Video Article

Isolation of Sensory Neurons of *Aplysia californica* for Patch Clamp Recordings of Glutamatergic Currents

Lynne A. Fieber[1], Stephen L. Carlson[1], Andrew T. Kempsell[1], Justin B. Greer[1], Michael C. Schmale[1]

[1]Division of Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Sciences, University of Miami

Correspondence to: Lynne A. Fieber at lfieber@rsmas.miami.edu

URL: https://www.jove.com/video/50543
DOI: doi:10.3791/50543

Keywords: Neuroscience, Issue 77, Neurobiology, Anatomy, Physiology, Cellular Biology, Molecular Biology, Environmental Sciences, Marine Biology, Receptors, Neurophysiology, Neurotransmitter, Neurotransmitter Agents, Patch Clamp Recordings, Primary Cell Culture, Electrophysiology, L-Glutamate, NMDA, D-Aspartate, dissection, ganglia, buccal ganglion, neurons, invertebrate, *Aplysia californica*, california sea slug, mollusk, animal model

Date Published: 7/10/2013

Citation: Fieber, L.A., Carlson, S.L., Kempsell, A.T., Greer, J.B., Schmale, M.C. Isolation of Sensory Neurons of *Aplysia californica* for Patch Clamp Recordings of Glutamatergic Currents. *J. Vis. Exp.* (77), e50543, doi:10.3791/50543 (2013).

Abstract

The marine gastropod mollusk *Aplysia californica* has a venerable history as a model of nervous system function, with particular significance in studies of learning and memory. The typical preparations for such studies are ones in which the sensory and motoneurons are left intact in a minimally dissected animal, or a technically elaborate neuronal co-culture of individual sensory and motoneurons. Less common is the isolated neuronal preparation in which small clusters of nominally homogeneous neurons are dissociated into single cells in short term culture. Such isolated cells are useful for the biophysical characterization of ion currents using patch clamp techniques, and targeted modulation of these conductances. A protocol for preparing such cultures is described. The protocol takes advantage of the easily identifiable glutamatergic sensory neurons of the pleural and buccal ganglia, and describes their dissociation and minimal maintenance in culture for several days without serum.

Video Link

The video component of this article can be found at https://www.jove.com/video/50543/

Introduction

The marine opistobranch mollusk, *Aplysia*, has been a useful neurobiological model for many decades. It is best known as a model of habituation and classical conditioning [7, 8]. Studies on learning and memory in this model won the Nobel Prize for Physiology or Medicine in 2000 for Eric R. Kandel, in a prize he shared with Arvid Carlsson and Paul Greengard [10]. Studies involving electrical recordings from reduced preparations, in which elements of the nervous system of this invertebrate are dissected from the animal with nerves and muscles left attached, have helped elucidate the roles of individual neurons in *Aplysia*. Identification of precise molecular mechanisms that constitute learning in *Aplysia* however, often employed another technique, long-term co-cultures of a sensory neuron and a motoneuron, obtained one by one from individual donor animals and allowed to form a synapse in the culture dish [21].

We and others [1, 3, 6, 14, 15, 16] have exploited the ease with which identified neurons can be targeted in this model as well as their endurance in long-term experiments to make dissociated short term cultures of clusters of nominally homogeneous neurons in which we study ionic currents under voltage clamp in the patch clamp configuration. Many *Aplysia* neurons stand up to repeated rounds of patch clamping to allow time for long-lasting experimental manipulations. The technique is useful for neurons such as the neurosecretory bag cells of the abdominal ganglion, and the sensory neurons of the pleural and buccal ganglia whose dissociation we describe here, but not for very large neurons >60 µm diameter, such as L7 or R2 of the abdominal ganglion. We do not employ *Aplysia* serum in our cultures, unlike the sensory-motoneuron co-cultures described elsewhere. Most neurons obtained using this procedure will be without processes for the first 48 hr in culture, facilitating whole cell voltage recording, but will then sprout and elaborate axons and other processes for approximately 14 days before dying from lack of nutrients and/or growth factors.

This technique produces primary cultures of 50-100 neurons per dish from physiologically documented regions of the buccal and pleural ganglia. This protocol is useful for researchers studying aspects of single cell physiology in experiments that require numerous experimental replicates per animal. It produces a matched pair of cultures due to the anatomical separation of the target cells into left and right hemiganglia, permitting studies that benefit from matched treatment and control cultures.

