Neuronal Responses to Water Flow in the Marine Slug *Tritonia diomedea*

Jeffry S. Blackwell, James A. Murray
Department of Biology, University of Central Arkansas, Conway, AR, 72035

The marine slug *Tritonia diomedea* must rely on its ability to touch and smell in order to navigate because it is blind. The primary factor that influences its crawling direction is the direction of water flow (caused by tides in nature). The sensory cells that detect flow and determine flow direction have not been identified. The lateral branch of Cerebral Nerve 2 (latCeN2) has been identified as the nerve that carries sensory axons to the brain from the flow receptors in the oral tentacles. Backfilling this nerve to the brain resulted in the labeling of a number of cells located throughout the brain. Most of the labeled cells are concentrated in the cerebral ganglion where the nerve enters the brain. The medial and lateral branches of CeN2 were backfilled for comparison of the pattern of cells from each nerve. A map of the cells innervated by latCeN2 reveals the location of the stained cells. Extracellular recording from latCeN2 revealed its involvement in the detection of water flow and orientation. The nerve becomes active in response to water flow stimulation. Intracellular recordings of the electrical activity of these cells in a live animal will be the next step to determine if these cells are the flow receptors.

Key Words: rheotaxis, navigation, tidal orientation, flow receptors, Nudibranchia, Gastropoda

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Introduction

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 could 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 neural connections to the brain been characterized.

The marine nudibranch gastropods *Tritonia diomedea* (Bergh) live on sandy bottoms in the turbid waters of the northern Pacific coast and probably have no visual sense (Chase 1974). The slug must rely on touch and smell in order to survive. It reaches lengths of up to 30 cm and weights of 1500 grams. *Tritonia* prefer to orient headfirst into the natural water flow caused by tides rather than to magnetic direction (Murray 1994, Murray and Willows, submitted). This slug is extremely sensitive to water flow and may use the headfirst orientation to reduce hydrodynamic drag when exposed to tidal currents (Murray and Willows 1996). In contrast to *Tritonia*'s magnetic field sensitivity, its sensitivity to water flow direction seems to be its primary sense, like vision is to

humans. *Tritonia* will often orient to water flow rather than attend to feeding and to mating. The slug turns its body and crawls into flow coming from the sides. Neurons located in the pedal ganglia are responsible for the movements that cause orientation to flow. Approximately 90% of the brain cells tested in the pedal ganglion responded to a gentle water flow stimulus applied to the oral tentacles (Murray *et al.* 1992). These neurons in the pedal ganglia are mainly motor neurons that cause muscle contraction, so their responsiveness to flow stimuli is a result of synaptic input from sensory neurons that are receiving information about water flow from the oral tentacles. The location and nature of these sensory neurons is the subject of this report.

Many of the 5000 - 10,000 neurons in the brain of *Tritonia* have been identified as having specific functions, and maps of the locations of these cells are available (Willows et al. 1973; Murray et al. 1992). The brain has 16 pairs of nerves that innervate all areas of the body. Cutting just one nerve branch on each side (lateral branch of Cerebral Nerve 2 a.k.a. latCeN2) eliminates the ability to orient to water flow (Murray and Willows 1996), although the slug can still crawl and turn. The latCeN2 nerve branch carries both sensory and motor axons from the lateral oral tentacles (Willows et al. 1973), and the sensory receptor endings are likely to be located there. The receptor endings may be attached to sensory cell bodies 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). Electrical activity was recorded from peripheral sensory 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.

It was hypothesized that flow-sensing cells in the oral veil have axons in latCeN2 nerve, and that stimulating the oral veil with water flow will result in an increase in the rate of action potentials recorded in latCeN2 nerves. Furthermore, it was predicted that some flow-sensitive cells would respond preferentially to one flow direction.

It is hypothesized that the encoded pattern recorded in both nerves gives clues to how the brain decodes the pattern to control the direction of orientation by the animal.

