Locating the Brain Region Responsible for the Withdrawal Effect in the Chinese Mud Snail, *Cipangopaludina chinesis*

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Abstract

Invertebrate nervous systems are commonly used as valuable models for the study of nervous system function due to their large, simple neurons; however, there is still much to learn about the various regions of the nervous system and what their functions entail. We have been working to describe the basic details of the withdrawal effect of one such animal, the Chinese mud snail, *Cipangopaludina chinensis*. These snails are large molluscs that have been introduced into the freshwater ecosystems of several countries outside of southeastern Asia, where they are natively from. We present evidence describing the location of the ganglia responsible for the retraction of the snail’s body into its shell. Two muscle groups work together to withdraw the snail into the shell. The foot Muscle condenses the body radially and the Columella pulls the condensed body into the cavity of the shell. Using 2 fluorescent tracing chemicals, Rhodamine B and 1,1’-dioctadecyl-3,3,3’33’-tetramethyl-indocarbocyanine perchlorate (DiI), we identified the population of motor neurons innervating these muscles. Innervation of the foot muscles is accomplished through 7 pair of bilateral nerve bundles exiting the lateral margin of the Pedal Ganglion. A single large nerve bundle exits to the medial margin of the Right Pedal Ganglion to innervate the Columella muscle. Our fluorescent microscopy results have shown that the anterograde tract tracer, appears to project to one specific region of ganglia when applied to the columellar nerve of each snail. The type of tracer as well as fresh versus fixed state of the tissue are important to consider when tracing neurons in the snail nervous system.

Introduction

•The Gastropoda contains between 40,000 to 100,000 species of freshwater, marine and terrestrial snails and slugs have long been used to study the nervous system (Bieler, 1992).
•The columella and foot muscles are responsible for bringing the soft body of snails in and out of the shell. They are comprised of a densely packed 3D array of muscular and connective tissue fibers (Thompson, 1998).
•Columella muscle and foot muscle firing rates can tell us a lot about the mechanism for how the withdrawal effect takes place.
•Acetylcholinesterase (AChE) is the enzyme that breaks down the neurotransmitter acetylcholine (ACh) after ACh has allowed some action to take place, such as contraction of smooth muscle (Sam, 2021)
•The goal of this study was to determine the specific ganglion responsible for the withdrawal effect in the Chinese Mud Snail by determining which brain region the anterograde tract tracer diffuses to. Measurements of muscle firing rates and acetylcholinesterase concentrations shed more light onto the mechanism behind the withdrawal effect.

Results

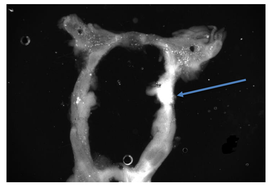

Figure 1. Bright region of interest in the upper right quadrant, pedal ganglia, indicating a large presence of tracer.

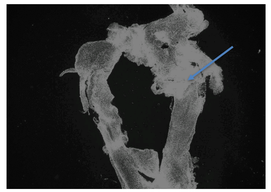

Figure 2. Bright region of interest in upper right quadrant, pedal ganglia, indicating a large presence of tracer.

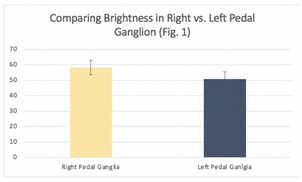

Figure 4. Comparing fluorescence intensity levels of right (yellow) versus left (blue) pedal ganglion in Fig. 1. Right is significantly brighter (t-test P = 0.002, n = 5).

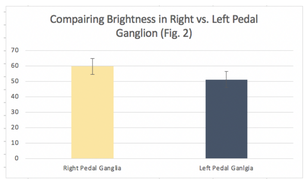

Figure 5. Comparing fluorescence intensity levels of right (yellow) versus left (blue) pedal ganglion in Fig. 2. Right is significantly brighter (t-test, P = 0.0006, n = 5).

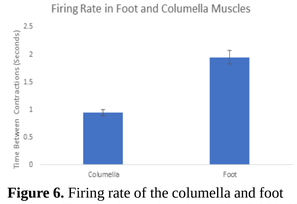

Figure 6. Firing rate of the columella and foot muscles. Ttest is highly significant (P < 0.001; *N* = 35 each).

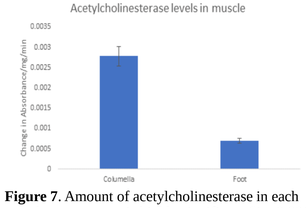

Figure 7. Amount of acetylcholinesterase in each muscle group. Ttest is highly significant (P < 0.001; *N* = 13 for columella; *N* = 15 for foot).