The protocol targets buccal S cluster (BSC) neurons of the buccal ganglion, and pleural ventrocaudal (PVC) neurons of the pleural ganglion. These cells are an appropriate size for whole cell voltage recordings and display robust glutamatergic responses. The discussed methodology is appropriate for most ganglia in the *Aplysia* nervous system.

Protocol

1. Cell Preparation

1. On Day 1, weigh and anesthetize animal.
    1. Weigh a 30 g-1 kg animal. Anesthetize in 5-10 animal volumes of 1:1 seawater:isotonic MgCl . $6H_20$ for 1 hr with aeration such as an electric aquarium air pump with attached airstone.

2. Prepare dissection supplies.
    1. Assemble clean dissection tray with stainless steel straight pins, such as fabric pins. Assemble several Petri dishes containing artificial seawater (ASW; see **Tables 1** and **3**) + penicillin/ streptomycin (P/S).
    2. Have ready a low power microscope. Have clean dissection instruments ready, such as ribbed nose and fine surgical forceps, and fine and large surgical scissors. Spray the instruments with 70% isopropol alcohol before use and allow to dry.

3. Position animal on dissection tray.
    1. Position animal ventral side down in the dissection tray. Pin animal through edges of body wall and parapodial borders, but avoid tail and head, to expose the dorsal surface and the genital groove
    2. Rinse the dorsal surface in slow-running cold tap water. Apply a light stream of 70% isopropol alcohol from a squeeze bottle along the genital groove and over the top of the head.

4. Make the initial incision.
    1. Using a low power microscope (see **Table 2**) and reflected light to observe the point where the genital groove originates from the anterior shell margin, tautly hold with ribbed-nose forceps a fold of tissue to the left of this origin, while snipping a shallow hole all the way through the smooth, flat area of body wall just to the right of the genital groove with fine angled scissors.
    2. Hemolymph should be observed to pour from the hole, sometimes carrying with it the abdominal ganglion and stomach.

5. Expand the incision.
    1. Use large scissors to expand the incision anteriorly to a point between the rhinophores, while pulling upward with the lower blade to avoid nicking the gut. The internal organs will be observed to spill out of the incision.

6. Remove the ganglia.
    1. Reposition the tray and refocus the microscope on the head region.
    2. Using clean forceps and fine scissors, remove the head ganglia by severing at one point the nerves that form the head ganglia into a ring around the esophagus to remove pleural-pedal and cerebral ganglia as a group.
    3. Remove the buccal ganglion that adheres to the ventral side of the esophagus.
    4. Remove the abdominal ganglion by cutting the 4 large nerve connectives that issue from this ganglion.

7. Isolate ganglia of interest.
    1. Trim head ganglia apart, leaving a length of connectives attached to each ganglion of interest that is equal to at least the diameter of the ganglion; this will retard enzyme over-digestion of the cells of interest in the next step.
    2. Put each target ganglion through 2 rinses of ASW + P/S, moving between rinses with clean forceps.
    3. If targeting the PVC cells, leave the right pleural hemiganglion attached to the right pedal hemiganglion, and similarly leave the left pleural hemiganglion attached to the left pedal hemiganglion.
    4. Discard unwanted ganglia and the rest of the animal according to accepted research practice.

8. Enzymatically digest the ganglia.
    1. For each ganglion, prepare 1 ml of enzyme solution consisting of 3.75 mg dispase, 1 mg hyaluronidase and 0.3 g collagenase type XI per ml of ASW + P/S in a 15 ml polypropylene conical tube.
    2. Place rinsed ganglia in enzyme solution and attach the cap tightly. Place the tube on its side on a rotary shaker (see **Table 2**) at slow speed for 13-5 hr overnight at approximately 23 °C (room temperature).

9. Prepare the culture dishes.
    1. Before microdissection (Day 2), prepare poly-D-lysine (PDL)-coated culture dishes in a tissue culture hood. Add ~0.5 ml PDL (0.2 mg/ml of sterile water) to the center of a 35 mm dish for ~25 min.
    2. Rinse PDL 3x with sterile water. Allow to dry. UV-sterilize the plates.

10. Day 2 Isolate neurons.
    1. Fill the center of 35 mm dishes that were PDL-coated and UV sterilized with approximately 0.5 ml of ASW + P/S to create an island of media inside the otherwise dry dish. This can be done on the bench, outside the tissue culture hood. One dish should be prepared for each cell cluster originating from a hemiganglion.
    2. Have ready a 100 mm diameter dissection dish made by sylgard-coating and curing a 5 mm deep dissection surface, outfitted with fine dissection pins of several sizes to be used as pins and probes (see **Table 3**). Rinse this dish with 70% isopropol alcohol, then with ASW + P/S, and finally fill it with ASW + P/S.

