

### C. Non-technical summary

I propose to identify the brain cells which are responsible for sensing water flow in the marine slug *Tritonia* (see photo).

Since *Tritonia* is blind, it must navigate by touch and smell. The primary factor that influences their crawling direction is the direction of water flow (caused by tides in nature). Although I have recorded the neural activity of some brain cells involved in crawling that are affected by water flow, no one has identified the sensory brain



cells which detect flow and determine flow direction. I will localize these flow receptor cells using selective cell staining, and electrical recording of brain activity while stimulating the slug with water flow. Once identified, I will determine how these flow receptor cells affect crawling direction via their connections to brain cells which effect turns.

The current situation is analogous to knowing that bats can avoid objects while flying in darkness, but having no idea of how they accomplish this except that it involves sounds. This is an opportunity to characterize the flow sensory modality of which we know very little.

This will also be a first step in establishing a method for investigating how animals make decisions based on conflicting sensory information.

### D. Problem statement

Many aquatic and marine species orient their bodies and their locomotion to the direction of water flow around them. The presence of flow can serve as a directional cue in navigation, or orientation to flow can serve as a mechanism to reduce hydrodynamic drag. For animals that are active at night, or in turbid waters, or those without vision, orientation to flow may be one of few potential sources of directional information. The physiological basis of how animals orient to water flow is not well understood. Putative water flow receptors (“rheoreceptors”) have been identified morphologically in a few animals (Boudko *et al.* 1999; Montgomery *et al.* 1997), but in no case have their projections to the brain been characterized. How rheoreceptors encode the direction of flow into neural signals is unknown, and few brain neurons that receive and process this information about water flow have been identified (Murray *et al.* 1992).

This research will be important in three ways. First, knowing how and why many animals sense flow and distribute themselves according to flow will help us better understand their ecology, which will have applications in disease vector control and in endangered species protection. Second, elucidation of this sensory modality will allow further investigation of behavioral choice in *Tritonia* by allowing one to compare how flow affects the motor centers for crawling along with chemical and tactile modalities. Third, understanding how these small, sensitive flow sensors works will help us understand more about the biomechanics of flow on biological sensors, as well as help us to design artificial sensors that could be used in future technologies.

*Tritonia* lives on sandy bottoms in turbid waters in the Pacific Ocean, and probably has no visual sense (Chase 1974). In this darkened world, the slug is challenged to navigate by touch or smell to find food, avoid predators, and mate. *Tritonia* can also

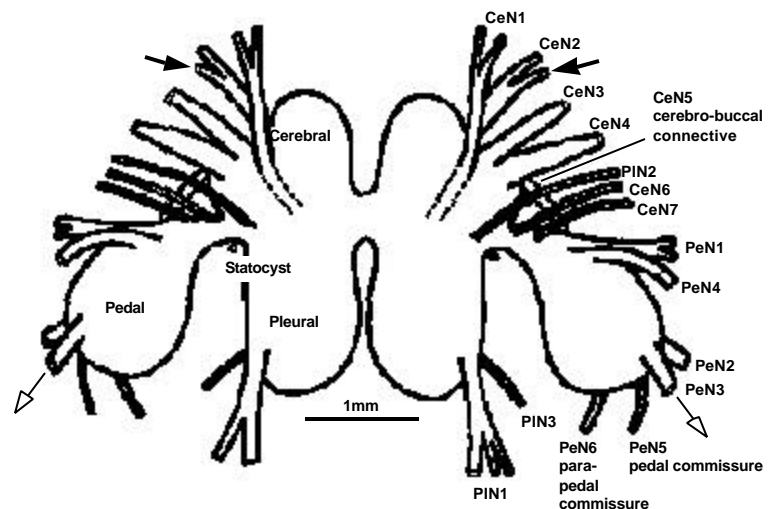
sense the earth's magnetic field, but has only been shown to orient its body to magnetic field direction in still water in the laboratory (Lohmann and Willows 1987). In nature, the tides keep water moving and *Tritonia* will prefer instead to orient headfirst into water flow, rather than to a magnetic direction (Murray 1994, Murray and Willows, in preparation). Since water flow in their habitat is almost always present, it is yet unclear if their magnetic sensitivity is currently useful, or merely an evolutionary remnant like our appendix.

