extremely fragile,

and despite continual attempts, the author was unable to achieve an optically clean preparate capable of producing an action potential.

When a separate giant axon is to be extracted from the abdominal cord, it is most convenient to begin the dissection procedure from the ventral surface and gradually advance towards the axons. The abdominal cord is pinned to the Sylgard dish, ventral surface up (Image 7). A cut is made on the connective tissue sheath covering an abdominal ganglion (Image 8), and starting from this cut the ventral intergangliar segment of the sheath is dissected away. The smaller axons located ventral to the giant axons are carefully removed, gradually exposing the dorsally located giant axons (Image 9). The shear forces applied on the abdominal cord during the dissection procedure often detach the giant axons from the dorsal connective tissue without any additional effort, but sometimes careful dissection work is required on the lateral and medial sides of the axons in order to finish their detachment. Finally, the proximal and distal ends of the intergangliar axonal preparate are cut and the axon is completely detached from its surroundings (Image 10).

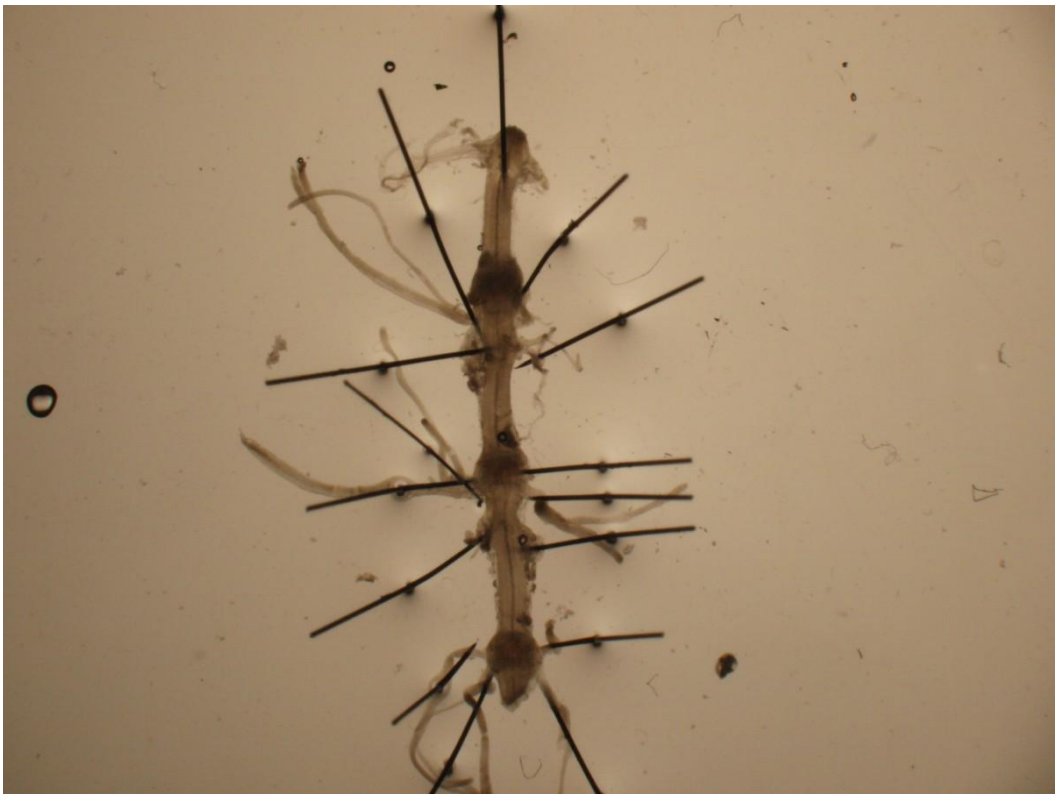

Image 7 - Section of abdominal cord pinned on a Sylgard dish

Image 8 - Making a cut on the connective tissue sheath

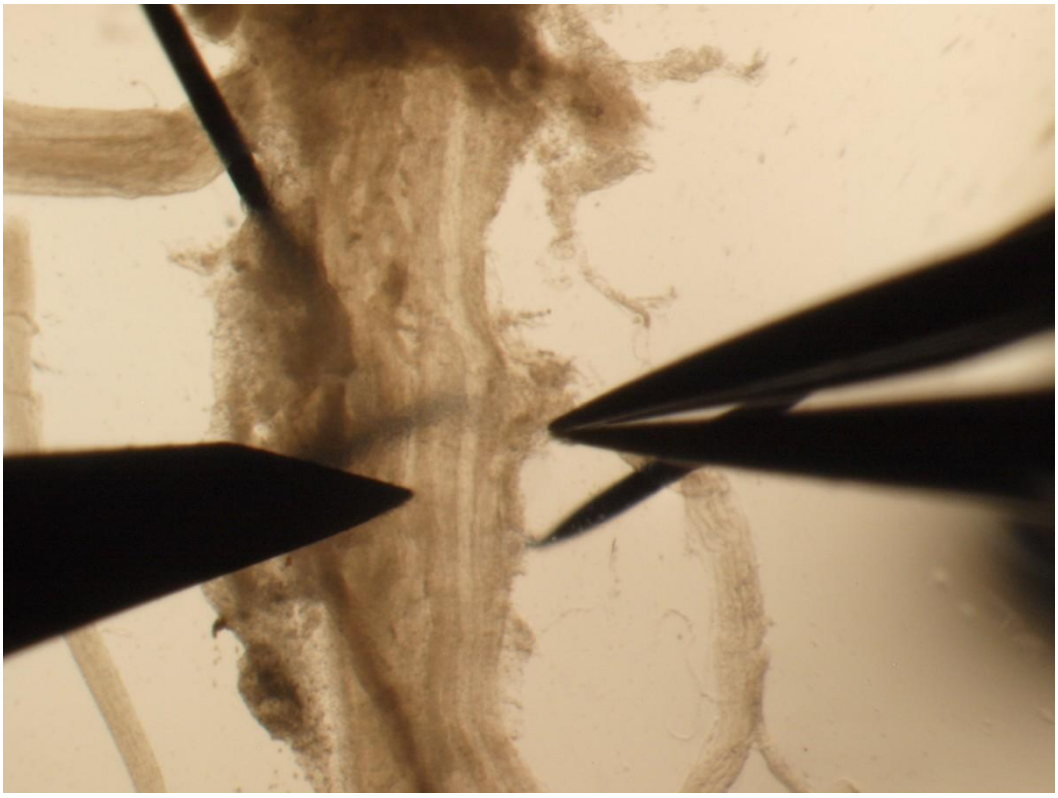

Image 9 - After removing layers of smaller axons, the giant axon is located on the ventral surface