Methods

Collection of Tritonia diomedea

In the experiments carried out in UCA's facilities, specimens of *Tritonia diomedea* were collected from Tofino, British Columbia (Canada) or from Dash Point (by SCUBA) or from Bellingham Bay (by trawling), Washington state (USA). In the experiments performed at the Friday Harbor Laboratories, specimens were collected from Dash Point or Bellingham Bay.

Aquaria

In the lab, the slugs were kept in a 90 gallon aquarium (Aquanetics Insulated Tank System) with carbon and biological filters as well as a UV sterilizer. Temperature was kept under 10°C, and the artificial seawater salinity was maintained near 28‰. Under these conditions, the slugs could be kept alive for months, although all the animals used in these experiments spent less than eight weeks in this tank. The slugs were fed with their natural prey, the cnidarian sea pen, *Ptilosarcus gurneyi*. A smaller tank (27.5 x 18 x 17 cm) was used during surgery. This tank was filled with 8 liters of artificial sea water maintained between 5°C and 12°C during the surgery.

Animals

Many of the slugs that were used for these experiments were used previously in the experiments another scientist in the lab. These animals often were left with an open incision for 24 hours before being used; however, the nerves used in these experiments were not damaged, and were not used for backfills if their brains were damaged. This had no obvious detrimental effects on the results, although some physiological changes may have occurred due to blood loss, posture, and pressure changes in the hydrocoel of the animal. Additionally, any

inactive animals that appeared to have been detrimentally affected by the open incision were not used for the experiments. Animals were anesthetized during surgery using 1-phenoxy-2-propanol diluted a thousand times in sea water. All the animals spent 20 to 60 minutes in that solution before the first incision was performed. This anesthetic reversibly reduces muscular tone, and allows surgery that is less stressful for the animal. This has the advantage of reducing blood loss. One hour after a 30 minute treatment, slugs seemed completely recovered from the anesthesia and behaved normally again.

Dissection

A small incision was made on the dorsal surface of the animal over the buccal mass to expose the brain. The slug was transferred to the smaller surgical tank (described above). The incision was held open with hooks, while a nonmagnetic metal platform covered with silicone elastomer (Sylgard 170B) was manipulated under the brain to support and immobilize it. The brain was secured to the platform with pins placed in its connective tissue sheath. Using small scissors, the outer layer of the connective tissue was removed from the nerves.

Cerebral nerve 2 (CeN2) attaches to the cerebral ganglion on the anterior side of the brain (Figure 1). On the left and right sides, CeN2 is the medial-most nerve that attaches to the cerebral ganglia. Near the brain, CeN2 splits into medial and lateral branches.

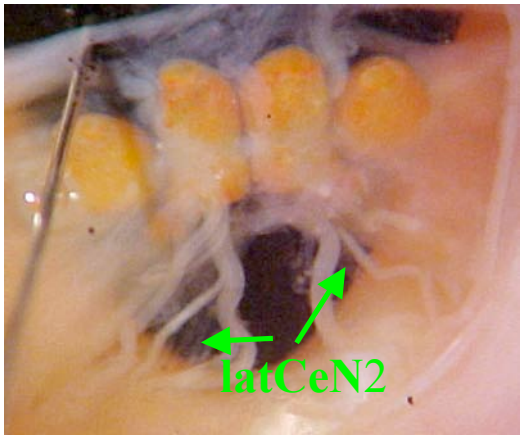

Figure 1: Lateral cerebral nerve 2 (latCeN2) exits the anterior surface of the brain below cerebral nerve 1. LatCeN2 was recorded from in the experiments. The orange ganglia comprise the brain of the animal. The black wax coated platform can be seen under the brain as well as the location of one of the holding pins.

Several millimeters of latCeN2 were exposed. In all experiments, the nerve was cut near the brain with extra length coming from the periphery. The cut peripheral end of the nerve was pulled into a suction electrode with a tip that fit firmly around the nerve. Activity from the right and left latCeN2 was recorded simultaneously while the oral veil of the slug was stimulated with water flow**.** The flow rate varied from 8 cm/s to 16 cm/s. The water flow was directed from the right to the left and from the left to the right on consecutive trials.