I have shown that this slug is extremely sensitive to water flow, and orients its body headfirst into flow to reduce its hydrodynamic drag when exposed to tidal currents (Murray and Willows 1996). In contrast to their magnetic field sensitivity, their sensitivity to water flow direction seems to be their primary sense, like vision is to humans. *Tritonia* will often orient to water flow rather than attend to feeding and to mating. One possible reason for this strong propensity to orient to flow is to reduce hydrodynamic drag, and thus to reduce the chance of being lifted from the bottom and carried away with the tide (Murray 1994). Water flow appears to be a powerful motivating stimulus that initiates crawling (Field and MacMillan 1973; Willows (1978) and causes turning (Murray and Willows 1996). One might predict that this powerful motivational stimulus should produce in the brain correspondingly strong and widespread neuronal signals, which in turn effect the water flow-initiated behaviors like crawling and turning. I have discovered that ~90% of the brain cells tested in the pedal ganglion responded to a gentle water flow stimulus applied to the oral tentacles (mustache-like structure in photo above) (Murray *et al.* 1992). These neurons in the pedal ganglia are motor neurons that cause muscle contraction, so their responsiveness to flow stimuli is a result of synaptic input from sensory neurons receiving information about water flow from the oral tentacles. The location and nature of these sensory neurons is unknown and is the subject of this proposal.

## E. Objectives

*Tritonia* is a species that has been used to study the basis of behavior at the cellular level for over 30 years. The cellular basis of neuronal function is similar in all species, and much of what we know about the mechanisms of learning were first elucidated in a related sea slug *Aplysia*.

Many of the ~5000 brain cells in *Tritonia* have been identified as having specific functions, and a map of the locations of these cells is available. The brain of *Tritonia* has 16 pairs of nerves that innervate all areas of the body (see drawing below). I have determined that cutting just one nerve branch on each side (lateral branch of Cerebral Nerve 2 a.k.a. latCeN2, indicated by **black arrows** on drawing) eliminates the ability to orient to water flow (Murray and Willows 1996). Since the slug can still crawl and turn, I hypothesize



that turning is no longer correlated with the direction of water flow because cutting latCeN2 has deprived them of sensory information from rheoreceptors. The latCeN2 nerve branch carries both sensory and motor axons from the outside-most oral tentacles (Willows 1973a), and the sensory receptor endings are likely to be located there. These receptor endings may be attached to sensory cell bodies that lie in the oral tentacle, as found in a different sea slug (Boudko *et al.* 1999), or attached to a long axon that connects to sensory cell bodies that lie in the brain (i.e. primary sensory receptors), as found with tactile receptors in *Tritonia* (Audesirk and Audesirk 1980). Recording from the sensory receptors in the oral tentacles may be technically difficult as they are likely to be very small. These peripheral receptors must synapse onto other neurons that then project an axon to the brain. I propose to record from brain cells that have an axon in the latCeN2 nerve branch, and that respond to water flow stimuli of a magnitude similar to that which causes oriented crawling to flow (i.e. rheotaxis). These brain cells may be primary sensory receptors like those found by Audesirk and Audesirk (1980), or may be neurons that receive synaptic input from sensory receptor cells located wholly in the oral tentacles. I will attempt to distinguish these two possibilities.

- **Objective 1:** I will localize the neurons in the brain that have axons in the latCeN2 nerve branch.
- **Objective 2:** I will identify which neurons identified above receive information from water flow receptors. I also determine how receptors encode the direction of water flow.
- **Objective 3:** I will determine whether a newly identified flow sensitive neuron is a primary receptor or is receiving synaptic input from a primary receptor in the oral tentacle.
- **Objective 4:** I will determine how appropriate turning and crawling is performed under various flow conditions.

This study will lay the groundwork for the study of *Tritonia* orientation as a model of behavioral choice. The cellular mechanisms by which animals make choices is poorly understood and a sensible approach will be to study decision making in simpler brains before proceeding to studying choice in animals with more complex brains. Water flow, food odors, magnetic field azimuth, and tactile cues influence orientation of *Tritonia*. I will study how these competing sensory influences affect the motor pathways and hence behavior. To date, only the tactile and chemosensory neurons have been identified in the central nervous system of *Tritonia*.

