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Young Rim (Rimi) Chang April 10, 2015

The Neuromuscular Transform between Heart Motor Neurons and the Heart in Leeches

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An abstract of
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Abstract

Central pattern generators (CPGs) produce motor patterns that control motor outputs. The Calabrese laboratory studies a variety of aspects of the leech heartbeat system, particularly researching how the CPG activity is ultimately translated to heart contractions. The left half of a single mid-body segment in a reduced preparation was electrically stimulated extracellularly in order to observe the effects of the stimulus protocol on the heart contractions. Variation in the duration and frequency of stimulus was reflected in the motor performance of the heart. To quantify the beat pattern, the Clampfit analysis program and the Excel program were used to calculate the peak heart contraction, the contraction force integral (area for the heart contractions), and the latencies to the onset of heart contraction and to the peak heart contraction from the onset of stimulus. These 4 parameters were used to study the neuromuscular transform in the leech, which was the goal of this project.

I measured the peak amplitude and the contraction force integral to assess the force of the heart contraction. Additionally, two latencies to the onset of the heart contraction and to the peak contraction amplitude from the onset of the stimulus were measured to assess the responsiveness of heart contraction. The data from a total of 9 experiments showed that usually longer stimulus durations at higher frequencies yield stronger correlations among the data values for the peak heart amplitude and the force integral. Moreover, only 2 s stimulus or longer showed a statistically strong negative correlation for latency to peak amplitude as frequency increased. All together, the results from this project will aid in comprehending how stimulus changes the heart contraction patterns. Additionally, these data can be used as a control in comparison with future experiments.

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Introduction

Movement is important. Movements such as crawling, flying, swimming, and beating of the heart are examples of rhythmic movements. The muscles executing these movements are controlled by motor neurons. Then, the motor neurons, in turn, are controlled by central pattern generators (CPG), which are an ensemble of interneurons (Thompson and Stent 1976a, b; Calabrese and Peterson 1983).

The present study focuses on invertebrates as an animal model. In vertebrate experiments, it is difficult to test the relationship between synaptic stimulus and the consequent motor patterns due to the sophisticated spinal networks in the CPG structures (Buschges et al). Compared to invertebrates, vertebrate spinal networks involve more neurons that are not as straightforward, which makes them less accessible. For this project, the leech was chosen as an animal model. Specifically, in the leech, the heart motor neurons are controlled by a well-characterized CPG that is found in simple nervous systems, which then can be easily manipulated in experiments (Kristan et al. 2005). Moreover, neurons and muscles in leeches are accessible for recordings. These traits thus make leeches ideal candidates for motor behavior research.

Adult leeches, *Hirudo* sp., have two bilateral heart tubes that run along the entire body length. A cross-section of a leech heart shows that the wall of each heart consists of an outer layer of circular muscle fibers and an inner layer of longitudinal muscle fibers. These two kinds of muscle fibers contribute to both the shortening the heart tubule length and constricting the heart tubule circularly at the same time (Hammersen and Staudte 1969). However, Maranto and Calabrese 1984 further demonstrated that the longitudinal and circular fibers are merely different regions of a single type of spindle-shaped muscle cell, thereby rejecting the previous notion of

two separate muscle fibers. Also, the spiral forms of these cells contribute to the shape of the heart wall and the efficient contraction of the heart (Maranto and Calabrese 1984b).

The leech's heartbeat CPG system is composed of 7 identified heart interneuron (HN) pairs and one yet unidentified pair of interneurons. The motor neurons are located in the chain of ganglia, connected by a nerve cord. A leech has a chain of 21 free segmental ganglia, or a total of 33 ganglia including the brain-fused ganglia in its body. On each side, each heart segment is entrained by an ipsilateral heart excitatory motor neuron (HE) in the corresponding segmental ganglion. Each HE motor neuron extends out to the ipsilateral heart through a bilateral set of segmental vascular nerves and Y nerves. The axon of each of these motor neurons inside the anterior nerve goes out and innervates the ipsilateral segmental section of the heart.

Maranto and Calabrese 1984 said that a leech heartbeat CPG coordinates an ensemble of 16 pairs of motor neurons into a functional heartbeat motor pattern. They showed that the electrical activity and contraction of the heart are correlated with the rhythmic discharge of segmental HE motor neurons (Fig 13). Since the HE motor neuron's activity is the output of the heartbeat CPG, the pattern generator must be the governing oscillator determining the leech's heartbeat. Finally, the contraction patterns of the hearts match closely to the activity patterns of the HE motor neurons, indicating that the heartbeat CPG ultimately controls the contraction pattern of the hearts (Maranto and Calabrese 1984b).

However, to gain an insight into how the HE motor neuron's activity sets the motor pattern of the heart contraction, a more quantitative approach is needed to address the following questions: *How will the force of a heart contraction change when the stimulus duration is changed? How will the heart contraction change if the stimulus frequency is changed? Is the*

response time affected by stimulus duration and/or frequency? Analysis on these questions will unveil more about neuromuscular transform: how the heart muscle contraction is related to the HE motor neuron activity. Under normal physiological conditions, HE motor neurons have a 4 second burst duration (~60% duty cycle) and fire at a 10 Hz frequency. The neuromuscular transform shows how heart contraction deviates from this baseline with a change in the stimulus duration and/or the stimulus frequency.

Hence, in order to study the neuromuscular transform, a reduced preparation is needed in which both the frequency and the duration of the stimulus of the HE motor neuron, i.e. its frequency and burst duration, are controlled, unlike intact preparation for intracellular recording from Maranto and Calabrese 1984. A method to directly control spike frequency and burst duration was needed, and this was possible through pulse trains (see Discussion for details). Then, the analysis of data on 1) peak amplitude of heart contraction and 2) the area under the curve of individual heart contractions (contraction force integral) is needed to assess the force of heart contraction. Also, the analysis of latency by measuring the time from the onset of stimulus to 3) the onset of heart contraction and to 4) the peak amplitude is needed to assess the response time.

Materials and Methods

Equipment:

- A displacement (semi-isotonic) transducer (model 1030, UFI, www.ufisevingscience.com): This force transducer was used to assess the contraction-relaxation of a single heart segment.

- A Flaming/Brown micropipette puller (P-97, Sutter Instruments, http://www.sutter.com) from borosilicate glass (1-mm OD, 0.75-mm ID; A-M Systems, http://www.a-msystems.com): This micropipette puller was used to pull suction electrodes.

- A suction electrode holder (E series, Warner Instruments, http://www.warneronline.com): A suction electrode was placed in a suction electrode holder in order to pull the anterior nerve into the electrode.

- Amplifiers for recording: Extracellular signals were monitored with a differential AC amplifier (model 1700, A-M Systems) at a gain of 1,000 with the low- and high-frequency cutoffs set at 100 and 1,000 Hz, respectively. Noise was reduced with a 60-Hz notch filter. A second amplifier (model 410, Brownlee Precision, http://www.brownleeprecision.com) amplified the signal appropriately for digitization.

- A Grass S88 stimulator (http://www.grasstechnologies.com/products/stimulators): This stimulator delivered either single pulses or trains of pulses through the electrode.

- The tension recordings were digitized (>5 kHz sampling rate) with a computer using a digitizing board (Axon DigiData 1550 Series Interface, http://www.moleculardevices.com) and acquired using pClamp software (Molecular devices, http://www.moleculardevices.com).

Preparation:

Adult leeches, *Hirudo* sp., were obtained from commercial suppliers Leeches USA (Westbury, NY, USA) and Niagara Leeches (Cheyenne, WY, USA), and kept in 0.05% (w/v) artificial pond water [0.05% (w/v) Instant Ocean sea salt diluted in spring water; Spectrum Brands Inc., Madison, WI, USA] at 16°C. To prepare an animal for dissection, leeches were cold-anesthetized by immersion in crushed ice for approximately 10 to 15 minutes. During the dissections and the following experiments, leeches were kept in leech saline that contained (in mmol l^{-1}) 115 NaCl, 4 KCl, 1.8 $CaCl_2$, 1.5 $MgCl_2$, 10 D-glucose, and 10 HEPES buffer, adjusted to pH 7.4 with NaOH (Wenning, Norris et al. 2014). All electrophysiological experiments were done at room temperature of 21-22°C (Wenning, Norris et al. 2014). Ganglion and segment numbers refer to mid-body segments. All of the experiments were involved with the left side of the leech body.

Leeches were pinned, dorsal side up, through the anterior and posterior suckers in a Sylgard-lined dish. Along the dorsal midline, an incision was made from segment 3 to segment 15. The leech's body was opened, and the edges of the dorsal body wall were pinned.

The left half of a single body segment between mid-body segments 7 to 13 was prepared for isolation. First, the crop was removed using fine forceps to expose the heart tube. With fine spring scissors, the junctions of the heart side vessels from the adjacent segments were cut, since these valve junctions where afferent vessels connect the hearts produce competing antidromic burst activity, or peripheral neurogenic rhythm (Jellies and Kuel 2012). Next, the segmental ganglion was exposed by removing the blood vessel lining around it. The anterior nerve was cleaned from the glia sheath in the same fashion. The ends of the heart tubule outside the

segment of interest were cut; portions of the heart tubes from the two adjacent segments were kept. Then, the ganglion's connectives were cut. This segment section was removed from the leech with broken pieces of razor blades. Finally, the cut-out segment was pinned in a smaller dish for recording.

