Characterization of the electrophysiology of neurons in the central nervous system of *Ciona robusta*

A Thesis
Presented to
The Academic Faculty

by

Paula Martinez-Feduchi

In Partial Fulfillment
of the Requirements for the Research Option in the
College of Sciences

Georgia Institute of Technology
May 2019

Characterization of the electrophysiology of neurons in the central nervous system of *Ciona robusta*

Approved by:

Dr. Alberto Stolfi, Advisor
School of Biological Sciences
Georgia Institute of Technology

Dr. Garrett Stanley
Coulter Department of Biomedical Engineering
Georgia Institute of Technology

Dr. Mary Holder
School of Psychology
Georgia Institute of Technology

Date Approved: May 2nd, 2019

This work is dedicated to my parents, Jorge Martinez-Feduchi and Clemencia Guijo, and my sister Alejandra Martinez-Feduchi, to honor the sacrifices they have made so I could have this opportunity, and their endless love, support, and encouragement. I'd also like to dedicate this to Bryce Watson who has excitedly and patiently listened to my rants about this project and encouraged my pursuit of science.

ACKNOWLEDGEMENTS

Many thanks to Dr. Alberto Stolfi, Susanne Gibboney, and Florian Razy-Krajka for their guidance. I'd also like to give thanks to Dr. Alberto Stolfi for conducting whole mount fluorescent *in situ* hybridization, and to Christopher Johnson for providing the *Crystallin-β-γ* construct and for performing its electroporation. Lastly, I'd like to give thanks to Bo Yang at the Neuroscience Core Facility in the Parker H. Petit Institute for Bioengineering and Bioscience for conducting the electrophysiology measurements.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

The objective of this study was to investigate the electrophysiological properties of *C. robusta* motor ganglion neurons—interneuron 2 (IN2) and the descending decussating neuron (ddN)— and the sensory cells of the papillae (Axial Columnar Cells, or ACCs). Primary cell cultures of mid-tailbud *C. robusta* larvae 24 hours after dissociation exhibited cell death and cell clumping, as well as neurons with short axons, typical of invertebrate larval neurons. In addition, cells did not attach to the poly-L-lysine glass coverslips. Improvements to the dissociation protocol reduced environmental stress and increased cell viability in plastic petri dishes only, with cells continuing to exhibit cell death when plated onto the poly-L-lysine coated glass coverslips. Future experiments should focus on refining the primary cell culture protocols to increase cell viability and cell attachment, as well as decreasing bacterial contamination.

CHAPTER 1

INTRODUCTION

The field of neuroscience is rapidly expanding, although the mechanisms of actions of neurons and the networks they interact through are still being studied. Thus, studying the electrophysiology of a neuron is essential in order to understand, predict and manipulate the communication between neurons to gain an understanding of the brain's functions and to improve them in diseased states. In 1952, Hodgkin and Huxley established the basis of neuronal electrophysiology through direct manipulation of ion currents in the giant squid axon using the voltage-clamp technique. The results of their experiments established the Hodgkin-Huxley model, describing action potential initiation and propagation based on Na+ and K+ ion conductance (Hodgkin and Huxley, 1952). In addition, the development of the patch-clamp technique to record current-voltage relations in a single ion channel allowed for the investigation of the functions of specific ions in neuronal activation and their effects on the resting membrane potential (Neher and Sakmann, 1976). Even though techniques have been developed in the field to study the electrophysiological properties of neurons, studies on the human brain are performed using model organisms due to experimental constraints.

Tunicates are model organisms for developmental biology and are the current sea invertebrates most closely related to vertebrates (Stolfi and Levine, 2015). Ciona robusta, a tunicate species, has become one of the primary model organisms for the study of the development of the nervous system due to its amenability to molecular perturbations, its

deterministic cell lineages its close genetic relatedness to vertebrates, and because it has its full genome sequenced (Veeman et al., 2011; Stolfi and Levine, 2015). In addition, *C. robusta* has a fully characterized connectome, a comprehensive map of the connections and cell structure of its larval nervous system, which is composed of only 177 neurons and 53 peripheral sensory cells (Ryan et al., 2016). Despite these findings, there is not a lot of literature on the neurons composing the nervous system, and it is unknown how they are involved in sensory or motor processes. Given the tunicates' metamorphosis from free-swimming larvae to sessile adults, it is of interest to study the anterior sensory papillae, conical protrusions in the epithelium required for the larva to settle on appropriate substrate, thus triggering metamorphosis (Wagner et al., 2014; Pennati and Rothbächer, 2015; Holland, 2016).

