Heartbeat Control in Leeches. II. Fictive Motor Pattern

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Wenning, Angela, Andrew A. V. Hill, and Ronald L. Calabrese. Heartbeat control in leeches. II. Fictive motor pattern. J Neurophysiol 91: 397–409, 2004. First published September 17, 2003; 10.1152/jn.00528.2003. The rhythmic beating of the tube-like hearts in the medicinal leech is driven and coordinated by rhythmic activity in segmental heart motor neurons. The motor neurons are controlled by rhythmic inhibitory input from a network of heart interneurons that compose the heartbeat central pattern generator. In the preceding paper, we described the constriction pattern of the hearts in quiescent intact animals and showed that one heart constricts in a rear-to-front wave (peristaltic coordination mode), while the other heart constricts in near unison over its length (synchronous coordination mode) and that they regularly switch coordination modes. Here we analyze intersegmental and side-to-side-coordination of the fictive motor pattern for heartbeat in denervated nerve cords. We show that the intersegmental phase relations among heart motor neurons in both coordination modes are independent of heartbeat period. This finding enables us to combine data from different experiments to form a detailed analysis of the relative phases, duty cycle, and intraburst spike frequency of the bursts of the segmental heart motor neurons. The fictive motor pattern and the constriction pattern seen in intact leeches closely match in their intersegmental and side-to-side coordination, indicating that sensory feedback is not necessary for properly phased intersegmental coordination. Moreover, the regular switches in coordination mode of the fictive motor pattern mimic those seen in intact animals indicating that these switches likely arise by a central mechanism.

INTRODUCTION

Rhythmic behaviors such as breathing, locomotion, and chewing are generated at least in part by rhythmically active neuronal networks, called central pattern generators that can operate in the absence of sensory feedback (Marder and Calabrese 1996). In isolated nervous system preparations, the pattern generator can produce a fictive motor pattern in motor neurons that often closely mimics the motor pattern observed in intact or semi-intact preparations. The relative roles of the central pattern generator and of sensory feedback in producing the behavior may be assessed by comparing the fictive pattern to the actual pattern (Kristan and Calabrese 1976; Pearson 2000; Wallén and Williams 1984). It is more difficult to assess the role of the central pattern generator by comparing the fictive motor pattern to the actual movements because muscular and biomechanical transformations intervene (Hooper and Weaver 2000; Morris et al. 2000; Williams et al. 1989).

The heartbeat central pattern generator in the medicinal leech has been extensively studied, and there is a wealth of knowledge about how this network of interneurons generates rhythmic activity and produces a coordinated pattern (Calabrese et al. 1995; Hill et al. 2002, 2003). Little is known about how this pattern contributes to the generation of the final motor program that drives the rhythmic pumping of the animal’s two tube-like hearts. Leech hearts are capable of producing myogenic activity but require phasic input from the heart motor neurons for proper timing and intersegmental coordination (Calabrese and Maranto 1984; Maranto and Calabrese 1984a,b). An intriguing aspect of the behavior is that the two hearts are coordinated differently and that regular switches between these coordination modes occur (Calabrese 1977; Krahl and Zerbst-Boroffka 1983; Thompson and Stent 1976a).

Although key switch interneurons have been identified, little is known about how they shift motor neuron activity phase to effect changes in coordination mode (Gramoll et al. 1994; Lu et al. 1999).

In the first paper of this series, we began the process of exploring how the heartbeat central pattern generator contributes to the behavior by describing in detail the constriction pattern of the hearts in intact, restrained, and quiescent leeches (Wenning et al. 2004). We showed that one heart constricts in a rear-to-front progression beginning at about the 14th midbody segment; this peristaltic wave (peristaltic coordination) produces high-systolic pressure and forward blood flow. The other heart constricts with a slight front-to-rear progression along its length; this near synchronous constriction (synchronous coordination) produces low systolic pressure and permits reaward blood flow. There are regular switches after which the formerly peristaltic heart becomes synchronous and vice versa.

Here we explore the fictive heartbeat motor pattern in denervated nerve cords and present evidence that the phase relations of the bursts of impulses in segmental heart motor neurons, along the nerve cord and side-to-side, match those of the phase relations found in heart constriction pattern. We also present an analysis of the duty cycle and intraburst spike frequency for the motor neuron bursts. We further show that the fictive motor pattern undergoes regular switches in the coordination pattern of the motor neurons that mimic the switches in heart coordination. Using data from the previous paper in this series (Wenning et al. 2004) obtained by recording motor neuron discharge in selected segmental motor neurons in fully innervated dissected leeches with simultaneous video monitoring of heart diameter, we provide an alignment of the fictive motor pattern and the constriction pattern.