The flow stimulation was controlled by an electric signal created using the Spike 2 software (Cambridge Electronic Design). This digital to analog converter (1401plus, Cambridge Electronic Design, CED) sent the command to a DC gear pump, assisted by a double-Darlington amplifier that supplied the current necessary to run the pump. A toggle switch directed the direction of the pump, creating left or right flow direction. The electric signal consisted of a 30 second period of no flow followed by a 30 second ramp up to a specified voltage between 4.5 and 7.0 volts (Figure 2). The maximum voltage was maintained for 30 seconds before ramping back down to 0 volts in 30 seconds where it remained for 1 minute. The total time for each trial was three minutes. Note that ~4V threshold of the pump restricts water flow to a period less than the entire 90 s duration of the ramping electrical command signal.

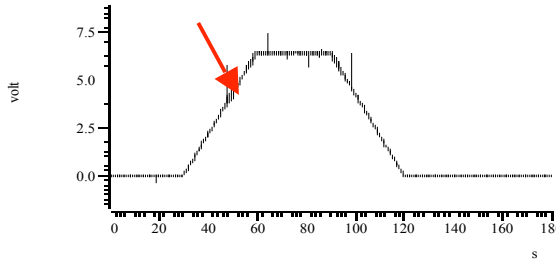

Figure 2: The changing level of electrical stimulation that the flow stimulation is driven by reaches 6.5 volts in this example. The voltage increases for 30 seconds to the maximum voltage, remains continuous for 30 seconds, then decreases for 30 seconds to 0 volts. Where the noise stops

marks the onset of flow (arrow). The noise was a result of feedback from the pump, and did not affect the pump speed.

Nerve recordings

The voltage difference between the recording and the reference electrode was amplified 10,000 times and filtered with a 100 Hz low cut-off and 1,000 Hz high cut-off, by an extracellular amplifier (A-M Systems model 1700). 60 Hz noise was reduced in the analog signal with an active noise eliminator (Hum Bug, Quest Scientific). A silver chloride ground electrode placed in the aquarium and connected to the amplifier reduced interference from extraneous electrical noise. Cell activity recordings were digitized through an analog to digital converter (1401plus, CED). This digital signal was captured and analyzed using Spike 2 software (CED).

Spike sorting

Successful discrimination between different spike types (action potentials from different axons) was achieved through computer software (Spike 2, CED). Spikes from the flow sensitive cells were distinguished from the activity of other cells that have axons in the whole nerve recordings. The spike sorting software allowed similar spike waveforms to be discerned from others and grouped into spike types. Recordings made from the water flow trials were analyzed and spikes were sorted into groups. Each group was then individually analyzed for its firing rate before, during, and after the flow stimulation. Spike types that occurred only during stimulation were determined to be caused by the flow sensitive cells. If a part of the spike group was present before or continued after the stimulation was ended, then it was excluded from the analysis.

Determining spike rate

Determining the spike rate increase or decrease in each trial was done by observing a recorded trace with the naked eye and/or measuring spike rate. All traces that showed activity were sorted into individual spike trains, so the change in activity was relatively easy to see with the eye (Figures 6 and 10). A comparison of spikes per second before, during, and after the stimulation with water flow was used in many trials and in all trials that left any doubt of a difference in activity to ensure accuracy in the reported results. Total counts were not made until all traces had been analyzed to ensure uniformity in the analysis and count.

Backfills to locate targets of latCeN2

When the central end of the nerve was soaked in a solution of cobalt chloride, the cobalt ions traveled to the neurons that the axons come from, and after processing, the location of the neurons with axons in latCeN2 was revealed.

The brain was removed from *Tritonia* along with several millimeters of Cerebral Nerve 2. The brain was cleaned by carefully removing the connective tissue sheath that surrounds the ganglia. Nerves that were not used were trimmed, leaving only the latCeN2 nerves long.

A line of petrolatum was placed in a small plastic petri dish using a syringe. The end of latCeN2 was carefully pulled over the petrolatum wall, leaving the brain on the opposite side. A second line of petrolatum was placed over the first to complete the impermeable wall with the nerve passing through it (Figure 3). Saline was placed on the brain side and alternately on the nerve side of the wall to test for leaks.