The purpose of this study is to characterize the electrophysiology of neurons, in particular interneuron 2 (IN2, A11.117), and the descending decussating neuron (ddN, A12.239) in the *C. robusta* motor ganglion, as well as the ACCs of the papillae, in order to gain a better understanding of the electrical properties of their membranes, which could provide some insight into their function in larval swimming and settlement. This research will involve creating and electroporating fluorescent constructs into larvae cells to identify the neurons of interest and conducting patch-clamp analysis in vivo to determine the electrophysiological properties of each cell. The data obtained from such analyses will be used to determine the patterns of inward and outward currents, the characteristics of the action potentials and spike trains, as well as to create current-voltage plots. This characterization will allow better understanding of the mechanisms underlying action potential initiation and propagation in the motor ganglion and papillae

and will establish the basis for future experiments on the functions of such cells. Additionally, this research will help expand the understanding of *C. robusta* as a model organism, as the identity and electrophysiology of the neurons in its central nervous system are still being investigated.

CHAPTER 2

METHODS AND MATERIALS

2.1 Model Organism

Adult Ciona robusta (formerly intestinalis Type A) were collected from San Diego, CA and shipped by Marine Research and Educational Products (M-REP).

2.2 Creation of Constructs

Plasmid cloning by restriction enzyme digest was conducted in order to create GFP constructs targeting the neurons of interest. Plasmid inserts were previously created based on previous probe screenings by the Stolfi Lab. DNA ligation was conducted to fuse each of the inserts into the recipient plasmid by adding 2.25 µL of the inserts Dmbx, Vsx and Crystallin-β-γ promoters for targeting ddN, IN2 and ACCs of the papillae, respectively, 2.25 µL of GFP vector |__| > nlsG, 5 µL of 2X Rapid Ligation Buffer, and 0.5 µL of T4 DNA ligase in an Eppendorf tube (Stolfi and Levine, 2011; Shimeld et al., 2005). After, 30 µL of competent E. coli cells were transformed with 2.5 µL of each of the constructs (*Dmbx*>GFP, *Vsx*>GFP, and *Crys-β-γ*>GFP), placed on a 42°C waterbath for 40 seconds, and then placed on ice for 2 min. Then, 50 µL of LB media without ampicillin was added to the cells, and they were plated on LB media + agar + ampicillin petri plates using sterile glass beads. After drying, the plates were incubated at 37°C overnight, with a maximum incubation time of 18 hours.

The next day, 8 colonies from each of the plates were picked into Eppendorf tubes containing 1mL of LB-ampicillin media and left in the 37°C shaker overnight (Eppendorf, Germany). Then, Colony Crack PCR was conducted to identify which cultures had successful ligations. 0.5µL of each culture was pipetted into a tube in an 8-tube PCR strip and 9.5 µL of Colony PCR Master Mix was added: 47.4 µL H2O, 47.6 µL 2X Quickload Taq Mix, 2 µL of Forward primer (Vsx = pFog, 20 µM & Dmbx = 298seq, 20 µM), and 2 µL of Reverse primer (GFP = uniq seq rev, 20 µM)). The CRACK program was run, and 1% agarose gel electrophoresis of the resulting products was conducted. Then, maxi culture was performed by pouring the rest of the successful cultures into 150 mL glass beakers containing 2XYT Broth + ampicillin and brewed overnight in the 37°C shaker. After, the cultures were transferred to 150 mL plastic bottles and centrifuged at 8°C for 30 minutes at 4,100 rpm. The supernatant was discarded, and the bottles were frozen with the pellet.