Parts of these results have appeared in abstract form (Wenning and Calabrese 2002; Wenning et al. 2000).
Methods

Leeches (Hirudo medicinalis L.) were obtained from commercial suppliers (Leeches USA, Westbury, NY; Carolina Biological, Burlington, NC). Leeches were kept in artificial pond water at 16°C. Experiments were carried out at room temperature (20–22°C).

For all experimental procedures, leeches were anesthetized by submerging them in ice cold leech saline [containing (in mM) 115 NaCl, 4 KCl, 1.8 CaCl₂, 10 glucose, and 10 HEPES buffer and was adjusted to pH 7.4 with NaOH or HCl]. Leeches were opened dorsally.

Anatomy

To visualize the innervation pattern of the circulatory system in whole mounts (Fig. 1A), the anterior segmental nerve of one segment was wick-filled toward the periphery with 6% hexaminecobalt(III)-chloride (Aldrich, Milwaukee, WI) for 24–40 h at 6°C. The hearts and their side vessels were dissected and developed with (NH₄)₂S and silver-intensified as described in Wenning and Cahill (1986). The preparations were fixed (30 min, 4% paraformaldehyde, pH 7.4), washed in distilled water, dehydrated in an ethanol series, cleared in salicylate methylester and embedded in Canada Balsam (Merck, Germany).

Denervated nerve cord preparations

The three different heart nerves, branching off from the median anterior segmental nerve (Fig. 1A) were exposed in four to six segments by removing the crop lining, the nephridial complex with the urinary bladder, and the lateral heart in that segment. All other peripheral nerves in that segment (the posterior nerve, the distal portion of the median anterior segmental nerve, the distal portion of the anterior anterior nerve) as well as the contralateral segmental nerve roots were cut. Finally, all segmental nerves of the ganglia in those segments that were not used were severed close to the ganglion. The head and tail brains and their peripheral nerves were left attached to the otherwise denervated ganglion chain (referred to as denervated nerve cord; Fig. 1C).

Dissections were completed within 60 min. The preparation was superfused at ~4 ml/min with leech saline for the duration of the experiment. Bath volume was ~30 ml.

Extracellular recordings from the heart nerves

The activity of the heart motor neuron present in a heart nerve was recorded extracellularly using a suction electrode made of a tapered glass capillary (1 mm OD) filled with leech saline. The respective vascular nerve or Y nerve was cut, and the tip of the recording electrode broken off to accommodate the cut nerve. Recording time from a given set of two to four nerves was 10–20 min. Electrodes were rearranged to record from a different subset of nerves for another 10–20 min. Total recording time was ~60–120 min.

In a separate set of experiments, we exposed and recorded extracellularly from the soma of a heart motor neuron while simultaneously recording from two heart nerves [anterior vascular nerve (AVN) and Y nerve] in the same segment (Fig. 1B) as described for heart modulatory neurons in Wenning et al. (2004).

Data acquisition and analysis

Extracellular signals were monitored with a differential A.C. amplifier (model 1700, A-M Systems, Carlsborg, WA) at a gain of 1,000. Filter settings were between 10 Hz (high-pass) and 1 kHz (low-pass) and included a notch filter (60 Hz). A second amplifier (model 410, Brownlee Precision, Santa Clara, CA) amplified the signal to facilitate digitization.

Data were digitized with the Digi-Data (1200 Series) Interface (Axon Instruments, Foster City, CA) and acquired using pClamp software (Axon Instruments) on a personal computer with a sampling frequency of 4 kHz. To decrease the processing time of the computation-intensive analyses used here (see following text), data were resampled at 100 Hz using the Min-Max function of Clampfit (Axon Instruments) in which the smallest and largest values in successive bins of data were retained. This resampling rate introduced an uncertainty of 0.1% phase for a heart period of 10 s. These reduced data sets were then analyzed using a spike train analysis program written in Matlab (Mathworks, Natick, MA). Spikes were detected if their peaks fell within an upper window defined by two thresholds, their troughs fell within a lower window defined by two thresholds, and the difference in time between the peak and trough events (peak minus trough) fell within specified minimum and maximum values. A refractory period of 30 ms prevented extracellular spikes with complex shapes from being detected more than once. Detected spikes are shown as triangles above each record (Fig. 1D).