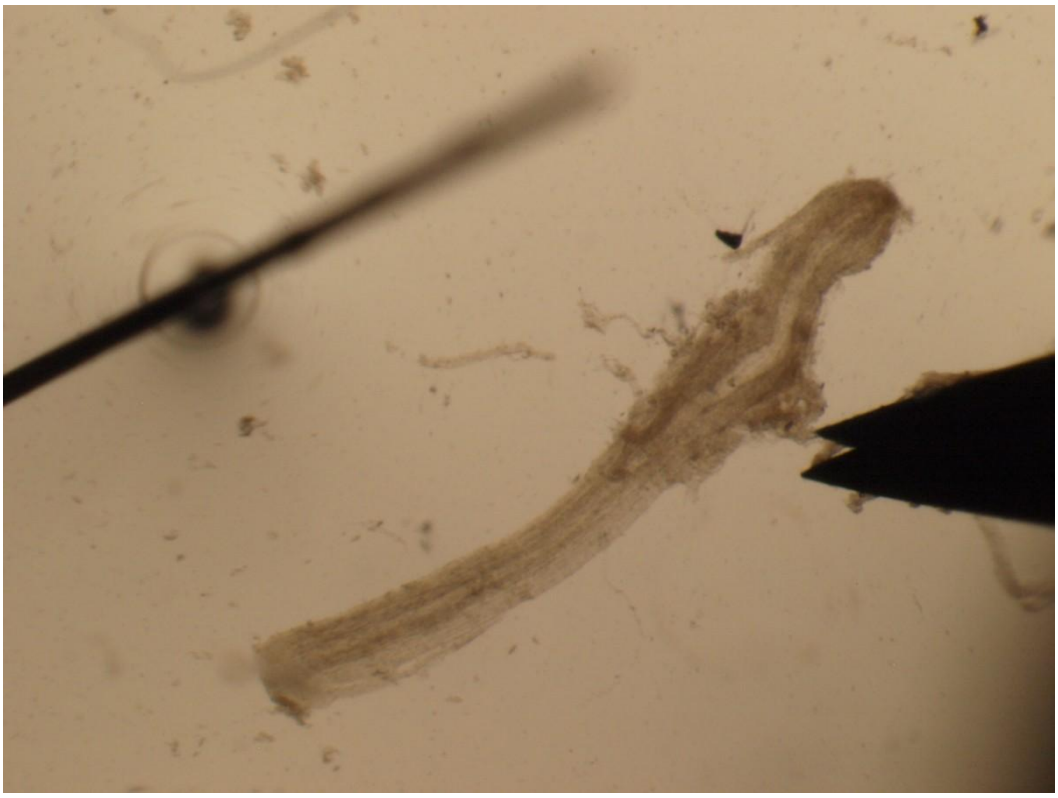

Image 10 - A giant axon with some connective tissue debris

To minimize the damage caused to the giant axons during dissection, a small strip of tissue debris and smaller axons may be left on the sides of the axonal preparate. This allows the moving of the preparate with dissection instruments without the need for directly disturbing the extremely fragile axon.

For experimental procedures, the axonal preparate can be transferred to another dish by a glass-tipped pipette. A convenient method for keeping the preparate in place during experiments is to carefully set a small segment of platinum wire on each end of the axon.

2.2 Preparing a neuronal culture of a sensory neuron from the pleural ganglion of California sea slug (*Aplysia californica*)

The protocols presented were acquired by the author during two visits to Dr Samuel Schacher's laboratory at Columbia University, New York, NY in August 2007 and 2010. Assistance and valuable advice on setting up a seawater aquarium was received from Markus Dernjatin, Sea Life Aquarium, Helsinki, Finland.

Aplysia californica can be ordered from the Aplysia facility of The University of Miami. Large (over 500 g) animals, needed for hemolymph extraction, must be caught in the wild, which is possible only during summer months (April-October). Smaller animals that are used for cell cultures are grown in aquariums and can be acquired year round.

The Aplysia are kept in a seawater aquarium with a water temperature between 13.5-15°C. The water is salinified with Instant Ocean®; the salinity level should be kept between 3.0-3.5 %. After delivery, the animals should be allowed to rest overnight before dissection or hemolymph extraction.

The solutions referred to in the protocols have the following compositions. Salt-enhanced Leibovitz medium (S-L15): 2000 ml L15, 30.4 g NaCl, 0.69 g KCl, 11.4 g $MgCl_2$, 12.48 g $MgSO_4$, 12.48 g dextrose, 0.38 g $NaHCO_3$, combined with 20ml penicillin-streptomycin solution (Sigma Cat. No. P0781) and 2.98g $CaCl_2$ that are mixed in a separate container. Protease solution: 7.3 ml S-L15 and 40 mg Dispase from Bacillus polymyxa (Invitrogen Cat. No. 17105-041). Glutamine-L15: 50 ml S-L15, 2 ml 200 mM glutamine. Culture medium: hemolymph : S-L15 : Glutamine-L15 = 2 : 1 : 1. Perfusion solution: artificial seawater (Instant Ocean) : S-L15 = 1 : 1.

2.2.1 Extracting Aplysia hemolymph

Aplysia hemolymph is a central component of the culture medium used in Aplysia neuronal cultures. Hemolymph is extracted through a lateral incision made with a razor to the side of the animal (Image 11). The animal is not anesthetized during this process, as anesthetization would affect the composition of the extracted hemolymph. Care should be taken not to let any ink excreted by the animal spoil the hemolymph, and it is recommended to wrap the animal in a paper towel when making the incision (Image 12).

The extracted hemolymph is centrifuged and the supernatant is filtered through a 450 nm filter. To prevent immunological reactions, it is important that hemolymph extracted from different animals is not mixed.