Maxi prep was then conducted to purify the plasmid DNA by following NucleoBond's DNA Xtra Midi/Maxi protocol (Takara Bio USA, Japan) for Midi cultures. The plasmid DNA was then measured in NanoDrop, and samples were diluted with H2O Ultra-Pure to reach a desired concentration between 2-3 µg/µL. Afterwards, whole mount fluorescent in situ hybridization was conducted to verify the constructs' specificity through the visualization of the location of the expressed RNAs in *C. robusta* embryos.

2.3 Electroporation

In order to conduct electroporation, a method of introducing the constructs into fertilized *C. robusta* eggs using a pulse of electricity, eggs had to be fertilized and dechorionated, as a modification of established protocols (Christiaen et al., 2009).

First, eggs were dissected from at least 2 adults and added to wells in a 6-well plate containing ASW (artificial sea water) using a glass pipette. Then, the sperm ducts were punctured using a new glass pipette and the sperm was pipetted onto an Eppendorf tube on ice. After, the eggs were transferred into a glass jar with ASW containing a basket and were rinsed 3 times by changing the ASW to mechanically break the mucus sac containing the eggs. The sperm was then transferred to basic ASW (2.5 µL of 10N NaOH in 10 mL of ASW) to become activated and poured into the basket containing the eggs. After 5 minutes, the eggs and sperm were imaged under an inverted microscope to ensure their quality and the success of the fertilization. The basket was then transferred to a new jar containing half of a pronase solution (pronase aliquot, 0.3 µg NaThioglycolate, 168 µL 10N NaOH, and 40 µL of ASW), with the rest of the solution being added on top of the basket. The eggs were immediately pipetted onto 15 cm ASW-agarose plates containing ASW and pipetted constantly for 4 minutes until the solution turned cloudy. The plate was manually swirled, and dechorionated eggs (purple/pink) were separated from undechorionated eggs (orange/yellow). The eggs were then rinsed by transferring 3 more times to 100 mm x 15 mm and twice to 60 mm x 15 mm ASW-agarose polystyrene petri dishes containing ASW (Fisher Scientific, New Hampshire). The eggs were electroporated 30 minutes post-fertilization and incubated at 20°C.

2.4 Dissociation

Dissociation of the embryos occurred in the mid-tailbud stage 14 hours post-electroporation at room temperature, a procedure adapted from established protocols from the Stolfi Lab. Larvae were collected and transferred into 12x75 disposable borosilicate glass culture tubes using glass Pasteur pipets (Fisher Scientific, New Hampshire). After the larvae settled to the bottom of the tube, the ASW was removed and replaced 3 times with 2 mL of Calcium/Magnesium-Artificial Sea Water (449 mM NaCl, 9 mM KCl, 33 mM Na2SO4, 2.15 mM NaHCO3 (0.5 M), 10 mM Tris-Cl (1M, pH 8.0), 2.5 mM EGTA (0.25 M, pH 8.0)). The supernatant was then removed, 2 mL of 0.2% trypsin solution was added, and the solution was pipetted vigorously 60 times with a glass Pasteur pipet (Sigma Aldrich, Missouri). Then, 2 mL of 0.05% BSA solution was added in order to deactivate trypsin (Sigma Aldrich, Missouri). The solution was then pipetted into 5 mL glass falcon test tubes with 35 µm cell-strainer caps (Fisher Scientific, New Hampshire). The filtered solution was then split into two 2 mL non-adhesive tubes, and centrifuged for 2 minutes at 800xg at 4°C. Then, the supernatant was removed, the pellet was resuspended in L-15 media, and the cells were seeded onto cell culture plates (Thermo Fisher Scientific, Massachusetts).

2.5 Cell Culture Media and Plates

The cell culture media used was Leibovitz's L-15 (Thermo Fisher Scientific, Massachusetts) adjusted to sea water salt concentration (13 mL MgCl 1M, 1.75 g MgSO4 anhydrous, 10.16 g NaCl, 489 ng CaCl anhydrous, and 185 mg KCl per 500 mL) with antibiotics added (2.5 mL of 100X penicillin, 100 U/mL and streptomycin, 0.1 mg/mL), adapted from established protocols (Hoechner et al., 2003; Zanetti et al., 2007).

