Video Article

An *In Vitro* Preparation for Eliciting and Recording Feeding Motor Programs with Physiological Movements in *Aplysia californica*

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Abstract

Multifunctionality, the ability of one peripheral structure to generate multiple, distinct behaviors[1], allows animals to rapidly adapt their behaviors to changing environments. The marine mollusk *Aplysia californica* provides a tractable system for the study of multifunctionality. During feeding, *Aplysia* generates several distinct types of behaviors using the same feeding apparatus, the buccal mass. The ganglia that control these behaviors contain a number of large, identified neurons that are accessible to electrophysiological study. The activity of these neurons has been described in motor programs that can be divided into two types, ingestive and egestive programs, based on the timing of neural activity that closes the food grasper relative to the neural activity that protracts or retracts the grasper[2]. However, in isolated ganglia, the muscle movements that would produce these behaviors are absent, making it harder to be certain whether the motor programs observed are correlates of real behaviors. *In vivo*, nerve and muscle recordings have been obtained corresponding to feeding programs[2,3,4], but it is very difficult to directly record from individual neurons[5]. Additionally, *in vivo*, ingestive programs can be further divided into bites and swallows[1,2], a distinction that is difficult to make in most previously described *in vitro* preparations.

The suspended buccal mass preparation (**Figure 1**) bridges the gap between isolated ganglia and intact animals. In this preparation, ingestive behaviors - including both biting and swallowing - and egestive behaviors (rejection) can be elicited, at the same time as individual neurons can be recorded from and stimulated using extracellular electrodes[6]. The feeding movements associated with these different behaviors can be recorded, quantified, and related directly to the motor programs. The motor programs in the suspended buccal mass preparation appear to be more similar to those observed *in vivo* than are motor programs elicited in isolated ganglia. Thus, the motor programs in this preparation can be more directly related to *in vivo* behavior; at the same time, individual neurons are more accessible to recording and stimulation than in intact animals. Additionally, as an intermediate step between isolated ganglia and intact animals, findings from the suspended buccal mass can aid in interpretation of data obtained in both more reduced and more intact settings. The suspended buccal mass preparation is a useful tool for characterizing the neural control of multifunctionality in *Aplysia*.

Video Link

The video component of this article can be found at http://www.jove.com/video/4320/

Protocol

1. Preparation of Solutions

1. To prepare magnesium chloride solution that is isotonic to the seawater in which the animals are kept (~ 1,000 millosmolar), mark a large jug at the level of the desired volume. Fill the jug with distilled water to approximately 80% of this level, and weigh out the appropriate amount of magnesium chloride hexahydrate to create a 333 mM solution in the final volume. Add the magnesium chloride to the jug, close the lid and shake vigorously until the magnesium chloride is fully dissolved, and then add distilled water until the final volume is reached.
2. To prepare *Aplysia* saline, again mark a large jug at the level of the desired volume, and fill with distilled water to approximately 80% of this level. Place the jug on a stir plate, place a stir bar in the jug, and turn on the stir plate.
3. One by one, weigh out and add to the jug the appropriate amounts of the following substances to create these concentrations in the final volume: 460 mM sodium chloride, 10 mM potassium chloride, 22 mM magnesium chloride hexahydrate, 33 mM magnesium sulfate heptahydrate, 10 mM calcium chloride dihydrate, 10 mM glucose, 10 mM MOPS.
4. Once the solutes are fully dissolved, use a pH meter to measure the pH of the solution. Slowly add 1 M sodium hydroxide to raise the pH to a final value of 7.5. If you accidentally add too much sodium hydroxide, you can add 1 M hydrochloric acid to lower the pH.
5. Add distilled water until the final volume is reached.

6. To slow bacterial growth, store solutions in the refrigerator until needed for an experiment. Note that temperature can affect *Aplysia* motor programs. We do not carefully monitor temperature, but after setup is complete, preparations are typically near room temperature.

2. Preparation of Recording Dish

1. During the experiment, the buccal mass will be suspended in solution in a round 100 mm (diameter) x 50 mm (height) Pyrex dish. This dish should have a front chamber for the buccal mass, a narrow, elevated middle platform where the buccal ganglia are pinned on Sylgard, and a separate, elevated back chamber where the cerebral ganglion is isolated. A notch in the Sylgard should connect the buccal ganglia platform to the cerebral ganglion chamber. See **Figure 1**, referring to the specified key dimensions when constructing the dish.
2. To create this dish, begin with a round 100 x 50 mm Pyrex dish. Prepare Sylgard following the instructions provided with the product. Construction of the dish will require several pours of Sylgard, between which the Sylgard must be allowed to set.
3. The first pour is to create the highest level of Sylgard in the dish, the wall in between the buccal and cerebral chambers (refer to **Figure 1**). Block off this portion of the dish from the other sections. Modeling clay can be used to build walls for blocking off sections. For a flat wall, a piece of cardboard can be used, with a modeling clay backing. For a curved wall (which reduces the size of the chamber containing the cerebral ganglion), use modeling clay alone as the basis for the wall. Coat all the walls with plastic wrap where they will contact the Sylgard for easier removal.
4. Once the space for the wall between the buccal and cerebral chambers has been blocked off as described in 2.3, ensuring tight seals at the edges to minimize leakage, pour Sylgard into this space nearly up to the top of the dish. Let the Sylgard set overnight, and make sure that it is fully set before continuing. Putting the dish in a warm place will induce faster setting.
5. Next, the buccal ganglia platform and cerebral ganglion chamber should be poured (again, refer to **Figure 1**, which shows the locations of these ganglia in the completed dish). Remove the previous blocking walls, and create a new wall a distance 0.5 cm from the flat Sylgard wall of the middle section to block off the buccal ganglia platform. No walls are necessary to create the back chamber which will hold the cerebral ganglion. Pour Sylgard in both sections up to a height of about 3 mm below the top level. The cerebral chamber should be just slightly lower than the buccal ganglia platform. Again, let the Sylgard set overnight.
6. Lastly, pour the front chamber (*i.e.*, the chamber in which the buccal mass will be suspended). No walls are necessary. Pour to the appropriate height as specified in **Figure 1**.
7. The final step is to cut a notch connecting the buccal ganglia platform and the cerebral chamber. The depth of the notch should be the same as the level of the buccal ganglia platform. A scalpel blade can be used to cut the notch. **Figure 1** illustrates the completed dish.