The analysis program was further used to determine the heartbeat cycle period, the duty cycle, and the phase for each recorded cell. Bursts were detected based on interspike intervals with long intervals indicating the beginning of each new burst. In addition, to eliminate the erroneous detection of burst due to extraneous spikes, a minimum number of spikes were required for the recognition of a burst (typically between 2 and 5). A diamond below each record indicates the median spike of a burst (Fig. 1D). The period of a given heart motor neuron x was defined as the interval (Tᵢ) between the median spikes of two consecutive bursts. Duty cycle (D) was defined as the fraction of the period occupied by a burst: D = (Tᵢ/Tₓ) × 100 where Tᵢ is the burst duration. Phase was determined on a cycle-by-cycle basis with the equation, φᵢ = (Δᵢ-10/Tᵢ) × 100, where Δᵢ-10 is the time difference between the median spike of a burst of a given neuron x and the median spike of a corresponding burst in the reference neuron, the heart motor neuron in segment 10. Tᵢ is the period of the reference neuron. A phase of 100 or 0% indicates a cell whose activity is in phase with the reference motor neuron, whereas a 50% phase indicates an antiphase (180°) relationship. A positive phase difference indicates that the activity of a motor neuron lags, whereas a negative phase difference indicates it leads the activity of the reference motor neuron. Vertical lines aid in visualizing the phase relationship in all recordings (e.g., Fig. 1D). The burst analysis program was further used to detect the number of spikes per burst and the mean spike frequency per burst.

Actograms illustrate the phase relationship between heart motor neuron bursts over time (see Fig. 2). Actograms were based on raster presentations similar to those used to display circadian activity patterns (Petersen and Calabrese 1982; Pittendrigh 1974). Each symbol represents the time of occurrence of the median spike of a burst in a heart motor neuron. The reference period of the actogram was chosen to be the mean period of an arbitrary stretch of record. This record was then broken into a series of segments of this length that were then arranged sequentially, one below the other. Periods equal to the reference period result in symbols that fall into a vertical line, while shorter periods causes a drift to the left and longer period drift to the right. For visual clarity, a duplicate copy of each segment is displayed to the right and shifted up one row.

A total of 47 preparations were analyzed. For each experiment, the average value (duty cycle, phase, period, spike frequency) for a given motor neuron was calculated. Values are expressed as means of these averages ± SD. A Student’s t-test (paired or unpaired, see text) assessed statistical significance. Segment numbers refer to midbody segments according to Kristan et al. (1974) (see Fig. 1C for convention).

Drugs

To test whether the observed phase differences among heart motor neurons along the body axis varied with period, myomodulin (Penin-
FIG. 1. A: innervation of the lateral heart and its side vessels in a left midbody segment. Heart motor neuron activity is found in 3 heart nerves: the anterior vascular nerve (AVN), the posterior vascular nerve (PVN), and the Y-nerve, all branching off from the median anterior (MA) segmental nerve. They give rise to a dense neural plexus that extends to all contractile parts (the heart, the incoming laterolateral and lateroabdominal vessels and the initial portion of the outgoing lateroabdominal vessel). Arrows indicate the direction of blood flow. AA, anterior anterior segmental nerve (see Kristan et al. 1974 for nomenclature). B: 3-point recording from the heart motor neuron soma in segment 8 on the left side [HE(L,8)] and from 2 outgoing heart nerves (Y-nerve and AVN). B1: the electrical activity of one heart motor neuron burst (41 action potentials) present in the Y-nerve (2nd trace) and in the AVN (3rd trace) when triggered from the heart motor neuron soma (1st trace). B2: the same set of action potentials but now triggered from the AVN (last trace). Traces are staggered with the 1st action potential on the bottom. C: extracellular recordings were done in denervated nerve cords shown here for 2 sites, 1 from the Y nerve in segment 8 [Y(L,8)] and one from the anterior vascular nerve in segment 10 [AVN(L,10)] on the left side. D: sample traces from the preparation shown in C to show spike train analysis. Heart motor neuron activity is characterized by well-defined bursts of action potentials. Action potentials are shown as triangles above each trace and bursts are shown as bars below each trace. The phase marker for each burst, its median spike, is shown as a symbol associated with the burst bar. The period (in s) is the interval between the median spikes of 2 consecutive bursts ($T_{10}$), shown here for the heart motor neuron of segment 10, the reference segment. The phase ($\phi$) of a given heart motor neuron, here that of segment 8, is defined as $\phi = [(\Delta t_{8,10})/T_{10}] \times 100$ where $\Delta t_{8,10}$ is the time difference between the median spike of the heart motor neuron in segment 8 and the median spike of the corresponding burst in the heart motor neuron in segment 10. See METHODS for a detailed description of the analysis.
sula Laboratories, San Carlos, CA) was added at $10^{-6}$ M to speed up the heart rate (Masino and Calabrese 2002b).

**RESULTS**

Identification of, and recording from, the nerves innervating the lateral hearts

Bilateral pairs of heart motor neurons occur in segments 3–18 (Thompson and Stent 1976a). Each heart motor neuron innervates the ipsilateral heart section in its segment of origin and extends branches along the nerve plexus on the heart (Maranto and Calabrese 1984a,b) and into neighboring segments (Jellies and Kopp 1995). We determined the heart innervation by wick-filling the segmental nerves and found that the posterior segmental nerve does not contribute. Moreover, the dense neural plexus of the hearts extends to the other contiguous contractile vessels, the two afferent (laterodorsal and -lateral) vessels and the initial portion of the outgoing (lateral abdominal) vessel, the latero-abdominal sphincter (Fig. 1A). We do not know whether the innervation of the hearts extends forward beyond segments 3 or backward beyond segment 18 nor where these vessels no longer have muscles in their vascular walls.