11. Prepare microdissection of digested ganglia.
    1. Pour the enzyme solution containing the digested pleural-pedal and buccal ganglia into the dissection dish.
    2. Place the dish on the stage of the microscope against a black surface under reflected light.

3. Using the attached connective tissue, pin down each pleural-pedal hemiganglion dorsal side up. The tissues will be soft and intolerant of stretching.

12. Microdissect PVC neurons.
    1. Using 2 pairs of clean fine forceps, and holding a fine dissection pin in one pair of forceps as a probe, isolate the PVC cells from a pleural hemiganglion. Enzyme digestion will have removed or broken apart the connective tissue sheath covering the PVC cluster, yet the cells will adhere to one another.
    2. Use the probe to detach these cells from the pedal-pleural connective[18], then let the cell cluster fall to the bottom of the dissection dish.

13. Transfer neurons to culture dish.
    1. Transfer the cell cluster to a prepared 35 mm culture dish by gently sucking up the cluster into the tip of a slightly fire polished disposable glass Pasteur pipette, then dispensing it slowly into the media island in a culture dish, being careful to avoid introducing air bubbles into the pipette. Repeat isolation of the PVC cluster of the other hemiganglion and place into a separate culture dish.

14. Microdissect and transfer BSC neurons.
    1. Pin out the buccal ganglion ventral side up, with care to spare the cells of interest. Repeat step 1.12 with the 2 BSC cell clusters of the buccal ganglion[10].
    2. Isolation of PVC and BSC neurons will produce 4 culture dishes containing neuron clusters: 2 dishes containing BSC clusters and 2 dishes containing PVC clusters. Discard the rest of the ganglia and set aside the 100 mm dissection dish.

15. Dissociate the cell clusters.
    1. Place a culture dish containing cells on the stage of the low power microscope and remove the cover.
    2. Dissociate the PVC cluster by gently flicking the bottom of the culture dish adjacent to the cell cluster with a fine dissection pin held in forceps. Flicking is digging the tip of the pin into the plastic of the bottom of the dish as though trying to dig out a fleck of plastic. When friction with the plastic releases the pin point, a percussion wave is produced through the medium that breaks apart the nearby clump of cells.
    3. 5-10 flicks may be needed to nearly completely dissociate the cells, but it is better to leave small clusters of cells rather than to flick too much lest the cells be destroyed by mechanical sheer.
    4. Replace cover and repeat with other culture dishes.

16. Store cell cultures.
    1. Place the culture dishes containing media islands in their centers with dissociated cells into a large container that permits air circulation, such as a 150 x 25 mm round plastic Petri dish, and place this dish in an incubator set to 17 °C.
    2. Install in the chamber an open dish of water as a source of humidity. Leave undisturbed overnight. The cells will adhere to the poly-D-lysine coating in the center of the dish.

17. Flood culture dishes.
    1. On Day 3, Gently flood the dishes with 2.5 ml of ASW + P/S. Examine and count the cells using an inverted cell culture microscope. Store in the 17 °C incubator.

2. Electrophysiology

The method is standard patch clamping that has been described in numerous texts (*e.g.* Sakmann and Neher, 1995)[16]. This protocol will work on cells ≤100 pF capacitance, or cells <60 µm diameter during days 3 and 4 of the protocol. Cells without processes are optimal for recording. The following special considerations apply:

1. Larger cells can be accommodated with the configuration setting on the Axopatch 200B amplifier set to β=0.1. Activation of very large currents may cause cells to escape voltage clamp. ASW solutions substituted for salt content (*e.g.* a fraction of the NaCl substituted with impermeable N-methy-D-glucamine chloride) may be used to reduce whole cell current amplitude.
2. Fiber-filled borosilicate patch pipettes pulled on a pipette puller and backfilled halfway with intracellular solution (ICS) consisting of 450 mM KCl and other salts (see **Table 1**) are suitable, and should have resistance of approximately 0.5-1 MOhms. If isolation of specific ionic currents is desired, for example, Na^+ currents in the absence of K currents, K^+ in this solution can be replaced with other ions such as Cs^+. Cl^- replacement of ICS with an impermeant anion is also warranted if a more natural ICS is desired[9].
3. The cells are robust with >1 hr-long recordings possible at room temperature. Recording is optimal on days 3 and 4 of the protocol, corresponding to 24-48 hr in culture. After 48 hr there is difficulty forming gigaOhm seals, perhaps because of elaboration of a glycocalyx on the cell surface, and space clamp problems caused by process outgrowth. The cells are useful, however, for non-patch clamp studies, such as toxicology, that can be completed in <14 d.
4. ASW and other solutions to be dispensed from the gravity fed solution device, as well as intracellular solution, should be filtered through a syringe-mounted 0.2 µm Acrodisk filter (**Table 3**) to eliminate fine particles that interfere with gigaOhm seal formation.
5. Desensitization occurs when using agonists such as D-Aspartate (D-Asp), therefore ~1-2 min between applications of agonists is recommended. Conversely, potentiation is often observed with rapid repeated application of agonists such as L-Glutamate (L-Glu).
6. Agonist activated currents such as L-Glu can be studied by applying agonists with the picospritzer pipette. This pipette is pulled the same as the patch pipette and contains agonist in ASW. When the tip of this pipette is positioned below the outflow pipe of the gravity fed solution device, and at a 45° angle from the outflow pipe (**Figure 2F**), agonist will be quickly washed away from the cell, and desensitization will be minimized. The agonist pipette should create an angle of 90° or more relative to the patch pipette.

Representative Results

The locations of the sensory neurons within the ganglia that are targeted in this protocol, the BSC and PVC neurons are shown in **Figure 1**. The BSC neurons are located in 2 symmetrical oval clusters on the ventral side of the buccal ganglion, the surface that faces away from the buccal mass in the intact ganglion (**Figure 1A**). The PVC neurons form bilateral, V-shaped clusters that wrap around the dorsal surface of the pleural ganglion toward the central axis (**Figure 1B**). These sensory neurons have the advantage of a stereotyped location within the ganglia, although the right or left cluster is missing in individual animals in approximately 1% of occurrences. The BSC and PVC neuron clusters are identifiable by the dark orange color of the cell membrane and relatively small size compared to nearby cells, as shown in the large circle outline of **Figure 1A**.

In culture, these neurons are often without processes the day after plating (**Figure 2A**) and are ideal for patch clamping that day, and one day later. It is sometimes possible to achieve adequate space clamp even when the cells appear to have process outgrowth (**Figure 2F**), suggesting that not all sprouting that seems to emanate from the cell body is part of the cell. If handling after enzymatic digestion has been gentle, cells arrive in culture with some processes intact, and these processes grow with time (**Figures 2B-2E**). Such cells are not suitable for patch clamping but intracellular recording is possible.

Whole cell patch clamp currents carried by voltage gated Na^+ and Ca^{2+} are readily recorded from PVC and BSC neurons in short term culture [6], and both cell types display a mixed K current. BSC neurons also respond to acetylcholine, serotonin and NMDA[4] (**Figure 3C**) with excitatory currents, while both BSC and PVC neurons respond to L-Glu and D-Asp[3] (**Figures 3A** and **3B**) with excitatory currents. The pharmacological characteristics of L-Glu- and D-Asp-induced currents in these neurons have been described[4].