3. Electrode Preparation

1. Pull extracellular electrodes from single-barreled capillary glass using a Flaming-Brown micropipette puller. The electrodes' inner diameters should be about 40 µm and their resistances should be about 0.1 MΩ. With the FT345B filament in the puller, our typical puller program settings are Heat 480, Pull 50, Vel 13, and Time 20, but note that settings will be different for different filaments. This program creates the electrodes in a single pull with no fire-polishing stage. Before beginning an experiment, backfill the extracellular electrodes with *Aplysia* saline.
2. Create hook electrodes for nerve and muscle recording[2] following a similar protocol to that described by Cullins and Chiel[4], except that individual electrodes do not have to be wrapped around each other. The steps to create these electrodes follow in 3.3-3.13.
3. Cut a piece of enamel-coated, 0.001 inch diameter stainless steel wire to a length of about 60 cm. Attach a ball of modeling clay to each end of the wire, hold the wire up in the air at its middle point, and spin the two balls of clay around each other to wrap the wires around one another (this reduces capacitative transients).
4. Hold the two ends of the wire together by joining the two balls of clay into one ball, and tape the other end of the wire to suspend the wire.
5. Coat the entire length of wire in household silicone glue by placing a glob of glue on your thumb, touching your thumb and index finger together on the wire, and running them over the wire. Let the glue dry overnight.
6. After the glue dries, place the wire on a flat surface under a binocular dissecting microscope. Cut the end of the wire that was previously the middle, resulting in two separate wires twisted together. This is a differential electrode.
7. Use tape to hold one of the ends of the electrode in place. Using forceps to strip off glue and enamel, prepare both wires at one end to have approximately 2 cm of free wire with about 1 cm of the wire stripped of enamel. Solder a gold connector pin to each of the wires, and place lab tape around the pins to keep them separated.
8. At the other end of the electrode, prepare the wires to have about 3 mm of free wire. Remove the enamel from the last approximately 1 mm of one wire, and bend this wire back and out of the way. This is the reference wire. It is critical that the two wires do not have bare wire touching each other.
9. Remove the enamel from the other wire all the way to the silicone glue. Twist this wire into a hook and cut it to a length of about 1 mm. This hook will attach to the nerve or muscle. Using a short hook provides greater stability when the nerve is being attached to the electrode.
10. Use a multimeter to verify that the electrode is properly constructed and intact. One gold connector should have a resistance reading of roughly 200-500 ohms with respect to the tip of one wire, and the other gold connector should have a similar reading relative to the other wire.
11. If there is not an intact connection on one wire, it may be possible to re-strip the end of the wire and to re-solder one or both gold connectors to create these connections. If both gold connectors have connections with both wires, the electrode may have two bare wires making contact. It may be possible to fix the other end to eliminate this problem; otherwise, the electrode should be discarded.
12. Repeat this procedure for as many electrodes as are needed for the experiment. Hook electrodes can be re-used from experiment to experiment by cutting off the end that attached to the nerve and creating new hooks, until the wire becomes too short after many uses. Each time this is done, use the multimeter to verify that the electrode is intact.
13. To keep track of the different electrodes during experiments, label electrodes and their cables. Using different colors of lab tape is helpful.

4. Animal Dissection and Buccal Mass Preparation

1. Choose a healthy animal of about 200-300 g weight. When presented with seaweed, the animal should produce bites with strong protractions at regular intervals of 3-5 sec.
2. Place the animal in a dissecting tray and anesthetize by injecting about 50% body weight isotonic magnesium chloride solution. Make the first injection with the syringe at about a 15 to 30 degree angle, near the middle and pointing toward the tail of the animal. After injection, gently massage the surface of the animal to spread out the magnesium chloride. Make additional injections as needed with the syringe pointing toward the head of the animal.
3. After the animal stops responding to gentle tactile stimuli, pin the animal to the tray, dorsal side up, with one pin in the tail and one pin in each anterior tentacle.
4. Use forceps to pinch and lift the animal's skin in the middle of the head, behind the rhinophores. Make a coronal incision across the animal's head, just behind the point being held. Then make a midsagittal incision going forward in between the rhinophores toward the mouth, exposing the buccal mass.
5. Use forceps to pull the flap of skin closer to you away from the buccal mass, and cut through the nerves and connective tissue that attach to the body wall to fully separate this flap of skin from the buccal mass. Be sure not to cut any nerves or tissue intrinsic to the buccal mass.
6. Use forceps to grab and hold the esophagus. Cut through the esophagus posterior to the point being held. Pull up on the esophagus to lift the buccal mass as the following cuts are made.
7. Behind the buccal mass, the cerebral, pleural, and pedal ganglia form a ring, with the cerebral ganglion attached to the buccal ganglia by the cerebral-buccal connectives (CBCs). Leave these ring ganglia attached to the buccal mass, and cut all nerves projecting from these ganglia to other parts of the body.
8. Cut through the connective tissue all around the buccal mass, continuing to lift the buccal mass up as it becomes less connected to the body. Once the buccal mass is only attached at the mouth, the esophagus and buccal mass should be held straight up.
9. Make a final cut just anterior to the jaws to free the buccal mass from the body. Place the buccal mass in a solution of 50% isotonic magnesium chloride solution and 50% *Aplysia* saline in order to maintain partial anesthetization while electrodes are attached.
10. Place the buccal mass, with solution, in a Petri dish with Sylgard bottom. Remove the pleural and pedal ganglia by cutting their connections to the cerebral ganglion. Leave the cerebral ganglion attached to the buccal ganglia and the buccal mass via the cerebral-buccal connectives (CBCs); sever the other connections between the cerebral ganglion and buccal mass. Trim the esophagus and salivary glands to short lengths so they do not get in the way.