Heart contraction recording:

A semi-isotonic, strain-gauge UFI Model 1030 Transducer (UFI Corp, www.ufisevingscience.com) was used to assess the contraction-relaxation of a single heart segment. The force transducer was placed at an angle of 45° on a micromanipulator stand. A cotton thread was connected to the end of the force transducer steel leaf. An L-shaped end hook was constructed from a 0.1 mm minutien pin. The force transducer steel leaf was placed over the heart tube segment by using the micromanipulator.

The hook was inserted into the heart tube initially from under the heart tube and penetrated through the top. The hook and the transducer arm were lifted and adjusted away at 45 degrees from the preparation just enough to overcome the slack of the thread until the heart contraction signals were regular and of a reasonable size.

Extracellular recording and stimulation from the anterior segmental nerve:

Electrodes were pulled on a Flaming/Brown micropipette puller (P-97, Sutter Instruments) from borosilicate glass (1-mm OD, 0.75-mm ID; A-M Systems, http://www.a-

msystems.com). For extracellular recordings and stimulation, suction electrodes were filled with normal saline and placed in a suction electrode holder (E series, Warner Instruments, http://www.warneronline.com). Then, the anterior segmental nerve of the ganglion, which contains the heart motor neuron axon, was cut close to the ganglion (Thompson and Stent 1976a). The electrode tip was brought in contact with the end of the nerve, and the tip was broken accordingly using forceps if the suction electrode's opening was too small for the nerve to fit inside. Light suction was applied to pull a portion of the anterior nerve inside the electrode. Extracellular signals were monitored with a differential AC amplifier (model 1700, A-M Systems) at a gain of 1,000 with the low- and high-frequency cutoffs set at 100 and 1,000 Hz, respectively. Noise was reduced with a 60-Hz notch filter. A second amplifier (model 410, Brownlee Precision, http://www.brownleeprecision.com) amplified the signal for digitization.

After recording several heart contractions and spontaneous nerve activity, the differential AC amplifier (model 1700, A-M Systems) was switched into Stimulation mode. A Grass S88 stimulator (http://www.grasstechnologies.com/products/stimulators) was used to deliver either single pulses or trains of pulses through the electrode. The length of a single stimulus was set to 0.5 ms, and the trains were presented every 10 seconds. The Grass stimulator allowed controlling the activity of the heart motor pattern by changing the frequency, amplitude, and train duration of trains of short duration (~1 ms) electrical stimuli that activates the heart motor neuron's axon in the anterior root.

Extracellular stimulation experiments lasted approximately 30 minutes.

Stimulus protocol:

First, the stimulus threshold was set using the Grass stimulator. With default of 4 s of train duration of 15 Hz pulses set by the Grass stimulator, amplitude of the electrical stimulus voltage, or stimulus threshold, was set at supra-maximal stimulus to display appropriate heart contraction amplitudes.

Once the threshold was found, a series of trains were delivered following the protocol detailed in Fig 4. This protocol was developed to mimic the firing of the HE motor neuron under physiological conditions: periodic bursts of action potentials at about the10 Hz and of 4 s in duration with a duty cycle of ~ 40%. One stimulus cycle consisted of 8 heart contractions produced by 8 stimulus trains repeated every 10 seconds. After the 8^{th} contraction, the next stimulus cycle was performed. The protocol chart shows that the sequence of stimuli moves from left to right within a row, and then downward for train duration (Fig.4). At the end of the stimulus protocol, the starting stimulus with 4 s train duration with pulses at 15 Hz was repeated to help estimate heart fatigue throughout the experiment. The 0.5 s train duration with pulses at 20 Hz was not performed in all of the experiments.

Data digitization and acquisition:

The tension recordings were digitized (>5 kHz sampling rate) with a computer, using a digitizing board (Axon DigiData 1550 Series Interface, http://www.moleculardevices.com) and acquired using pClamp software (Molecular devices, http://www.moleculardevices.com). On a personalized computer in the gap free acquisition mode, sampling rate per signal (Hz) was set at

5000, so total data throughput was 15000 samples per second. To remove low-frequency body wall movement signals, the signals were low-pass filtered at Gaussian of 3 Hz.

Data analysis:

The cursors in the ClampFit 10.5 analysis program were used to measure the absolute value of the peak heart contraction (cursor 2) and the absolute value of the antipeak, or baseline (cursor 1) for each heart contraction (Fig 5). Then, in the Excel program, the difference of peak and antipeak was calculated (Fig 6).

Similarly, the cursors in the ClampFit analysis program were used to measure the time of the onset of the stimulus train (cursor 1), the time of the onset of heart contraction (cursor 3), and the time of peak heart amplitude (cursor 2), as seen in Fig 5. These 3 values were used to calculate the two latencies. In the Excel program, the difference of the onset of the stimulus train (cursor 1) and the onset of the heart contraction (cursor 3) was calculated (Fig 7). Also, the difference of the onset of the stimulus (cursor 1) and the peak heart amplitude (cursor 2) was calculated (Fig 7).

The contraction force integral for one stimulus cycle was measured by cutting print-outs of the heart contractions (Fig 8). The contraction evoked by each train in a stimulus cycle was printed on a single paper with identical y-axis and x-axis within one experiment. In order to compare force integrals among different experiments, a rectangular calibration of 20 μV for 20 s was also cut for each experiment. The cut-outs and calibrations were then weighted on a chemical balance. The contraction cut-outs for each stimulus cycle were weighted together and

then divided by the number of heart contractions in the stimulus cycle (N=8). This individual contraction weight and was this further divided by the weight of the calibration.

Plots and statistics:

The following values were plotted: peak heart amplitude vs. pulse frequency, peak heart amplitude vs. train duration, contraction force integral vs. pulse frequency, contraction force integral vs. train duration, latency to the onset of heart contraction vs. pulse frequency, and latency to the peak heart amplitude vs. pulse frequency. In addition, plots with averages were also done. Also, standard deviations were calculated from the averaged data values for each set of stimulus duration and frequency. Standard deviation is shown as vertical lines above and below each point in the graphs. For statistics, t-score was generated from the correlation value, r, of the same stimulus duration or stimulus frequency in the Excel (Microsoft) program. This correlation analysis showed whether correlations were significant, and the values were considered significant when $p \leq 0.05$.

Results

The goal of this project was to analyze the neuromuscular transform by answering the following question: How are heart contractions affected if the train duration and/or the pulse frequency are changed? I used a reduced preparation consisting of a heart tube from a left single body segment and severed anterior nerve (Fig 2). I measured the peak amplitude and the contraction force integral to assess the force of the heart contraction. Additionally, two latencies

to the onset of the heart contraction and to the peak contraction amplitude from the onset of each stimulus train were measured to assess the responsiveness of heart contraction (Fig 7).

The same stimulus protocol (Fig 2) was used for all experiments for 7 animals, using 2 preparations from the same animals (see Materials and Methods for details). At the end of the stimulus protocol, the initial stimulus cycle with 4 s train duration and 15 Hz pulse frequency was repeated to help estimate heart fatigue throughout the experiment, and this stimulus cycle was also plotted in graphs (black •).

Peak contraction amplitude:

The absolute values of peak amplitudes of single heart contractions ranged from 4.0 μV to 15.8 μV, about a 4 fold difference. Not surprisingly, this variability was most likely caused by different factors such as placement of transducer hook, quality of preparation, and etc. The averaged data values were normalized to compare peak amplitude values among all 9 experiments. Values were normalized to the value of the peak amplitude for the first stimulus cycle (Fig 9a) which had the longest train duration (4 s) and highest pulse frequency (15 Hz). This value was set at 100% for each experiment. For each experiment, the peak amplitudes measured throughout the course of an experiment were normalized to the value of the peak amplitude of the first stimulus cycle (4 s, 15 Hz).

When averages of peak amplitude were plotted over pulse frequency, the 2 s train duration displayed a positive correlation (Fig 9a). Additionally, 2 s train duration showed a

statistically significant increase when individual data values were plotted ($p \leq 0.008$) (Fig 9b). Fig 9b shows a high variability among individual data points.

Averages of peak amplitude were plotted over train duration (Fig 9c). For short train durations (0.5 and 1 s) at lower pulse frequencies (5 Hz), the peak amplitude did not change. Not surprisingly, at higher frequencies (15 Hz) and for 2 and 4 s long trains, the peak amplitude increased ($p \leq 0.043$) (Fig 9d). Between the 0.5 and the 2 s train duration, peak amplitude increased greatly, and the peak amplitude already reached close to 100% at the 2 s train duration (Fig 9d).

Contraction force integral:

As explained above for assessing peak amplitudes, the values for the contraction force integrals were normalized to the value of the force integral for the longest train duration and highest pulse frequency (4 s; 15 Hz). When the force integrals were plotted over pulse frequency, a positive correlation was observed for 4 s and 2 s train durations (Fig 10a). The 4 s train duration had a steeper slope for its positive correlation than the 2 s train duration. Only 4 s train duration showed a statistically significant increase when individual data values were plotted ($p \leq 0.024$) (Fig 10b).

When averages of force integral were plotted over train duration (Fig 10c), a positive correlation was found for the 15 Hz pulse frequency ($p \leq 0.034$) (Fig 10d).

