UNIVERSITY OF CALIFORNIA, SAN DIEGO

The effects of alpha-conotoxin Im1 on the intact, swimming medicinal leech

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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2008

The thesis of David Christopher Ries is approved and it is acceptable inquality and form for publication on microfilm:

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Chair

University of California, San Diego

2008

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ABSTRACT OF THE THESIS

The effects of alpha-conotoxin Im1 on the intact, swimming medicinal leech

By

David Christopher Ries

Master of Science in Biology

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William B. Kristan, Jr., Chair

We are studying the effects of α-conotoxin Im1 on the swimming behavior of the intact medicinal leech. Injection with Im1 leads to abnormal, circular swimming patterns. First, we observed the change in amplitude of the swimming movements of toxin treated leeches, and then we recorded the motor output of dorsal longitudinal excitor Cell 3 in an intact animal. Our purpose was to characterize the effects of Im1 both behaviorally and physiologically, and then decide whether the toxin was affecting the swim CPG at the muscle, the motor neuron input, or higher up in the swim circuit.

We discovered that Im1 affects behavior by increasing the amplitude of troughs during swimming and increasing the delay before the onset of the following peak. By observing firing patterns in the DP nerve, Cell 3 was seen to fire for longer durations after treatment with Im1. These observations have led us to conclude that Im1 is affecting the swimming CPG at a higher level, altering the timing between ventral and dorsal muscle contractions.

INTRODUCTION

The nervous system of an animal produces a variety of different behaviors by activating muscles in a specific and deliberate way. To coordinate muscle activation, a large network neurons connected by inhibition, excitation, and modulation must coordinate the neurons responsible for motor output. One type of network found across species is a central pattern generator (CPG). A CPG generates a repeated pattern of muscle contraction and relaxation to produce a coordinated repeated behavior (Calabrese, 1995). CPGs are most commonly seen in cyclic activities such as respiration and locomotion. CPGs are useful to animals because they provide a system that produces a reliable, repeated behavior that can be modified by sensory feedback. Such autonomy means that the animal does not need to focus on swimming or walking, and can instead focus on searching for food or avoiding danger in its environment.

From a neurobiological point of view, obtaining an in-depth understanding of these rhythmic circuits is an important step towards understanding how the nervous systems of animals reliably control behavior. By understanding how these circuits are wired and how input modulates and interacts with the circuit, one can understand how different circuits interact with one another to form larger networks, like those seen in higher vertebrate nervous systems.

Invertebrates have been used to study CPGs because their nervous systems are experimentally accessible and are made up of far fewer neurons. Thus, by studying these simpler systems, one can gain insight to the general layout and function of

different circuits for generating rhythmic behaviors. Circuits involved in locomotion have been studied in a variety of invertebrates (Kristan and Calabrese, 1976; Stent et al., 1978; Katz and Frost, 1995; Katz, 1996). Circuits involved in locomotion are useful to study because they can be triggered by external stimuli, continue to incorporate input from the outside world to modify direction and speed of movement, or stop the animal from moving altogether. For this reason, these types of circuits are also useful in studying how a given circuit is involved in making a choice. The decisions to change direction of movement or to stop are made on the basis of a variety of sensory input and often involve modulation within the circuit to alter its output (Brodfuehrer et al., 1995; Yu et al., 1999).

Hirudo medicinalis, the medicinal leech, has been useful in studying the neurobiology of behavior because of the size and experimental accessibility of its nervous system. It uses different pattern generators in order to produce different locomotive behaviors (Kristan et al., 1988). One such circuit is the one responsible for generating the contractile rhythm during the swimming (Brodfueher et al., 1995). Recordings of swimming and other behaviors have been made from neurons in the central nervous system of the medicinal leech in both semi-intact and isolated cord preparations. These recordings have helped to understand how the leech is able to produce the movements and decisions necessary to start, stop, and continue swimming.

However, even though the leech is capable of producing swim behavior in both of intact and semi-intact preparations, the timing of swim cycles varies depending on

how much of the animal is intact (Pearce and Friesen, 1984; Cang and Friesen, 2000). Thus, to know the pattern under normal conditions it is sometimes necessary to record from the nervous system of a fully intact leech. By recording the motor output from specific nerves with a cuff electrode, one can see the pattern that is being produced by the oscillatory circuit with all sensory input intact (Yu et al., 1999).

A variety of carnivorous cone snails produce venom to attack their prey. These venoms contain different neurotoxins with specific molecular targets. *Conus imperialis*, a cone snail that hunts worms, produces a neurotoxin, α-conotoxin Im1 (pronounced *eye-em-one*), that binds to nicotinic acetylcholine receptors. More specifically, in mammals it acts as an antagonist to α7-subunit-containing nAChRs, which are located on neurons in the central nervous system (McIntosh et al., 1999). In *Aplysia*, Im1 targets nAChRs with similar pharmacological properties. However, in mammals, the effected receptor is responsible for gating a cation channel, where as in *Aplysia* the receptor gates an anion channel (Kehoe and McIntosh, 1998; McIntosh et al., 1994). In both cases, Im1 blocks a rapidly desensitizing current.

In the medicinal leech, injection with Im1 causes the leech to swim in circles towards its dorsal side. Because Im1 targets mainly neuronal nAChRs in other invertebrates, it is likely that its target is within the central nervous system of the leech as well. Also, because Im1 induces a recurring change to the swimming pattern of the leech, Im1 probably acts on neurons within the swim-generating CPG. Thus, by studying Im1's effect on the swim pattern and its target within the nervous system, we can better understand the role of the target neuron or neurons in the swim circuit.

To study Im1's effect on the nervous system, we will first observe and characterize the change in behavior. We will look at the amplitude of the body movements created by the leech to see how they are affected by Im1. To look at the change in the activity of the nervous system, we will record from the DP nerve in a fully intact animal. The DP nerve innervates the dorsal longitudinal muscles, and recordings will show what change in motor output Im1 has invoked. Through these experiments, we will hypothesize at what level in the swimming circuit Im1 is acting.

MATERIALS AND METHODS

Behavioral Amplitude Analysis

Medicinal leeches were obtained from Carolina Biological Supply (Burlington, NC) and kept in normal pond water at 15ºC. Leeches used were between 3-6 grams. We used three groups of leeches in this study. A control group was injected with 250 μL of normal leech saline. A second group was injected with 20 nmol of α-conotoxin Im1, a toxin produced by the snail *Conus imperialis*, in 250 μL of normal leech saline. A third group was confined in small containers, 5.5 centimeters in diameter, for a period of six to ten weeks, a treatment that caused them to swim in small circles when they were put into a larger tank. We placed each leech individually into shallow water so that it turned on its side to swim. This condition does not appear to affect the swimming movements of the leech, and it allowed us to videotape swimming behavior from above because the movements were in only two dimensions.

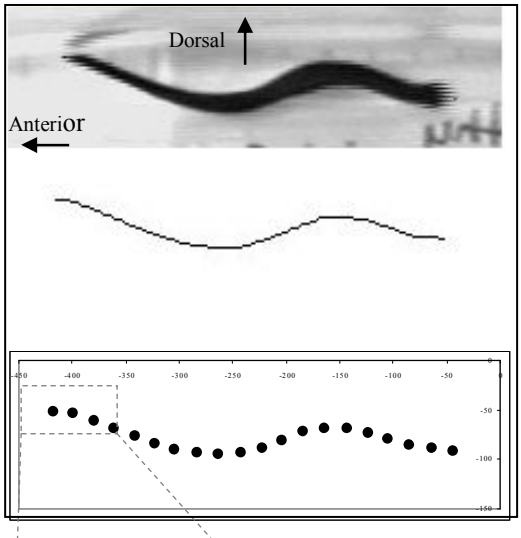
We used a Hitachi HV-C20 color video camera and recorded at 30 frames per second to take video sequences from various animals of each swim type. An analysis was made of a total of four video clips of three different control animals swimming, two video clips of two control animals swimming while turning, three video clips of a confined leech recorded at the sixth, seventh, and tenth weeks of confinement, and a total of seven video clips of two animals injected with α-conotoxin Im1.