5. Hook Electrode Attachment

1. For recording and stimulation, hook electrodes can be attached to a number of different nerves and muscles, as appropriate for the experiment (**Figure 2**).
2. To characterize patterns as demonstrated *in vivo* by Cullins and Chiel[4], attach electrodes to the radular nerve, the I2 muscle, and buccal nerves 2 and 3. Together, these recordings show the activity of motor neurons for the major muscles intrinsic to the buccal mass[3,7,8], and allow identification of the protraction phase from the I2 recording[3], the retraction phase from the buccal nerves 2 and 3 recordings[2,5,8], and timing of closure of the food grasper from the radular nerve recording[2]. Attach an additional hook electrode to branch *a* of buccal nerve 2 for pattern stimulation. Attachment of electrodes follows a procedure similar to that demonstrated by Cullins and Chiel[4], and is described in the following steps.
3. For each electrode attachment, place the electrode on a coarse manipulator. To the manipulator, tape a wooden stick with an alligator clip on its end and use the clip to hold the end of the electrode. The clip should attach to the silicone-coated section of the electrode, about 1 cm before the end of the silicone coating. Use the manipulator to position the hook electrode near the buccal mass.
4. With the buccal mass on its side and placed against the side of the dish for stability, begin with the radular nerve, which presents the most difficult electrode attachment. There are two options for accessing this nerve.
5. The radular nerve projects from underneath the buccal ganglion and goes under the I2 muscle in two branches. First, see whether the radular nerve can be accessed without cutting the muscle. Gently push aside the white sheath attaching the buccal ganglia to the I2 muscle; do not cut this sheath. If the nerve is accessible, use forceps with the tip bent into a hook to lift one branch of the nerve.
6. If the radular nerve is not accessible in this way, a second option is to locate the nerve beneath the thin I2 muscle, and make a small incision in I2 to expose the nerve. This incision should go through two layers of muscle. Use the hooked forceps to pull a branch of the radular nerve out from beneath the muscle.
7. Use the manipulator to position the electrode's hook next to the nerve. To place the nerve into the hook, it is helpful to hold the nerve in the hooked forceps while simultaneously using another pair of forceps to create separation between the two halves of the nerve being held.
8. After the nerve is securely in the hook, use the manipulator to lift the nerve away from the buccal mass until it is taut, but do not overstretch.
9. Take a Kimwipe and tightly twist one corner to form a small wick. Use this wick to dry the end of the electrode and the section of nerve that is hooked.
10. Use another set of curved-tip forceps to apply a glob of Quick Gel super glue to the hooked nerve. Begin the application at the point of contact between wire and nerve. Make sure that the hook is completely covered with glue, and that the tip of the reference wire is not covered with glue (**Figure 3**).
11. Use a syringe with a polyethylene tubing attachment to apply solution from the dish onto the glue, which will induce the surface of the glue to set. Be sure to thoroughly bathe the glue with solution to ensure that the glue sets properly.
12. Use the manipulator to release the tension on the nerve, and then release the electrode from the clip. This completes the procedure of attaching one hook electrode.
13. The I2 muscle electrode should be attached next. We record EMG activity from the I2 muscle rather than ENG activity from its innervating nerve because the I2 nerve is very small and difficult to access in an intact buccal mass. Near buccal nerve 2, use the hooked forceps to lift one or two bands of the I2 muscle, and use another pair of forceps to help separate them from the rest of the muscle. The portion of I2 separated should be similar in thickness to buccal nerve 2 or 3. Be careful not to over-separate (which may denervate the muscle). Attach a hook electrode with the same procedure used for the radular nerve.

14. The next electrode to attach is that for branch *a* of buccal nerve 2. This branch can be electrically stimulated to induce motor programs[9]. Before buccal nerve 2 goes underneath the I1 muscle at the lateral groove, the nerve trifurcates; branch *a* is the first branch to split off. The branches are quite small, so the electrode attachment should be done with care. Follow the same procedure to attach the electrode.
15. Buccal nerves 2 and 3 are easily accessed. Follow the same procedure, attaching the electrodes roughly halfway between the buccal ganglion and lateral groove.
16. To help distinguish neurons with unilateral vs. bilateral projections, it is also useful to attach electrodes to buccal nerves 2 and 3 on the other side of the buccal mass, as well as buccal nerve 2 branch *a*, because some neurons respond differently to ipsilateral vs. contralateral BN2-*a* stimulation.

6. Positioning the Ganglion and Thinning the Sheath

1. The buccal mass will be moved to the round 100 x 50 mm Pyrex dish described in section 2. See **Figure 1**.
2. Apply a thin layer of vacuum grease to the notch between the cerebral and buccal chambers, using a pipet tip to pick up a glob of vacuum grease and spread it over the notch. We have found that vacuum grease minimizes leakage better than Vaseline. Fill the front chamber with *Aplysia* saline. Carefully move the buccal mass from the Petri dish to the front chamber of this larger dish, making sure that none of the electrodes are pulled tightly, which could break the nerves.
3. If the dish must be transferred to another binocular dissecting microscope for thinning the sheath, be very careful with the hook electrodes. Group the electrodes on one side of the buccal mass together, and also group the electrodes on the other side of the buccal mass together. Carefully hold the electrodes by grasping the lab tape that covers the connector pins, again making sure that none of the electrodes are pulled tightly.
4. When the dish is positioned under the microscope, the electrodes should be draped gently over the sides of the dish and rest on the platform next to the dish.
5. During breaks and between stages of the experiment, aerate the saline in the buccal mass chamber using an aquarium airstone.
6. Pin the cerebral ganglion in the back with the cerebral-buccal connectives (CBCs) running through the notch. Apply more vacuum grease over the CBCs, then add more *Aplysia* saline to both parts of the dish, so the ganglia are completely submerged. Ensure that the amount of vacuum grease added is higher than the level of saline in either the cerebral or buccal chambers so no leakage occurs between the chambers. At this stage, the buccal mass should rest on the bottom of the front chamber so as not to pull too forcefully on the nerves and ganglia.
7. Pin the buccal ganglia on the middle platform. To avoid damaging the nerves that are still attached to the buccal mass, only place pins on the sheath between two nerves. Add two more pins on the side of the CBCs to stretch and anchor them.
8. Keep the buccal ganglia flat or rotated based on the location of the neurons of interest. To rotate the buccal ganglion, use fine forceps to grab some excess sheath of the CBC and pin it down between buccal nerves 2 and 3. Then most of the cell bodies that are proximate to the back chamber and cannot be easily accessed from the top of the buccal ganglion can be seen. Lastly, add two pins on the sheath of the buccal ganglion proximate to the buccal mass side to minimize the movement of the buccal ganglion. See **Figure 4**.
9. Use fine forceps to grab the sheath of the buccal ganglion proximate to the back chamber, and then cut away the excess sheath with fine scissors without exposing the cell bodies. In order to minimize damage, only remove the amount of sheath necessary to see the cell bodies.