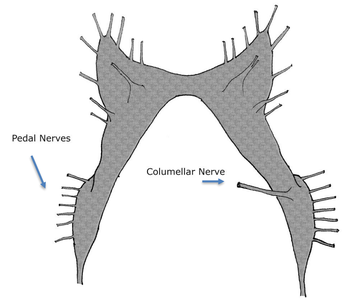

Figure 3. Representation of the central ganglia of Gastropda with labels (Satterlie, 1995).

Materials and Methods

Collection and Maintenance
•*Cipangopaludina chinesis* snails were collected at two sites in Newington, CT, Churchill Park Pond (Lat. Long. = 41.676, -72.721) and Mill Pond Park (41.693, -72.730).
•The specimens were collected between April and October from 2009 to 2012.
•The species of all snails collected was identified using the key, *Freshwater Snails of Connecticut* (Jokinen, 1983).
•Upon collection, the snails were maintained in lab aquaria kept at 20°C +/- 3°C on a 16:8 dark/light cycle until used.
Basic Measurements and Classification
•On dissection day, one snail is removed from the tank. Then, using a Dremel tool, a section (spanning approximately ¾ inch by ¼ inch) of the first whorl located near the first suture directly above the foot is removed to expose the columella
•Once the columella is exposed, one of the two anterograde tract tracers, more commonly DiI, was applied to the columella muscle using a sterile needle. The dye is allowed to trace for 20 minutes through the live nervous system before the brain of the snail is carefully removed.
•After the brain is removed and intact it is stored in formalin for 48 hours before going through a series of alcohol washes.
•The brain is then washed twice with 70% ethanol for 3 minutes each, twice with 95% ethanol for 3 minutes each, twice with 100% ethanol for 3 minutes each, once with Citrisolv for 3 minutes, and one final time with 100% ethanol for 3 minutes.
•Once the alcohol washes are complete, pronase is applied to the brain for 1 minute in order to get rid of any excess tissues that may be attached to the brain. The pronase is then washed off with water, and a DAPI fluorescent dye is applied in order to see where the tracer has travelled once under the fluorescent microscope. After the DAPI has been applied for approximately 1 minute, the brain is put on a slide for viewing under the microscope. Using ImageJ analyses, the average brightness was taken for each side of the pedal ganglion and compared using t-tests.
Electrophysiology
•Muscle firing rate was determined using the AM Systems 3000 Extracellular amplifier Iworx 214 DAQ.
•Access was gained to the columella and foot muscles using a Dremel tool to cut a window in the shell. Then, two silver chloride electrodes were placed – one in the body of the snail and one in either the columella muscle or the foot muscle.
•The snails were then allowed to relax and exit their shells. Once they were out of their shells, "record" was hit on the computer, and the snail was touched with a blunt wooden stick to initiate withdrawal behavior.
•Frequency of the motor neuron input was recorded and counted.
Acetylcholinesterase (AChE) Measurement
•Acetylcholinesterase was measured by applying an Ellman’s reagent, which digests acetylthiocholine iodide. This reaction leads to the production of a colored product which can be measured using spectrophotometry. The more product released from the reaction, the less light will be able to pass onto the spectrophotometer, and the higher acetylcholinesterase concentration there is in the muscle.

Discussion

Dissection and tract tracing indicates there is one major nerve bundle innervating the Columella muscle and it originates unilaterally from the anterior end of the right Pedal Ganglion (Figures 1-3). This makes sense as the columella muscle is a fairly compact midline structure that inserts on the center column of the shell then descends and interdigitates within the body of the snail and within the muscle fibers of the foot muscle complex. Similar innervations are known from closely related snails (Thompson 1998). The foot muscles are a much larger complex of fiber bundles arranged in several layers and with vertical, horizontal/radial and interdigitating orientations (Ferguson and Benjamin 1991). We found 7 nerve bundles exiting the lateral margins of both Left and Right Pedal ganglia and innervating the different components of the foot muscle. The organization of these Pedal Nerves is most likely organized by muscle orientation and lateralization. Further tracing, particularly anterograde tracing is necessary to understand the organization of foot muscle innervation.
The foot has a faster firing rate (Fig. 6) as it allows the body to compact laterally into the shell, then the columella pulls it up vertically before the shell closes off. As for acetylcholinesterase (AChE), there may be less present in the foot than in the columella for a few reasons (Fig. 7). The first being that AChE breaks down ACh faster in the foot muscle, and thus is needed for a shorter period of time. The second being that AChE stays around for longer in the columella muscle because the columella does not fire as quickly or for as long as the foot muscle, indicating that more AChE is needed to break down the ACh around. Likewise, the foot muscle fires at a faster rate and for a longer period of time, so there may be a lower AChE concentration in the foot muscle since it takes longer for the smaller amount of AChE to breakdown the ACh that is around. More research will have to go into determining the precise reason for decreased firing rate but increased AChE concentration in the columella muscle.

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