Latency to the onset of the heart contraction and to the peak contraction amplitude from the onset of the stimulus train:

Averages of latency values to the onset of the heart contraction were plotted over pulse frequency (Fig 11a). Latency to the onset of contraction was the same for all stimuli (train durations and pulse frequencies) and had a high variability.

When averages of latency values to the peak contraction amplitude were plotted over pulse frequency, generally a negative correlation was shown (Fig 12a). Only 2 s train duration showed a statistically significant decrease when individual data values were plotted ($p \leq 0.036$) (Fig 12b).

Discussion

Assessing the force of heart contraction:

The data values from stimuli with a 2 s train duration showed a strong positive correlation between contraction amplitude and pulse frequency: as pulse frequency increased, peak amplitude increased (Fig 9b). Hence, from 2 s stimulus duration, the effects of stimuli began to show a strong correlation. Also, only 4 s train duration showed a strong increasing correlation for force integral as pulse frequency increased (Fig 10b). In order to measure the effects on force integral due to pulse frequency, a 4 s train duration would be ideal. Moreover, only 15 Hz pulse frequency showed a strong positive correlation with the peak amplitude and the force integrals as train duration increased (Fig 9d). Hence, for future experiments, longer train durations (2 s and 4 s) and higher pulse frequency (15 Hz) will be more important to assess the force of heart contraction compared to 0.5 s and 1 s, and 5 Hz and 10 Hz.

Assessing the latencies of heart contraction:

Statistically, only the 2 s train duration showed a negative correlation with latency to peak amplitude as pulse frequency increased ($p \leq 0.036$) (Fig 12b). So, this observation indicates that train durations 2 s or longer would impact the latency more. Train durations of 0.5 s and 1 s might not have produced a strong correlation simply because their durations were too short to trigger a heart contraction. A shorter train duration at lower pulse frequency releases smaller amounts of neurotransmitter, which then will take a longer time to initiate heart contraction.

Additionally, there was simply too much variability among individual data points, weakening the strength of correlation. This was especially problematic for latency to the onset of the heart contraction (Fig 11a). It was somewhat subjective to measure the time of onset of the heart contraction, because the heart sometimes contracted gradually, instead of a sharp rise. Therefore, latency to the peak amplitude is probably a better method of measurement for future experiments. Unlike the onset of heart contraction, peak amplitude can be measured objectively.

Latencies were not plotted over train durations. The time difference to either the onset of contraction or the peak amplitude is determined by the pulse frequency, not train duration, as the duration cannot be anticipated. The pulse frequency determines how fast the heart will start contracting and reach the peak amplitude. At higher stimulus frequency, more neurotransmitter is released per second, thus overcoming the contraction threshold faster. In turn, reaching the threshold faster causes the heart to contract faster, too. Hence, the latencies are already determined in the beginning of the stimulus, due to the release of neurotransmitters. The heart tube cannot predict when the stimulus will end. Thus, the length of train duration does not affect the latency of heart contraction, but rather affect the contraction amplitude and the force integral.

Importance of reduced preparation:

Maranto and Calabrese 1984 examined the electrical activity of the heart through intracellular recording in a reduced preparation (Fig 13) (Maranto and Calabrese 1984b). Their data showed that the electrical activity of the heart is correlated with the rhythmic discharge of segmental HE motor neurons. Additionally, they also asserted that the HE motor neuron's activity is the output of the heartbeat CPG, and that the CPG ultimately governs the contraction pattern of the hearts. However, to gain an insight into how the HE motor neuron's activity sets the motor pattern of the heart contraction, a more quantitative approach was needed: measurement of force and latency of heart contractions.

My goal for this project was to analyze the neuromuscular transform in leeches, which was done through extracellular stimulation in reduced preparations. In the past, however, I also attempted to achieve this same goal for my previous project through intracellular recordings of reduced experiments with attached ganglion, which is similar to the methods done by Maranto and Calabrese 1984. This past method did not remove the cell soma and input neurites (dendrites), and instead separated intact HE motor neuron in a ganglion from the heart tube by using a split bath. I used $MnCl_2$ to isolate the motor neurons from their synaptic input from the interneurons. However, this split bath method did not always effectively suppress the inhibitory post synaptic potentials (IPSP), which was essential to obtain analyzable data. With IPSPs present, my stimulation from the protocol could not take its full effect. Moreover, it was difficult to control spike frequency with intracellular stimulation.

Methods for reduced preparation evolved for this project by removing the cell soma and dendrites (HE motor neuron) and stimulating the HE motor neuron axon directly. This new

method not only fixed the problem with IPSPs by removing the cell soma and dendrites, but also allowed me to have more control with the stimulation. When I had to perform stimulus protocol on the intact HE motor neuron in the past, I was not able to control spike frequency effectively. However, stimulating the HE motor neuron axon enabled me to control the spike frequency directly with the pulse frequency.

Importance of the data for future research:

Wenning et al. 2014 extracellularly recorded from 2 ipsilateral HE motor neurons along with the heart contraction movement *in vivo* (Wenning, Norris et al. 2014). The results from Wenning et al. 2014 can be compared with my data in order to analyze the difference between *in vivo* and reduced preparation. For example, the effects of blood load *in vivo* experiments can be revealed by this comparison, since reduced preparation has no blood load in the heart tube. The latency from the onset of the stimulus train to the peak heart contraction ranged from 1.8 s to 3 s in my reduced preparation, whereas the latency from the beginning of an HE burst ranged from 3 s to 4 s in vivo (Fig 12a). Wenning et al. 2014 observed this longer latency even when the spike frequency was above the 10Hz, which they proposed to be due to the heart blood load (Wenning, Norris et al. 2014).

Hence, the data from this thesis can be set as a baseline to be compared in the future. For instance, future research can involve neuromodulators that influence the heart contraction pattern. Then, the modulator's effect on the heart can be compared to data from my experiments, which will be set as a control.

Presence of mini-contractions:

From the data, I observed "mini-contractions," which are heart contractions with peak heart amplitudes only half as big as normal contractions or peak heart amplitudes (see a red arrow in Fig 5). They were frequently observed at low pulse frequencies and were prominent in 2 of the 9 experiments. To understand where these mini-contractions come from, a short video imaging showing the junction of the segment's main heart tube and the side vessel was done along with the transducer recording to observe the movement of the heart tube. The stimulus was recorded on the audio channel to synchronize the data with the transducer recordings. The video imaging observation of side vessel contraction matched with the presence of mini-contractions. According to Wenning and Meyer 2007, the side vessels are afferent vessels that constrict to bring the blood into the main heart tube (Wenning and Meyer 2007). Furthermore, contraction of side vessels was seen before contraction of the heart tube according to the video imaging. Simultaneous video imaging will provide information whether the contraction is actually due to the main heart tube or the side vessels. Hence, eliminating the contraction of the side vessels and analyzing only the main heart tube contractions may eliminate mini contractions in the future.

Potential pitfalls:

One major potential pitfall is the subjective measuring of the onset of heart contraction. The onset of heart contraction is gradual, so it is hard to pinpoint a precise time for the onset of heart contraction. In addition, the noise in the recording and shifting baseline made measuring the onset of heart contraction more difficult. This subjective measurement contributed to the

variability of individual latency data values. Hence, a less subjective measurement of latency is from the onset of a stimulus train to the peak heart amplitude.

There is a need to explore pulse frequency of 20 Hz in depth. A stimulus protocol with trains of 2 s at 20 Hz was performed for only 5 of 9 experiments. Fig 9b and Fig 12b showed a strong correlation for the 5 values using 20 Hz pulse frequency. However, this correlation should be interpreted carefully, because the number of experiments is low.

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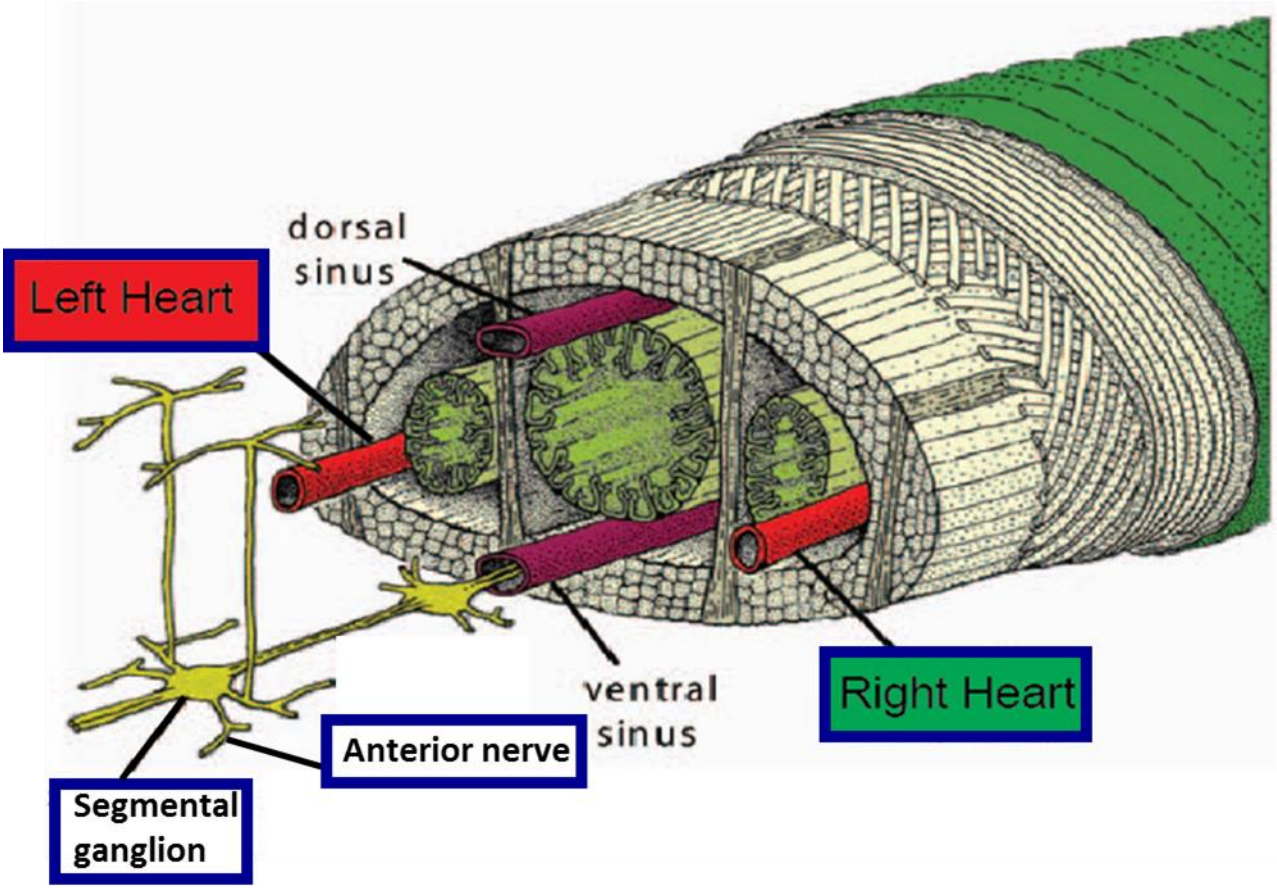