7. Stimulation and Recording

1. After thinning of the sheath is completed, attach all electrode pins to their sockets on the cables that connect to the amplifiers. Again, make sure that the electrodes are not pulled tightly while doing this. Make sure that the electrodes are correctly attached to their appropriate cables and that the polarities are correct.
2. To wash out any remaining magnesium chloride, replace the *Aplysia* saline in the buccal mass chamber with fresh *Aplysia* saline.
3. Thread a silk suture through the soft tissue at the front of the buccal mass, anterodorsal to the jaw cartilage, and use two pieces of modeling clay to attach the suture to the edge of the dish, thus suspending the buccal mass from its anterior end. Note that the posterior end is suspended by the buccal nerves attached to the buccal ganglia.
4. Position a manipulator holding a glass extracellular electrode so that the electrode tip is near the buccal ganglion.
5. To locate and identify a neuron, use the manipulator to gently press the tip of the extracellular electrode down onto the sheath over the neuron soma (**Figure 5**). Apply a stimulating current, and then switch the channel that is being used to excite the soma to recording mode in order to record the activity on the extracellular electrode and corresponding activity on the nerves (**Figure 6**). Refer to published ganglion maps and neuron descriptions, including nerve projections and muscle innervations, (*e.g.* [7,8]) for help in identification of neurons.
6. For video recording of feeding programs, position a mirror at the side of the dish so that front and side views of the buccal mass can be seen simultaneously (location of the mirror is shown schematically in **Figure 1**). Position a video camera with both front and side views in its field of view.
7. Video and electrophysiological recordings can be synchronized by sending a TTL pulse both to an electronic timer in the camera's view and to a channel in AxoGraph, the computer program used to record and analyze electrophysiological data. This pulse provides a time point that is exactly the same in the AxoGraph and video recordings, based on which the files can be synchronized.
8. After a neuron is located, its activity can be recorded in different feeding-like behaviors, which can be elicited as described below.
9. To induce rejection-like motor programs, stimulate BN2-*a* with a long train of 2 Hz, 1 msec pulses[9] (our experience is that this stimulation reliably generates egestive patterns in this setting). Patterns continue for the duration of the stimulation and may continue until shortly after the stimulation ends.
10. To induce biting-like motor programs[12], place a few crystals of solid carbachol directly on the cerebral ganglion. Alternatively, if a controlled level of carbachol exposure is necessary, use a solution of between 1 and 10 mM carbachol in *Aplysia* saline. Higher concentrations are more likely to yield responses. Repetitive patterns generally begin within 5 min, and last for roughly 10 to 15 min before beginning to run down.
11. To induce swallowing-like motor programs[13], apply carbachol, and wait until the buccal mass generates strong bites. Then, during a bite, place a strip of seaweed (dried laver) in the animal's mouth so that the radula grasps the seaweed (**Figure 7**). Strips are usually 0.5 cm wide by 5 cm in length.

12. After the first series of carbachol-induced patterns, it is often possible to obtain additional carbachol responses. Thoroughly wash out the *Aplysia* saline in the cerebral ganglion chamber, removing and replacing the saline at least three times. Wait at least 20 min before applying carbachol again. Waiting up to 40 min may yield more reliable results.

At this time in the United States, invertebrate animals do not require formal approval by an institutional animal use and care committee. However, we have ensured that all treatments of *Aplysia* minimize harm and suffering to the animal, and that all dissections are done while the animal is fully anesthetized.

Representative Results

When an extracellular electrode is positioned above a neuron's soma and used to stimulate the neuron, a one-for-one correspondence between spikes on the soma channel and on the nerve(s) the neuron projects to can be observed (**Figure 6**, left panel, stimulation of identified neuron B9). The soma channel (top channel) is set to stimulating mode when the current is applied (time 1 in the figure), and is then quickly switched to recording mode (time 2). By maintaining the position of the electrode, the activity of the neuron can be recorded during motor programs (**Figure 6**, right panel), along with activity from the I2 muscle, radular nerve, and bilateral buccal nerves 2 and 3.

Figure 7 shows video images taken from a series of swallowing patterns induced by application of carbachol to the cerebral ganglion and the subsequent placement of a seaweed strip into the grasper during carbachol-induced biting. In **7A**, the radula is protracted to grasp the seaweed, and in **7B**, the radula has retracted, pulling seaweed into the mouth. Videos and electrophysiological recordings can be directly synchronized to each other; the still images in **Figure 7** correspond to times A and B indicated in **Figure 6**'s right panel.

