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Pre-neural vs Neural: Neurotransmitter Signaling Development in *Lottia scutum*

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ABSTRACT

Metazoans use nervous systems to transmit, interpret, and respond to sensory information from across their bodies using neurotransmitters. Neurotransmitters such as the serotonin-precursor 5-hydroxytryptophan convey signals using the synaptic pathways present in neural networks that result in physical reactions. They can also function as molecular messengers outside of the nervous system such as in the human digestive tract. The introduction of molecular messengers has been known to incite changes in cilial behavior within the phylum Mollusca. This change is seen in both adult and neural larval specimens. However, it is known that many in the family Gastropoda have a larval stage that lacks a nervous system yet possess cilia. Whether molecular messengers can impact ciliary behavior in the pre-neural stage of larva is yet unknown. Here we aimed to show that some molecular messengers do cause behavioral changes in both pre-neural and neural larvae of *Lottia scutum*. For example, our in-situ tests revealed an increase in the ciliary swimming behavior in specimens exposed to ATP compared to control specimens in filtered seawater. The resulting increase in the velocity of the organisms' movement was similar for both pre-neural and neural larvae. This suggests that ATP functions similarly in the pre-neural stage to how it does in the neural stage. Our results suggest that in the case of *Lottia scutum*, signaling using molecular messengers does occur in the absence of the nervous system. We anticipate that by studying the function of molecular messengers in pre-neural and neural larvae, we can gain insight into the evolutionary development of neuronal signaling.

INTRODUCTION

Lottia scutum is a species of marine limpet which spawn larvae whose swimming is based on ciliary action. These larvae begin swimming prior to the development of their nervous system, a developmental stage which lasts a few hours, and continue to swim after its development until they undergo metamorphosis and settle into a benthic life in rocky intertidal regions.

During previous work with Lottia scutum Dr. Eric Edsinger formed the hypothesis that the serotonin precursor 5-hydroxytryptophan may elicit changes in their larval swimming behavior. 5-hydroxytryptophan has been seen to have an impact on cilia action in adult limpets which prompted an investigation into whether there would be a similar impact on the pre-neural and neural Lottia scutum larvae. In order to evaluate the utility of future tests, screening of additional drugs was performed. The potential for changes in receptor proteins available on ciliated cells during the development of the larvae, encouraged the inclusion of a variety of molecular messengers.

The primary investigation was to determine if molecular messengers played an active role in larvae before and/or after the development of a nervous system. Furthermore, to test the hypothesis that pre-neural signaling is possible with molecular messengers used in neuronal signaling.

Figure 1 (a-c): 1a. Lime Kiln intertidal area, location of collections. 1b. *Lottia scutum* adults in their custard dishes post collection, during gamete release (see top left dish). 1c. 4 Custard dishes with fertilized gametes in the sea table.

Collection Location: Lime Kiln Point State Park *Friday Harbor, WA*
Laboratory Habitat: Sea Tables. Sea water pumped in from Friday Harbor through the tables and drained back into the harbor. Care was taken to ensure no contaminants, including cleansers were used in or around the tables. Custard dishes were used to separate animals, collect gametes, and hold larvae. Running water ensured the animals were kept at comfortable temperatures, close to 11°C.

Proposed Future Experiments/Observations:
Observations of cilia motion of immobilized specimen during drug screen. Effects of tidal schedule on spawning of L. scutum. Time differences of spawning behavior based on collection location. Temperature effect on spawning and larvae development.

METHODS AND MATERIALS

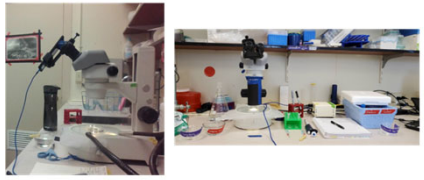

Figure 2 a & b: a. Prototype rig with side lighting rather than from below. B. A later prototype with the icebox.