Figure 1

A cross-section of a leech. The leech has 21 midbody segmental ganglia connected by a nerve cord. Two heart tubes run along the entire length of the leech body on its lateral sides. Each heart is innervated by heart motor neuron called HE cell, which is placed inside the segmental ganglion. The HE motor neurons extend out to the heart through a bilateral set of segmental vascular nerves. (Modified after Nicholls and van Essen 1978)

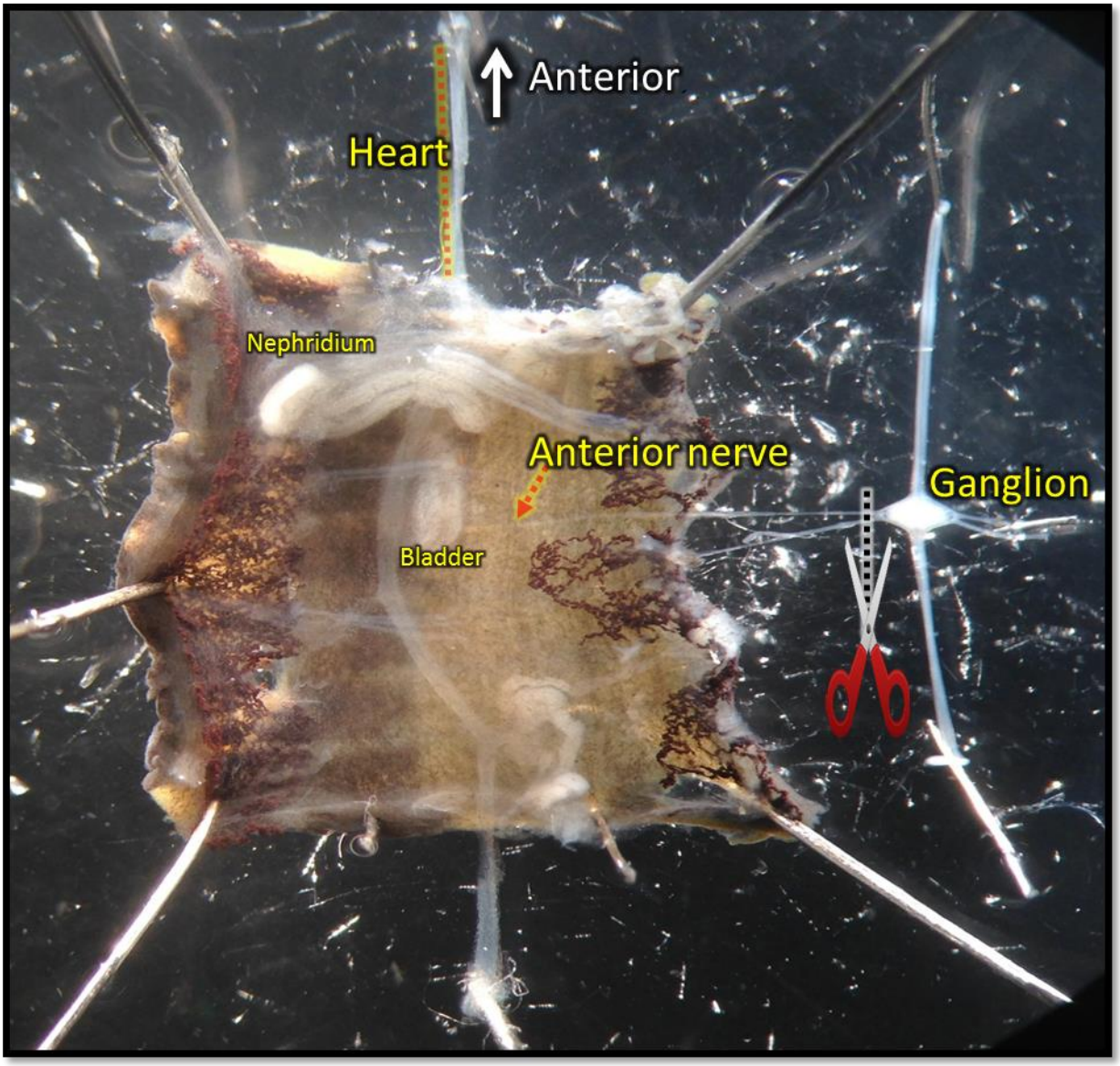

Figure 2

A photo of a reduced preparation of a left half of a single body segment with its ganglion still attached. A cut on the anterior nerve root of the ganglion was made near its exit from the ganglion. The nerve contains the HE motor neuron which will be stimulated by suction electrode.

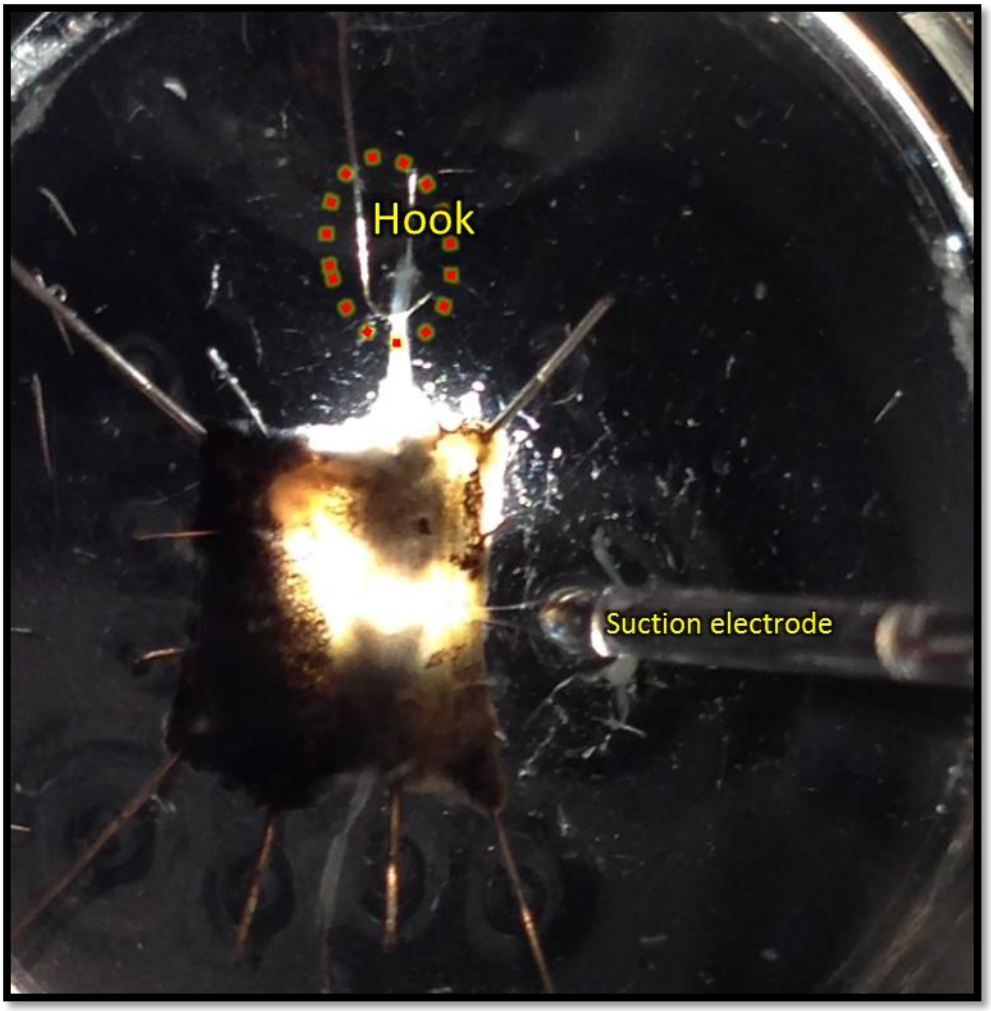

Figure 3

A photo of a reduced preparation ready for extracellular recording. A small hook attached to a fine thread was used to connect the exposed heart to a force transducer. The anterior nerve is pulled into the suction electrode.