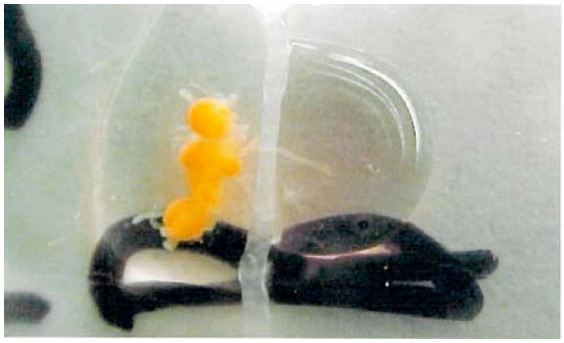

Figure 3: The backfill setup with the nerve (latCeN2) passing through the wall of petrolatum has normal saline on the side with the brain and 5% cobalt chloride on the opposite side. The cobalt chloride travels into neurons of the brain via the axons of latCeN2. The black area is a label on the opposite side of the dish written with a marker.

The nerve was cut near the tip with small, sharp scissors and was osmotically shocked for 10 seconds in distilled water to push the axons open. The water was dried using a Kimwipe and

replaced with a small amount of 5% cobalt chloride to immerse the nerve (sometimes the solution was lowered to 2.5% if a lighter staining result was desired). The brain was left in the refrigerator for 24 hours to allow time for the cobalt to be drawn through the narrow axons into the neurons and was surrounded by moist paper towels to prevent the liquids from evaporating.

The brain was removed from the refrigerator after 24 hours, and the cobalt chloride was removed. The brain was then removed and placed in saline for at least 10 minutes to wash away any contaminants on the surface. The brain was placed in fresh saline with 4-5 drops of ammonium sulfide $[(NH_4)_2S]$ to reduce the cobalt and turn it black. After about 10 minutes, the brain was transferred to 50% ethanol in a small centrifuge tube.

The brain was run through a dehydration series to remove water. The 50% ethanol was replaced by 70%, 90%, 95%, and finally 100%. The brain remained at each step in the process for about 10 minutes to avoid dehydrating too quickly.

The brain was transferred to a glass dish containing methyl salicylate (oil of wintergreen). The brain remained immersed until the tissues cleared and became ready to view. The brain was then mounted between two cover slips and viewed on a compound microscope. Digital photographs were taken of the entire brain, focusing on stained areas. The brain was stored in methyl salicylate.

Results

The data presented are used to illustrate the results of the experiments and do not include all the data that had similar results.

Backfilling of latCeN2

The backfilling of latCeN2 in several brains resulted in the staining of approximately 180 cells in the brain of *Tritonia.* The cells that were filled could be sensory or motor neurons. The filled cells were more highly concentrated in the cerebral ganglia than in any other area of the brain (Figure 4). Most of the cells filled (50-60%) were in the cerebral ganglion on the side that the nerve was filled. Cells in the pleural and pedal ganglia were filled in the right and left sides of the brain in most backfills. The number of cells filled in the pedal ganglia did not appear to be dependent on the side of the backfill, with about the same number of cells filled each time in the pedals. Figure 5 is a map summarizing the locations of the cells that were filled.

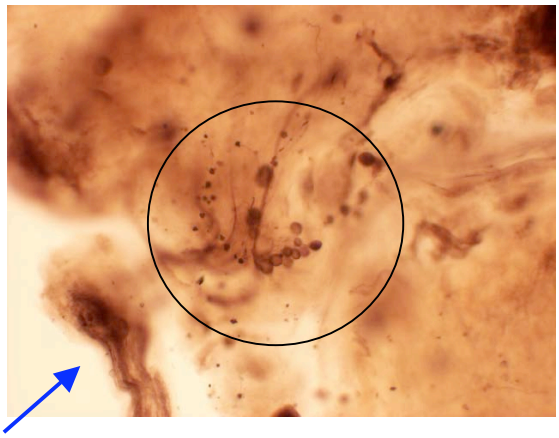

Figure 4: The cells filled with cobalt from latCeN2 are mostly located in the cerebral ganglia (circle). The nerve can be seen well in this image (arrow). The areas appear black after the cobalt is reduced using ammonium sulfide.