Two anastomosing vascular nerves branch off from the median anterior nerve (MA) distal to the nephriopore, one anterior (AVN) and one posterior (PVN). These nerves run near the heart giving rise to smaller branches extending toward the heart along the way (Thompson and Stent 1976a). The anterior-going vascular nerve of one segment is anatomically continuous with the posterior-going vascular nerve of the next anterior segment (Fig. 1A). The third nerve, the “Y-nerve,” which has not been described before, has the smallest diameter and takes off either from the median branch (MA; Fig. 1A) or from the anterior (AA) branch of the anterior segmental nerve, close to its bifurcation. Initially running in a small bundle of dorsoventral muscles, the Y-nerve eventually peels off and gives rise to an anterior-going and a posterior-going branch that feeds into the nerve plexus on the heart and side vessels. Backfills from the Y-nerve into the segmental ganglion showed among 4–8 other cells (data not shown) a cell body in the position of the heart motor neuron with its characteristic pattern of primary branches (data not shown).

The electrical activity of the individual heart motor neurons was easily recognized in these three heart nerves in segments 3–18 due to the large spikes and characteristic bursting pattern (Fig. 1C) (Thompson and Stent 1976a). We used three-point recordings, recording extracellularly from the soma of one heart motor neuron, the anterior vascular nerve, and the Y-nerve, to confirm that heart motor neuron activity is present in the newly identified Y-nerve. The example shown in Fig. 1B is a series of all 41 action potentials from a single burst of the heart motor neuron in segment 8. When sweeps were triggered from action potentials recorded at the heart motor neuron soma (Fig. 1B1, 1st trace), action potentials were then recorded first on the Y-nerve, which is closer to the soma (2nd trace), and next on the anterior vascular nerve (3rd trace). Latency increased systematically throughout the burst but similarly in both nerves. Using the same burst but triggering from the anterior vascular nerve (Fig. 1B2, 3rd trace), action potentials appeared with a fixed latency on the Y-nerve (2nd trace) and in a staggered fashion in the soma (1st trace). We attribute the progressive shift in latency to a shift in the spike initiation zone.

We identified the individual action potentials from a given heart motor neuron (Fig. 1D; triangles above each burst) as described in Methods. Action potentials were grouped into bursts based on interspike intervals and stray action potentials were eliminated (Fig. 1D; bars below each burst).

Phase relations of heart motor neurons in the two coordination modes

We first analyzed the phase relations among heart motor neurons on one body side in segments 3–18. We used a preparation in which all segmental nerves were severed and which was therefore deprived of all segmental afferent activity (Fig. 1C). The recorded efferent activity of a heart motor neuron was characterized by regular bursts of action potentials (Fig. 1D). This motor neuron discharge pattern recorded from the denervated nerve cord is referred to as the heartbeat fictive motor pattern. All recordings included the motor neuron from segment 10, which served as a reference. The recording shown in Fig. 1D from the two heart motor neurons in segments 8 and 10 also illustrates our method for determining period and phase differences.

The heartbeat fictive motor pattern displayed two coordination modes characterized by different phase relations (Fig. 2 for heart motor neurons in front segments; Fig. 3 for those in rear segments), confirming earlier results (Calabrese 1977, 1993; Thompson and Stent 1976a). The peristaltic coordination mode was characterized by a clear rear-to-front progression, whereas the synchronous coordination mode was characterized by an almost synchronous firing of the heart motor neuron bursts. The actograms associated with the recordings display only the phase markers of heart motor neurons and illustrate the regularity of the period and the stability of the phase relations encountered in our recordings. These actograms also demonstrate that switches occurred regularly between these two coordination modes.

To compare different preparations effectively, it was first necessary to demonstrate that the phase relations among motor neuron in the two coordination modes did not co-vary with period, which varied considerably across preparations. The maintenance of constant intersegmental phase differences across changes in cycle period, phase constancy, is an important feature of many segmentally organized motor patterns such as lamprey swimming (Grillner et al. 1993; Sigvardt 1993), leech swimming (Friesen and Cang 2001), and crayfish swimmeret beating (Mulloney et al. 1993; see Hill et al. 2003 for a recent review). Our measurements of heart constriction in intact juvenile leeches suggested that phase constancy is a property of leech heartbeat (Wenning et al. 2004). Figure 4 shows that the phase relations of motor neurons in the fictive motor pattern appear independent of period over a broad range. In the denervated adult preparations used here, the period of the fictive motor program varied from ~6 to 15 s, average 9.1 ± 1.5 s.