We videotaped the movements and converted each frame into a digital image for analysis. The first stage of analysis was a program provided by John Feng that detects the outline of the leech image and compresses it into a single line. It then

divides the line into twenty equally-spaced points (Figure 1a). The coordinates of these points were exported into a Microsoft Excel worksheet. We then calculated the angle (θ) formed by each set of three consecutive points (Figure 1b) and recorded the angle *i*, which equals 180-θ (Figure 1c). The value of *i* was then assigned to the middle point of the angle. This angle describes the degree of curvature of the leech's body. For example, when the leech body is flat in a given section (θ = 180), then the angle *i* is equal to zero. As the curvature of the body increases, angle *i* also increases. The value of *i* is positive for angle in one direction and negative for angles in the opposite direction.

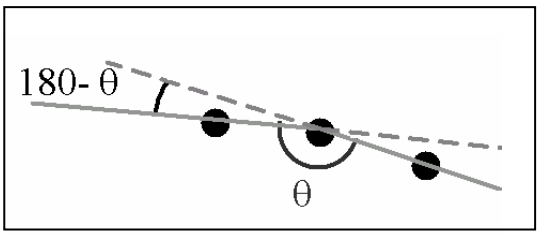
As the leech swims, it creates peaks and troughs with its body. When it bends toward its dorsal side, the curve is termed a *trough*, and when it bends towards its ventral side the curve is termed a *peak*. Thus, a series of positive *i* values represent all of the angles in a peak, and the following series of negative *i* values represent a trough. To measure the total curvature for a peak or trough, we summed the consecutive angles in a trough or peak of the leech's body (Figure 1d). The spot on the leech where a peak and trough meet is a *transition point*. A transition point was detected as a point along the leech body where the value of angle *i* is zero or between two consecutive points with negative and positive *i* values. In Figure 1c, angle 3 is a transition point because it equals zero. There is also a transition point between angles 14 and 15. From simple geometric considerations, the sum of angles within a given peak or trough is equal to the angle crated by the lines tangent to the leech body at consecutive transition points. The amplitude of each peak and trough was calculated

Figure 1: *Measuring amplitude of peaks and troughs from video images*. A. Individual frames of the video image were condensed to a single line and then converted to 20 equally-spaced points. B. The angle created by each set of three consecutive points (θ) was used to create the angle *i* ($i = 180 - \theta$). C. The *i* values are assigned to the middle point of each angle. Consecutive groups of points with positive or negative *i* values denote a single peak or trough. Transition points are either points with an *i* value of zero or between consecutive points with negative and positive *i* values. D. The sum of the individual angles within a single peak or trough (θ_{sum}) is equal to the angle created by the lines tangent to the body line at the transition points. The value used to describe the amplitude to of the peak or trough is equal to $180 - \theta_{sum}$.



Point #	Angle #	Angle[i]
1		
2	1	14.30
3	2	3.73
4	3	0.00
5	4	-6.34
6	5	-6.19
7	6	-8.84
8	7	-8.06
9	8	-7.92
10	9	-6.74
11	10	-1.76
12	11	10.76
13	12	5.67
14	13	8.30
15	14	5.87
16	15	-0.53
17	16	-8.06
18	17	-8.17
19	18	10.63
20		

B. Body angles



D. Summed angles

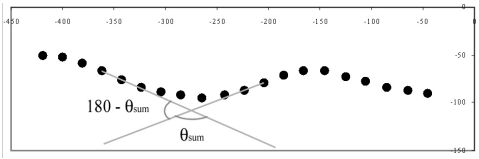
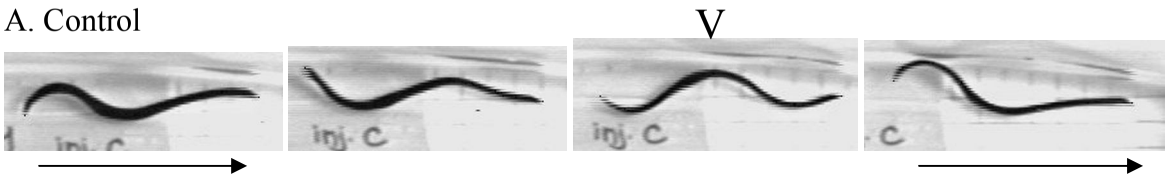
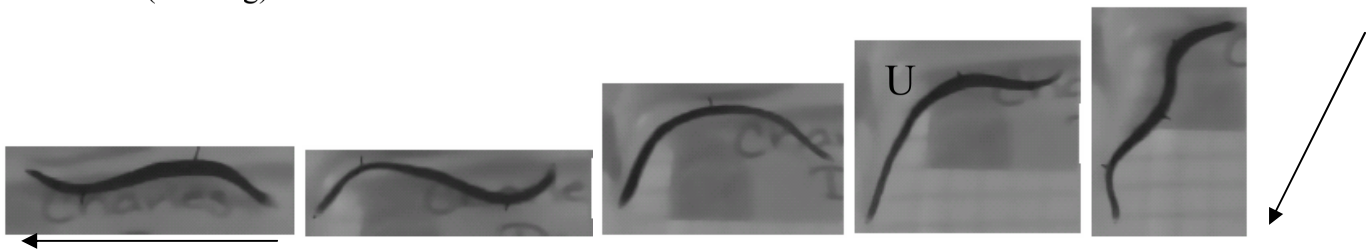


Figure 2: *Leech body shapes during different swim behaviors.* (A) When swimming straight, control animals produce equally sized peaks and troughs, creating a shallow "V" shape when either is at the center of the body. (B) When turning, control animals produce a very shallow trough between two peaks, creating a "U" shape with their body. (C) After being confined, animals make larger peaks and smaller troughs, resulting in a "W" shape with the trough is midway through the animal and a "V" shape when the peak is at the animal's center. (D) Animals that have been treated with Im1 make larger troughs, and thus produce an OMEGA shape when a trough is at the middle of the leech and a "V" shape when a peak is at the middle. Arrows point towards the anterior end of the leech.