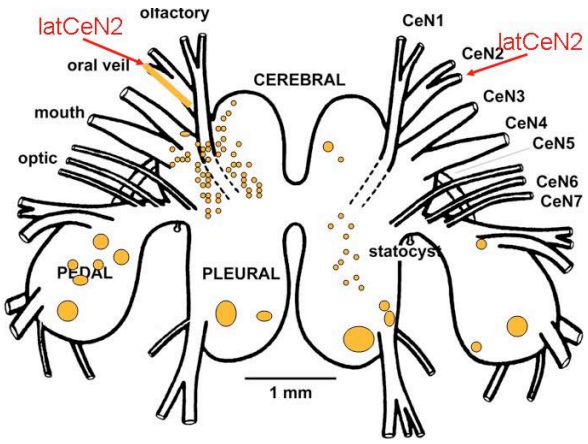

Figure 5: The brain with filled cell locations marked (anterior at top). The left latCeN2 was backfilled in this brain. A mirror image pattern also occurs with the fill of right latCeN2. A one-sided fill is depicted to clearly show which cells have an axon in left latCeN2. The marked cells demonstrate relative sizes, numbers, and locations.

Effects of water flow stimulation on activity in latCeN2

Stimulation of the sensory structures of the oral veil with water flow resulted in a number of different responses recorded in latCeN2. A total of nine sea slugs were used in the experiments (9 experiments). In a total of 178 trials recording from the left and right latCeN2, 65 of the trials showed an increase in the activity (spike rate) of individual receptor cells with axons in latCeN2 during flow stimulation on the ipsilateral side, while 8 showed a decrease in activity (Figures 6 and 7) with flow from the ipsilateral side. The remaining 105 trials showed no change in activity. The change in activity was usually seen only during the time that the pump was running. At the conclusion of the ramp when the flow was discontinued, the activity generally returned to a level very similar to the level seen before the flow occurred. An increase was seen on the contralateral side in 27 trials (Figures 6 and 8). A decrease in the spike rate was also seen on the contralateral side in 29 trials (Figure 6). The change in activity on the contralateral side was usually seen in trials with higher flow rate.

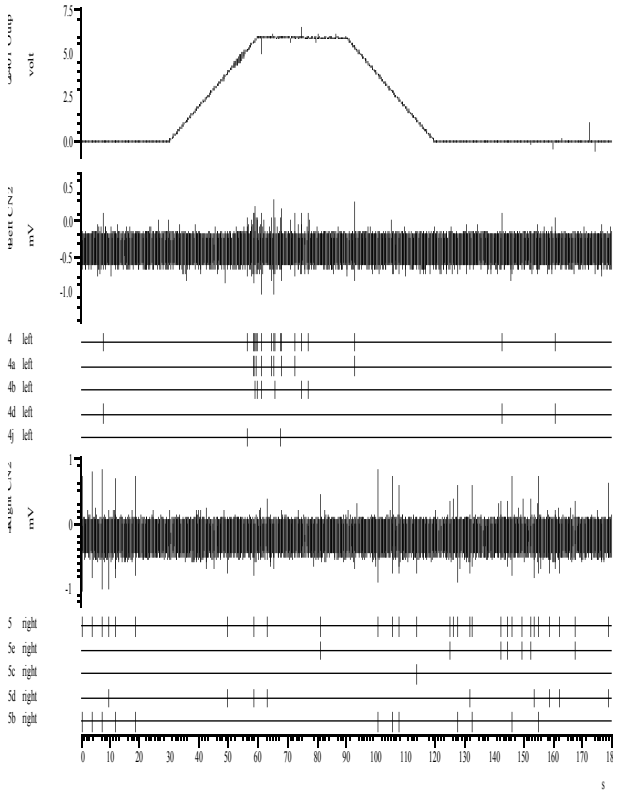

Figure 6: The increased activity in left latCeN2 (top) in a single trial while flow was coming from the left side is seen here. The flow rate was 13 cm/s. The spike rate in the left side increased during flow (top) as the overall activity decreased during flow in the right latCeN2 (bottom). The spikes have been sorted by type (based on voltage magnitude and spike shape) below the recording and the top line includes all spikes sorted from the trace. Four individual units can be seen in each trace.