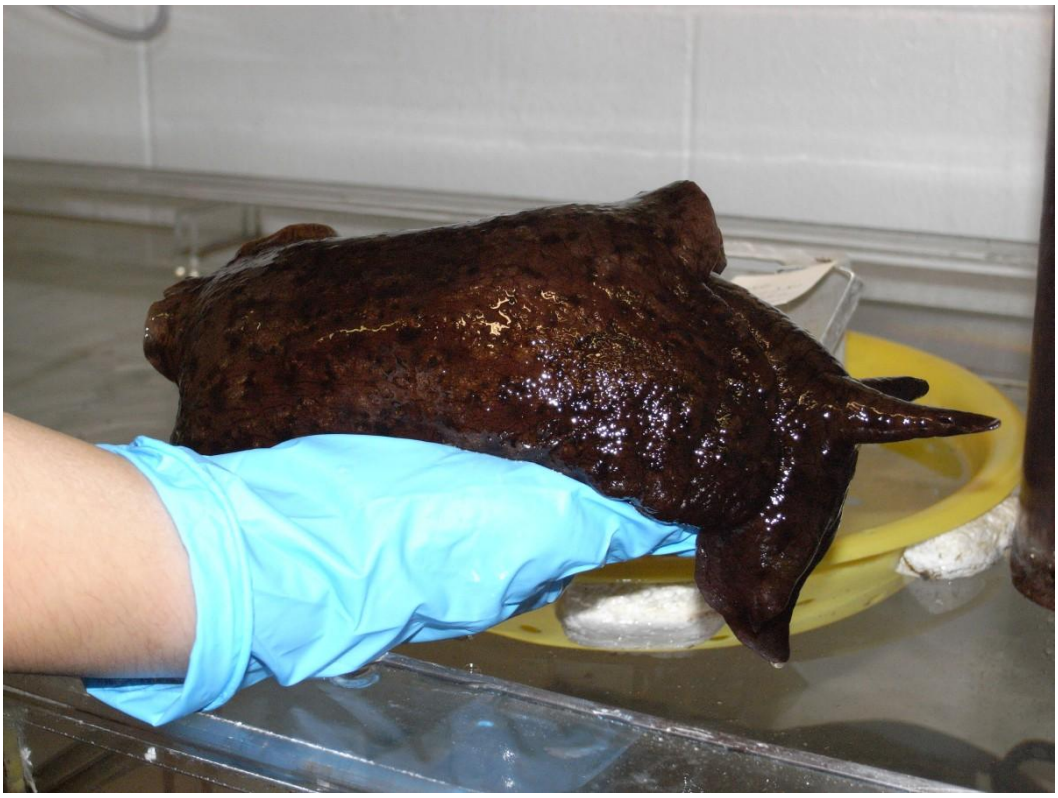

Image 11 - A large Aplysia. The incision is made laterally on the side facing the camera.

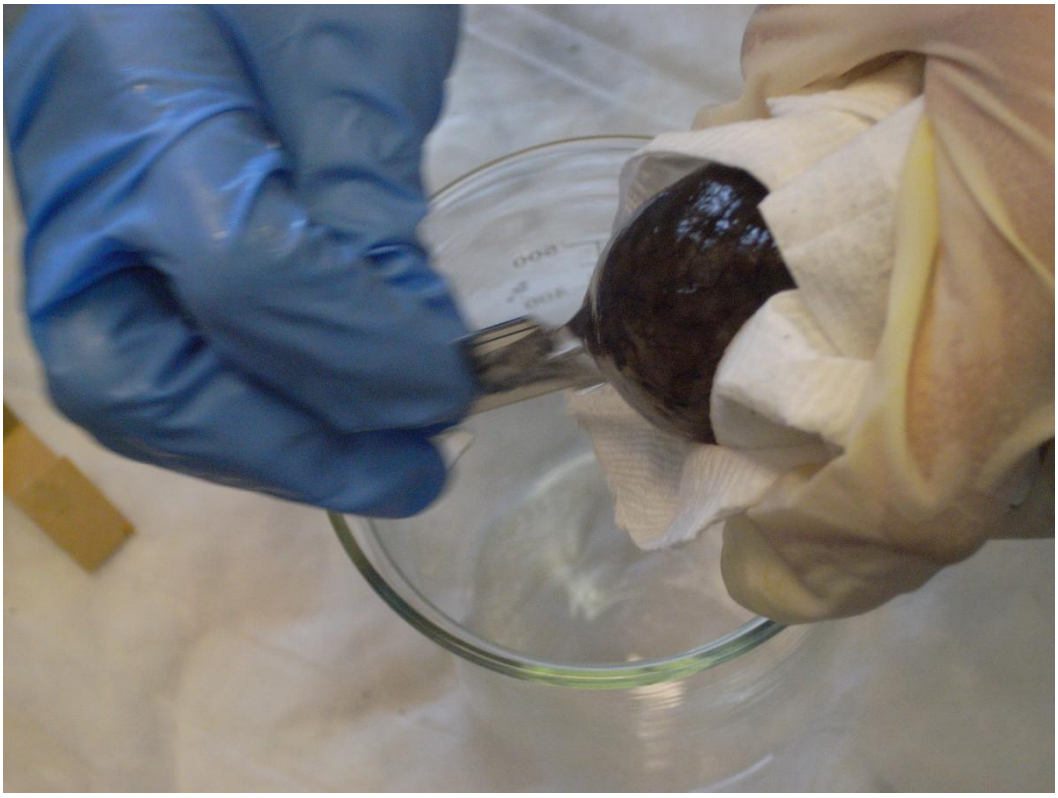

Image 12 - To prevent contamination of the hemolymph by ink, the Aplysia is wrapped in a paper towel.

2.2.2 Dissection and cell culture preparation

A method is presented for preparing a one-cell culture of a VC mechanosensory neuron from the pleural ganglion. Contrary to many other Aplysia neurons these cells are intrisically silent; they do not fire action potentials without external stimulation. VC mechanosensory neurons are also abundant in the pleural ganglion and their detachment from the surrounding tissue is relatively easy.

Prior to dissection, the Aplysia are anesthetized by injecting $MgCl_2$ isotonic to sea water into the body cavity of the animal (Image 13). The animal is pinned on a dissection tray, tail before head in order to prevent contraction of the animal. The body cavity is cut open (Image 14) and the rostrally located pleural and pedal ganglia (Image 15 and Image 16) are removed by cutting the connectives extending from the ganglia. The ganglia are transferred into a dish filled with protease solution and incubated for 120 min at 33.8 °C. After incubation, the ganglia are rinsed twice with S-L15. The pleural ganglion is separated from the larger pedal ganglion by cutting through the pedal ganglion at the location where the connective between these two ganglia is attached. It is important not to damage the pleural-pedal connective as it contains the axons of the VC mechanosensory neurons.