Experimental Equipment

Equipment included our cell phones for video recording, phone mounts, dissecting microscopes with adjustable magnification (0.8 to 5x), micropipette, Styrofoam ice box with ice and internal Styrofoam block suspending the larvae/sea water in cool air around 11 degrees Celsius, fresh sea water, *L. scutum* larvae, custard dishes for spent larvae, fresh larvae, and sea water, Lab notebook, dropper to acquire the right number of larvae to mix with fresh sea water for a total volume of 2 mL, and 2x solutions of the transmitters to be tested.

Ethogram

Behavioral Assays were performed prior to experimental trials to familiarize researchers with typical larvae behavior and development. Our observations included the 6th, 9th, 12th, 15th, 24th, and 42nd hours post-fertilization (HPF). These enabled comparisons to be made between the behavior of untreated larvae at the timepoint and the behavior of the treated larvae.

Drug Screen

Our final drug assays consisted of examining the impact of the following neurotransmitters: 200uM serotonin, 200uM dopamine, 1mM glutamate, 1mM glycine, a 1mM glutamate-glycine combination, 1mM ATP, 1mM acetylcholine, 1mM GABA, and 1mM histidine on pre-neural and neural L*ottia scutum* larvae.

Organisms were drawn from one fertilization for each experimental session. Generally, four or five drugs were screened per session, with each session lasting approximately three hours. The difference in timing between the beginning of the sessions and the end could account for developmental differences between the first organisms imaged and the last. To account for this, controls were recorded immediately prior to every drug screen.

RESULTS

Example of Quantitative Analysis of Data

Figure 3 is an example of how data analysis is performed using Fiji (ImageJ). Each still shot shows a colored path that maps the organism's movement over 500 frames of video. The time the larvae were moving for in each image is the same, therefore a longer path such as those seen with some of the ATP-treated organisms, would indicate movement at a higher velocity in addition to any changes in movement patterns depicted by the track shapes.

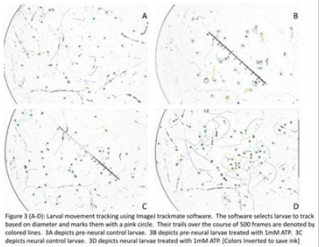

Figure 3 (A-D): Larval movement tracking using ImageJ trackmate software. The software selects larvae to track based on diameter and marks them with a pink circle. Their trails over the course of 500 frames are denoted by colored lines. 3A depicts pre-neural control larvae. 3B depicts pre-neural larvae treated with 1mM ATP. 3C depicts neural control larvae. 3D depicts neural larvae treated with 1mM ATP. [Colors inverted to save ink]

Summary of Drug Screen

Serotonin Precursor: Similar impact on pre-neural and neural larvae; increase in swimming velocity and shorter rest duration.
Dopamine: Increase in the neural larvae's tendency to swim in loops, change reverted five minutes post-drug addition. Pre-neural larvae did not display behavioral changes in response to dopamine.
Glutamate: No impact on the behavior of pre-neural or neural larvae.
Glycine: Abridgement of movement for around 4 minutes post drug addition (pda) in pre-neural larvae. Neural larvae movement shortening only apparent for 3 minutes pda.
Glutamate & Glycine: No visible change in behavior of pre-neural larvae. Neural larvae display an increase in the frequency of bursts of acceleration and looping behavior, however returned to control-like behaviors by 3 minutes pda.
ATP: Immense increase in swimming activity, particularly looping, in pre-neural larvae. Exhibit decreased swimming behavior compared to control around 12 minutes pda. In neural larvae, increase in the continuity of their swimming (pause less frequently) but by 9 minutes pda returned to control-like behavior.
Acetylcholine No effect on pre-neural larvae. Apparent increase in overall activity of the neural larvae.
GABA Pre-neural larvae to swim slightly longer distances between pauses. Neural larvae near-immediate increase in frequency of acceleration bursts and after 6 minutes PDA return to control-like behavior.
Histidine May increase looping behavior in pre-neural larvae for 3 minutes PDA. Neural larvae movements become truncated for about 1 minute PDA before returning to control-like behavior.

Disclaimer: The results garnered during the experimental window are best described as preliminary. Full analysis of the data has been ongoing for the past 6 months in addition to more robust testing. The results above are a summary of what was observed during the behavioral drug assays. They may not be supported by the quantitative analysis being performed but are the initial observations of the researchers.

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