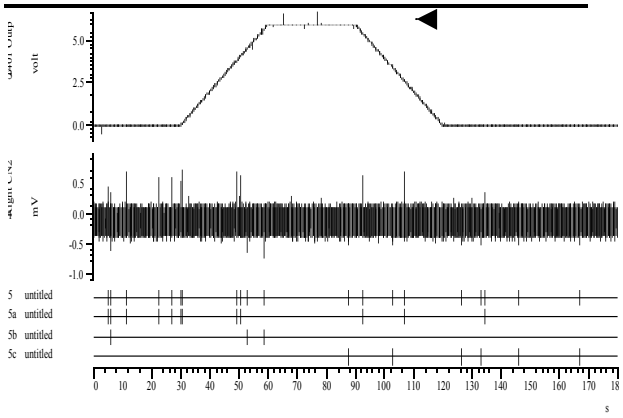

Figure 7: This is a trace of right latCeN2 activity while flow was coming from the right and the activity in latCeN2 decreased. This pattern of activity was seen in 8 trials.

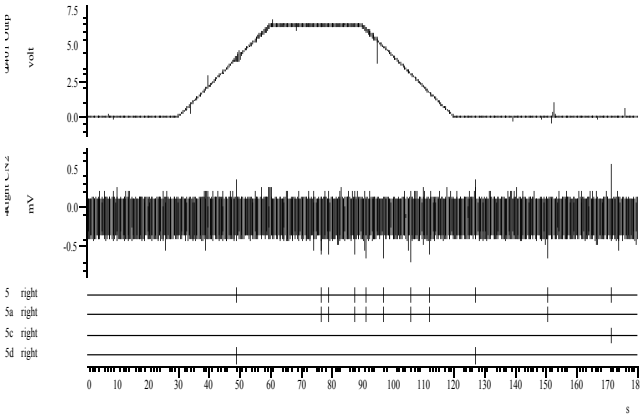

Figure 8: This trace is a recording of the right latCeN2. Flow coming from the left here caused an increase in overall activity on the right side of the animal. Three individual units (5a,5c,5d) are indicated by the breakdown at the bottom of the trace. This pattern was seen in 27 trials and could indicate directional sensors active on both side or could be due to turbulence among other alternatives.

Table 1 summarizes the results of the recordings from the water flow sensitive nerve.

Table 1: Summary of the trials that produced changes in activity during recording from latCeN2. The trials were categorized using the overall spike rate in each individual trial. Note that water flow caused increased spike rates more often in the ipsilateral nerve. Conversely, water flow caused decreased spike rates more often in the contralateral nerve.

	Rate increases		Rate decreases	
Flow Direction	**Right latCeN2**	**Left latCeN2**	**Right latCeN2**	**Left latCeN2**
Right to Left	32	15	6	10
Left to Right	12	33	19	2

Discussion

Backfilling of latCeN2

The final map is a summation of cells labeled from eleven total backfills. Some backfills were not used for the map because of partial filling of medCeN2, but they could be used for comparison. While most of the cells shown on the map were seen in multiple backfills, a few were seen in only one. These were included because some backfills have much better results than others and very few are excellent. The actual number of brain cells that have axons in latCeN2 is difficult to determine because there will usually be some cells that are not filled due to axon damage, not enough cobalt concentration, not enough time to backfill, etc. So summation of the best fills was used to elucidate a useful map of these cells. There was always a risk that the dye would fill the sheath that surrounds latCeN2 and possibly get into the axons of medCeN2. The backfills were examined thoroughly to avoid this problem. The amount of cobalt that reached the soma was also a consideration. Some cells had small, long axons that needed more time to backfill completely.