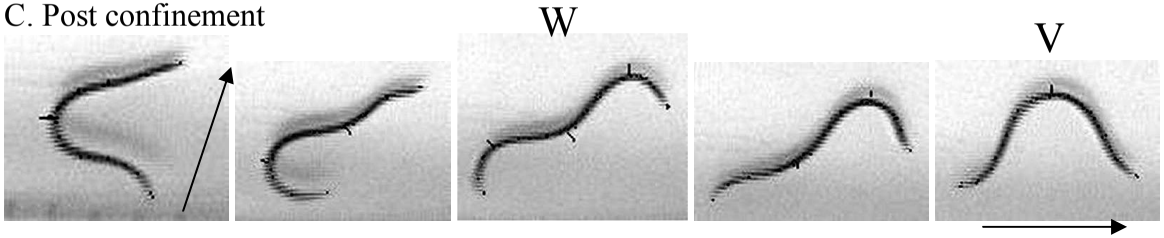
A. Control



B. Control (Turning)



C. Post confinement



D. Im1-Treated

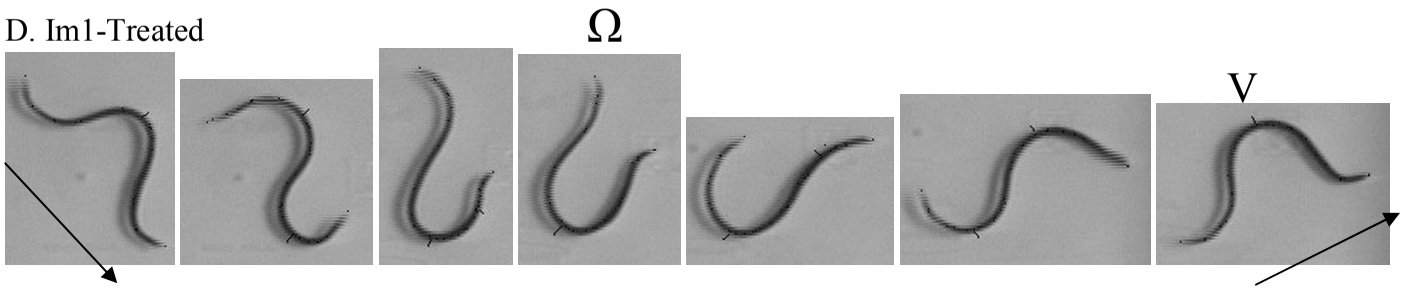
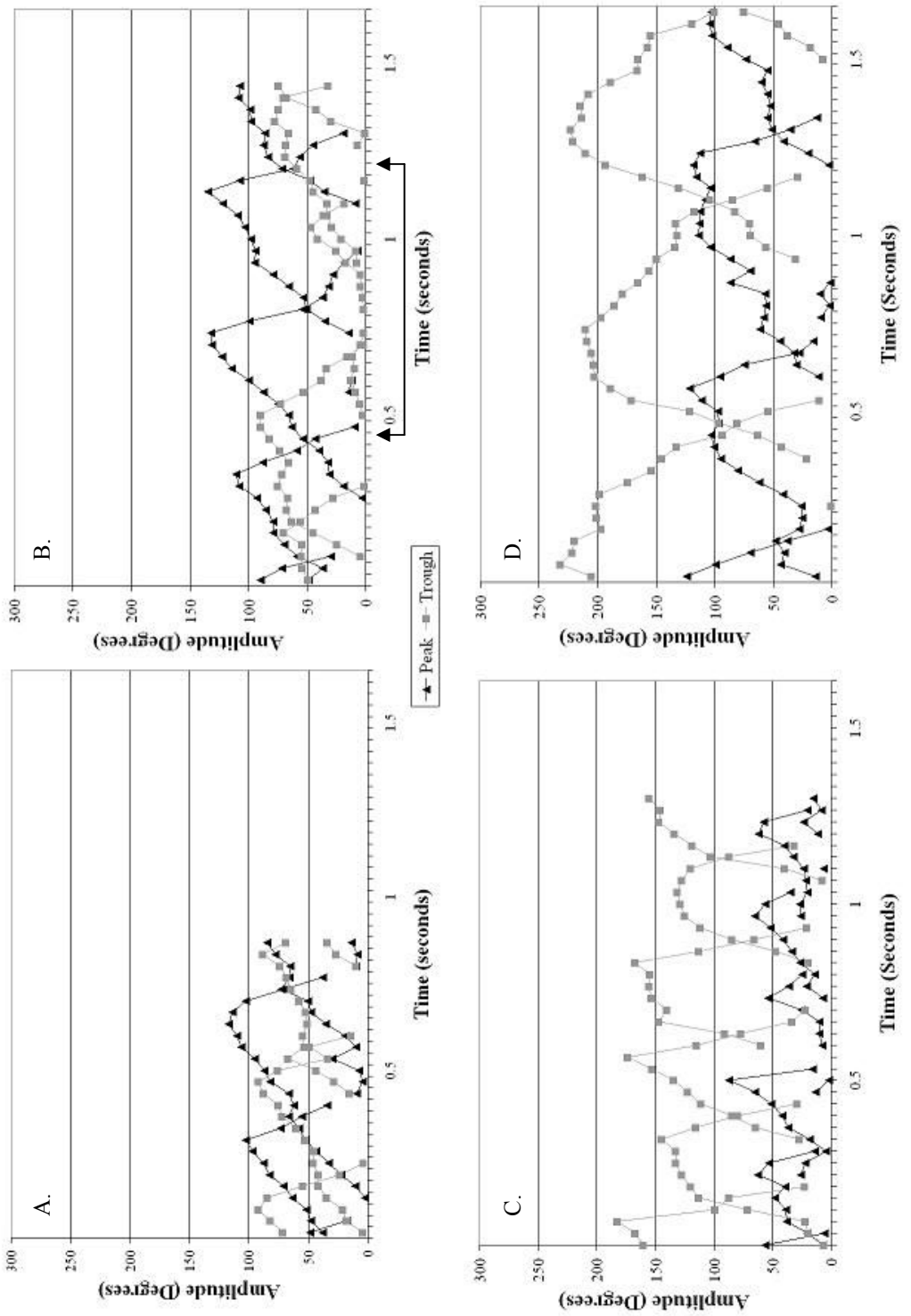


Figure 3: *Peak and trough amplitude during different swim behaviors.* The amplitudes of peaks and troughs are plotted against time. These patterns are characteristic of amplitudes created during (A) straight swimming in control animals, (B) turning in control animals, (C) circular swimming in animals post-confinement, and (D) animals that have been injected with α-conotoxin Im1. Arrows in (B) indicate the period of time during which the animal was turning.


A.
B.
C.
D.
Amplitude (Degrees)
Time (seconds)
Time (Seconds)
Peak
Trough
0
50
100
150
200
250
300
0.5
1
1.5

for each frame of a video sequence and plotted against time (Figure 3).

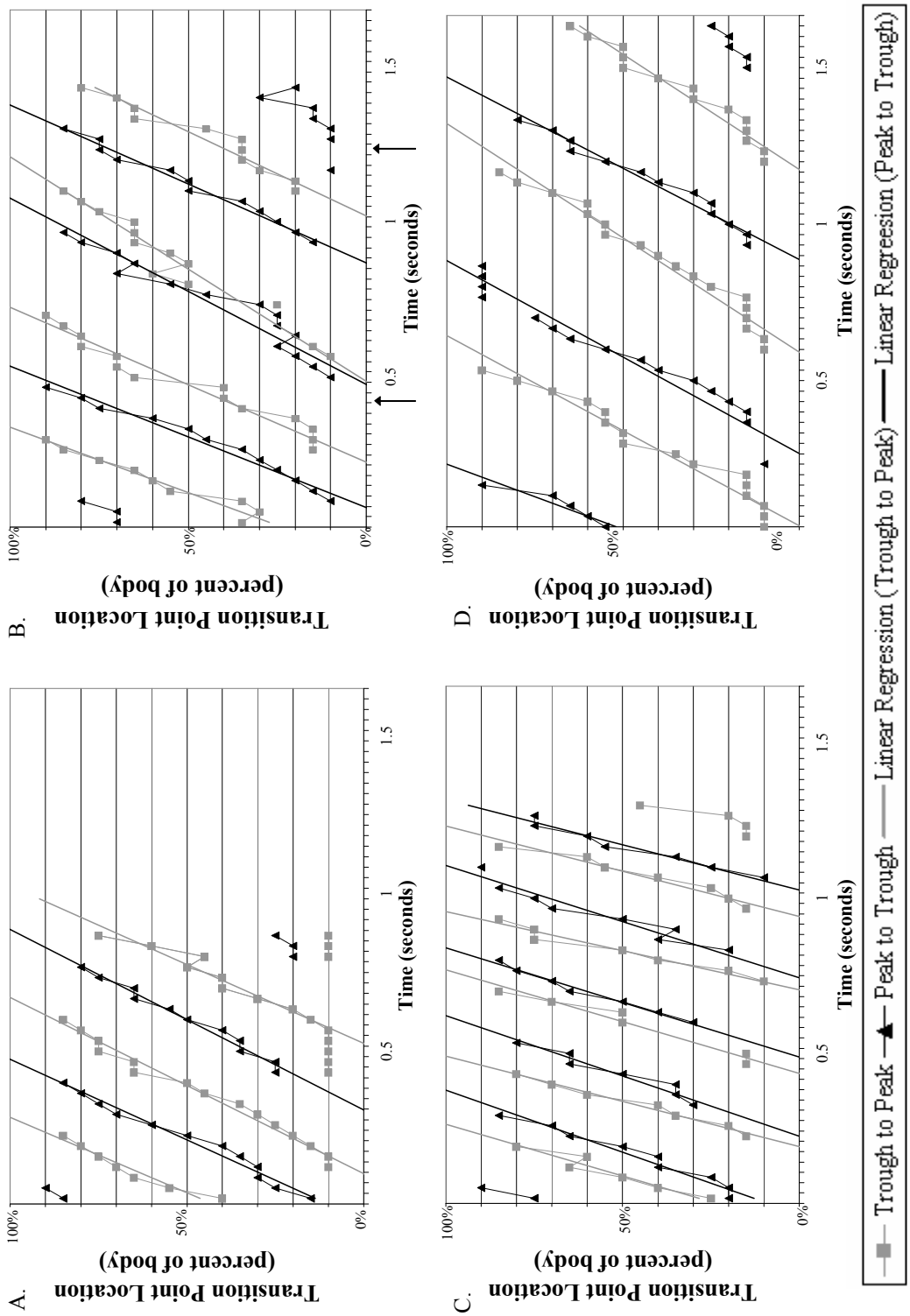
In addition, we plotted the position of the transition points on the leech body. When a transition point was between two points, the more anterior of the two points was used as the transition point. The locations were plotted as a percent of the leech body length. The head of the leech is 0% and the tail is 100%. This procedure generates graphs such as those seen in Figure 4.