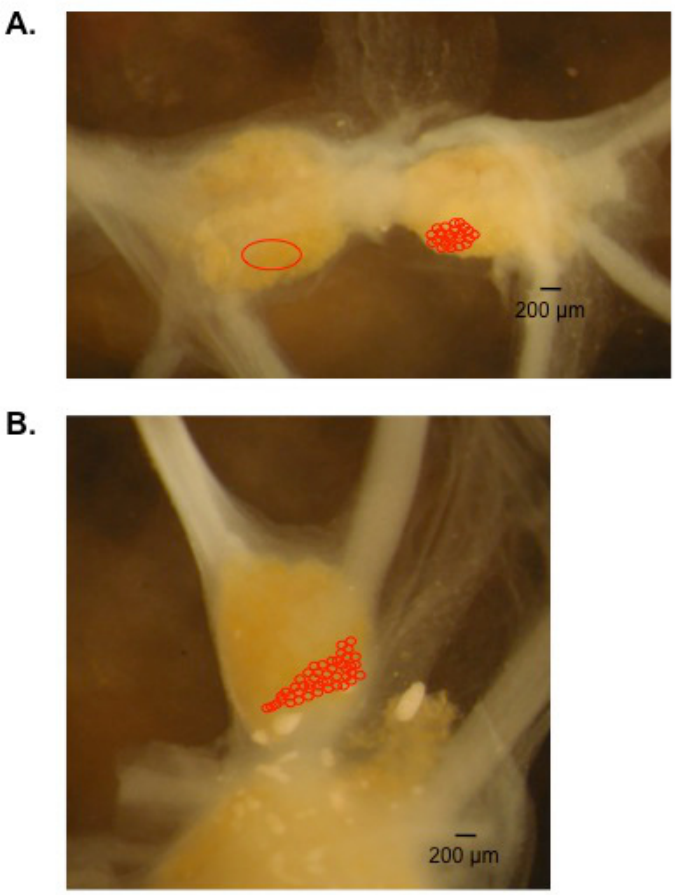

Figure 1. Photomicrographs showing the position of the glutamatergic sensory neurons of the buccal and pleural ganglia (red circles). **A.** The buccal S cluster (BSC) neurons, identifiable by their dark orange color (large circle on left hemiganglion) and the corresponding position in the right hemiganglion (smaller circles). **B.** V-shaped, dark orange pleural ventrocaudal (PVC) cell cluster of the right pleural hemiganglion (left hemiganglion not shown).

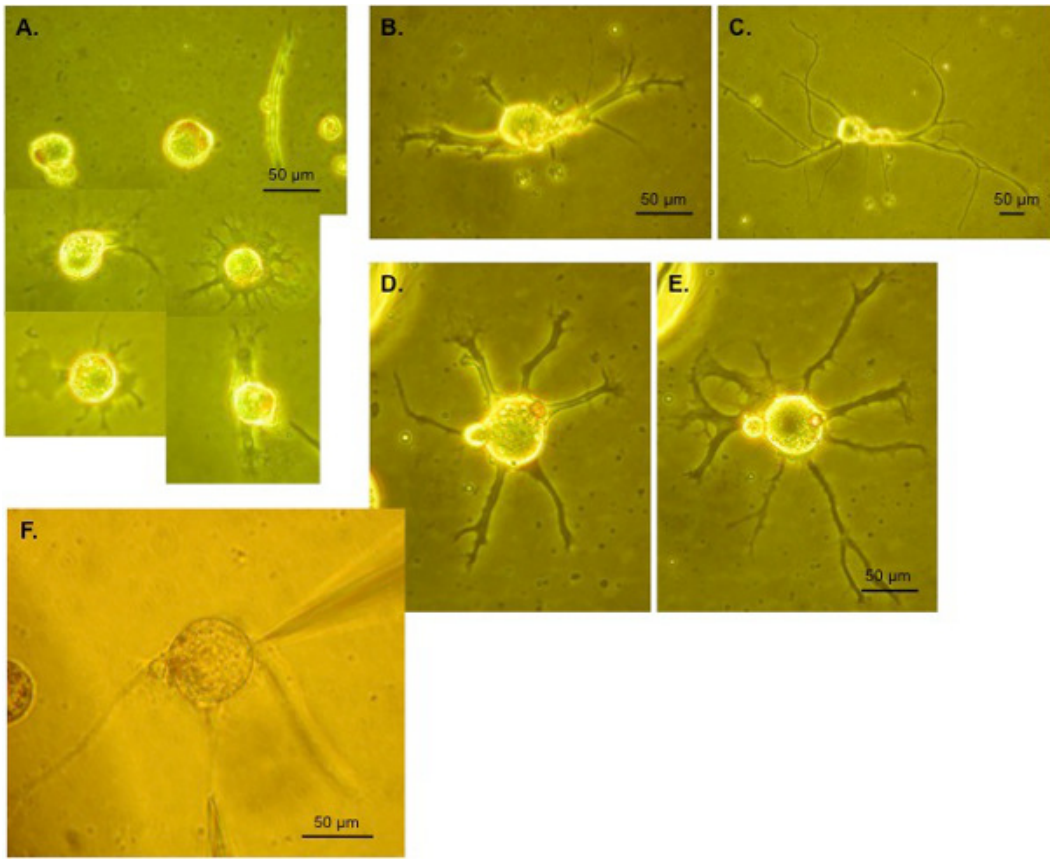

Figure 2. Photomicrographs of glutamatergic sensory neurons in culture. **A.** Cells from the PVC cluster 24 hr after plating in culture dish showing 2 sensory neurons and other cells; other PVC cells from separate cultures at 24 hr are shown as insets. **B**, **C**. A BSC neuron in culture at 24 hr and the same neuron at 96 hr, respectively. **D**, **E.** A PVC neuron in culture at 24 hr and the same neuron at 96 hr, respectively. **F.** A PVC neuron at 48 hr in a patch clamp experiment. The patch pipette is contacting the cell from the right edge, while the picospritzer pipette is at the bottom. The large dark shadow visible to the left of the cell is the opening of the flowing bath pipette. Click here to view larger figure.