Most of the cells filled were located in the cerebral ganglia near the attachment of latCeN2 to the brain. The cerebral ganglia have been identified as the location of cells that receive sensory information from the head (Willows et al. 1973, Murray et al. 1996), so it is logical that these cells would be filled. On a physiological level, it is reasonable that the cells would be concentrated in an area near the nerve rather than located throughout the brain so that the axons do not take up more space or risk damage.

Only one of the latCeN2 nerve branches was filled in each experiment. Preparing the brain sometimes resulted in the damage of one of the nerves. Filling both nerves at the same time added much difficulty to the setup and more than doubled the chances of a problem in the backfill or contamination of the brain. The biggest problem with filling both sides at once was that it would be difficult to see which cells were filled by each nerve, because each nerve fills cells on the ipsilateral and contralateral sides of the brain. Labeling only one nerve resulted in cells labeled in the contralateral pedal ganglion (Figure 9). This was observed in some of the backfills. Murray (1996) observed that *Tritonia* are able to orient to flow when the ipsilateral latCeN2 is cut as long as the contralateral is still intact. The existence of labeled neurons in the contralateral pedal ganglion might explain how information about

water flow direction gets across the midline of the animal's brain, allowing the animal to turn into flow when the ipsilateral nerve is cut. Cells were also filled in the ipsilateral pedal ganglion. The information in those axons may help the slug to turn into flow from the ipsilateral side. Further experiments are necessary to determine which of the cells are sensory and which are motor neurons.

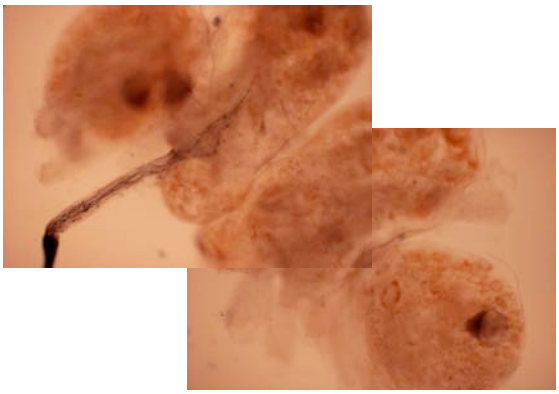

Figure 9: Notice the cells in the pedals that have been filled on both sides. Two pictures of the brain have been combined to show the entire brain.

The locations of several neurons that have axons in latCeN2 have been located in the brain of *Tritonia*. Cells were filled in each ganglion of the brain. The concentration and location of cells is consistent with previous findings in the physiology of the animal and flow orientation. The determination of which of these cells play a role in water flow detection will require intracellular recording in an animal being stimulated by water flow.

Effect of water flow stimulation on activity in latCeN2:

Increase and decrease in spike rate

The increase in spike rate can be attributed to action potentials produced by flow sensitive cells in the oral veil of *Tritonia*. The dynamic pressure caused by the water flow increased activity in many cells, and might alert the animal that water flow is present. These cells could contribute to the animal's ability to orient to water flow if the cells respond differently to different flow directions. The activity of these cells would be received by interneurons that would relay the information to motor neurons and elicit turning of the animal. Increases in activity were observed in response to flow from the ipsilateral side and the contralateral side of the animal. The activity in the contralateral side of the animal was not surprising. As mentioned previously, *Tritonia* has shown the ability to orient to flow coming from either direction even with one latCeN2 cut. There may be single sensory cells that have the ability to detect the direction of flow. Thus, different subpopulations of cells on one side of the oval veil may respond differently to flow from the ipsilateral or contralateral directions. The increased activity on the contralateral side of the animal was observed at higher flow rates. The sensory cells were probably "shadowed" from significant flow by the animal at the lower flow rates.

Decrease in the activity on the contralateral side was an interesting observation (Figure 6). The decrease occurred when one or more cells had tonic firing activity that was decreased by flow. The decrease in activity in the contralateral nerve, coupled with the increase in activity in the ipsilateral nerve, could enhance the differential sensory signal to help the animal determine flow direction.