This demonstration of phase constancy not only allowed us to construct the composite phase diagram of Fig. 5 but also permits us to compare the fictive motor program to the constriction measurements made in the previous paper (Wenning et al. 2004). Those studies were performed in intact juvenile leeches.
and adult leeches; the intersegmental phase relations of their constriction patterns were also independent of period. Juveniles had substantively shorter periods (~4–6 s) as compared with adult leeches (~7–15 s) and the denervated nerve cord preparations used here (see preceding text).

The phase diagram shown in Fig. 5 summarizes the average phase relations with respect to the reference heart motor neuron in segment 10 as well as the average duty cycles. To analyze these differences, we used pair-wise comparisons to determine which motor neurons were different from the reference motor neuron.

In the peristaltic coordination mode, heart motor neurons traverse 63% of phase in a rear-to-front progression between segments 15 and 18. The rear-to-front progression starts in segment 15 and proceeds with gradually increasing segmental phase steps. The motor neuron of segment 12 is the first posterior one to lead that of segment 10 significantly, and the motor neuron of segment 8 is the first anterior one to lag significantly with respect to that of segment 10 (Fig. 5). Phase differences between heart motor neurons are ~10% between segments 7 and 6, 7% between segments 6 and 5, and 11% between segments 5 and 4. The heart motor neurons of segments 3 and 4 are nearly in phase (Fig. 5).

In the synchronous coordination mode, heart motor neurons traverse 43% in a front-to-rear progression. Most of the phase progression occurs in the front and the rear segments. While heart motor neurons of segments 3 and 4 are nearly in phase, the motor neuron of segment 3 leads that of segment 5 by ~12% (Fig. 5). Heart motor neurons of segments 5–14 fire nearly in phase. Between segments 15 and 18 intersegmental phase differences get gradually larger with a conspicuous jump in phase by the heart motor neuron in segment 18. This phase difference is ~15% and is the largest phase difference observed between neighboring segments (Fig. 5).

In our recordings, heart motor neuron activity was robust and regular for hours. An exception was the heart motor neuron in segment 3. Bursting activity was detected in only 7 of 21 preparations, and it was active, it fired more reliably on the synchronous side (Fig. 6).

Side-to-side coordination

To establish the phase relations of the fictive motor pattern between the sides, we recorded from the bilateral pair of reference heart motor neurons (segment 10). Figure 7 shows a recording in segments 9, 10, and 13 on the left side and in segment 10 on the right side (green). The mean phase difference between these two reference heart motor neurons was 62.0 ± 6.7% with the peristaltic side leading (n = 6; ranging from 55.0 to 70.4%). In the bilateral phase diagram (Fig. 8), the median spike of the burst in the heart motor neuron in segment 10 on the peristaltic side was assigned 0% phase and that on the synchronous side +62%. To better show the phase relations in the rear segments, the phase diagram of the peristaltic side is duplicated and shifted by 100%.

Heart motor neuron discharge comes together in phase in the front and in the rear with the synchronous heart motor neuron 4 bursting slightly later than that of the peristaltic side. In the rear, the heart motor neurons come increasingly closer together in phase with the synchronous side leading up to segment 16. The heart motor neurons of segment 17 fire almost simultaneously. Phase relations reverse in segment 18 due to the conspicuously large segmental phase step of the heart motor neuron on the synchronous side. The heart motor neuron in segment 18 on the synchronous side lags the peristaltic side by ~15% (Fig. 8).

The bilateral phase diagram predicts that the heart motor neurons in midbody segment 8 should burst in antiphase (Fig. 8), and consequently, their phase relations should not change across switches in coordination modes. This prediction is borne out by the recording shown from the bilateral pair of heart motor neurons in segment 8 (Fig. 9).
Heart motor neuron burst architecture in the two coordination modes

Heart motor neurons in the anterior and posterior segments had shorter duty cycles than those in the midbody (Fig. 5). Along the body axis, between segment 4 and 11, mean duty cycles doubled in both coordination modes. When comparing the same heart motor neuron in the two coordination modes, the duty cycles in segments 4 and 5 were on average longer (Figs. 2, 3, and 5; \( P < 0.01 \); paired \( t \)-test), and that in segment 18 was shorter in the synchronous mode (Fig. 5; \( P < 0.01 \)).

We determined average intraburst spike frequencies and the number of spikes per burst for the heart motor neurons in both coordination modes (Table 1). Spike frequencies in segments 4, 5, 10, and 18 were not different between the two coordination modes (ANOVA, \( P > 0.05 \)), but intraburst frequencies were significantly higher in front segments 4 and 5 and in rear segment 18 compared with segment 10 (ANOVA post hoc; \( P < 0.01 \) for all tested segments). The higher spike frequency at the two ends compensates for the shorter duty cycle so that spike number per burst remained approximately equal.