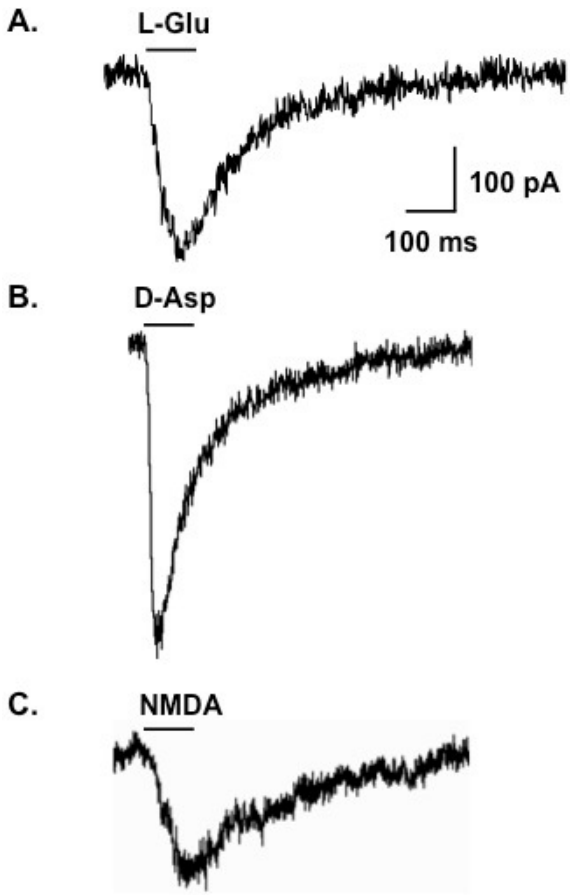

Figure 3. Glutamatergic currents in whole cell patch clamp recordings from BSC and PVC neurons. A. Whole cell current in a BSC neuron in response to pressure application of L-Glu (1 mM, 100 msec). **B.** Whole cell current in a PVC neuron in response to pressure application of D-Asp (1 mM, 100 msec). **C.** Whole cell NMDA current in the same BSC cell as in **A** (1 mM, 100 msec).

Discussion

The dissociation techniques described here yield sensory neuron cultures containing 50-100 isolated neurons interspersed with small numbers of glia and other unidentified cells. The most critical steps in the protocol are the time the ganglia remain in enzyme solution, and flicking, the dissociation of the digested cell clusters to break apart the cluster into individual cells. Enzyme digestion (step 1.8) must be optimized at the available temperature. At 23 °C with slow shaking, 13 hr is sufficient for digestion of the BSC clusters while15 hr is optimal for PVC clusters. Low cell yields in the culture dish can be attributed to insufficient time in enzyme, or too much mechanical disruption during either the cell transfer step (1.11 and 1.12) or during the flicking step (1.13). Failure of the cells to stick down to the culture substrate, as well as brief survival in culture is usually due to too much mechanical disruption. Contamination of the cultures can also occur, and is best addressed by ensuring that the anesthetized animal is well rinsed, that the dissection instruments are clean, and that the ganglia of interest are put through several rinses in ASW + P/S. It may be necessary to wash and alcohol-clean instruments between different parts of the dissection procedure, or have enough instruments ready so that clean ones can be used for each step.

Aplysia neurobiological studies are most often conducted on reduced preparations that preserve nerve connections between neurons or between neurons and muscles [2, 18]. These preparations facilitate the assignment of control of muscular processes to specific neurons and neuronal circuits and also permit studies of the potentiation and facilitation of repetitive reflexes. Intact preparations are not suited to certain types of voltage clamp protocols and the study of effects of agonists that rapidly desensitize their receptors.

The long-term neuronal co-culture is an additional tool to study potentiation and facilitation under highly controlled conditions. Long term co-cultures also lend themselves to biochemical studies on single neurons. Success of the co-culture preparation is often attributed to the addition of up to 50% *Aplysia* hemolymph to cultures [17]. However, the success of this approach often depends on batch-to-batch variability of this hemolymph.