Experiments showed that the activity in latCeN2 increases when stimulated with water flow. However, in 105 trials there was no perceptible change in activity in the animal. This could have been due to several factors including a very low flow rate (Figure 10), an animal that was in poor health, low signal from the recording, or high interference noise. This did not suggest that the animal is unable to sense flow.

Number of cells affected by flow

The number of individual cells that were actually excited or inhibited in each trial varied. Some trials showed that a higher rate of flow caused more cells to become active (Figure 10).

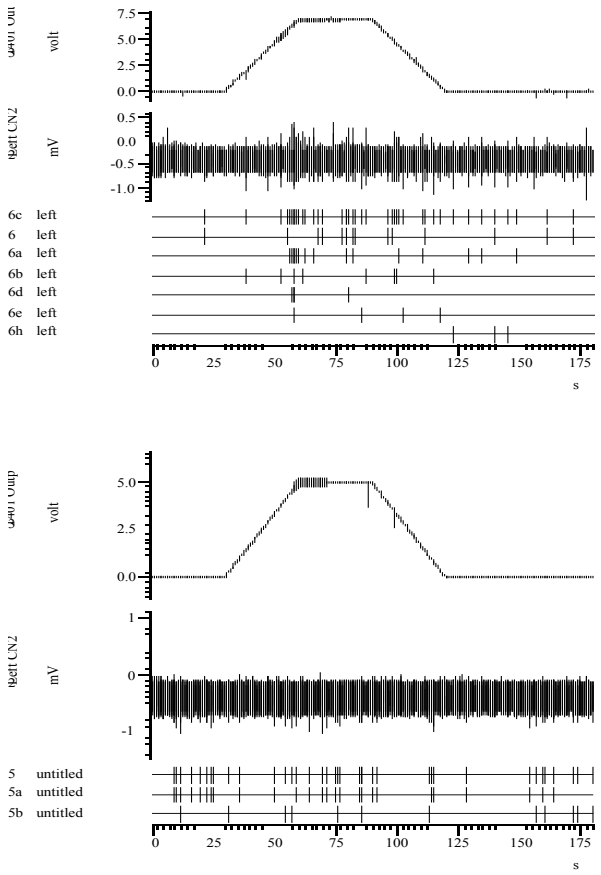

Figure 10: The top trace shows a high flow rate recording with many spike types, while the bottom has a lower flow rate with fewer spike types. The flow stimulation is from the ipsilateral side in both of the trials shown. A high flow rate recruited more flow sensing cells. Many cells being stimulated may indicate a higher flow rate to the animal.

The location of the sensory endings and the sensitivity of the individual cell might cause cells to be able to detect flow at different rates. Higher flow rate would cause higher recruitment of cells. This would presumably lead to greater motor neuron recruitment and cause the animal to turn more sharply. Each cell has a threshold level of stimulation that must be reached in order for the cell to produce an action potential. The minimum threshold for the animal was not determined because the animal could detect the lowest flow rate that the pump could produce (8 cm/s).

When the spikes are sorted, the computer groups spike types of similar shape. Often, some of these groups were combined by the experimenter because they were very similar in size and shape and probably from the same cell. Each different spike type presumably corresponds to a different cell. They differ due to the size and position of the axon in the nerve. Most trials that showed activity allowed us to distinguish 4 to 6 different cells. A higher amplification of the signal, or a reduction in electrical noise might reveal that more cells are active. Future experiments could correlate the spike rate of each cell to the speed of water flow.

The experiments supported the hypothesis that the cells would show an increase in activity during flow stimulus. However, many more interesting results were provided by the experiments. These results lead to more questions about *Tritonia's* nervous system.

The next steps…

This experiment is the first step toward learning the way in which many animals encode directional stimuli. This will lead to an answer for the general question of how distributed receptors all over the body encode a directional stimulus to lead to an unambiguous decision by the animal. The next step in this research will be to record intracellularly from the neurons located by the backfilling of latCeN2. The goal is to determine how the flow stimulus is perceived, processed, and ultimately results in the turning of the animal into the water flow. The motor neurons involved in turning have been identified, (Murray, unpublished) so this will help determine how flow sensors affect these motor neurons synaptically. This will be a step toward understanding how animals navigate.

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