4 s		15 Hz	10 Hz	5 Hz
2 s		5 Hz	10 Hz	15 Hz
1 s	20 Hz	15 Hz	10 Hz	5 Hz
0.5 s	5 Hz	10 Hz	15 Hz	20 Hz *
4 s (final stimulus, control)		15 Hz		

Figure 4

A stimulus protocol sequence over the course of extracellular recording. The protocol chart shows that the sequence of stimulus moves from left to right within a row, and then downward for stimulus duration. The stimulus protocol started with 4 s of stimulus duration at 15 Hz, at 10 Hz, and then at 5 Hz. After then, 2 s of stimulus duration at 5 Hz was performed. At the end of the protocol, another cycle of 4 s at 15 Hz was repeated as a control to establish heart fatigue by comparing with the first identical stimulus cycle. Each cycle consisted of 8 heart contractions.

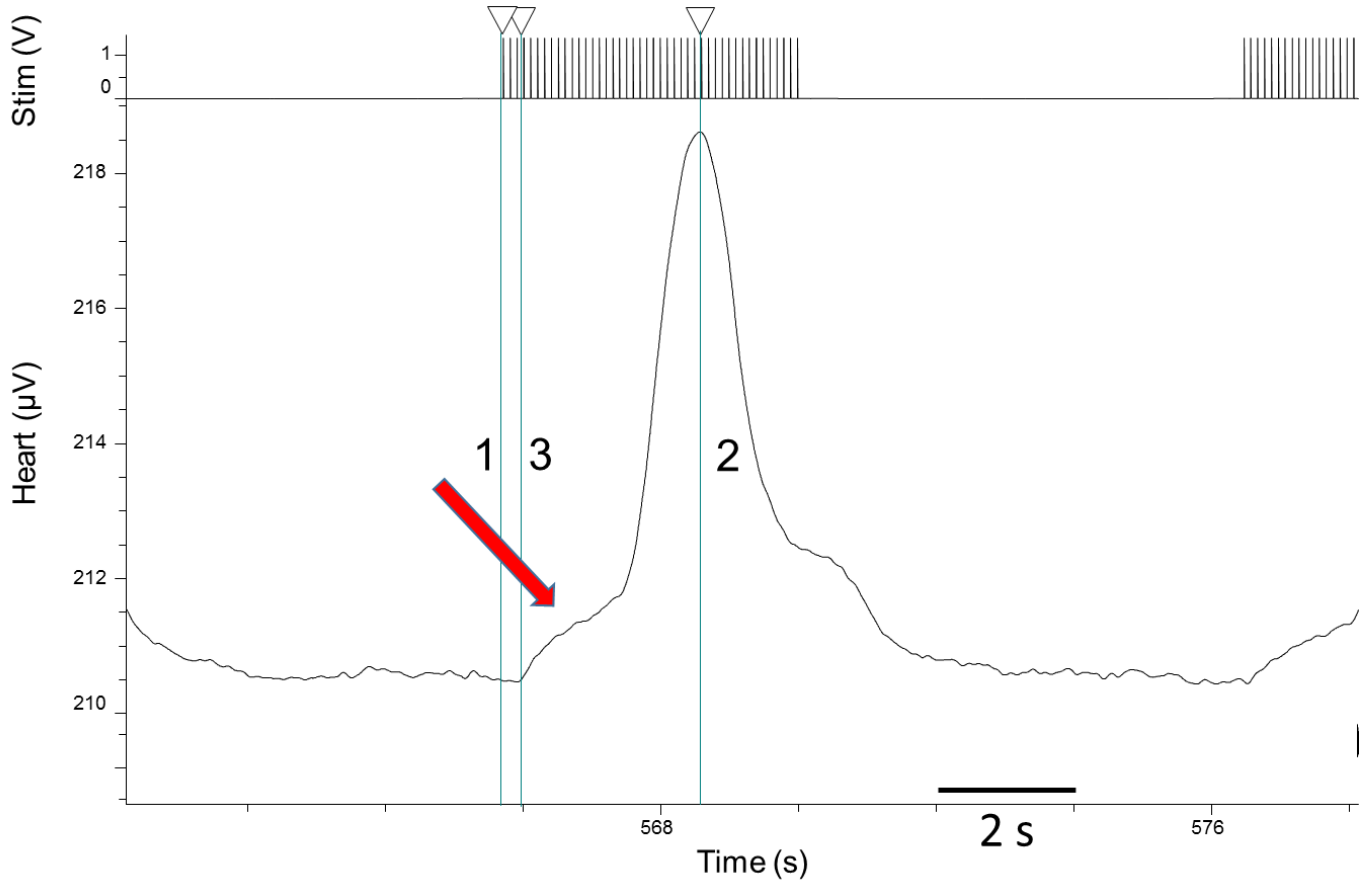

Figure 5

A sample extracellular recording that shows frequency and duration of a stimulus pulse train (4 s, 10 Hz). The recording shows the heart contraction measured by the force transducer and the frequency and duration of a stimulus train. In the ClampFit analysis program, cursor 1 was placed at the onset of the pulse train, cursor 3 at the onset of heart contraction, and cursor 2 at the peak heart contraction (See Materials and Methods for details). The red arrow indicates a "mini contraction" (see Discussion).

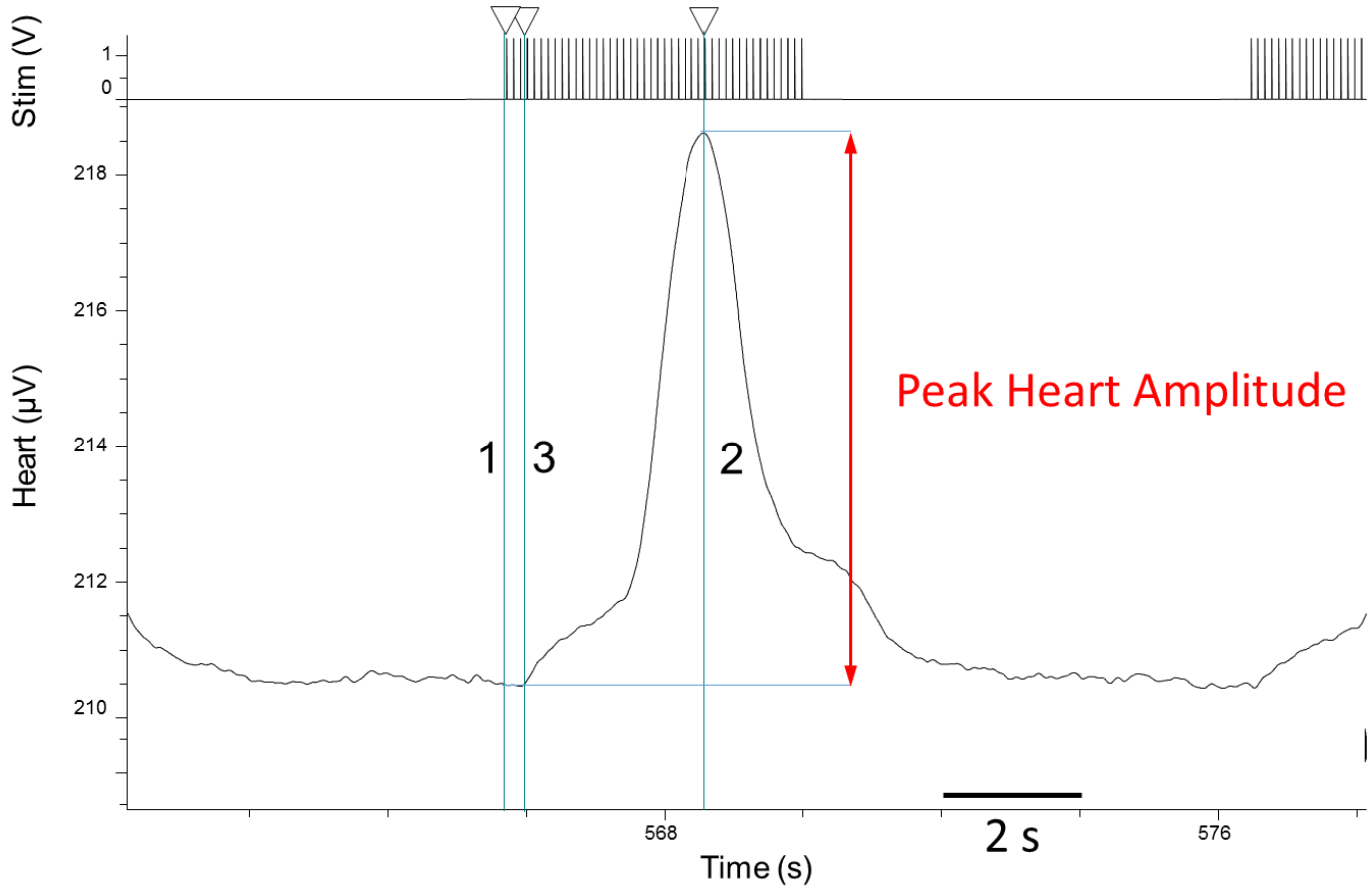

Figure 6

A sample extracellular recording. The peak heart amplitude was calculated by the difference of cursor 2 (µV) and cursor 1 (µV).