Matching the heart constriction pattern to the heartbeat fictive motor pattern

Leech hearts require phasic input from the heart motor neurons for coordination, timing, and switching between the two coordination modes. To understand how the motor pattern is executed at the level of constrictions of the hearts, we need to map the fictive motor pattern onto the constriction pattern described for intact leeches (Wenning et al. 2004).

Each burst in a heart motor neuron leads to a constriction in the corresponding heart segment (Maranto and Calabrese 1984b). Using simultaneous video imaging of a heart section and en passant recording from its corresponding heart motor
neuron, we determined when a heart segment constricts with respect to the burst of its corresponding heart motor neuron for segments 4, 7, 10, and 14 in innervated, dissected preparations (described in Wenning et al. 2004). We showed that the phase relation between the median spike of the heart motor neuron burst and the heart constriction (systole) is consistent within and across preparations and the same in both coordination modes, but it varies from segment to segment along the body axis (Wenning et al. 2004).

Because these preparations were fully innervated, we were able to determine the outlines of the heartbeat motor pattern with afferent feedback. This innervated motor pattern is shown for the motor neuron in segments 4, 7, and 14 (all with respect to a simultaneously recorded motor neuron in ipsilateral segment 10) in Fig. 10 (dark gray bars). Because it is similar to the fictive motor pattern which itself is independent of period, we superimposed the patterns by aligning the median spikes of the reference motor neurons in segment 10.

Figure 10 also shows the alignment of the constriction pattern of intact adult leeches with the fictive motor pattern. The phase relations within and among segments match closely in these patterns, and similar matching was obtained using the constriction pattern of intact juvenile leeches (data not shown). We chose the phase relation between systole and the median spike in segment 4 to align the fictive motor pattern with the constriction pattern in intact adult leeches because this alignment gave the greatest congruence between the patterns (Fig. 10). The other possible alignments placed constrictions in the

![Fictive Heartbeat Motor Pattern](image)

**FIG. 5.** Phase diagram of the heartbeat fictive motor pattern showing the duty cycles and the phase relations of the heart motor neurons activity along the body axis for segments 3–18 (indicated on the left) for the peristaltic coordination mode (dark boxes) and the synchronous coordination mode (light boxes). Normalized duty cycles are displayed as box plots. Each box represents the average duty cycle of heart motor neuron bursts from different recordings with the number of preparations for a given motor neuron indicated on the right (N; left number: peristaltic coordination mode; right number: synchronous coordination mode). Error bars represent the SD of these averages for the beginning and the end of the burst as well as for the median spike (vertical line inside each box). The heart motor neurons of segment 10 served as the phase reference (bold boxes). Their median spikes (phase markers) were assigned 0 phase (emphasized by the vertical dotted lines). Small asterisks in the boxes indicate differences between the lengths of the duty cycles with respect to segment 10 (unpaired t-test; *P < 0.05). Large asterisks placed near the median spike note the 1st occurrence of a statistically different phase with respect to segment 10. For example, on the peristaltic side, segments 8 and anterior lag significantly, and segment 12 and posterior lead, segment 10. On the synchronous side, segments 3 and 4 lead, and segments 14 and posterior lag, segment 10 (unpaired t-test; *P < 0.02). The mean period was 9.1 ± 1.5 s. Data are from 47 preparations.

![Recordings of heart motor neuron activity](image)

**FIG. 6.** Recordings of heart motor neuron activity from the heart nerves in segments 3 and 10 (left body side) across a switch in coordination modes. The heart motor neuron of segment 3 fires only occasionally in the peristaltic coordination mode but is more active in the synchronous mode. Its bursts are considerably shorter than those of the motor neuron in segment 10.Abbreviations and symbols: see Fig. 1. Mean cycle period: 7 s.
and motor neuron bursts in segment 10 (Fig. 10) are both ~60%, for example. Front and rear segments come together in phase both in the fictive motor pattern (Fig. 8) and in the constriction pattern in intact animals (see Fig. 8 in Wenning et al. 2004). Segments 3 and 4 on the peristaltic side lead slightly those on the synchronous side, and segments 16–17 on the synchronous side lag slightly those on the peristaltic side. In segment 18, however, the conspicuous “crossing over” observed in the fictive motor pattern—with the peristaltic side now leading the synchronous side—was not apparent in intact leeches (Fig. 8, this study) (see also Fig. 8 in Wenning et al. 2004).

We conclude from these results that the fictive heartbeat motor pattern closely matches the constriction pattern of the heart in intact restrained leeches and that this centrally generated motor pattern may therefore play the dominant role in timing heartbeat.