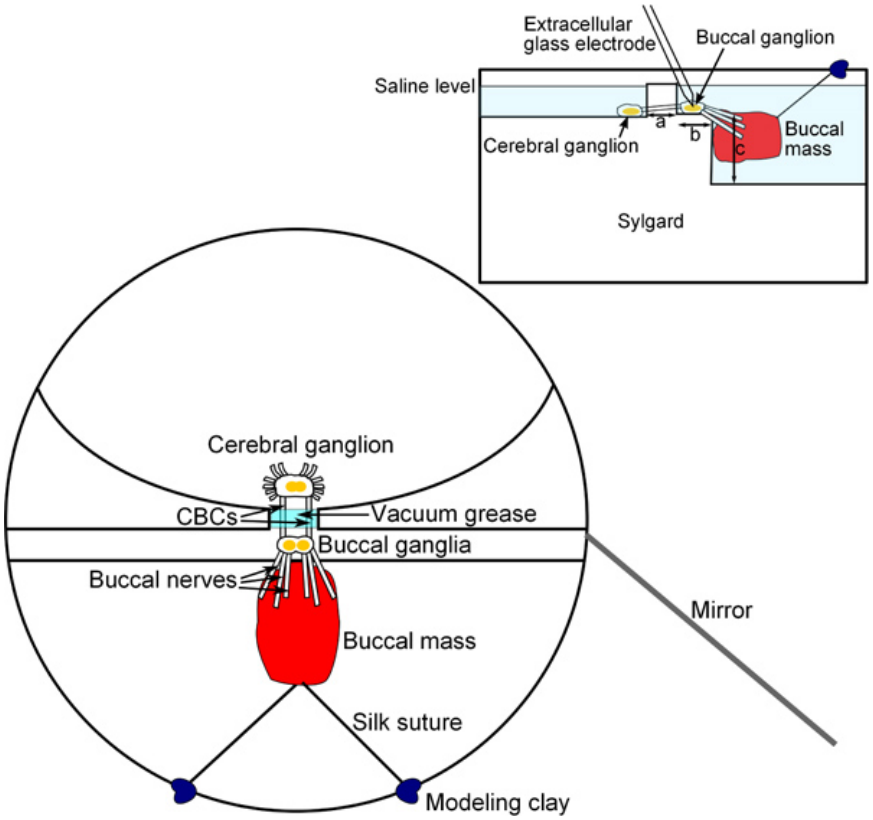

Figure 1. Schematic of overall setup for experiment. The main image shows an overhead view. The cerebral ganglion and buccal ganglia are pinned to Sylgard. The cerebral ganglion is in an isolated chamber so that carbachol can be applied to it. The cerebral-buccal connectives pass through a notch in the Sylgard, which is sealed with vacuum grease. The buccal mass, in the front chamber, is suspended by the buccal nerves from its posterior and by a silk suture at its anterior edge. An inset shows a side view of the setup, including an extracellular electrode positioned above the buccal ganglion. The entire structure within the dish is constructed from Sylgard. Dimensions indicated within the inset are: a. Length of notch connecting cerebral and buccal chambers, 0.5 cm. b. Width of buccal ganglia platform, 0.5 cm. c. Height of buccal mass chamber from bottom to buccal ganglia platform, 1.7 cm. Note that the height of the buccal mass chamber can be adjusted, depending on the size of the buccal mass, by placing a small platform made of Sylgard underneath the buccal mass.

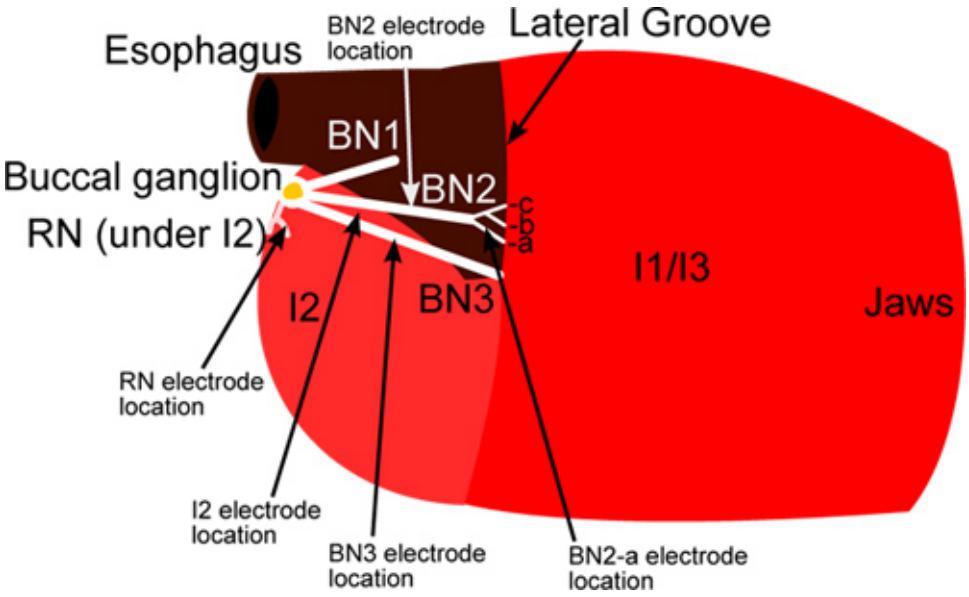

Figure 2. Schematic of the *Aplysia* feeding apparatus, the buccal mass, showing the locations of key structures, including buccal nerves (BN) 1, 2, and 3, the radular nerve (RN), and the I2 muscle, and the attachment points for hook electrodes. The radular nerve projects ventrally from the rostral surface of the buccal ganglia and goes beneath the surface fibers of the I2 muscle. Buccal nerve 2 trifurcates into branches *a*, *b*, and *c* before going beneath the I1 muscle at the lateral groove. Branch *a* is the first branch to separate from the main trunk, and is adjacent to buccal nerve 3. The nomenclature of branches *a*, *b*, and *c* was used by Warman and Chiel[5]. Branches *a*, *b*, and *c* correspond to branches 3, 2, and 1, respectively, in the nomenclature of Nargeot *et al.*[9] Furthermore, the RN, BN1, BN2, and BN3 correspond to nerves 1, 6, 5, and 4, respectively, in the nomenclature of Kandel[10] and Scott *et al.*[11]

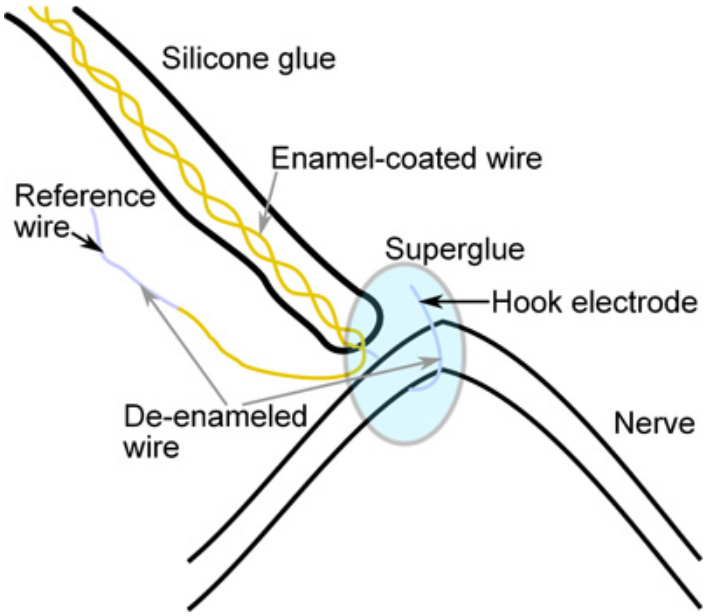

Figure 3. Attachment of hook electrode to nerve. The nerve is placed within the hook and lifted, and superglue is applied to the nerve and hook. The glue must completely cover the hook, and must not cover the end of the reference wire.