The connective tissue sheath is removed and the ganglion is rinsed once more with S-L15 before transfer on a Sylgard dish filled with culture medium.

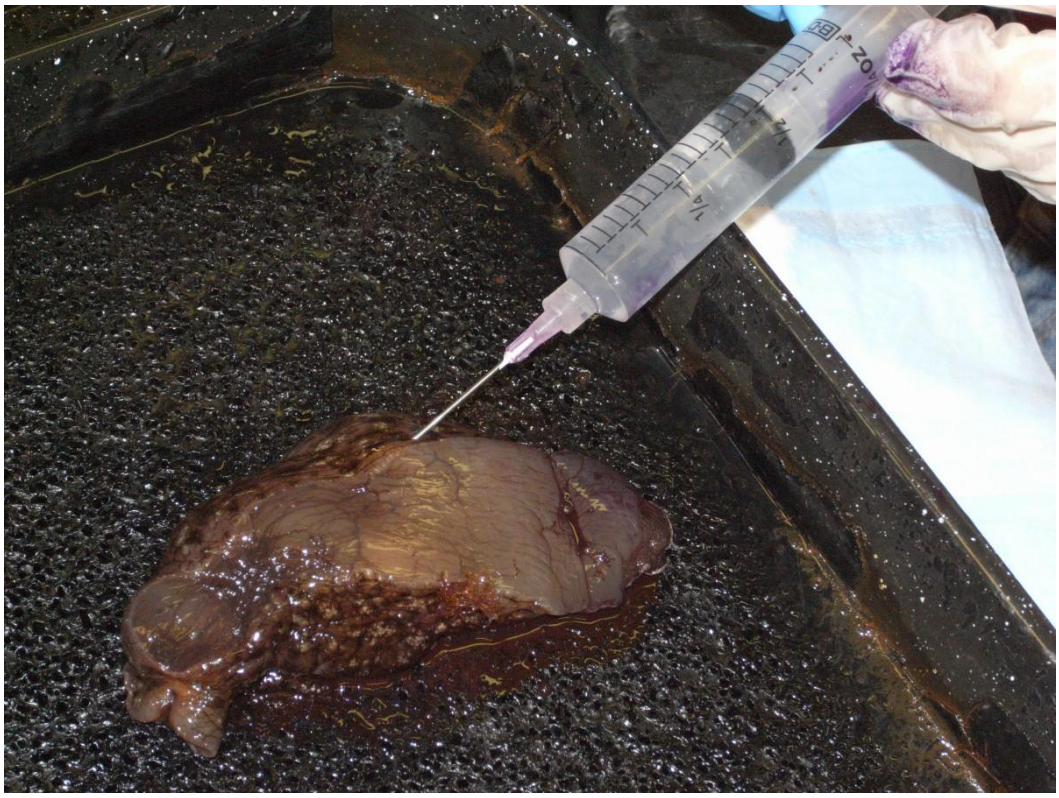

Image 13 - Anesthetization of Aplysia

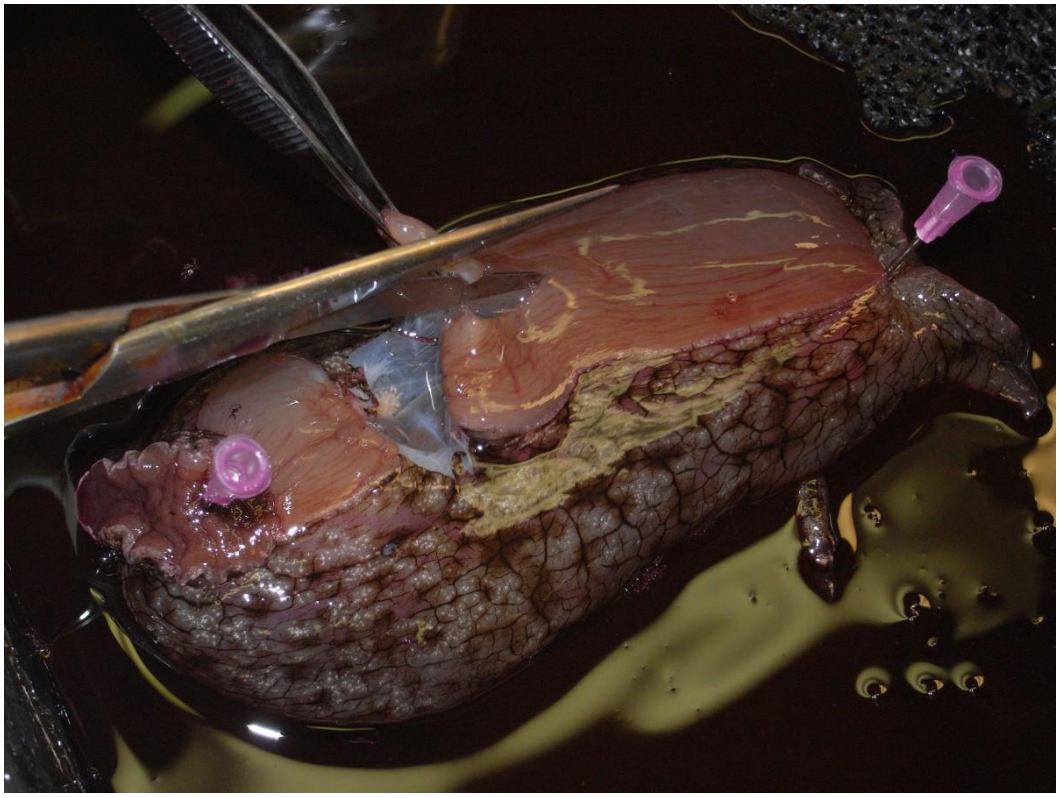

Image 14 - Opening the Aplysia body cavity

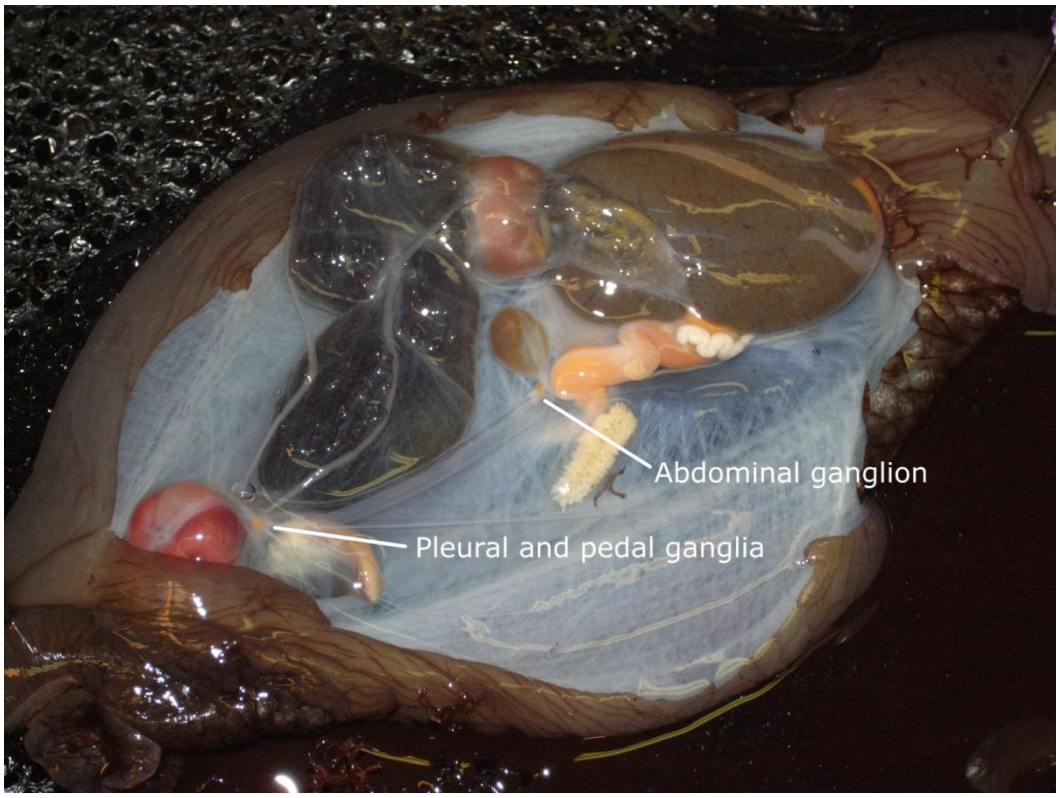

Image 15 - Aplysia cut open, with location of major ganglia indicated

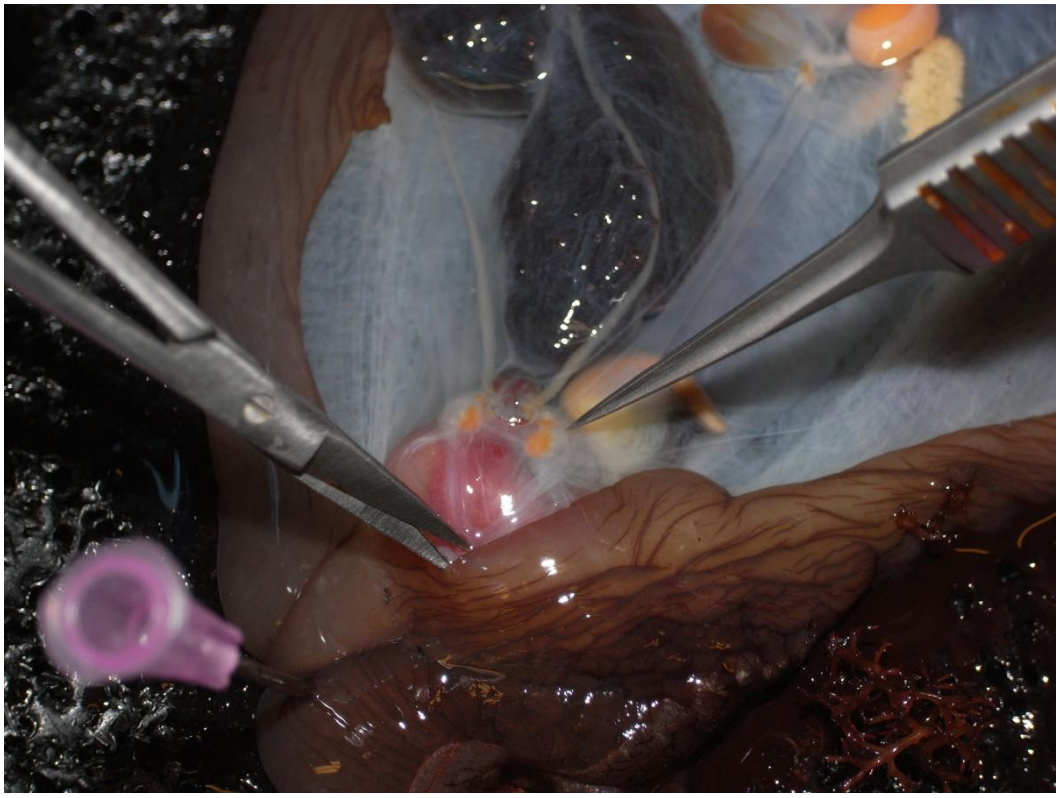

Image 16 - Close-up of the two pleural-pedal ganglion clusters

The ganglia are pinned down on the Sylgard through the middle of the ganglion (Image 17). The somas of the VC mechanosensory neurons are located around the proximal end of the pleural-pedal connective into which they project their axons. The neurons are separated from the ganglion by gently pushing on the axon hillock by a flexible instrument, e.g. a glass microelectrode. The viability of the separated neurons can be observed immediately: if a neuron is damaged during the separation process, its axon degenerates and the soma gets deformed (Image 18).