I would also like to pursue a comparative project in which I compare the functions of the tentacles of *Tritonia* with those of the sea slug *Aplysia*. A master's thesis on the subject of the function of head tentacles in *Aplysia* was recently submitted from a student in the laboratory of Prof. Paul Hamilton in Biology here at U.C.A. Prof. Hamilton's extensive experience with the sensory biology of *Aplysia* (Hamilton and Russell, 1982) could provide for a fruitful collaboration that may involve U.C.A. students.

I also intend to study the structure and function relationship of the flow receptors that exist in the oral tentacles. Not only will this help us to understand how animals detect outside water flow, but may help us to design better and smaller tools for measuring blood flow for medical applications in humans. If *Tritonia* flow receptors are on the surface of the skin, as has been found in other animals (Boudko *et al.* 1999; Montgomery

*et al.* 1997), then I would propose to visualize them using a scanning electron microscope (SEM). An SEM is available at the marine station where this work will be done, and the College of Natural Sciences and Mathematics is currently seeking funds to bring an SEM facility to U.C.A.

## F. Methodology

I started at U.C.A. this semester, and am just now beginning to talk to students about working in my laboratory, which should be operational next semester. One student has plans to accompany me on my summer research. Jay Vacca is an outstanding young student working in Prof. Deborah Kreiss' laboratory in Biology. He has experience with neurophysiology and would be a welcome addition to this project.

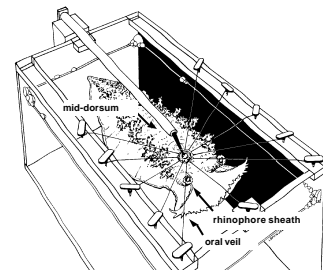
**Methods for Objective 1:** I will stain the nerves that project to the flow sensing tentacles (latCeN2) on its head so that the stain will be carried inside neuronal axons back to the brain cells that receive this sensory information. The result will be a brain with about 200 brain cells stained. I will remove the brain from *Tritonia* along with a several millimeter length of Cerebral Nerve 2. I will place a Vaseline dam over the nerve between the brain and the cut end of the nerve, and then place a drop of filtered sea water over the brain to keep it healthy, and a drop of solution containing the stain neurobiotin over the cut lateral branch of the nerve. Neurobiotin is taken up by the cut ends of the axons and both diffuses and is actively transported back to the cellular structures in the brain. After 2-6 hours in neurobiotin, the brain will be transferred to a solution containing fluorescein-conjugated avidin.

Neurobiotin will bind to the chemical avidin readily, and I will use an avidin molecule that is bound to a fluorescent marker molecule called fluorescein. The brain will be fixed, dehydrated, cleared, and mounted on a microscope slide. The stained brain cells will be visualized on a fluorescent microscope and drawn, photographed or have images stored on computer. This will be done on at least five brains to determine the extent of inter-individual variability. Preliminary results from less efficient staining has indicated that I should expect to see nearly 200 brain cells stained using this method, with many of them clustered in specific locations in the brain that are consistent between individuals.

These neurons will include some motor neurons, which cause muscle contraction, and also sensory receptors, likely of three modalities—chemical, tactile, and water flow. This objective will allow me to proceed with Objective 2 efficiently by allowing me to ignore neurons which are in locations in the brain that make them unlikely to participate in water flow sensitivity.

The results of the staining of the brain will be summarized as a map showing the locations, grouping, numbers, sizes, and shapes of brains cells with axons in the lateral branch of Cerebral Nerve 2 (latCeN2).

**Methods for Objective 2:** With this staining “map” as a guide to the probable locations of brain cells with axons in latCeN2, I will pierce brain cells in those areas with fine glass electrodes so as to record their internal voltage signals while the tentacles are being stimulated with a gentle flow stimulus. This will allow me to identify and stain specific brain cells that are responsible for sensing flow.