The period for each swim type was found by taking the linear regression of transition points located within 20-100% of the leech body (Figure 4). We measured cycle period as the distance from the 50% mark of one linear regression for a peak-to-trough transition point to the 50% mark of the next peak-to-trough transition point. These were then averaged for each period within a clip, and all the clips were averaged for each swim type.

Intact Physiology

Electrode Assembly: The cuff electrode that we used was assembled with PE-50 tubing and 75 micron Teflon-coated silver wire. A small hole was made in the side of the tubing, about 2mm from the end. The wire was inserted through this hole and out the end of the tubing. A knot was placed near the end of the wire, and the Teflon was stripped off the end of the wire. A hook was then formed out of the stripped portion of the wire. The other end of the tubing was attached to a microinjector filled with an oil/petroleum jelly mixture. After cleaning all of the surrounding muscle off of the nerve, we hooked the wire around the nerve, pulled the hook and nerve up into

Figure 4: *Location of transition points.* The location of transition points are plotted for (A) a control leech, (B) a control leech while turning, (C) a leech after 6 weeks of confinement, and (D) a leech that has been injected with Im1. Arrows in B show the time during which the leech is turning. Location is plotted as percent of the leech body. 0% represents the anterior end of the leech and 100% represents the posterior end.

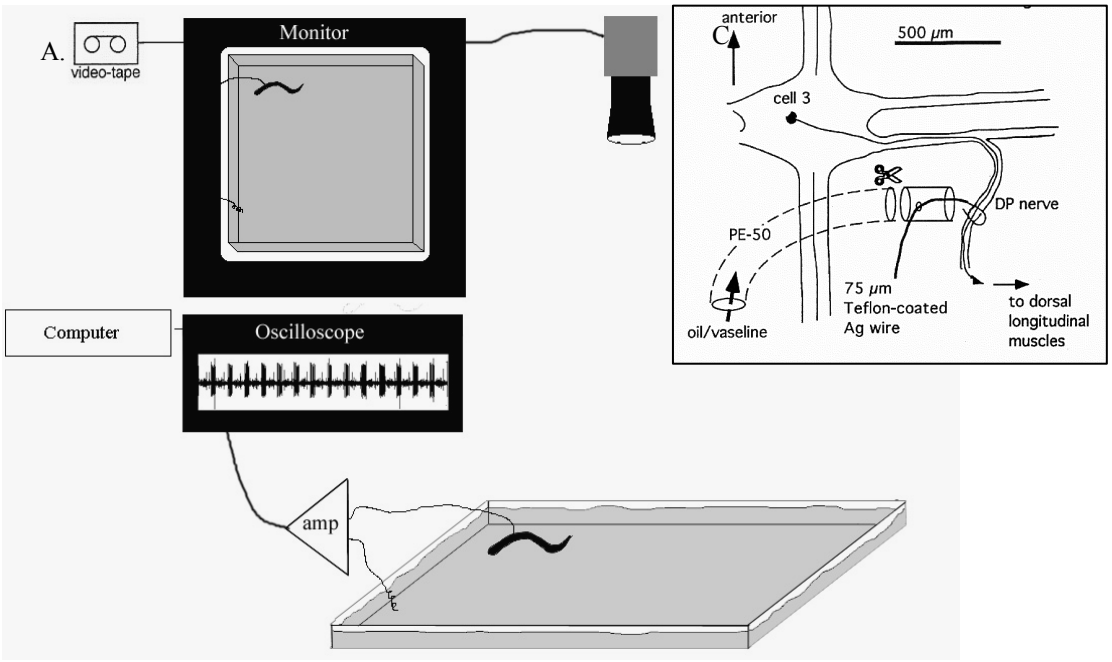

A.
B.
C.
D.
Transition Point Location (percent of body)
100%
50%
0%
0.5
1
1.5
Time (seconds)
Trough to Peak
Peak to Trough
Linear Regression (Trough to Peak)
Linear Regression (Peak to Trough)

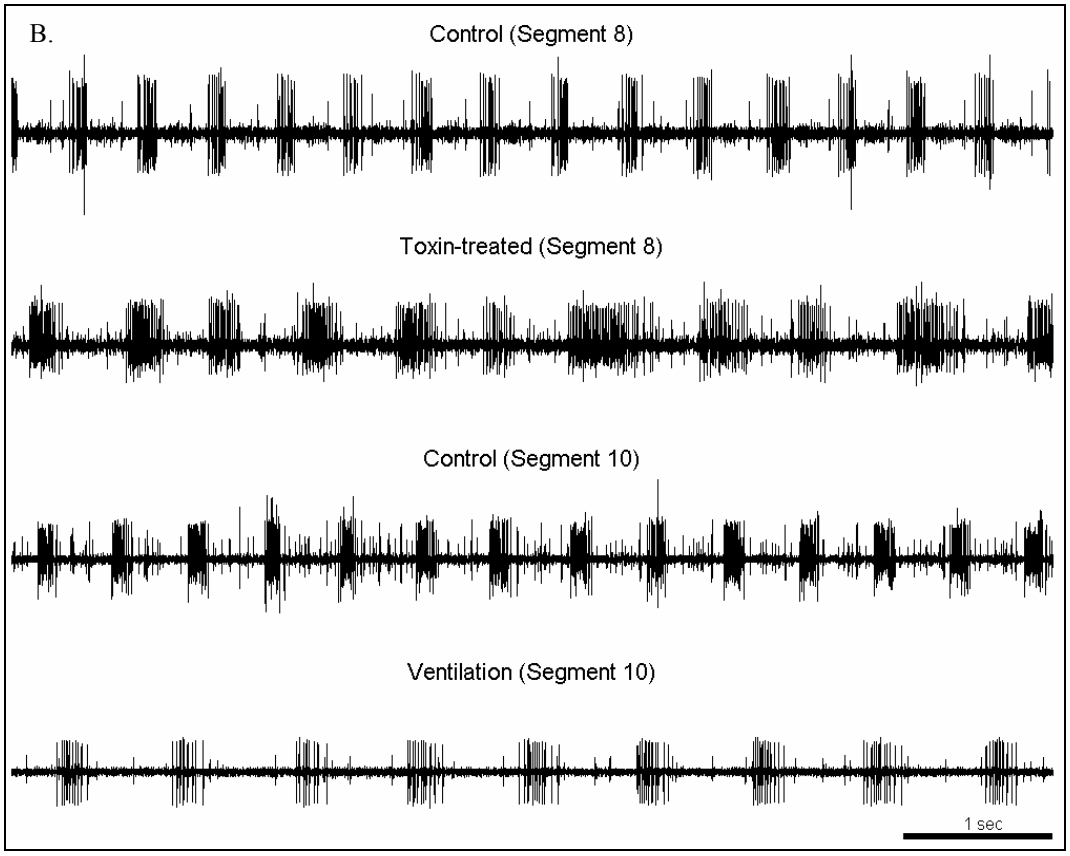
the tubing, and filled the tube with the insulating mixture. The end of the tube was then cut off, leaving a small piece of tubing around the electrode and nerve (Figure 5c).

Electrode Insertion: The electrode was placed on the DP nerve because it contains the axon to motor neuron Cell 3, which is a dorsal longitudinal excitor. To insert the cuff electrode onto the DP nerve of a leech, the leech was first anesthetized in cold normal leech saline with 8% ethanol for 60 to 90 minutes prior to insertion. The leech remained in the same saline during the procedure, which took 40 to 60 minutes. We made a small incision ventrally, about 2 to 3 annuli in length. The incision was one annulus posterior of the annulus containing the sensilla, between the ventral midline and the most medial sensilla. The electrode was placed either in segment 8 or 10. After stripping muscle and tissue away from the DP nerve, we attached the cuff electrode to it. The electrode was laid down along the incision, and the body wall was sutured up around the electrode, using about one suture per annulus. A loop was made in the wire near the tubing, allowing it to be sutured to the body wall. This prevents the electrode from moving while the leech is swimming. We then placed the leech in cold normal saline for about an hour to recover from the ethanol. After recovering, we placed the leech in a tank measuring 50cm x 40cm in 2cm of pond water.

Data Acquisition: We attached the electrode to an A-M Systems differential AC amplifier (gain = 10,000; low cutoff = 300Hz; high cutoff = 500Hz). This was attached to a National Instruments model AT-M10-16E-10 data acquisition PC board,

Figure 5: *Electrode assembly and recording set-up.* (A) When making recordings, the leech was placed in a shallow tank with a video camera placed above to record its movements. The electrode attached to the DP nerve of the leech and a wire placed in the water of the tank were attached to a differential AC amplifier. (B) Sample recordings from two different animals. The first two samples are from an animal before and after injection with Im1. The last two samples are from the same animal during normal swimming and during ventilation.