Cells were plated onto 12 mm #1 thickness poly-L-lysine coated glass coverslips placed in 100 mm x 15 mm petri dishes with L-15 media (Fisher Scientific, New Hampshire; Neuvitro Corporation, Washington).

2.6 Patch Clamp Analysis

Patch-clamp analysis was performed by Bo Yang at the Neuro Design Suite of the Neuroscience Core Facility, Parker H. Petit Institute for Bioengineering and Bioscience. Equipment used for the electrophysiology recordings included: an Axon MultiClamp 700B Amplifier—a computer-controlled microelectrode amplifier for patch voltage-clamp—, a Slice Scope Pro 1000 system—a compact upright microscope with a motorized fixed stage, electrode manipulators, and a 780 nm LED system—, a Digidata 1500A Digitizer, and a Teledyne Dalsa Intrinsic High Speed Imaging Camera (Molecular Devices, San Jose, California; Scientifica, United Kingdom; Teledyne Technologies, Thousand Oaks, California).

CHAPTER 3

RESULTS

3.1 Primary Cell Culture

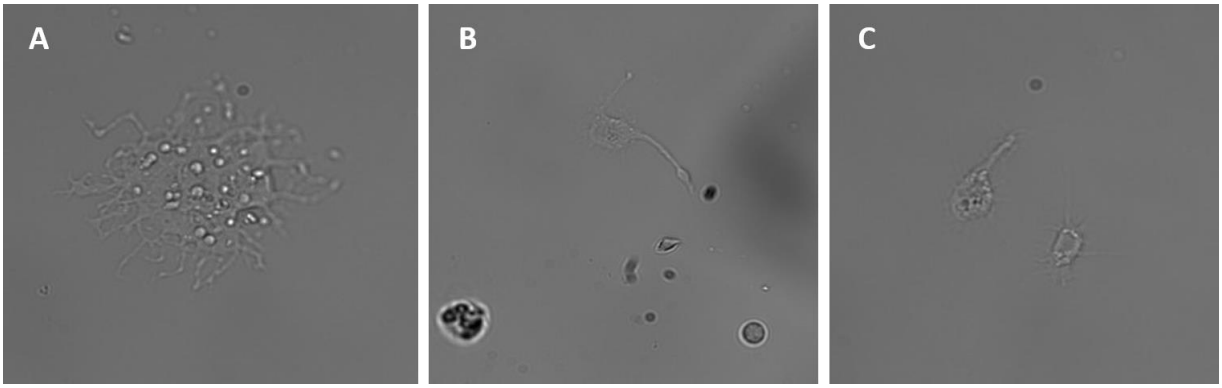

Figure 1 – Cell cultures of C. robusta exhibiting typical larval neurons. Initial cell cultures of *C. robusta* imaged 24 hours after dissociation suggest issues in the cell culture protocol, with most cells exhibiting signs of cell death. (A) Cell undergoing apoptosis. (B) Neuron with short axon, typical of larval brain neurons. (C) Neuron with short axon (left) next to a cell undergoing apoptosis (right).