Switches in coordination mode

Switches in coordination mode are regular, reciprocal, occur about every 13–36 heartbeat cycles, or 102–348 s, and need only one or two cycles to complete. Similar switch characteristics are observed for heart constriction in intact leeches, blood pressure profiles recorded in quiescent, minimally dissected leeches (Krahl and Zerbst-Boroffka 1983; Wenning et al. 2004), and the electrical activity seen in heart interneurons in isolated ganglia chains consisting of as little as one segmental ganglion (Calabrese 1977; Lu et al. 1999). Long-term anterior heart segments before the onset of the burst of the corresponding heart motor neuron. Mean periods were similar in the fictive motor pattern (9.1 ± 1.5 s) and in intact adults (10.0 ± 3.5 s) (Wenning et al. 2004).

In the peristaltic coordination mode, the fictive motor pattern and the constriction pattern in the hearts of intact animals traverse the same phase difference between segments 15 and 4 (Fig. 10). Moreover, they faithfully match the intersegmental phase relations. We attribute the larger variation in the constriction pattern in the rear (segments 16–18) to more irregular constrictions and more error in our measurements due to low contrast in the video recordings (Wenning et al. 2004).

The constriction pattern and the fictive motor pattern are also similar in their side-to-side coordination. The phase difference between the bilateral hearts constrictions (Wenning et al. 2004)
TABLE 1. Number of spikes per heart motor neuron burst and intraburst spike frequencies

<table>
<thead>
<tr>
<th>Midbody Segment (No. of Preparations)</th>
<th>Peristaltic Coordination Mode</th>
<th>Synchronous Coordination Mode</th>
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<tbody>
<tr>
<td></td>
<td>No. of Spikes per Burst</td>
<td>Intraburst Spike Frequency, Hz</td>
</tr>
<tr>
<td>4 (10)</td>
<td>23.5 ± 3.2</td>
<td>11.8 ± 2.9</td>
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<tr>
<td>5 (7)</td>
<td>24.9 ± 8.2</td>
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<td>7 (8)</td>
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<td>7.8 ± 1.2</td>
</tr>
<tr>
<td>9 (3)</td>
<td>25.3 ± 2.5</td>
<td>5.8 ± 0.5</td>
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<tr>
<td>10 (27/30)</td>
<td>26.9 ± 7.3</td>
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<td>13 (1/2)</td>
<td>19.9</td>
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<tr>
<td>15 (1)</td>
<td>29.1</td>
<td>5.3</td>
</tr>
<tr>
<td>16 (1)</td>
<td>31.0</td>
<td>6.6</td>
</tr>
<tr>
<td>17 (2)</td>
<td>37.8</td>
<td>7.3</td>
</tr>
<tr>
<td>18 (665)</td>
<td>29.7 ± 8.2</td>
<td>9.6 ± 1.4</td>
</tr>
</tbody>
</table>

Values are means ± SD.
the fictive motor pattern, this co-variation was not observed. Here, the switch period increased significantly even though the heartbeat period decreased slightly by $\frac{\text{H}11011}{\text{H}11006}$ 4 – 13% (control: $\frac{\text{H}11006}{\text{H}11006}$ 179 s; myomodulin: 218 s; $P = 0.03$; $n = 5$).

**DISCUSSION**

**Fictive motor patterns and behavior**

Stereotyped rhythmic behaviors such as breathing, locomotion, and chewing arise in part through the actions of central pattern generators. Those who study these oscillatory neuronal networks face the challenge of relating fictive motor activity to behavior. Sensory feedback signals the realization of rhythmic patterns and adjusts movements to the prevailing external conditions, providing smoothness and flexibility to movements (Pearson 2000). Therefore the fictive motor pattern may not accurately represent the motor output that actually produces the behavior. On the other hand, fictive motor patterns are often very close to the motor pattern observed in intact unperturbed animals (Wallén and Williams 1984). The degree to which sensory feedback modifies the fictive pattern on a cycle-by-cycle basis may be related to the environmental heterogeneity encountered when performing the behavior. Thus for swimming in lampreys, sensory input generated by the swimming movements appears to play a reinforcing modulatory role (Ekberg and Grillner 1999; Wallén 1997), but for insect flight and terrestrial locomotion in insects and mammals, such feedback provides critical timing cues (Pearson 2000; Pearson and Ramirez 1997). The situation for leech swimming presents an interesting contrast; sensory feedback is more or less reinforcing for the basic alternating pattern between dorsal and ventral motor neurons within a segment, but proper intersegmental coordination (phase delays) requires sensory feedback (Cang and Friesen 2000, 2002; Yu et al. 1999). The dependence on feedback for intersegmental coordination may reflect the unique problems associated with a hydrostatic skeleton as in leeches.