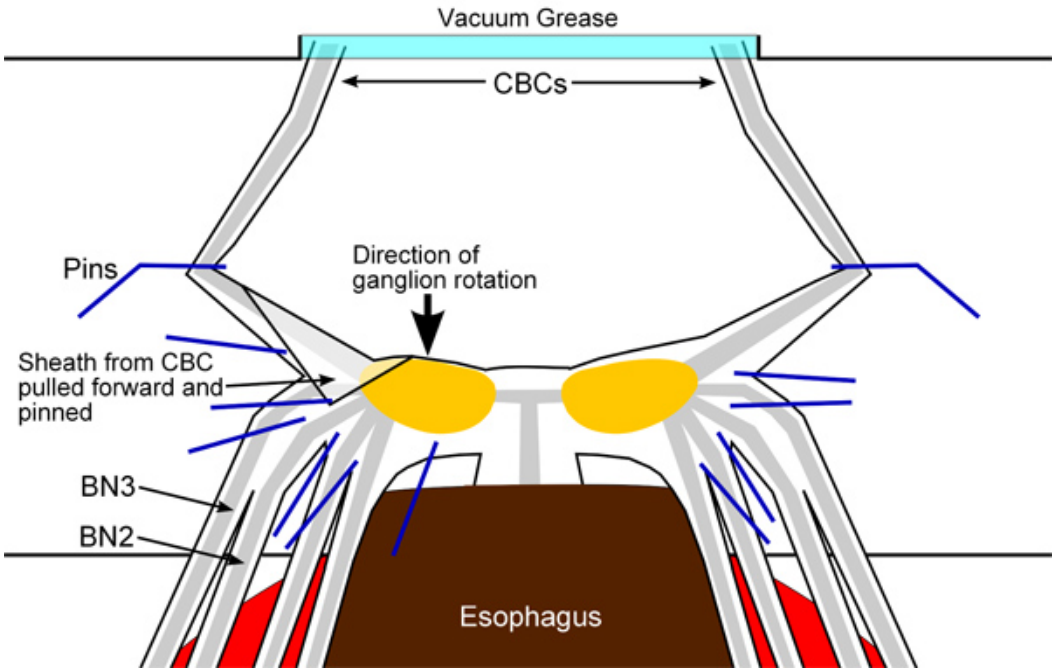

Figure 4. Setup of buccal ganglia, showing the arrangement of pins to position and secure the ganglia. Pins must be placed in the sheath surrounding the nerves and ganglia, being careful not to damage nerves or neurons. The left ganglion is shown as rotated forward to provide access to neurons that are proximate to the back chamber. To accomplish this rotation, excess sheath from the CBC is pulled forward and pinned between buccal nerves 2 and 3. Note that the caudal surface of the buccal ganglion (which is rounded, rather than flat) is schematically shown in the figure.

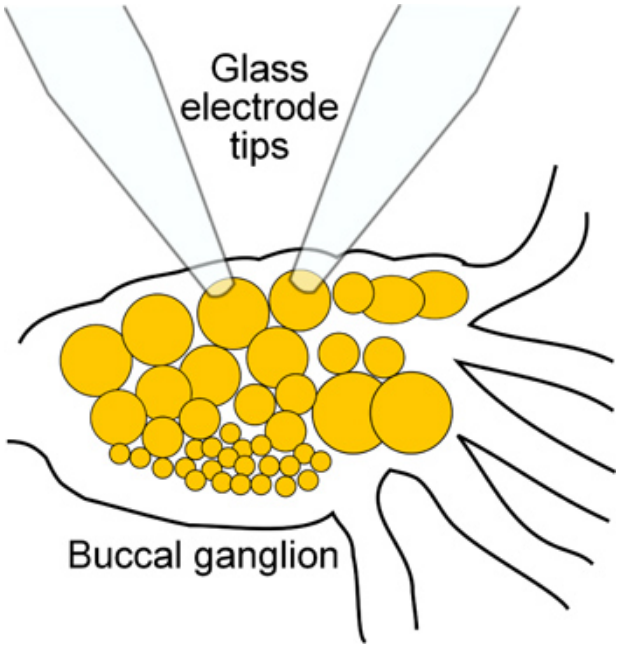

Figure 5. Positioning of extracellular electrodes over neuron somata in the buccal ganglion. Glass electrode tips should be gently pressed, using manipulators, into the thinned sheath above target cell bodies. Two electrodes can be simultaneously positioned above different cells, as pictured.