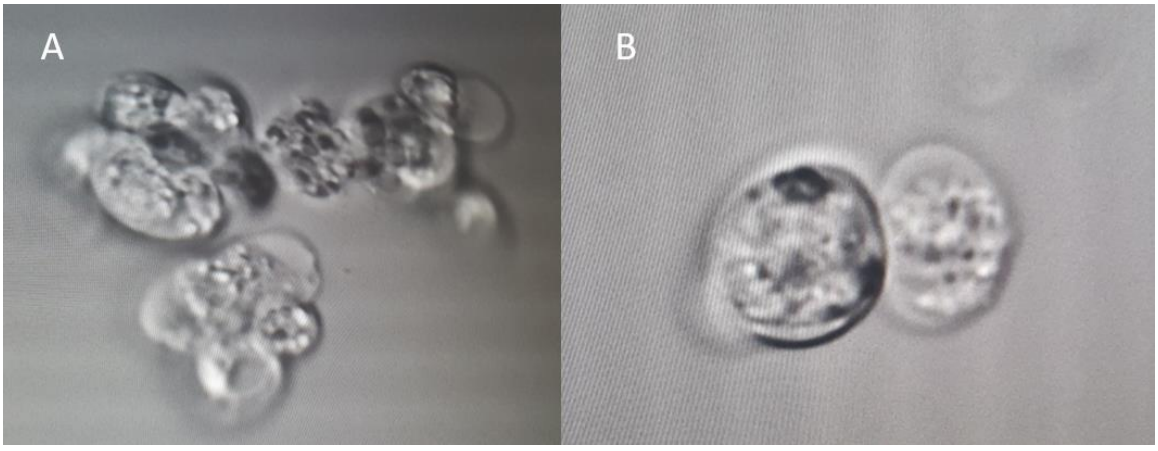

Figure 2 – Cell cultures of C. robusta exhibiting cell clumping and lack of attachment. C. robusta cell cultures 24 hours after dissociation exhibited clumping (left) and lack of attachment (right) to the poly-L-lysine coated glass coverslips, suggesting that the cells may be experiencing environmental stress.

C. robusta larvae cells were transfected with the *Dmbx*>GFP, *Vsx*>GFP, and *Crys-β-γ*>GFP constructs using the electroporation method. To determine successful localization, intact larvae were visualized using fluorescence microscopy. Results of such analysis revealed correct labelling of the IN2s, ddNs, and ACCs by the GFP constructs (data not shown).

Initial primary cell cultures of *C. robusta* 24 hours after dissociation exhibited cell death, with the observed cells undergoing apoptosis, cell debris throughout the cell culture media, and neurons with short axons, typical of larval brain neurons 14 hours after fertilization (Fig. 1). In addition, cells did not adhere to the glass coverslips, and were moving throughout the culture media, with some cells clumping together (Fig. 2). Bacterial contamination was also present in all the analyzed coverslips, despite the addition of antibiotics (penicillin and streptomycin).

Modifications to the established dissociation protocol from the Stolfi lab were applied, with the dissociation being conducted at room temperature instead of on ice. In this experimental trial, cells were not plated onto poly-L-lysine coated glass coverslips. Instead, cells were seeded onto a 100 mm x 15 mm petri dish containing the L-15 media with antibiotics added. Cells were then analyzed under an inverted microscope. These cells were viable, and as expected, did not adhere to the petri dish, although bacterial contamination was still present.

Primary cell cultures with these modified protocols yielded the same results as initial cell cultures, with cells undergoing apoptosis and clumping, and not adhering to the glass coverslips.

3.2 Electrophysiology Recordings

Electrophysiology recordings were unsuccessful, as there was a lack of viable cells and the few neurons exhibiting typical larval growth did not attach to the glass coverslips. In addition, we were unable to identify GFP-labelled cells using the GFP fluorescence filter on the electrophysiology rig at the Neuro Design Suite. This could be due to the relatively low percentage of neurons expected to express each cell type-specific GFP reporter in the larva (< 0.1% of all cells).

CHAPTER 4

DISCUSSION

4. 1 Principal Findings

The purpose of this study was to characterize the electrophysiology of neurons of *C. robusta*, specifically IN2 and ddN, as well as the papillae, in order to gain a better understanding of the electrical properties of their membranes. Difficulties maintaining the viability of the primary cell cultures of *C. robusta* larvae 14 hours post-fertilization prevented electrophysiological recording and analysis. Primary cell cultures exhibited signs of cell death including cell debris in the media, and cells undergoing apoptosis. One possible reason for the decreased cell viability could be due to exposure to environmental stress. Improvements to the dissociation protocol, with the dissociation being conducted at room temperature instead of ice, support this hypothesis, as cells cultured under this modification were viable and did not exhibit cell clumping. However, once primary cell cultures were seeded onto poly-L-lysine coated glass coverslips, the cells were once again observed to be undergoing apoptosis. In addition to decreased viability, cells were also clumped in the media, and did not attach to the glass coverslips.