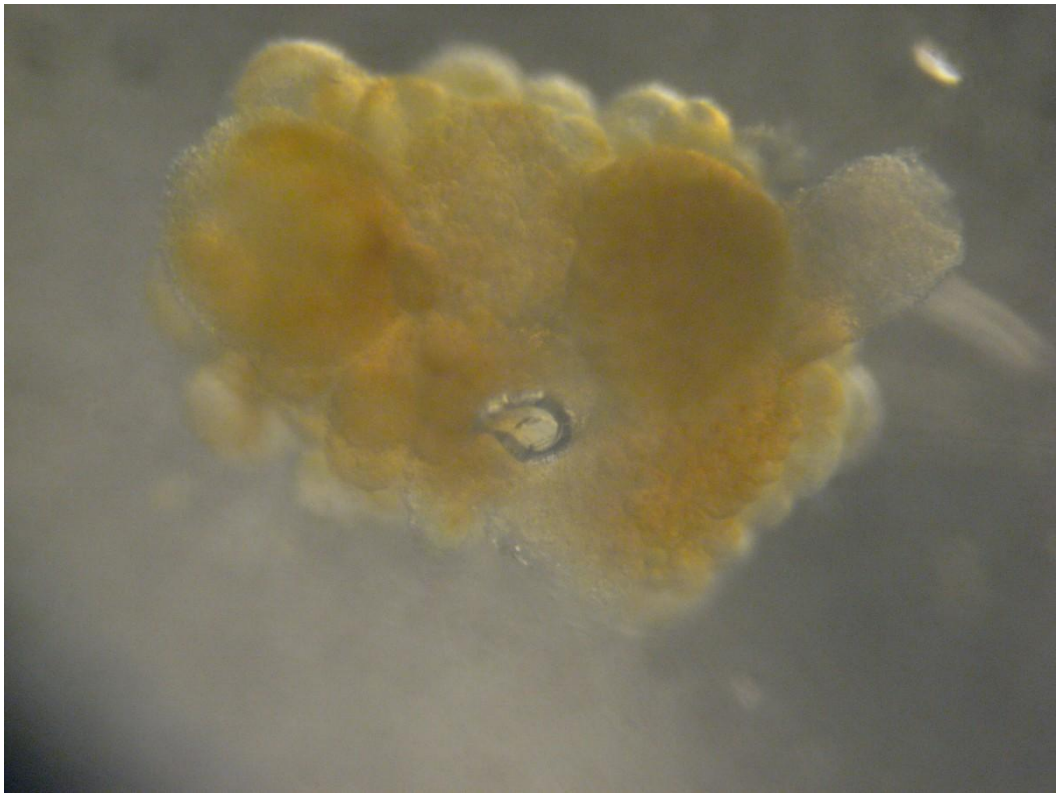

Image 17 - Pleural ganglion pinned on a Sylgard dish

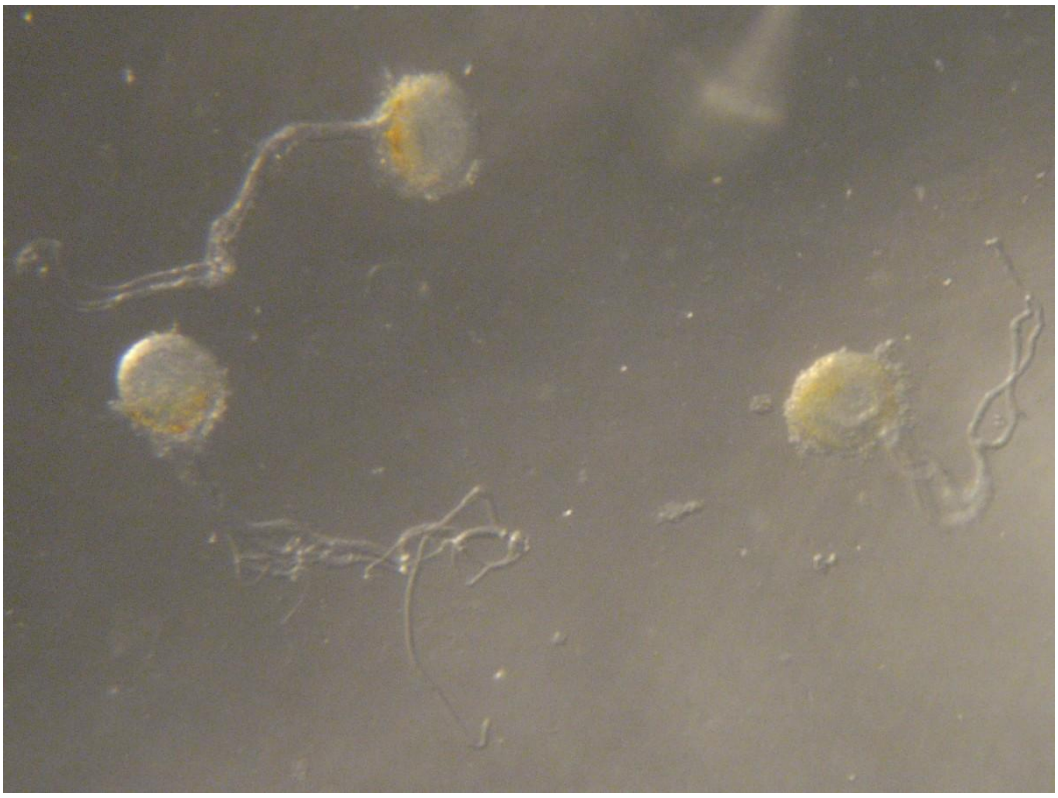

Image 18 – Three viable Aplysia neurons with intact axons

A separated neuron is transferred with a pipette on a poly-lysine coated glass-bottom culture dish filled with culture medium. By gentle handling with glass microelectrodes, the neuron is set to the desired position on the glass-bottomed area of the dish. The culture is incubated for 10 h in 33.8 °C.

When electrophysiological experiments are performed, the nutrient-rich culture medium is replaced with a perfusion solution that does not interfere with the measurement setup.

2.3 Studying the effect of phloretin on action potential

During the development of the crayfish preparation technique, preliminary experiments were performed on an abdominal chord preparate to test the applicability of the model animal for our purposes and also to screen for a possible effect of phloretin on the excitability of the giant axon. The experiments were performed only once, with a single preparate, and the significance of the experiments is thus preliminary only.