I will use a preparation in which the brain has been exposed *in vivo* (see figure to right, used by permission). I will expose the brain with a 1-cm incision in the body wall, and immobilize the brain by placing pins around the nerve into a wax-covered platform. I will record from a candidate neuron, and stimulate the oral tentacles with a flow stimulus. Those neurons that respond with a low threshold (behavioral threshold is under 2 cm/s), and that have an axon in latCeN2 (determined with an extracellular electrode), will be stained, drawn, and photographed.

The flow stimulus will be controlled by a computer-controlled valve that turns on water flow from a 2-mm inside diameter Tygon tube placed 1 cm away from the oral tentacles. The flow rate will be adjusted to not exceed flow rates that reliably induce turning in freely behaving *Tritonia* (~ 2 cm/s).

While recording from brain cell bodies, I will also monitor axon activity in the nerve with an extracellular electrode. If I find a brain cell that responds to flow, I will stimulate the cell to produce action potentials that should be visible in the nerve recording. If an action potential is visible in the nerve record correlated to my stimulation of the brain cell, I can conclude that the brain cell has an axon in latCeN2, and may be a neuron responsible for receiving flow information mediating flow orientation behavior. I will also observe and videotape the slug to determine if the stimulated flow responsive neuron is a motor neuron that receives reflexive input from a sensory neuron. Neurons that produce movement when stimulated will be excluded as flow receptor candidates.

I will determine how flow stimuli of different intensities and directions are encoded in the brain cell signals. *Tritonia* orients to flow with the left or right nerves intact, so determining flow direction is not accomplished only by comparing stimulation intensities on each side of the head. Previous nerve recordings show multiple spike heights, therefore many units likely carry flow information to the brain. Since *Tritonia* can orient to flow with sensory information from the oral tentacle on one side of the head, I predict that there exist flow-sensitive neurons of at least two directional selectivities, allowing the animal to determine if flow is coming from the left or the right. I will first test each flow receptor to determine if it responds differently depending on whether water flow comes from the left or the right. If each receptor responds similarly to both flow from the left or right, then I would predict that some receptors from one side of the head will respond more strongly to flow from the left, and others on that side of the head will respond best to flow from the right. Which result is found will affect the nature of the synaptic connections to motor neurons to be investigated in Objective 4.

**Methods for Objective 3:** Although primary mechanosensory neurons have been identified in *Tritonia* CNS (Audesirk and Audesirk 1980), central flow neurons may not be primary sensory neurons. Electron microscopy in another slug suggests that putative ciliated rheoreceptors synapse on peripheral neurons in the anterior sensory structures. The third goal of the study is to determine if the flow-sensitive neurons identified are primary receptors. Primary sensory neurons will be identified as those that produce no visible muscular contractions (as determined in Objective 2), respond to stimuli even in high  $Mg^{2+}$ /low  $Ca^{2+}$  artificial sea water (which blocks synaptic transmission), and that receive only spikes with a constant amplitude (as opposed to receiving synaptic input which can vary in its amplitude with stimulus intensity).

The brain can be isolated from the body with a Vaseline dam built around the brain in which one can place isotonic  $Mg^{2+}$ /low  $Ca^{2+}$  artificial sea water to block synaptic transmission.

**Methods for Objective 4:** I will record from both the flow sensing brain cells, and the brain cells which are responsible for controlling the muscles that cause turning to determine how flow direction is used by the brain to guide crawling direction. I will determine how central flow-sensitive neurons synaptically affect two types of motor neurons: pedal ciliary motor neurons that propel crawling (Pedal neuron 21 [Pe21]), and turning neurons [Pe3]. This will involve placing a microelectrode in two neurons simultaneously, which is considerably more difficult than placing only one since either electrode may become displaced if the brain moves at all. Recording from multiple neurons has been accomplished previously in *Tritonia*, often involving as many as 4 neurons.

Pe21 is a motor neuron that increases the rhythmic beating of cilia on the bottom of the foot of *Tritonia*. The activity of Pe21 increases with increased crawling (Audesirk 1978). Since *Tritonia* crawls faster in higher flow (Willows 1978), I predict that flow-sensitive neurons will excite Pe21. At very high flow crawling ceases (unpublished observations), so I will determine if these flow rates cause Pe21 to be inhibited.