4.2 Future Work

Future work should focus on refining the primary cell culture protocol. First, future experiments should plate the cells onto two 35 mm plastic petri dishes, one containing poly-L-lysine coated glass coverslips and one without, in order to assess if the

cells are sensitive to the material they are plated on. In addition, future experiments should be carried out at different temperatures to determine if there is an optimum for cell growth, as previous research that has successfully developed primary cell cultures of *C. robusta* cites an incubation temperature between 16 and 18°C (Francone et al., 2007). Moreover, the observed bacterial growth could be due to incubating the cell cultures at room temperature. In order to further reduce contamination, the media and other reagents used for the electroporation, dissociation, and cell culture media should be regularly checked to ensure their sterility, and other drugs such as antifungals could be added. Another possible cause for decreased cell viability could be the use of serum-free media. The addition of fetal bovine serum (FBS) and L-glutamine, could also increase cell growth and viability rates, as these provide the necessary growth promoting factors and amino acids needed by cells to grow (Gstraunthaler, 2003; Francone et al., 2007). Lastly, to increase the odds of finding a suitable neuron in culture for patch-clamp analysis, a pan-neural reported with much higher expression numbers could be used. Optimization of culture and recording should be conducted with such pan-neural reporters before attempting more specific reporters.

REFERENCES

1. Brunetti, R., Gissi, C., Pennati, R., Caicci, F., Gasparini, F., and Manni, L. Morphological evidence that the molecularly determined Ciona intestinalis type A and type B are different species: Ciona robusta and Ciona intestinalis. *J. Zool. Syst. Evol. Res.* **53:** 186-193 (2015).
2. Christiaen, L., Wanger. E, Shi, W., and Levine, M. Isolation of Sea Squirt (Ciona) Gametes, Fertilization, Dechorionation, and Development. *Cold Spring Harb Protoc.* 2009. doi: doi:10.1101/pdb.prot5344.
3. Francone, M., Ristoratore, F., and Zanetti, L. Development of primary cell cultures from *Ciona intestinalis* larval stage. ASSEMBLE. 2007. https://www.assemblemarine.org/assets/Uploads/Documents/tool-box/Development-of-primary-cell-cultures-from-Ciona-intestinalis-larval-stage.pdf
4. Gstraunthaler, G. Alternatives to the Use of Fetal Bovine Serum: Serum-free Cell Culture. *ALTEX*. 2003; **20**(4/03): 275–281.
5. Hochner, B., Brown, E. R., Langella, M., Shomrat, T., and Fiorito, G. A learning and memory area in the octopus brain manifests a vertebrate-like long-term potentiation. *J Neurophysiol.* 2003; **90**: 3547-3554. doi: 10.1152/jn.00645.2003
6. Hodgkin, A., and Huxley, F. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117:** 500–544 (1952).
7. Holland, L. Z. Tunicates. *Current Biol.* **26:** R146-152 (2016).
8. Neher, E., and Sakmann, B. Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature* **260:** 799–802 (1976).
9. Pennati, R., and Rothbächer, U. Bioadhesion in ascidians: a developmental and functional genomes perspective. *Interface Focus.* **5(1):** 20140061 (2015).
10. Ryan, K., Lu, Z., and Meinertzhagen, I.A. The CNS connectome of a tadpole larva of Ciona intestinalis (L.) highlights sidedness in the brain of a chordate sibling. *eLife* **5:** 1-34 (2016).
11. Shimeld, S. M., Purkiss, A. G., Dirks, R. P. H., Bateman, O. A., Slingsby, C., and Lubsen, N. H. Urochordate βγ-Crystallin and the Evolutionary Origin of the Vertebrate Eye Lens. *Current Biology.* 2005; **15**(18): 1684-1689. doi: doi.org/10.1016/j.cub.2005.08.046.
12. Stolfi, A., and Levine, M. Neuronal subtype specification in the spinal cord of a protovertebrate. *Development* **140:** 480–480 (2013).
13. Wagner, E., Stolfi, A., Choi, Y. G., and Levine, M. Islet is a key determinant of ascidian palp morphogenesis. *Development* **141:** 3084-3092 (2014).

14. Zanetti, L., Ristoratore, F., Francone, M., Piscopo, S., and Brown, E.R. Primary cultures of nervous system cells from the larva of the ascidian Ciona intestinalis. *J. Neurosci. Methods* **165:** 191–197 (2007).