The dissociated cell culture preparation described here expands the use repertoire of the *Aplysia* model. Dissociated cell cultures are not suitable for deducing neural circuits or studying the intact synapse as the methods mentioned above do. The dissociated cell culture preparation has its greatest utility in confirming the existence of specific ionic conductances and their kinetic and pharmacological properties in single cell voltage clamp experiments. Several unique currents have been discovered using single cell preparations, including an excitatory cation current in bag cells [19] and the D-Asp receptor current in BSC neurons [4]. Studies on *Aplysia* rarely focus on the ionic currents of individual cells, in part because of the often unwieldy size of such cells and the existence of numerous other suitable model cell types that lend themselves well to patch clamp techniques. As a result, the existence of certain currents in *Aplysia*, such as those activated by alpha-amino-3-hydroxyl-5-methyl-4-

isoxazole-propionic acid (AMPA) are usually inferred from block of putative AMPA receptor (R)-related behaviors by AMPAR antagonists applied to the reduced nerve preparation, rather than directly recorded. Thus verification of the existence of certain currents is lacking. Isolation of cultured glutamatergic neurons and single cell voltage clamp methodology provides a direct test for the existence of different types of L-GluR channels in this model organism.

Another use of *Aplysia* neurons in single cell patch clamp experiments is in dissecting second messenger cascades. *Aplysia* neurons are particularly well suited to studies of second messenger induced modulation because they are stable for long periods of recording at room temperature [3, 12, 20, 21], and are sufficiently robust that individual cells can be saved after recording and recorded from again after 24 hr [5]. These buccal and pleural sensory neuron clusters conveniently yield a pair of matched cultures from each ganglion, so that one dish can be designated a control culture while its mate receives a treatment.

An additional advantage of this preparation is in studies of the morphological status of individual neurons. For example, we discovered that cell bodies of *Aplysia* neurons from senescent animals were larger than those from younger animals, but did not elaborate cell processes after several days in culture [6]. These isolated neuron preparations also facilitate studies using vital dyes to assess levels of intracellular Ca^{+2}, pH, reactive oxygen species, mitochondrial membrane potential and other physiological traits that can then be compared with neurophysiological features of individual cells. While some of these approaches are applicable in intact or co-culture preparations, data collection is much more straightforward using isolated neurons in this easy culture system. In the future we hope to use these cells for single cell molecular profiling [13].

The dissociated short term culture provides ideal material for investigating fundamental neurophysiological processes, and may provide a particularly powerful tool when used in conjunction with studies involving reduced or co-culture preparations. The dissociated *Aplysia* neuron culture extends the utility of this venerable animal model to new and interesting areas of neuroscience.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

Funded by NIH P40 OD010952, the Korein Foundation, a University of Miami Fellowship to SLC and a Maytag fellowship to ATK. The authors gratefully acknowledge the staff of the National Resource for *Aplysia*, as well as Lauren Simonitis and Hannah Peck, who provided micrographs for a figure.