A.
video-tape
Monitor
Computer
Oscilloscope
amp
C.
anterior
500 μm
cell 3
DP nerve
PE-50
75 μm
Teflon-coated
Ag wire
to dorsal
longitudinal
muscles
oil/vaseline


B.
Control (Segment 8)
Toxin-treated (Segment 8)
Control (Segment 10)
Ventilation (Segment 10)
1 sec

and data were recorded using the Labview program. Data were collected at 5000Hz for a period of 60 seconds for each trial. At the same time a Hitachi HV-C20 color video camera was placed about 1.5 meters above the tank. All trials were recorded at 30 frames per second on professional grade VHS tape. The video and electrophysiology were synchronized using a flash of light (Figure 5a).

Data Analysis: We analyzed all data using Matlab and Microsoft Excel. Cell 3 spikes were selected manually on the basis of amplitude and temporal relation to other spikes. Bursts were defined as groups of spikes that occurred during swimming and were separated from other groups by at least 100 milliseconds. We determined the duration of swimming bursts as the time from the first spike to last spike within a burst. The period of the burst cycle was measured as the distance from the median spike of one burst to the median spike of the following burst. The rate of spikes within a burst was calculated as the number of spikes in the burst divided by the duration of the burst. The duty cycle of the burst cycle is defined as the percent of the period during which the burst occurs ([100*duration]/period).

We plotted data on axes of data value versus cumulative fraction of data under that value. This group of data was then fitted to a third degree polynomial curve using Matlab. A different curve was fitted to each animal to illustrate the variance between animals.

Im1 injection: We obtained α-conotoxin Im1 from the laboratory of Dr. Baldomero Olivera at the University of Utah. It was stored at –80ºC. We injected 20 nmol of Im1 in 250 μL of saline into the dorsal muscles. Leeches were then allowed

to swim and explore as the toxin took effect. Once irregular swimming patterns typical of Im1 commenced, we made physiological recordings. We made control recordings from the 8th segment of four leeches. We made recordings from three of these leeches after injection with Im1. We made control recordings from the 10th segment of one other leech.

RESULTS

Behavior

To compare the relative amplitudes of the peaks and troughs in the leech body during the swim cycle, we plotted them as they progressed along the leech (Figure 3). In control swimming, both peaks and troughs increase in amplitude gradually, reach a similar maximum value, and then decrease in amplitude rapidly as they run off the back of the animal (Figure 3a). Peak and trough maximum amplitude was not significantly different. Peaks had an average maximum amplitude of 107.3 ± 4.5º (mean ± S.E.M.), and troughs had an average maximum amplitude of 100.0 ± 5.7º (Figure 6a). In control animals during turning, peak amplitude increased slightly (115.1±6.7º), while trough amplitude decreased significantly (48.8 ± 6.8º) (Figures 3b & 6a). The opposite was also seen, depending on the direction of the turn.

For comparison with the swimming of Im1-treated animals, we analyzed the swimming of leeches that had been confined to a small container for several weeks. After weeks of confinement, these leeches swim in small circles with their dorsal side facing towards the outside of the circle. The leech appeared to turn a corner with every peak that it made. It would then swim in a relatively straight line while making a shallow trough, and make another sharp turn as it formed a new peak (Figure 2c). Analysis showed that confined animals form peaks with larger amplitude than control (171.6 ± 3.8º) and troughs with smaller amplitude (47.3 ± 4.0º) (Figures 3c).

Animals that had been injected with Im1 followed a different swimming path. When making a trough, the leech would bend backwards. Then the following peak

would bring its head part way back around. The repetition of this cycle formed a path as if the leech were swimming around a cross (+), with the ventral side of the leech facing the exterior (Figure 2d). Finally, after analyzing animals that had been injected with α-conotoxin Im1, we saw that the average maximum amplitude of the troughs was much larger (229.7 ± 4.2º) while the average maximum amplitude of the peaks was only slightly larger than that of control animals (135.1 ± 6.5º) (Figures 3d).

Comparing the shapes created by the body of the leech while swimming under these conditions accentuates the differences in swimming patterns. In post-confinement swimming, the large peaks and small troughs result in repeated "W" and "V" shapes. The "W" is created when a trough is in the center of the body, and the "V" is created when a peak is in the center (Figure 2c). Im1-injected animals, on the other hand, produce a hook shape when a trough is at the front of the body, followed by an "Ω" shape when the trough reaches the center of the body. When a peak reaches the center of the body of the animal, a "V" shape similar to the one produced during post-confinement swimming is made (Figure 2d). Control swimming yields a repetition of much shallower "V" shapes in both directions when either the peak or trough is at the mid-body of the leech (Figure 2a). When the control animals turn, they mostly produce a "U" shape, which is the result of an almost nonexistent trough in between two large peaks (Figure 2b).

To examine the rate of progression of peaks and troughs through the body, transition points were plotted as a percent of the body of the leech against time. Following the body of the leech from posterior to anterior, where a peak develops into

a trough is a T transition point, and where a trough develops into a peak is a P transition point. The slopes of the linear regressions through these points show the rate at which transition points moved along the animal (Figure 4). The period of the swim cycle was measured as the distance from the linear regression of one series of T transition points to the next at 50% of body length. This period can be split into two *half periods*. A half period is the distance from the regression of a series of T transition points at 50% of body length to the following series of P transition points at 50% of body length (T-P period), or it is the distance from a series of P transition points to the following series of T transition points (P-T period).

In control animals, the P and T transition points are evenly spaced. The P-T period is 194 ± 12.6 msec and the T-P period is 202.6 ± 9.2 (mean ± S.E.M.). This leads to an overall average period of 386.9 ± 15.3 msec.

During turning, the P transition point follows the T transition point very closely, resulting in the trough taking up a very small percentage of the body (Figure 4b). This results in the shallow "U" shape that causes the animal to turn (Figure 2b). The periods were not measured for normal turning because a turn usually lasts for less than an entire period.

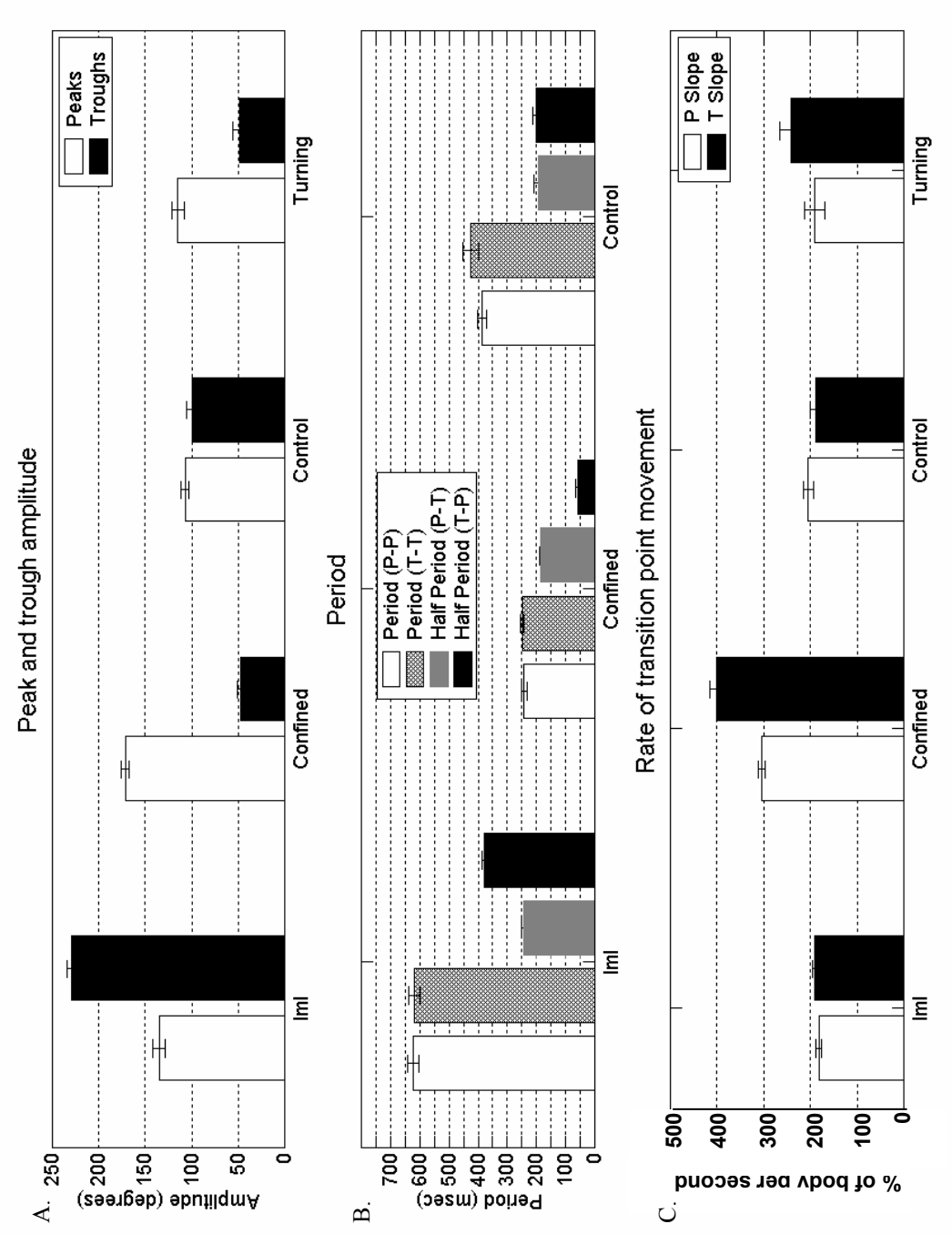
Post-confinement swimming is similar to that of control turning in that the P transition point follows the T transition point very closely (Figures 2b & 4c). This makes the P-T period only 185.4 ± 4.3 msec while the T-P period is 58.3 ± 7.8 msec. However, because it does not follow as closely, a small trough is able to form, resulting in the previously mentioned "W" shape. Post-confinement swimming also