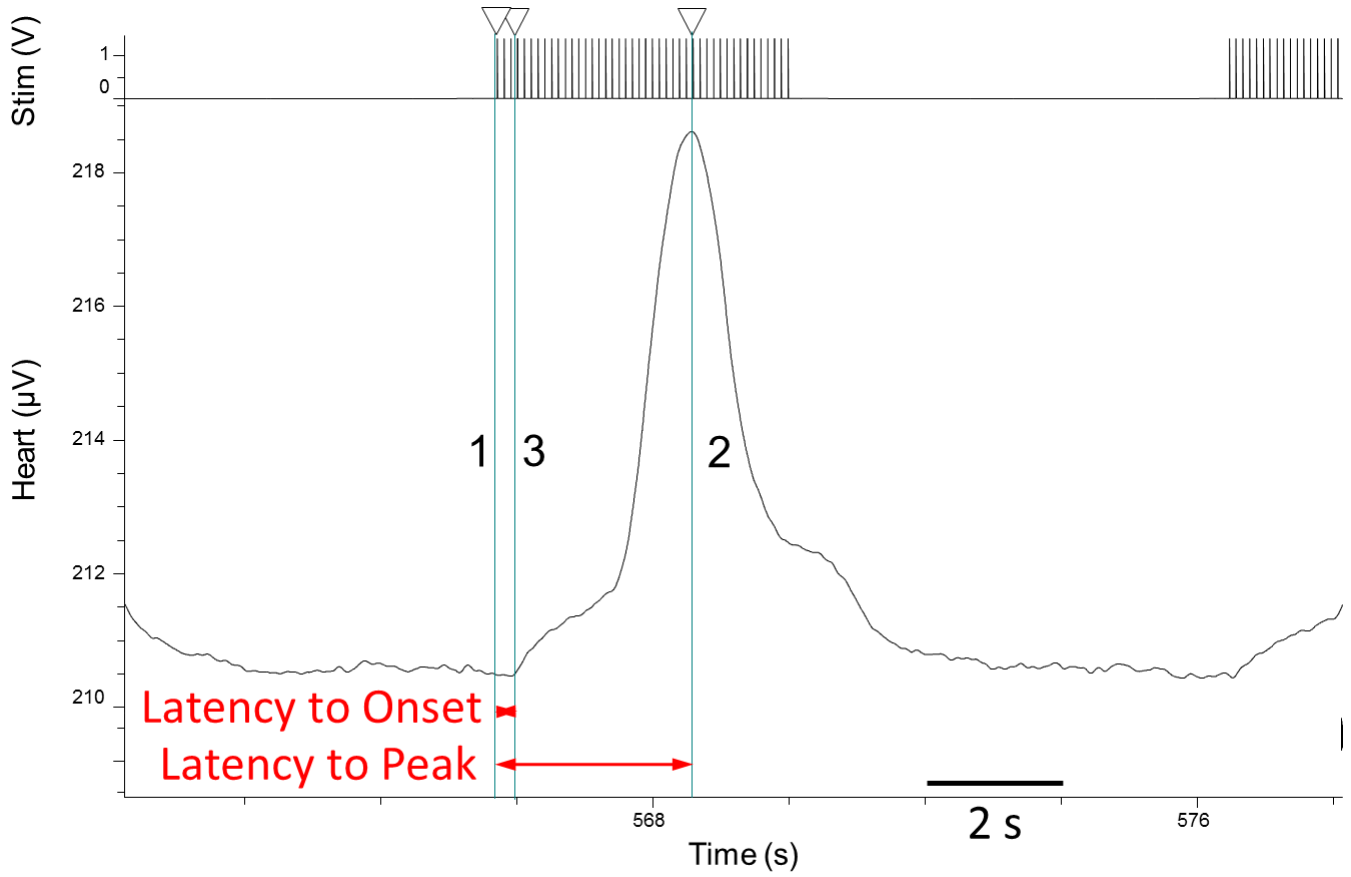

Figure 7

A sample extracellular recording. Latency from the stimulus pulse train onset to the onset of heart contraction was calculated by taking the difference of cursor 1 (s) and cursor 3 (s). Latency to the peak heart amplitude was calculated by taking the difference of cursor 1 (s) and cursor 2 (s).

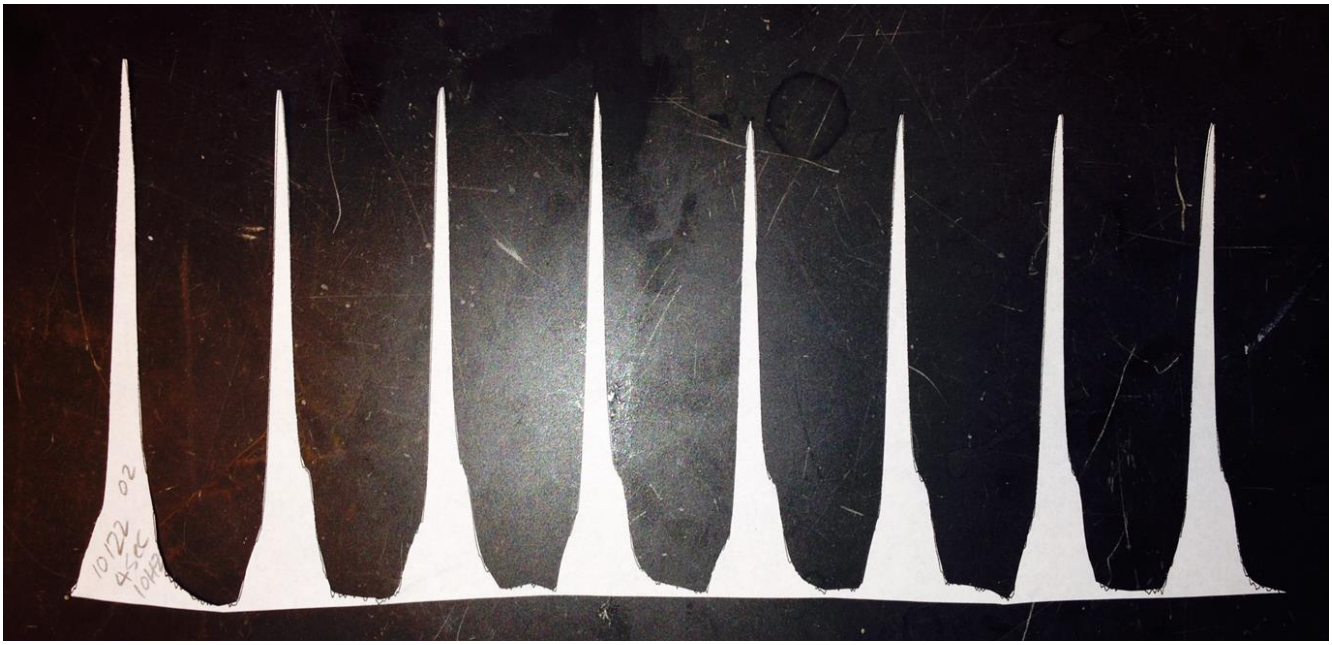

Figure 8

A photo of printed cut-outs for one stimulus cycle of 4 s duration trains with pulses at 10 Hz. One stimulus cycle consists of 8 heart contractions. This weight was used to calculate the contraction force integral.

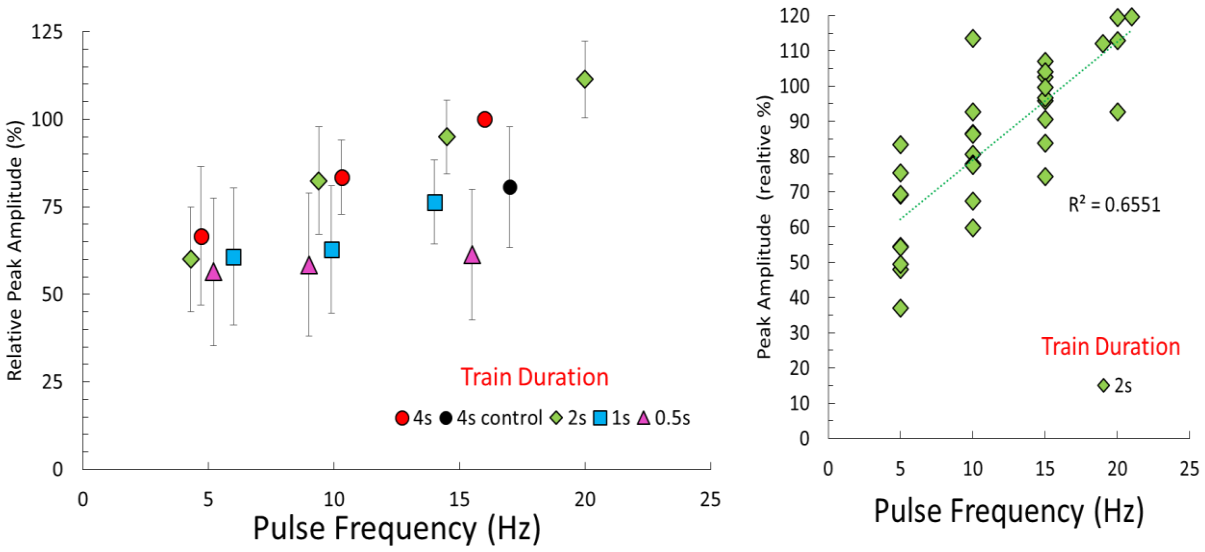

Figure 9a

Relation between peak amplitude and pulse frequency. Values for all experiments were averaged for each stimulus cycle. Values were normalized to the peak amplitude for the first stimulus cycle with the longest train duration and highest pulse frequency (4 s; 15 Hz) for each experiment. Thus, 4 s at 15 Hz was set to 100% (red •; see Results for details). The same stimulus cycle (4 s, 15 Hz) was repeated at the end (black •). Note that the 1s train duration does not increase the contraction amplitude with pulse frequency between 5 Hz and 10Hz. Note also that there is only a slight increase for the 0.5 second train duration from 5 Hz to 15 Hz. The train duration is color-coded and represented by different symbols.