The dissection of the crayfish was performed as described in section 3.1. The giant axons were not dissected apart; the abdominal cord was left intact and a small section of the peripheral connective tissue sheath was removed to enable electrode penetration and stimulation. The preparate was incubated in room temperature for 30 minutes in a 1 mg/ml collagenase solution consisting of Collagenase Type I (from Millipore) dissolved in crayfish buffer. The proximal end of a medial giant axon between the second and third abdominal ganglia was stimulated externally by a blunt-tipped glass microelectrode attached to Digitimer DS2A Mk.II Constant Voltage Isolated Stimulator. The stimulus protocol consisted of a single monophasic block with a constant voltage of 50 V, and the stimulus durations used were 0.1, 1, 10 and 100 ms. Voltage over the axonal membrane was monitored distally at the same interganglíar region by an intracellular glass microelectrode with a resistance of 5-10 MΩ. If the stimulus artifact detected on the voltage measuring intracellular electrode started with a positive deviation from the base level, the stimulus was considered positive. If the stimulus artifact started with a negative deviation, the stimulus was considered negative. The excitability of the axon was studied systematically with both positive and negative stimuli.

The number of action potential spikes peaking over 0 mV that were generated by a single stimulus with a particular duration and sign were registered. The same stimulus was applied 5-20 times depending on the variability of the number of spikes generated (large variability led to more repeats) before altering the stimulus parameters. The experiment was monitored and the raw data was analyzed with WinWCP electrophysiology software from University of Strathclyde.

The excitability of the axon was studied first under 1 ml/min infusion of crayfish buffer, then under a 1 ml/min infusion of the buffer with 0.1 mM phloretin added. Finally, the phloretin was rinsed away with plain crayfish buffer and the excitability was studied once more under a 1 ml/min crayfish buffer infusion without added phloretin.

3 Results

We recorded the number of action potential spikes after 0.1 ms, 1 ms, 10 ms and 100 ms positive stimuli before, during and after perfusion with phloretin (Figure 1). The number of spikes shown is an average from 5-20 recordings after an applied stimulus with a specific duration and sign with the calculated standard deviation.

In the absence of phloretin (open circles), very few action potentials were generated with stimuli less than 10 ms, and only 100 ms stimuli generated a profound burst of spikes. After the addition of phloretin (red circles), a significant increase in the number of spikes was detected with the 10 ms and 100 ms stimulus, but the effect was only moderate with the shorter simuli. The stimulus series was repeated again without washing out the phloretin (green squares). Now even a 1 ms stimulus caused a major burst of spikes, but the increase in number of spikes when advancing to longer stimuli was not as steep as in the previous stimulus series. After washing out the phloretin with plain crayfish buffer (blue triangles) the axon did not react to the 1 ms stimulus, but with the 10 ms stimulus the axon fired a similar number of action potentials when compared to the previous stimulus series. The 100 ms stimulus now led to significantly fewer action potentials than before.

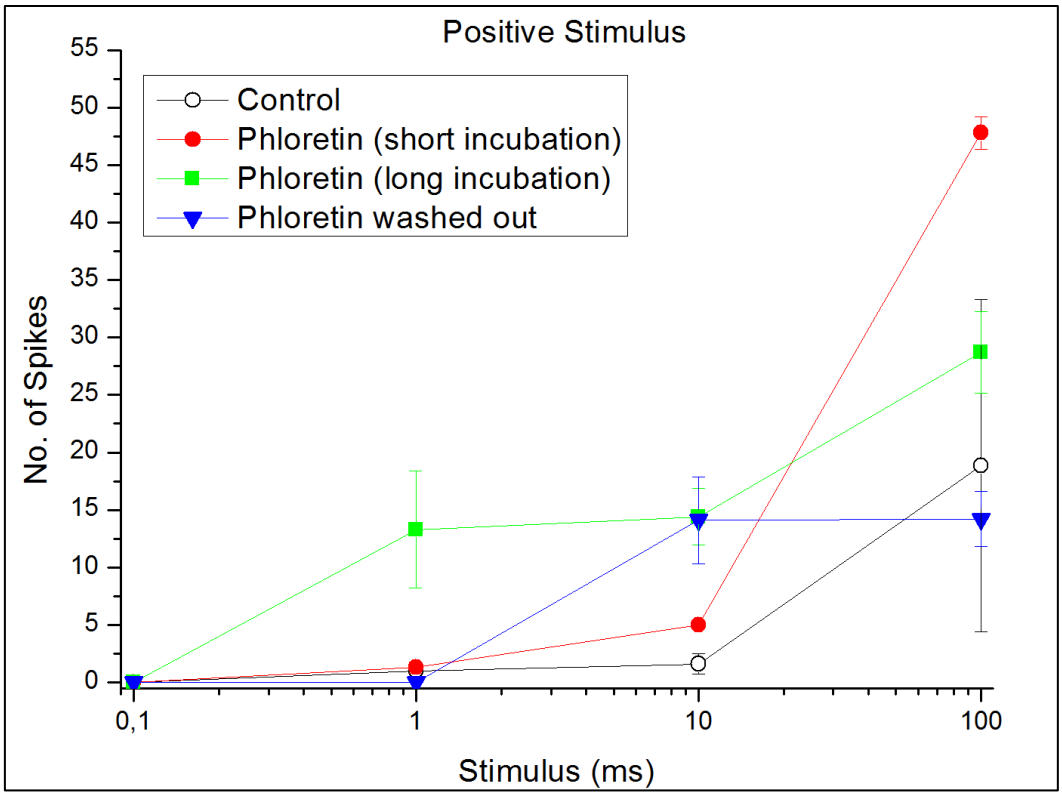

Figure 1 - No. of action potential spikes after positive stimulus

The effect of negative stimuli to the axon was also studied (Figure 2). Without phloretin (open circles) and during the first stimulus series after phloretin addition (red circles) the axon fired only a few action potentials even after a 100 ms stimulus. The second stimulus series with phloretin (green squares) showed a remarkable increase of excitability from 1 ms stimulus length onward. After washing out the phloretin (blue triangles), a 10 ms stimulus was needed for action potential generation and the number of action potentials fired was diminished by half on this stimulus length. The 100 ms stimulus resulted in a similar number of action potentials both before and after the washing.