results in a shorter total period (242.3 ± 8.8 msec) as compared to control.

In Im1-injected animals, the P transition point trails behind the T transition point at a greater distance (Figures 2b & 4d). This makes the T-P period is 377.6 ± 9.6 msec while the P-T period is 242.8 ± 10.0 msec. This increases the overall period of the swim cycle (623.0 ± 19.7 msec), which is the opposite effect of that produced by confinement. This results in large percentages of the animal being in either a single peak or trough at a time, which corresponds with the "Ω" shape caused by a large trough and the "V" shape caused by a large peak.

The rate at which transition points moved through the body is measured by their the slope of the line they create when their position on the body of the leech is plotted against time (Figure 4). In control animals, the P-T and T-P transition points appear to progress through the leech at the same speed. Their speeds were 205.2 ± 10.8 and 188.2 ± 13.0 percent/sec respectively (Figure 6c). In both control turning and post-confinement swimming, the T-P transition point moves faster through the body than the P-T transition point. This allows for a trough that is small or almost nonexistent at the front of the animal to increase in size at the back of the animal (Figure 4b & 6c). The speed of the P-T and T-P transition points during control turning is 190.8 ± 22.5 and 241.6 ± 23.5 percent/sec respectively and during post-confinement swimming is 304.5 ± 10.8 and 402.2 ± 13.0 percent/sec respectively. In Im1-treated animals, the rates of the P-T and T-P transition points are similar to one another (182.3 ± 7.0 and 190.5 ± 6.0 percent/sec respectively)

Figure 6: *Summary graphs of behavioral data.* (A) Comparison of peak and trough amplitudes during different swim behaviors. (B) Comparison of period lengths of different swim behaviors. Whole periods are shown as measured from P transition point to P transition point at midbody and from T transition point to T transition point. Half periods are measured from both P to T transition point (signifying the front and back of a peak) and from T to P transition point (signifying the front and back of a trough). (C) Comparison of the speeds at which P and T transition points move through the body, showing that confinement swimming exhibits faster T transition point movement than P transition point movement. Error bars = ±S.E.M.


A.
Peak and trough amplitude
Amplitude (degrees)
Peaks
Troughs
Iml
Confined
Control
Turning
B.
Period
Period (msec)
Period (P-P)
Period (T-T)
Half Period (P-T)
Half Period (T-P)
Iml
Confined
Control
C.
Rate of transition point movement
% of body per second
P Slope
T Slope
Iml
Confined
Control
Turning

Physiology

To outline the differences in the firing patterns produced by the DP nerve during swimming under different conditions, we looked at burst duration, spike count per burst, firing rate with each burst, and period between bursts (Figure 7 & 8). The average burst duration among the control animals was 143.7 ± 3.6 msec in segment 8 and 143.1 ± 5.0 msec in segment 10 (mean ± S.E.M.) (Figure 7a & 8a). Burst duration became much more variable once the leech was injected with Im1. The average burst duration for the Im1-injected animal was 288.3 ± 9.9 msec. The vast majority of bursts was considerably longer than control. There was also no recognizable pattern in burst length.

Recordings were also made from the DP nerve while the leech ventilated. This was not done as a control for this experiment, but rather as a side experiment made possible by the intact preparation. Ventilation resembles swimming except that the leech keeps its rear sucker attached. The leech produces waves with its body to force water to flow over its skin. The movements are slower and much less forceful than swimming. The wave created by the body of the leech does not progress all the way to the rear of the animal, but rather it stops about two thirds of the way to the tail end. Burst duration during ventilation was longer than in control, 223.4 ± 4.3msec. This recording was only made in segment 10. Examples of the recordings made during different conditions is shown in Figure 5b.

The number of spikes per burst in control animals was also similar in segments 8 (12.1 ± 0.3) and 10 (14.1 ± 0.3) (Figure 7b & 8b). In this case, ventilation also

produced a similar number of spikes per burst (10.4 ± 0.3), with only a slight decrease from the control for segment 10. However, Im1-treated animals had an average of 22.4 ± 0.6 spikes per burst. Not only is this greatly increased from control, but the variability between bursts has greatly increased as well.

Looking at average spike rate, the control animals for segment 8 were 87.7± 2.0 Hz, but the average spike rate for segment 10 was higher (101.0 ± 2.4 Hz) (Figure 7c & 8c). The spike rate during ventilation was significantly decreased to an average of 46.8 ± 1.4 Hz. In Im1 however, the spike rate was 80.1 ± 1.5 Hz, similar to that of the control. Even the distribution is similar to that of the control animals.

The average period length in the 8^{th} segment was 457.7 ± 6.0 msec and in the 10^{th} segment was 467.5 ± 5.1 msec. The period length in the 10^{th} segment during ventilation was much longer (797.1 ± 6.8 msec). During swimming in the Im1-treated leech was much longer and much more variable than control animals (629.3 ± 11.8 msec)

Figure 7: *Individual plots of physiological data, by animal.* Points are plotted for each animal both before and after Im1 injection. Points are plotted as either (A) duration, (B) number of spikes, (C) rate, or (D) period versus the fraction of bursts less than or equal to that value. The number associated with each line represents a different animal (e.g. Normal 1 and Im1 1 are both from leech 1).