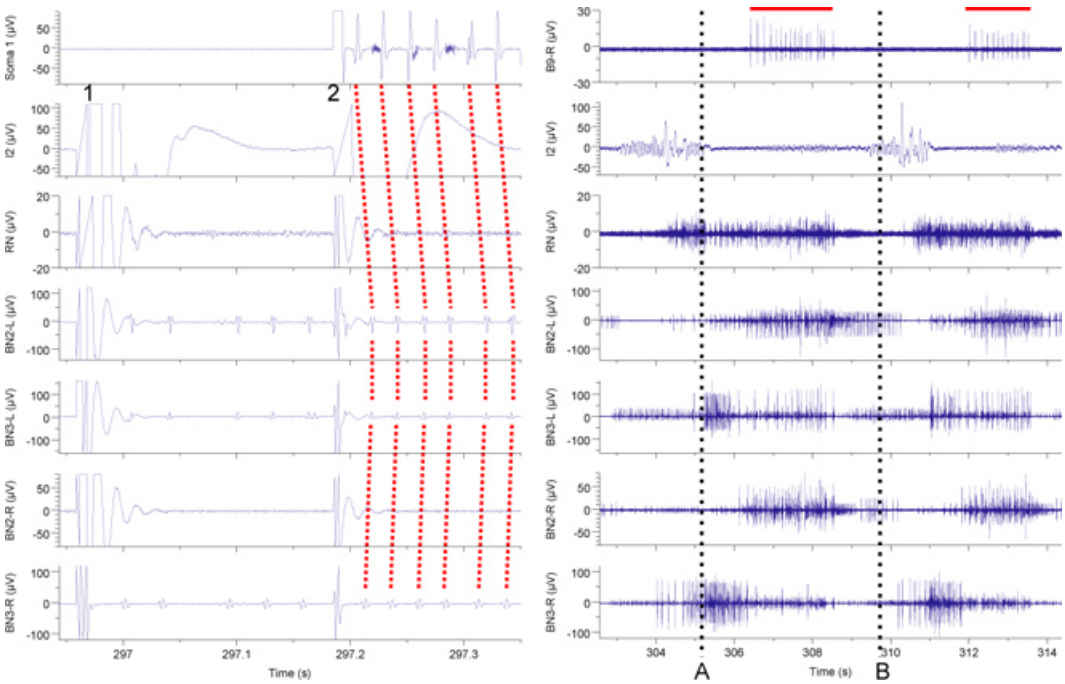

Figure 6. Example AxoGraph recordings. Channels are shown for one extracellular soma electrode (top channel) and (in sequence from the top) for hook electrodes on the I2 muscle, radular nerve, left buccal nerve 2, left buccal nerve 3, right buccal nerve 2, and right buccal nerve 3. At left, identification of a neuron by extracellular stimulation. The soma channel is used to apply a stimulating current (time 1, note stimulation artifact in all channels other than the soma channel, which is not recording), and is then switched to recording mode (time 2). Note the one-for-one correspondence between spikes on the soma channel and the BN2-L, BN3-L, and BN3-R channels. At right, recording of two cycles of feeding programs as the buccal mass swallows a strip of seaweed. Note that the activity of the neuron previously stimulated can be visualized on the soma channel (these spikes are highlighted by red bars above the soma channel). This is identified neuron B9 in the right buccal ganglion. Times A and B correspond to video frames shown in **Figure 7**. Click here to view larger figure.

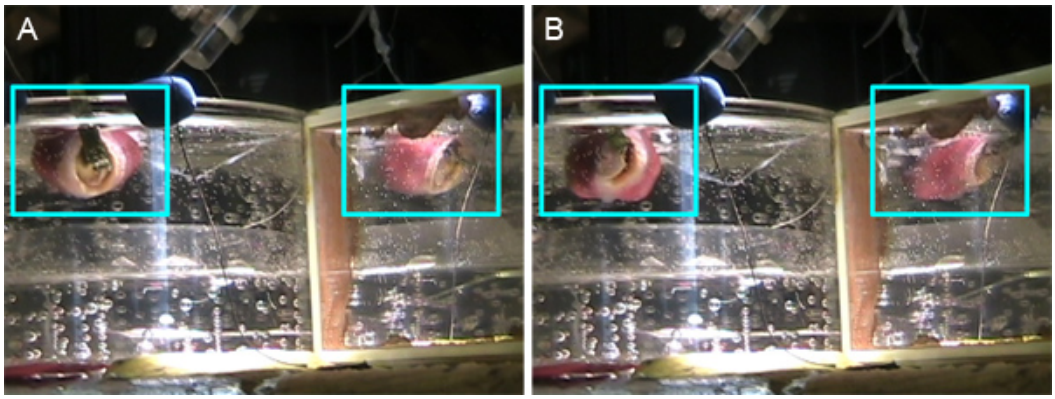

Figure 7. Example video images corresponding to the recording shown in **Figure 6**. The suspended buccal mass is shown in a front view (left) and side view (right, a mirror positioned adjacent to the dish). A strip of seaweed has been placed in the mouth and the radula is in the process of swallowing the seaweed. A. The radula is protracted to grasp the seaweed. B. The radula has retracted, pulling seaweed into the mouth. Note that A and B correspond to the same times labeled A and B in **Figure 6**.

Discussion

Previous work has characterized *Aplysia* motor programs in the intact animal and in reduced preparations, such as isolated ganglia. In the intact animal, although recordings of individual neurons have been obtained[5], such experiments are very difficult, and electrodes cannot be moved from neuron to neuron during feeding. In isolated ganglia, the feeding movements induced by neural activity cannot be observed. The suspended buccal mass preparation bridges the gap between these two extremes.

Other semi-intact preparations have been used in previous studies, but the suspended buccal mass preparation has significant advantages. In one semi-intact preparation, the lips and buccal mass were still attached, but the preparation was too reduced for full feeding movements to be observed [14]. A feeding head preparation[15,16] allowed observation of feeding movements *in vitro*. However, this feeding head preparation was more complicated to set up than the suspended buccal mass described here, and only provided access to individual neurons in the cerebral

ganglion, not in the buccal ganglion. There is an advantage to the feeding head preparation, in that the presence of the lips and anterior tentacles allowed the generation of biting patterns through a natural stimulus, application of seaweed. Although it would make the experiment more difficult, it may be possible to combine the preparations, leaving the lips attached to the buccal mass while also pinning the buccal ganglia for access to individual neurons.

Feeding patterns in *Aplysia* can be classified as ingestive or egestive based on the timing of activity on the radular nerve, which closes the food grasper, relative to the protraction and retraction phases of motor programs. If radular nerve activity occurs at the same time as the retraction phase, patterns are ingestive, because the grasper is closing and retracting as it attempts to pull food into the buccal cavity. If radular nerve activity occurs during the protraction phase, patterns are egestive, because the grasper is closing and protracting to push material out of the buccal cavity[2].

Ingestive patterns can be divided into at least two distinct sub-types: biting, attempts to grasp food, and swallowing, transport of already grasped food[1,2]. However, most *in vitro* work (*e.g.* [14,17,18]) has focused only on the ingestion/egestion dichotomy, not attempting to distinguish biting from swallowing. Two studies[16,19] have classified *in vitro* ingestive patterns in biting-like and swallowing-like groups. In the first of these studies, the patterns were observed in isolated ganglia; if the associated feeding movements of the buccal mass could be observed, this would strengthen the argument that these patterns represent the biting and swallowing seen *in vivo*. In the second study, the patterns were observed in a feeding head preparation, so patterns were classified based on movements observed, but no recordings of buccal ganglion neurons were obtained.

The suspended buccal mass preparation provides a way to observe all three types of behaviors - biting, swallowing, and rejection - while simultaneously recording activity from key buccal nerves and muscles, and both stimulating and recording from individual neurons. The extracellular neuron technique[6] is another key advance in this preparation, because the strong feeding-like movements elicited would almost certainly dislodge an intracellular electrode. With extracellular recordings of individual neurons, the timing of these neurons' activity during motor programs can be ascertained when this would be difficult with nerve recordings alone, because many different units are firing simultaneously.