References

1. Buttner, N. & Siegelbaum S.A. Antagonistic modulation of a hyperpolarization-activated Cl(-) current in *Aplysia* sensory neurons by SCP(B) and FMRFamide. *J. Neurophysiol.* **90** (2), 586-598 (2003).
2. Byrne, J.H., Castellucci, V.F., & Kandel, E.R. Contribution of individual mechanoreceptor sensory neurons to defensive gill-withdrawal reflex in *Aplysia*. *J. Neurophysiol.* **41** (2), 418-431 (1978).
3. Carlson, S.L. & Fieber, L.A. Physiological evidence that D-Aspartate activates a current distinct from ionotropic glutamate receptor currents in *Aplysia californica*. *J. Neurophysiol.* **106** (4), 1629-1636 (2011).
4. Carlson, S.L., Kempsell, A.T., & Fieber, L.A. Pharmacological evidence that D-Aspartate activates a current distinct from ionotropic glutamate receptor currents in *Aplysia californica*. *Br. Behav.* **2** (4), 391-401 (2012).
5. Fieber, L.A. Characterization of Na+ and Ca2+ currents in bag cells of sexually immature *Aplysia californica*. *J. Exp. Biol.* **201** (5), 745-754 (1998).
6. Fieber, L.A., Carlson, S.L. Capo, T.R., & Schmale. M.C. Changes in D-Aspartate ion currents in the Aplysia nervous system with aging. *Br. Res.* **1343**, 28-36 (2010).
7. Glansman, D.L. Habituation in *Aplysia*: The Cheshire Cat of neurobiology. *Neurobiol. Learn. Mem.* **92**, 147-154 (2009).
8. Hawkins, R.D., Abrams, T.W., Carew, T.J., & Kandel, E.R. A Cellular Mechanism of Classical Conditioning in *Aplysia*: Activity-Dependent Amplification of Presynaptic Facilitation. *Science, New Series.* **219** (4583), 400-405 (1983).
9. Ichinose, M., Sawada, M., Maeno, T., & McAdoo, D.J. Effect of acetylcholine on ventrocaudal sensory neurons in the pleural ganglia of *Aplysia*. *Cell Mol. Neurobiol.* **9**, 233-245 (1989).
10. Kandel, E.R. Cellular basis of behavior. W.H. Freeman, New York, 1976.
11. Kandel, E.R. The molecular biology of memory storage: a dialog between nerves and synapses. *Science*. **294** (5544), 1030-1038 (2001).
12. Kupfermann, I., Castellucci, V., Pinsker, H., & Kandel, E. Neuronal correlates of habituation and habituation of the gill-withdrawal reflex in *Aplysia*. *Science*. **167** (3926), 1743-1745 (1970).
13. Magoski, N.S. Regulation of an *Aplysia* bag-cell neuron cation channel by closely associated protein kinase A and a protein phosphatase. *J. Neurosci.* **24** (30), 6833-6841 (2004).
14. Moroz, L.L., Edwards, J.R., Puthanveettil, S.V., Kohn, A.B., Ha, T., Heyland, A., Knudsen, B., Sahni, A., Yu, F., Liu, L., Jezzini, S., Lovell, P., Iannucculli, W., Chen, M., Nguyen, T., Sheng, H., Shaw, R., Kalachikov, S., Panchin, Y.V., Farmerie, W., Russo, J.J., Ju, J., & Kandel. E.R. Neuronal transcriptome of *Aplysia*: neuronal compartments and circuitry. *Cell.* **127**, 1453-1467 (2006).
15. Nick, T.A., Kaczmarek, L.K., & Carew, T.J. Ionic currents underlying developmental regulation of repetitive firing in *Aplysia* bag cell neurons. *J. Neurosci.* **16** (23), 7583-7598 (1996).
16. Sakmann, B. & Neher, E., eds. *Single-channel recording.*, Plenum Press, NY, (1995).
17. Tam, A.K., Geiger, J.E., Hung, A.Y., Groten, C.J., & Magoski, N.S. Persistent Ca2+ current contributes to a prolonged depolarization in *Aplysia* bag cell neurons. *J. Neurophysiol.* **102** (6), 3753-3765 (2009).
18. Schacher, S. & Proshansky, E. Neurite regeneration by *Aplysia* neurons in dissociated cell culture: modulation by *Aplysia* hemolymph and the presence of the initial axonal segment. *J. Neurosci.* **3** (12), 2403-2413 (1983).

19. Walters, E.T., Byrne, J.H., Carew, T.J., & Kandel, E.R. Mechanoafferent neurons innervating tail of *Aplysia*. I. Response properties and synaptic connections. *J. Neurophysiol.* **50** (6), 1522-1542 (1983).
20. Wilson, G.F., Richardson, F.C., Fisher, T.E., Olivera, B.M., & Kaczmarek, L.K. Identification and characterization of a Ca(2+)-sensitive nonspecific cation channel underlying prolonged repetitive firing in *Aplysia* neurons. *J. Neurosci.* **16** (11), 3661-3671 (1996).
21. White, B.H., Nick, T.A., Carew, T.J., & Kaczmarek, L.K. Protein kinase C regulates a vesicular class of calcium channels in the bag cell neurons of *Aplysia*. *J. Neurophysiol.* **80** (5), 2514-2520 (1998).
22. Wilson, G.F., Magoski, N.S., & Kaczmarek, L.K. Modulation of a calcium-sensitive nonspecific cation channel by closely associated protein kinase and phosphatase activities. *Proc. Natl. Acad. Sci. U.S.A.* **95** (18), 10938-10943 (1998).
23. Zhao, Y., Wang, D.O., & Martin, K.C. Preparation of *Aplysia* sensory-motor neuronal cell cultures. *J. Vis. Exp.* (28), e1355, doi:10.3791/1355 (2009).