(Mean ± s.d., N=9 experiments; N=5 for 2 s stimulus length at 20 Hz; N=8 for the control).

Figure 9b

Individual data values for 2 s stimulus duration shows a significant positive correlation as stimulus frequency increases ($p \leq 0.008$). Values were normalized to the peak amplitude for the first stimulus cycle with the highest stimulus duration and frequency (4 s; 15 Hz).

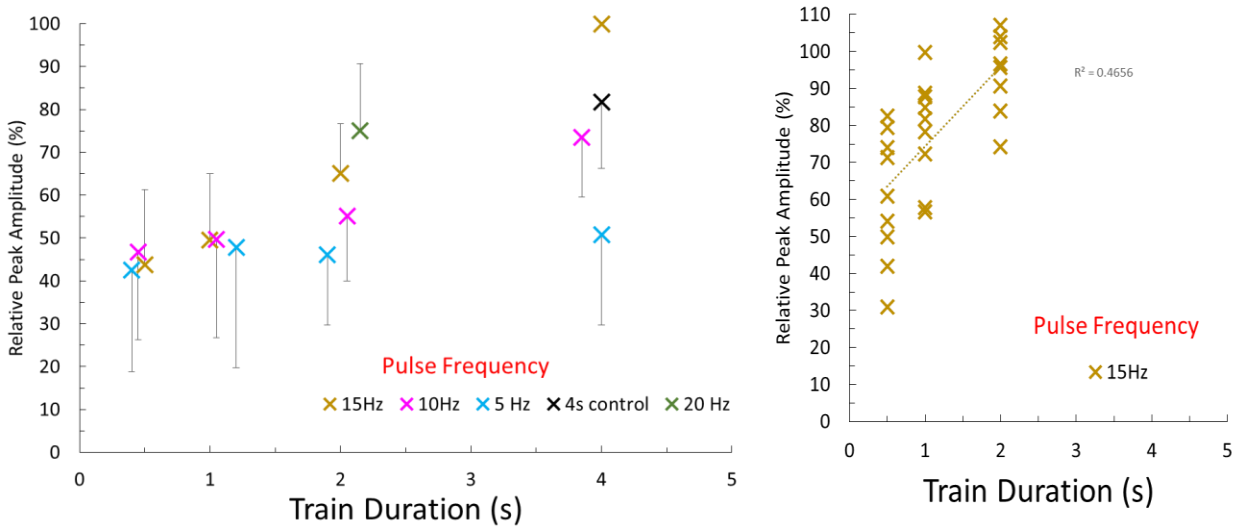

Figure 9c

Relation between peak amplitude of heart contraction and train duration. Values for all experiments were averaged for each stimulus cycle. Values were normalized to the peak amplitude for the first stimulus cycle with the longest train duration and highest pulse frequency (4 s; 15 Hz) for each experiment. Thus, 4 s at 15 Hz was set to 100% (red •; see Results for details). The same stimulus cycle (4 s, 15 Hz) was repeated at the end (black •). Although the relative peak amplitude appears to increase as the train duration increases, no pulse frequency showed a statistically significant increase in amplitude with train duration (p values>0.05). The stimulus frequency is color-coded and represented by different symbols.
(Mean ± s.d., N=9 experiments; N=5 for 2 s stimulus length at 20 Hz; N=8 for the control).

Figure 9d

Individual data values for 15 Hz pulse frequency shows a significant positive correlation as stimulus duration increases. Values were normalized to the contraction force for the first stimulus cycle with the longest train duration and highest pulse frequency (4 s; 15 Hz). The t-test for 15 Hz stimulus gave $p \leq 0.043$.

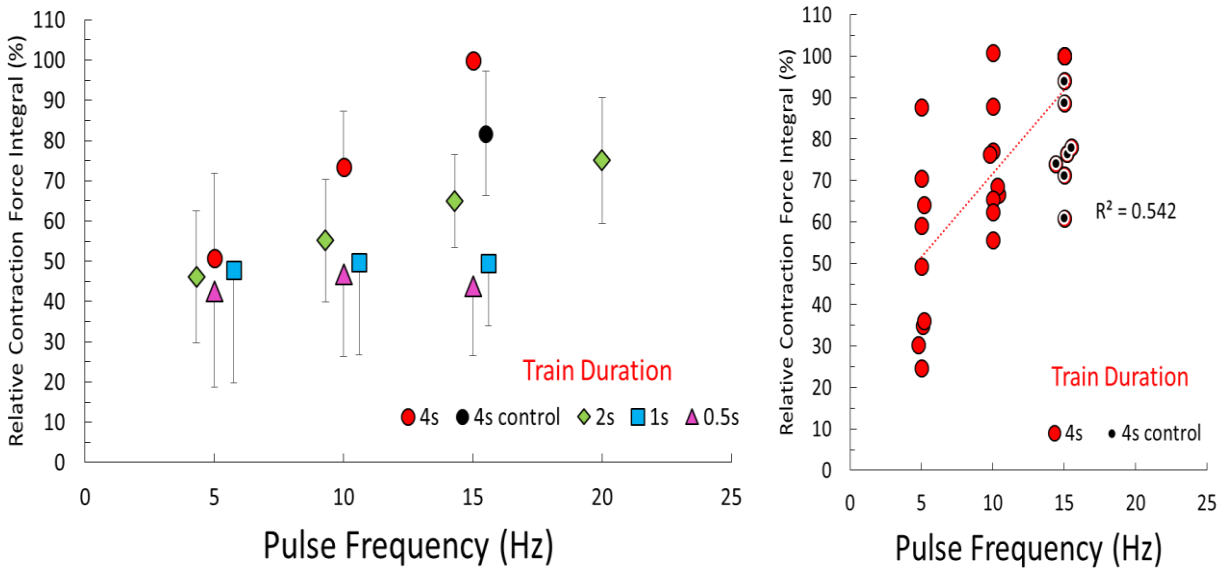

Figure 10a

Relation between the force contraction integral and stimulus pulse frequency. Force integrals (μV x s; area under the curve) were normalized to the contraction force for the longest train duration and highest pulse frequency (4 s; 15 Hz) for each experiment. Thus, 4 s at 15 Hz was set to 100% (See Results for details). Note that there is a steeper increase for the 4 s train duration in the relative contraction force as pulse frequency increases compared to the 2 s train duration. Color code and symbols are as in Fig. 9a.

Figure 10b

Individual data values for 4 s train duration shows a significant positive correlation as pulse frequency increases. Values were normalized to the contraction force for the first stimulus cycle with the longest train duration and highest pulse frequency (4 s; 15 Hz). The t-test for 4 s stimulus gave $p \leq 0.024$.

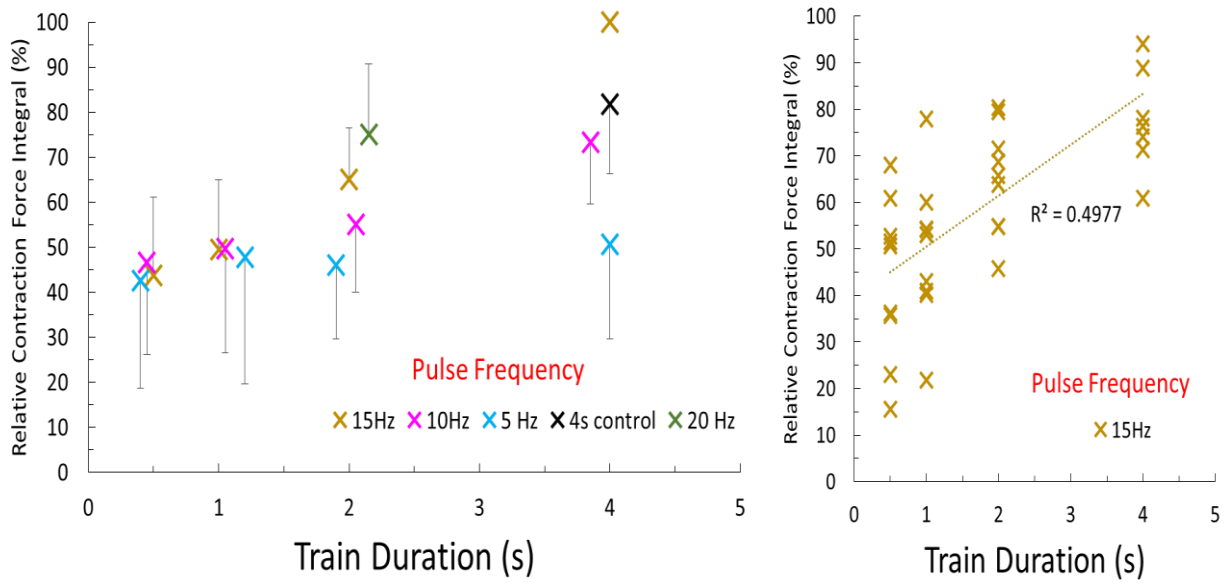

Figure 10c

Relation between the force integral of the heart contraction and stimulus train duration. Force integrals (μV x s; area under the curve) were normalized to those of the longest train duration and highest pulse frequency (4 s; 15 Hz) for each experiment. Thus, 4 s at 15 Hz was set to 100% (See Results for details). Note that at a pulse frequency of 15 Hz, the relative force integral increases as the train duration increases. Color code and symbols are as in Fig. 9c.