We have observed that feeding movements in the suspended buccal mass appear qualitatively similar to those seen *in vivo*. Importantly, stimuli used to generate biting and rejection patterns (carbachol and buccal nerve 2 branch *a* stimulation, respectively) have been used in more reduced preparations, and our finding that the stimuli generate physiological movements lends credibility to their use in other settings. By contrast, to generate swallowing patterns, we combined an artificial stimulus (carbachol) with a natural food stimulus (a seaweed strip) which could not be applied to isolated ganglia. We have also observed that the neural patterns in the suspended buccal mass appear to be more similar to *in vivo* patterns than are patterns obtained in more reduced preparations, suggesting sensory feedback may be important in shaping the patterns from the CPG. For example, motor programs in isolated ganglia are typically several times longer in duration than motor programs in intact animals, whereas motor programs in the suspended buccal mass are typically much closer in duration to motor programs in intact animals (unpublished data). Another advantage of the suspended buccal mass is that simultaneous side and front views of the feeding apparatus can be obtained. The front view provides a similar view of the mouth to that which would be seen *in vivo,* while allowing better observation of jaw muscle contraction. The side view allows observation of buccal muscle contractions and the forward and backward movement of the grasper underneath the muscles, making it easier to understand biomechanical changes during the different behaviors.

In this preparation, we have recorded from up to five nerves (radular nerve and bilateral buccal nerves 2 and 3) and the I2 muscle simultaneously, and placed up to two extracellular electrodes over identified nerve cells in the ganglion. It would be possible to record from additional nerves and/or muscles, if the experimenter desired. It would also be possible to stimulate and record from neurons in the cerebral ganglion, which could give insight into whether stimulation of cerebral buccal interneurons, a common technique[17,19], induces motor programs similar to *in vivo* behavior. Although doing so increases the technical difficulty of the preparation, perfusion of the buccal artery[15] could allow the preparation to generate stronger and longer-lasting behaviors (unpublished observations).

Disclosures

No conflicts of interest declared.

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References

1. Neustadter, D.M., Herman, R.L., Drushel, R.F., Chestek, D.W., & Chiel, H.J. The kinematics of multifunctionality: comparisons of biting and swallowing in *Aplysia californica*. *J. Exp. Biol.* **210**, 238-260 (2007).
2. Morton, D.W. & Chiel, H.J. *In vivo* buccal nerve activity that distinguishes ingestion from rejection can be used to predict behavioral transitions in *Aplysia*. *J. Comp. Physiol. A.* **172**, 17-32 (1993).
3. Hurwitz, I., Neustadter, D., Morton, D.W., Chiel, H.J., & Susswein, A.J. Activity patterns of the B31/B32 pattern initiators innervating the I2 muscle of the buccal mass during normal feeding movements in *Aplysia californica*. *J. Neurophys.* **75**, 1309-1326 (1996).
4. Cullins, M.J. & Chiel, H.J. Electrode fabrication and implantation in *Aplysia californica* for multi-channel neural and muscular recordings in intact, freely behaving animals. *J. Vis. Exp.* (40), e1791, doi:10.3791/1791 (2010).
5. Warman, E.N. & Chiel, H.J. A new technique for chronic single extracellular recording in freely behaving animals using pipette electrodes. *J. Neurosci. Methods.* **57**, 161-169 (1995).
6. Lu, H., Chestek, C.A., Shaw, K.M., & Chiel, H.J. Selective extracellular stimulation of individual neurons in ganglia. *J. Neural Eng.* **5**, 287-309 (2008).
7. Church, P.J. & Lloyd, P.E. Expression of diverse neuropeptide cotransmitters by identified motor neurons in *Aplysia*. *J. Neurosci.* **11**, 618-625 (1991).

8. Church, P.J. & Lloyd, P.E. Activity of multiple identified motor neurons recorded intracellularly during evoked feedinglike motor programs in *Aplysia*. *J. Neurophys.* **72**, 1794-1809 (1994).
9. Nargeot, R.N., Baxter, D.A., & Byrne, J.H. Contingent-dependent enhancement of rhythmic motor patterns: an *in vitro* analog of operant conditioning. *J. Neurosci.* **17**, 8093-8105 (1997).
10. Kandel, E.R. *Behavioral biology of Aplysia*. Freeman, San Francisco, (1979).
11. Scott, M.L., Govind, C.K., & Kirk, M.D. Neuromuscular organization of the buccal system in *Aplysia californica*. *J. Comp. Neurol.* **312**, 207-222 (1991).
12. Susswein, A.J., Rosen, S.C., Gapon, S., & Kupfermann, I. Characterization of buccal motor programs elicited by a cholinergic agonist applied to the cerebral ganglion of *Aplysia californica*. *J. Comp. Physiol. A.* **179**, 509-524 (1996).
13. Kupfermann, I. Feeding behavior in *Aplysia*: A simple system for the study of motivation. *Behav. Biol.* **10**, 1-26 (1974).
14. Morton, D.W. & Chiel, H.J. The timing of activity in motor neurons that produce radula movements distinguishes ingestion from rejection in *Aplysia*. *J. Comp. Physiol. A.* **173**, 519-536 (1993).
15. Weiss, K.R., Chiel, H.J., Koch, U., & Kupfermann, I. Activity of an identified histaminergic neuron, and its possible role in arousal of feeding behavior in semi-intact *Aplysia*. *J. Neurosci.* **6**, 2403-2415 (1986).
16. Jing, J. & Weiss, K.R. Generation of variants of a motor act in a modular and hierarchical motor network. *Curr. Biol.* **15**, 1712-1721 (2005).
17. Jing, J. & Weiss, K.R. Neural mechanisms of motor program switching in *Aplysia*. *J. Neurosci.* **21**, 7349-7362 (2001).
18. Morgan, P.T., Jing, J., Vilim, F.S., & Weiss, K.R. Interneuronal and peptidergic control of motor pattern switching in *Aplysia*. *J. Neurophysiol.* **87**, 49-61 (2002).
19. Jing, J., Cropper, E.C., Hurwitz, I., & Weiss, K.R. The construction of movement with behavior-specific and behavior-independent modules. *J. Neurosci.* **24**, 6315-6325 (2004).