Comparison of physiological results - Individual

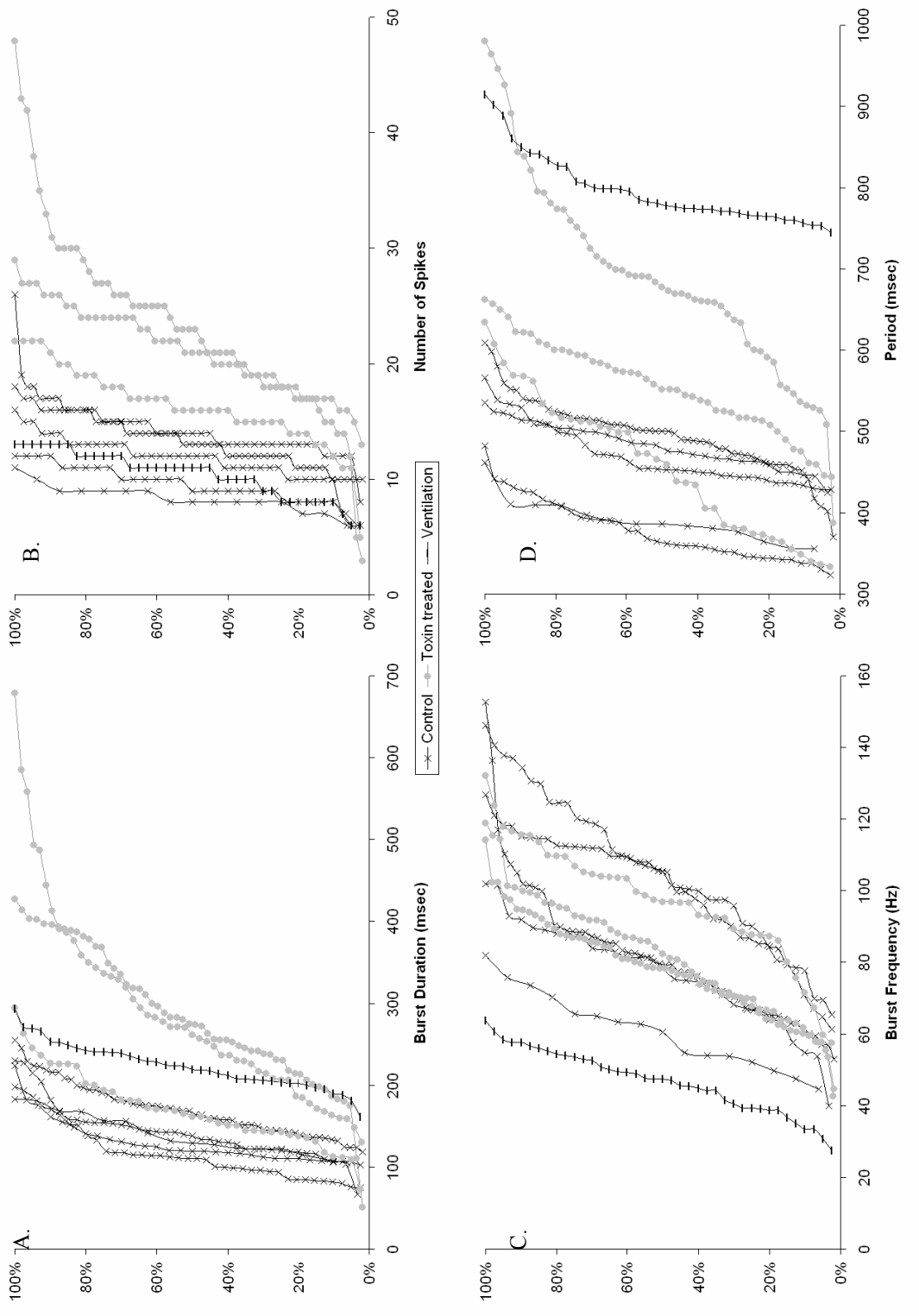
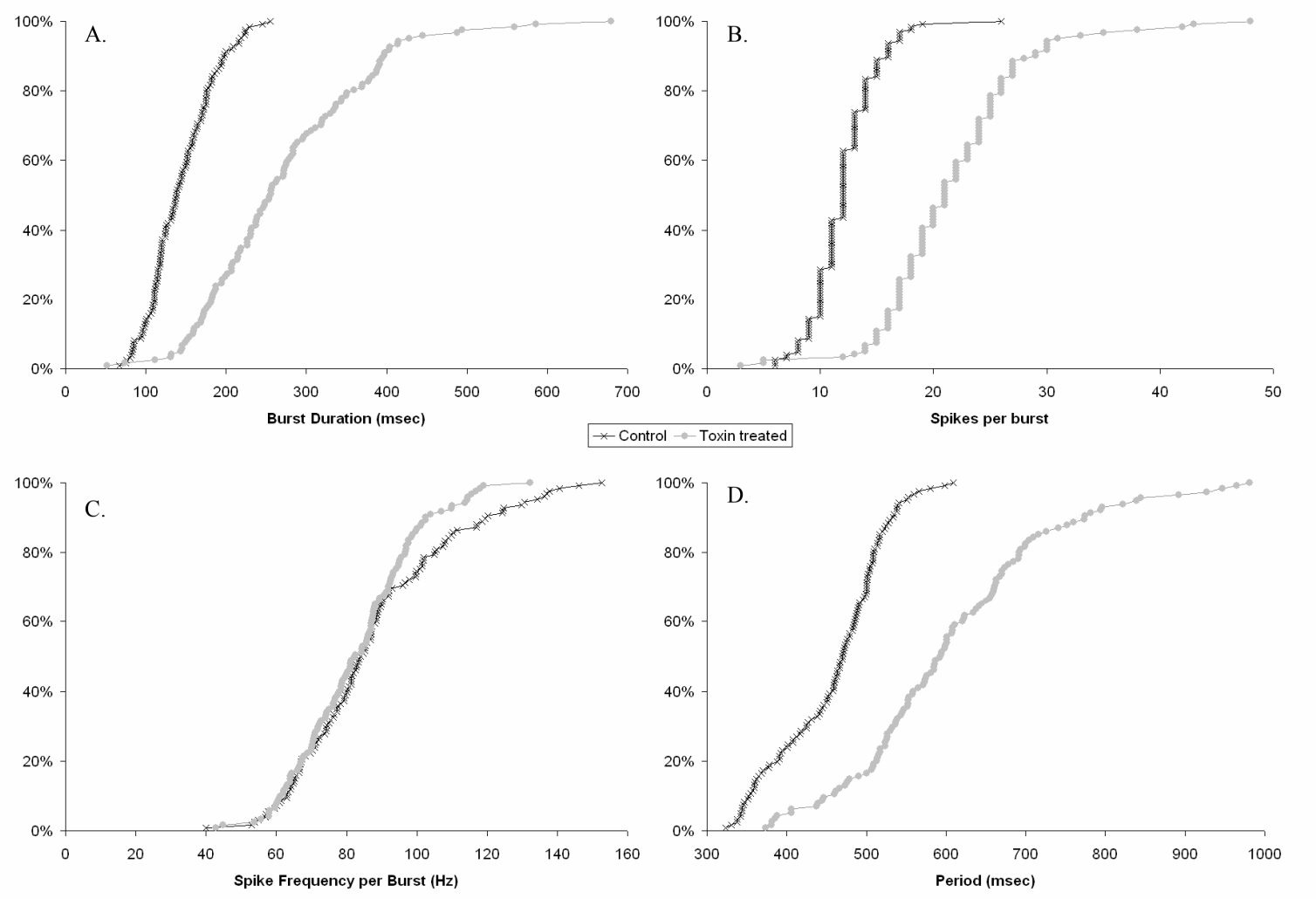


Figure 8: *Cumulative plots of physiological data.* All burst from control animals are plotted as a cumulative fraction of total control bursts (red). The same is done for total Im1 bursts (black). Individual plots for each control animal (light red) and each Im1-injected animal (grey) are also shown. Points are plotted as either (A) duration, (B) number of spikes, (C) rate, or (D) period.

Comparison of physiological results - Cumulative



DISCUSSION

Peak and trough amplitude appears to be the method through which the swimming leech changes direction. Amplitude of peaks and troughs remains equal when the leech is swimming in a straight line (Figure 3). When the leech turns ventrally, the peaks increase in amplitude, and the troughs decrease in amplitude. This changes the direction that the head of leech points, allowing for the leech to continue swimming in this new direction. When a leech is swimming in a shallow arc with only a gradual turn in the ventral direction, even this is accompanied by a small increase in peak size over trough size (data not shown). This makes sense when looking at how the leech is able to push itself through the water. The leading wall of a peak pushes against the water in both the posterior and dorsal directions. The leading wall of a trough pushes against the water in both the posterior and ventral directions. When peaks and troughs are of equal size, they push in the ventral and dorsal directions with the same force, canceling one another out. This leaves only a push directed towards the animal's posterior that propels the animal forward through the water. When a peak (or trough) becomes larger than its counterpart, the net push against the water shifts more towards pushing in the dorsal (or ventral) direction. This can lead to a turn in the ventral (or dorsal) direction.

While this will work to explain a gradual arc, the sharp turns that have been studied here seem to be caused for a different reason. During normal turning, when the leech makes a sharp turn, almost the entire body is taken up by large peaks. This results in the front end of the animal pointing perpendicularly to the direction the tail

is pointing (Figure 2b). By repositioning the direction the head is facing, when the next series of waves progress through the body, they are now providing a net push against the water parallel to the line that the head is on. Thus, it seems that the large peaks created by the leech during a normal turn are for the purpose of reorienting the head, rather than pushing harder against the water in one direction.