Figure 10d

Individual data values for 15 Hz pulse frequency shows a significant positive correlation as train duration increases. Values were normalized to the contraction force for the first stimulus cycle with the longest train duration and highest pulse frequency (4 s; 15 Hz). The t-test for 15 Hz stimulus gave $p \leq 0.034$.

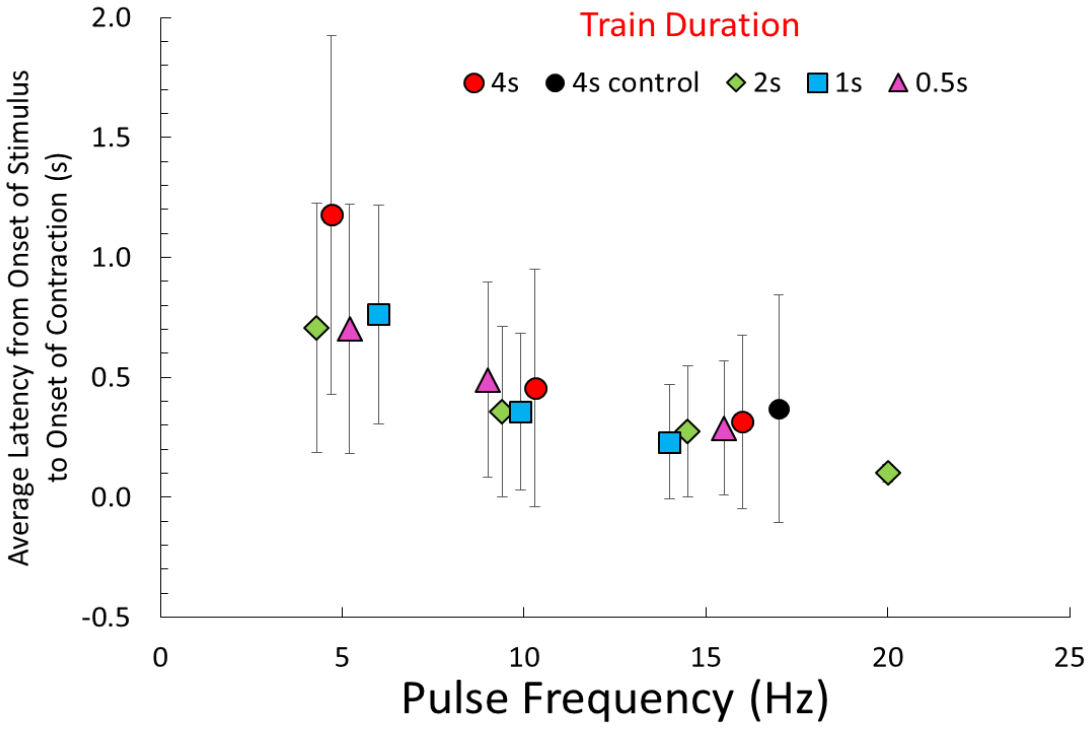

Figure 11a

The average latency from the stimulus train onset to the onset of the heart contraction does not change (p values>0.05). Color code and symbols are as in Fig. 9a.

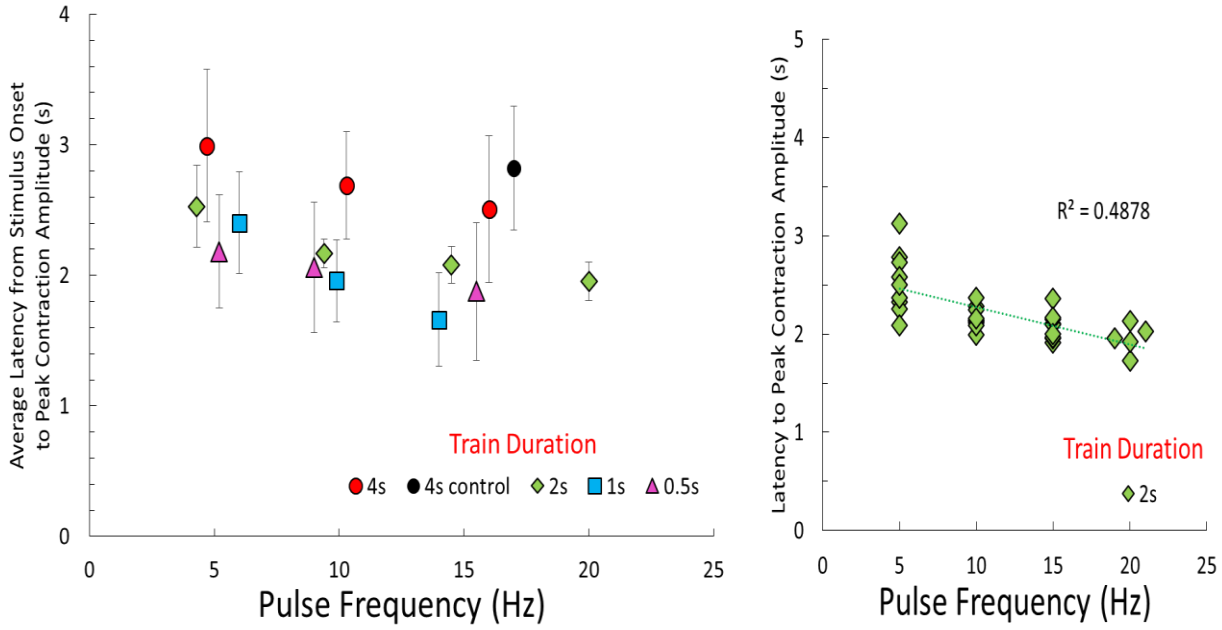

Figure 12a

Average latency from the stimulus train onset to the peak of the contraction amplitude. Note that the latency for the stimulus with a train duration of 4 s and a pulse frequency of 15 Hz did not change over the course of the experiment (compare the red with the black circle). Note that train durations of 4 s have longer latencies than those of shorter train durations. Color code and symbols are as in Fig. 9a.

Figure 12b

The latencies for a stimulus train duration of 2 s decrease as pulse frequency increases. The t-test for 2 s stimulus gave $p \leq 0.036$.

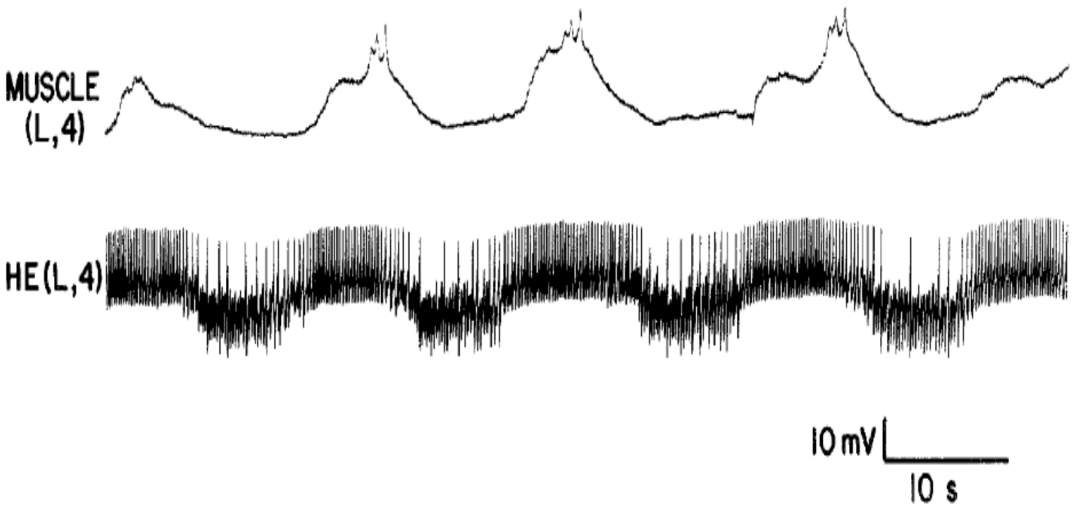

Fig. 2. Semultaneous intracellular recordings from an HE motor neuron and a heart muscle cell. These and all subsequent recordings of heart activity were taken from the in situ preparation described in Methods, unless otherwise noted

Figure 13

A figure from Maranto and Calabrese 1984. An HE motor neuron and the heart are intracellularly recorded at the same time. With each HE cell burst, the heart shows a plateau potential that would induce heart contraction.

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