Since turning to one side is effected by motor neurons on the same side of the brain, some of the flow-sensitive neurons must send signals across the midline to the contralateral Pe3. For instance, a flow receptor responding best to flow from the right, should cause excitation of the Pe3 motor neuron in the right pedal ganglion. These predictions will be tested with pairwise recordings of flow-sensitive and motor neurons. First, I will record from the flow receptor and Pe3 in the left ganglion, and then move the Pe3 electrode to the corresponding Pe3 motor neuron in the right ganglion.

My long term objective is to characterize the mechanisms by which water flow is sensed, how directional information is processed in the brain, and how that directional information is employed in producing adaptive behavior. Further goals include using the water flow sensory modality together with the better described tactile and chemosensory modalities to determine how an animal makes decisions when two sensory modalities provide conflicting motivations. These results will also provide the basis for a comparative approach to understanding how animals detect flow.

## G. Evaluation and Dissemination of Results

This project will have achieved its objectives if I am able to reliably demonstrate the location, structure, and function of flow-sensitive neurons in the brain of *Tritonia*. These flow receptor cells must receive flow sensory information from the periphery, and must (directly or indirectly) relay this information to the motor neurons that cause turning and crawling. Publication of these results in a high-quality, peer-reviewed journal in the field of neuroscience and behavior will be the final determination of success.

Results from these studies will be presented at the annual Society for Neuroscience convention, to be held November 4-9, 2000 in New Orleans, LA. Approximately 25,000 neuroscientists from around the world attend the Neuroscience convention. The results will also be presented at the annual meeting of the Arkansas Chapter of the Society for Neuroscience, held a few weeks before the meeting in New Orleans. In addition, the results will be presented at the annual U.C.A. Student Research Symposium, held in the spring, as U.C.A. undergraduate students may conduct a significant portion of this research. It is my intention that Jay Vacca present his results from next summer at the

national Society for Neuroscience meeting, and to eventually prepare his results for publication.

Results from these studies will be submitted for publication into peer-reviewed journals pertinent to neuroscientists. Possible venues of publication include the Journal of Comparative Physiology A– Sensory, Neural, and Behavioral Physiology (in which I have published thrice previously), the Journal of Neuroscience, the Journal of Experimental Biology, or Invertebrate Neuroscience.

Results from these studies will also be used to apply for additional funding for further research endeavors. To successfully compete for research monies on a national level, I must have preliminary results with which to justify my requests. Funding of this research stipend will enable me to accomplish this goal.

## H. Facilities and equipment

This research will be done at the Friday Harbor Laboratories, a marine research station belonging to the University of Washington, located in Friday Harbor, WA, to which I will be sending an application for the rental of laboratory space. I am familiar with these facilities and have done similar research there from 1989-1994, and on a Grass fellowship in 1997. I chose this location because it is close to the only known source of my species of sea slug, and because most of my colleagues in research on *Tritonia* will also be in Friday Harbor next summer. Laboratory fees there will cover the laboratory space for myself and my student, which includes access to running filtered sea water, aquaria for keeping slugs, electricity, computing facilities, microscopes, an electrode puller, a computer-controlled magnetic coil system for magnetic stimulation, a flume aquarium for water flow stimulation, a stock room of chemicals and supplies, a dive locker with tanks and emergency equipment, and a library. I plan to be at the marine station from May 22<sup>nd</sup> until August 10<sup>th</sup>.

I will transport UCA equipment purchased with my start-up funds for recording from the brains of *Tritonia*. This equipment will include a vibration isolation table, a dissecting microscope, an acrylic aquarium for holding the slug during recording, a fiber optic lamp for illuminating the brain, two micromanipulators to precisely position electrodes into specific brain areas, a Faraday cage to insulate the sensitive recordings from interference from the AC electrical systems, an intracellular amplifier to amplify signals from inside brain cells, an extracellular amplifier to amplify the signals from the nerves, an oscilloscope to monitor these electrical signals, an analog-to-digital converter to transfer the signals to a computer, a computer to record and analyze the signals, a tape recorder to record large amounts of data, and a camcorder for video analysis of behavior.

## Citations

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