While the back half of the animal (especially the whip of the tail) appears to be for the purpose of propulsion, the head of the animal seems to act as a rudder as the leech moves through the water. It is the amplitude of the peaks and troughs that the body creates that determines where that rudder points. During control swimming, the head points in angles that, when added together, create a straight line. During post-confinement swimming, the head forms an acute angle with the body during a peak, but then the head barely changes direction when forming a trough (Figure 2). This leads to a circular swim path, because the added angles sum up to around a 70º dorsal turn per swim cycle. Leeches injected with Im1 follow a pattern as well. The peaks causes the head to a right angle with the body, and the troughs cause the head of the animal point in the opposite direction of the rest of the body, a change of 180º. These add up to about a 90º ventral turn per cycle, which is what was observed in the behavioral studies. Therefore it is the amplitude of the peaks and troughs that steers the animal.

This leaves the question of how it is that the animal makes these changes in the amplitude of its peaks and troughs. It appears the period between transition points is associated with amplitude. Across all of the types of swimming that have been looked

at here, half period length and amplitude of either peaks or troughs have a linear relationship. It seems that as the period between transition points increases, the amplitude between those transition points increases as well. This implies that the amount of bend per segment remains somewhat constant and that as an increasing number of segments are involved, the overall bend increases. Thus it is the distance between transition points that determines the degree of the turn.

There is some variability though in the relationship between transition point distances and amplitude of peaks and troughs. This is explainable through the physiological data obtained. The behavioral data obtained from the Im1-injected animals shows that the average peak and trough had slightly higher ratio of amplitude to percentage-of-body than did the control animals. The recordings from the DP nerve of an Im1-injected animal show an increase in the duration of bursts, and therefore the number of spikes per burst, while the spike firing rate remains the same as in controls. This increased burst duration causes the dorsal muscles to contract for a longer period of time, increasing the bend in that segment per burst. Also the duty cycle (duration/period) increases from 31.6 ± 0.7% to 45.3 ± 1.4%. Thus, the bursts in the DP nerve are taking up a considerably larger portion of the swim cycle. Because the muscles are contracting at the same rate as control, but for a larger percentage of the cycle one would expect the troughs to increase in amplitude.

The same idea applies to ventilation. While no behavioral analysis was done of the peak and trough amplitude during ventilation, they are visibly much smaller in amplitude than the peaks and troughs created by swimming. Looking at the DP nerve

recordings during ventilation, one sees a dramatic decrease in the rate of firing and therefore a decrease in the number of spikes per burst. This is presumably the reason behind the low amplitude of the peaks and troughs during ventilation.

The main physiological changes seen in the Im1-injected animal are increases in both period and burst duration. These two changes work in tandem to cause the circular swimming seen in the behavior. The longer period increases the amount of time between the start of a trough and the start of the following peak, and the increase in burst duration increases the amount of bend per segment and the length of time that each segment remains bent.

Possible effects of Im1 on the circuit

Because Im1 does not cause a large change in the firing rate of Cell 3 during swimming, it is unlikely that Im1 acts on the motor neuron itself. If the electrical or chemical properties of the motor neuron were altered, this would most likely alter the rate at which it could fire action potentials.

Because the period between transition points is affected, this points to a change in the timing between contraction of the dorsal muscles and ventral muscles. A transition point signifies the point at which a given segment has gone from bending in one direction to bending in another. These transition points are therefore manifestations of the time between the appearance of a burst of dorsal motor excitation and a burst of ventral excitation. The part of the period that is most changed by injection with Im1 is the T-P period. This part of the period is equivalent

to the time during which dorsal motor neurons are active and the time afterwards, before ventral motor neurons are firing. This implies that the period after a dorsal burst but before a ventral burst is longer than the period after a ventral burst but before a dorsal burst. In control animals both intervals are equal. Im1, it seems, must then alter the oscillatory circuit in such a way as to create this temporal variation. This could be tested by recording from a PP nerve, which contains the axons of ventral excitatory motor neurons, as well as a DP nerve. This, however, would be difficult due to the close proximity of the two nerves.

It also seems that Im1 does not behaviorally affect the intersegmental delay of burst generation from one ganglion to the next. This is illustrated by the rate of progression of transition points down the body of the animal. In control animals, the P-T and T-P move through the body at a rate of around 200 percent/sec. The rate that bursts travel through the body remains similar in Im1-treated animals (Figure 4c). This implies a similar intersegmental delay in control and Im1-injected animals. Similar experiments with multiple electrodes in adjoining segments would help to verify if this is true.

A recent study of swimming leeches *in situ* with both intact and severed nerve cords has demonstrated the importance of sensory feedback in the coordination of swimming (Yu et al.). They showed that even if the nerve cord of an animal is severed in an intact animal, bursts still propagate down the cord in phase with only a slightly longer intersegmental delay. The propagation of burst past the point where the nerve cord is severed is explained by the ability of the central nervous system to

use sensory feedback to coordinate motor output. This ability helps to coordinate the swimming of Im1-treated leeches as well. Isolated cords that have been treated with Im1 show a breakdown in the pattern of bursts as they progress down the nerve cord, even as early as segment 8 (Ruben Gonzalez, results not published). The bursts begin to occur at increasingly irregular intervals with varied durations. By the time the swim pattern has progressed to the posterior end of the animal, the pattern is mixed up, with some bursts of action potentials having run together to form abnormally long bursts of activity. It is likely that sensory feedback in the intact leech is responsible for enhancing the coordination of the motor output of the central nervous system to allow the leech to continue to swim in a patterned way.

The cells in the swim circuit that are affected by Im1 are currently unknown. However, Im1 has been shown to bind to specific α7-containing nAChRs in vertebrates (Broxton et al., 1999; McIntosh et al., 1999) and nAChRs in invertebrates with similar properties (Kehoe and McIntosh, 1998; van den Beukel et al., 1998). α-bungarotoxin affects α7-containing nAChRs, making it a useful starting point for determining cells that might be affected by Im1. There is a range of possibilities, seeing as there are a few different cells in the leech nervous system which have been shown to have α-bungarotoxin-sensitive nAChRs (Kehoe and McIntosh, 1998; Broxton et al., 1999). Because α-bungarotoxin binds to α7-containing nAChRs, it is likely that the receptors on these cells will also be sensitive to Im1. These include Retzius, AP, and P cells. It is possible that any (or all) of these cells could be the target of Im1. Retzius cells have modulatory effects on various motor neurons, so

modifying them could possibly result in the changes seen in motor neuron output during swimming. Also, the excitatory current produced in Retzius cells by stimulation with ACh is rapidly-desensitizing and from neuronal nAChRs, like those seen in *Aplysia* (Szczupak et al., 1993). The P cells are pressure sensitive cells, so a change in their firing rate could lead to "hallucinations" which result in circular swimming. The function of the AP cell is unknown, providing for any range of possibilities as to the effect of a change in its sensitivity to ACh.

There are probably more cells within the swim circuit that have α7-containing nAChRs which have not been identified yet. Thus, it seems that at this time it would be impossible to accurately speculate as to the mechanism through which Im1 acts. However, through the use of optical imaging techniques (Cacciatore et al., 1999; Taylor et al., 2003), one could better understand how the overall activity of the cells in a ganglion is affected by Im1 during swimming. Also, by studying the physiological properties of individual cells and pairs of cells that have been treated with Im1, one could see what cells are affected by Im1 and how they are affected. These experiments would show the exact activity that Im1 elicits as well as the method of how it does so.

CONCLUSIONS

The length of the half-period is linked to peak and trough amplitude. A longer half-period accompanies a larger amplitude. Also, the T-P period varies a great deal between different swim conditions while the P-T period stays relatively constant. With respect to half-period length, Im1 and confinement have opposite effects. Confinement leads to the decrease in T-P period length and thus a decrease in overall period length, while Im1 injection leads to an increase in T-P period length and thus an increase in overall period length. Both of these conditions lead to circular swimming because they both result in a large difference in T-P versus P-T period length.

Physiologically, Im1 causes an increase in the average duration of bursts produced by dorsal excitor Cell 3 as well as an increase in the average period length of Cell 3 bursts. These increases are accompanied by larger variability in both duration and period, leading to a large range of burst durations and period lengths that are not seen in controls. Nevertheless, all of these changes are not sufficient to cause the circular swimming that is observed behaviorally. Im1 would also need to alter the output of ventral motor neurons in such a way as to affect half-period lengths. Thus, Im1 does not simply affect motor output, but rather it appears to affect coordination of the circuit as a whole

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