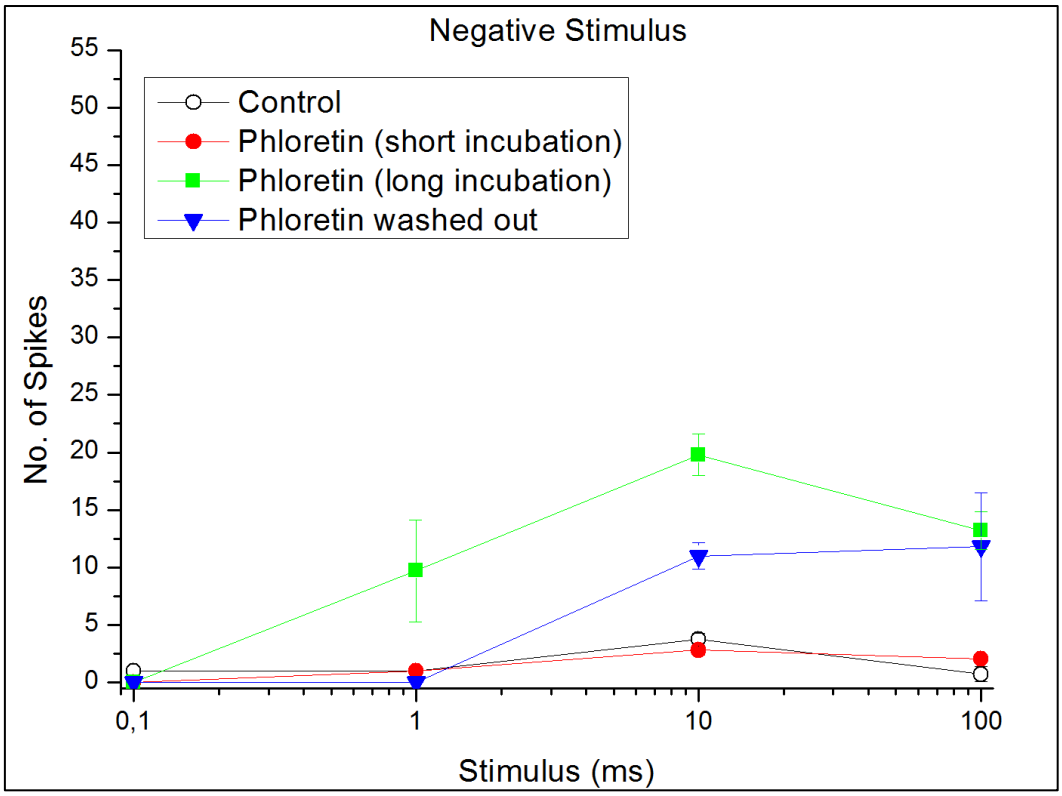

Figure 2 - No. of action potential spikes after negative stimulus

4 Discussion

A technique for the isolation of a giant axon from the abdominal cord of Pacifastacus leniusculus was developed. The fragility of the axon is a major issue, as the ability to generate and propagate action potential is severed when the axon is subjected to even the slightest damage. As previously mentioned, the author was not able to dissect a giant axon from the abdominal cord without destroying its ability to generate action potential.

A major difference between the preparation techniques of Pacifastacus leniusculus and Aplysia californica is the use of dispase from Bacillus polymyxa that breaks down the connective tissue around the Aplysia neurons and eases their separation from the pleural ganglion. During the development process of the Pacifastacus dissection techniques, a variety of enzymes were tested for their applicability in separating the giant axons from their surrounding connective tissue, but these also increased the fragility of the axon and no dissective actions could be performed on the preparate after enzyme incubation. A collagenase was used only after the dissection process was complete to ease the

penetration of microelectrodes through the axonal membrane. It is possible that a suitable enzyme that acts on the connective tissue but does not lead to the breaking of the axon itself indeed exists. The author decided not to perform a tedious search for an optimal enzyme, but instead tried to develop an enzyme-free dissection method. Unfortunately, the resulting method did not keep the axon intact for action potential generation.

A single-cell culture of an Aplysia neuron is an alternative model setup for deciphering the role of axonal membrane in action potential, and it has various assets compared to the Pacifastacus preparate: One Aplysia pleural ganglion can provide over 10 viable VC mechanosensory neurons, which makes the dissection process more time-efficient than the tedious preparation of a Pacifastacus giant axon. The Aplysia neurons are nearly devoid of extracellular debris and connective tissue, this makes them excellent candidates for studying the optical changes in the membrane. Anatomical cleanness of the separated neurons is a major asset for experiment repeatability as well, as penetration of drugs and fluorescent membrane probes is not hindered by variable amounts of connective tissue debris as is the case with the Pacifastacus preparate. A cultured Aplysia neuron is viable over a period of several days during which extended experimental procedures can be carried out. When a dissected Pacifastacus giant axon is used, experiments must be performed within a few hours after the dissection procedure.

The study on the effect of phloretin on the excitability of the Pacifastacus giant axon strongly suggests that phloretin lowers the functional treshold for action potential generation, and a possible mechanism for this is the lowering of the membrane dipole potential. Addition of phloretin initially increases the number of spikes generated by a stimulus, but the reached maximal excitability is gradually decreased during prolonged incubation by phloretin. A reasonable explanation for this would be that phloretin penetrates through the axonal membrane and lowers the dipole potential in the inner membrane leaflet, thus countering the effect of the dipole potential modification in the outer membrane leaflet and the initial increase of excitability. On the other hand, the repeated stimulation might have weakened the axon and thus decreased its general excitability. It should be noted that after the phloretin was washed out, the numbers of spikes generated after 10 ms positive stimulus and 100 ms negative stimulus were not

diminished but remained near-maximal. This suggests that the possible weakening of the axon would not thoroughly explain the changes in the excitability of the axon. The data acquired from the negative stimulus series further supports the role of dipole potential modification in the excitability changes of the axon. For a negative stimulus to induce a major burst of spikes, phloretin needs to reach the inner membrane leaflet and decrease its dipole potential. This is contrary to the positive stimuli, which induce fewer spikes as the phloretin reaches the inner membrane leaflet.

The significance of the phloretin experiments is preliminary only as the measurements were performed only once and with one axonal preparate. As discussed, Aplysia cultures should represent a more reliable model for these studies than the Pacifastacus preparate with which the preliminary experiment was performed. The phloretin study will be repeated with an array of Aplysia single-neuron cultures before further conclusions will be made about the effects and mechanisms of action of phloretin on the excitability of neurons.

Further information on the effect of phloretin, and more generally on biophysical phenomena taking place during the action potential that have been left unexplained by the HH model will be acquired by studies combining electrophysiological and fluorescence spectroscopy methods. The Aplysia neuron culture is an ideal model for these future experiments. The studies will also provide insight into the pharmacological mechanisms of anesthetics and other lipid-soluble drugs that affect neuronal function.

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