Here we demonstrated that the fictive motor pattern for heartbeat closely matches the constriction pattern of the two hearts. Both intersegmental and side-to-side coordination among motor neurons show similar phase relations to the constriction pattern of segmental heart-tube sections (Fig. 10). This finding indicates that sensory feedback is not necessary for coordination of heart motor neurons and that it arises from the temporal pattern of activity from the heartbeat central pattern generator and the spatial pattern of its synaptic connectivity with heart motor neurons. Sensory influences on the heartbeat pattern generator appear to be mainly modulatory, adjusting heartbeat period (Arbas 1984; Davis 1986), and no receptors capable of providing phasic sensory feedback about cardiac performance have been described. On the other hand,
intersegmental phase relations were less variable in the fictive motor pattern than in the constriction pattern (compare Figs. 5 and 10); this may indicate a role for sensory input in producing variation in the motor program. The timing of the constriction in an individual heart segment with respect to the corresponding heart motor neuron burst might additionally depend on muscle dynamics and the hydrodynamics of the vascular system. Earlier reports on blood flow in minimally dissected leeches stress that movement of the body wall cause perturbations in the local circulation (Hildebrandt 1988).

### Phase constancy

Many wave-like behaviors such as swimming in lamprey (Ekeberg and Grillner 1999; Wallén and Williams 1984) and swimmeret beating in crayfish (Mulloney et al. 1993, 1998) are characterized by having central pattern generators that produce equal phase lags between successive sets of segmental motor neurons. These phase lags are maintained in the face of considerable changes in cycle period and thus assure a constant number of wavelengths in the motor pattern and ultimately in the behavior. Such systems are said to be phase constant. It is tempting to analogize the wave-like peristalsis of leech hearts to these behaviors. Nevertheless, the heartbeat central pattern generator appears to share a property with these others: phase constancy. The intersegmental phase differences among motor neurons in the fictive motor pattern for both coordination modes are independent of period (Fig. 4), and our limited data on the constriction pattern in intact animals suggest the same independence from period (Wenning et al. 2004). Therefore the same global pattern of heart constriction is maintained despite changes in period, as one might expect for efficient circulation in a closed system.

Previous studies of phase relations among the interneurons of the central pattern generator have focused on the two segmental oscillators located in the third and fourth segments (Hill et al. 2002; Masino and Calabrese 2002a–c). This timing kernel of the network is characterized by highly flexible phase relations between the oscillators. It will be interesting to determine how this flexibility is harnessed to produce the more stable fictive motor program.

### Switching

The regular switching in coordination modes observed here in the fictive heartbeat motor pattern (Table 2) has been the focus of intensive investigation. Key heart interneurons, which implement this switch, have been identified (Calabrese 1977; Gramoll et al. 1994; Lu et al. 1999), but little is known about the timing of these switches. Lu et al. (1999) concluded from limited recordings of switch interneurons during spontaneous switches that the timing arose outside the identified neurons of the heartbeat pattern generator and possibly from an independent oscillatory network. Our work here with entire denervated nerve cords suggests that switch period scales with heartbeat period (Fig. 12) [this scaling was not observed previously in short chains of ganglia (Lu et al. 1999)], which could be interpreted as a link between switch timing and heartbeat timing. On the other hand, we did observe that in those denervated nerve cords where heartbeat period was accelerated by application of myomodulin that switch period paradoxically elongated, suggesting independent timing. Although the ori-

![Figure 12. Correlations between the switch period, the heartbeat period, and the number of heartbeat cycles per switch period of the fictive motor pattern are shown for 117 switch cycles from 46 animals. The correlation between the switch period and the heartbeat period is also shown for intact juvenile leeches (△: 50 switch cycles from 9 animals) with their heartbeat period scaled to that of the adults (factor: 1.94).](image-url)
gins of switch timing must therefore remain an open question, the results presented here support the previous conclusions that switches do not require sensory input, are regular, and can be centrally mediated (Calabrese 1977; Gramoll et al. 1994; Lu et al. 1999).

Matching the fictive motor pattern and the constriction pattern

A primary goal of our research is to determine how the central pattern generator for heartbeat contributes to the coordinated contractions of the hearts and thus circulation. In the first paper of this series, we were able to define the constriction pattern in intact juvenile and adult leeches that were physically restrained and quiescent (Wenning et al. 2004). In this paper, we define the fictive motor pattern in denervated nerve cords. To meld these two patterns, we made en passant recordings from heart motor neurons with simultaneous video-monitoring of the heart in selected segments in dissected leeches in which the dorsal vessel was damaged, and thus over time the blood in all the major vessels and heart was replaced by saline presumably through the action of the hearts. Under these conditions, it is doubtful that the hearts experience normal loads. These recordings allow us to align motor neuron discharge to constriction (movement) in each segment for an innervated preparation, but one that experiences uncertain loading and that is unlikely to provide appropriate feedback should the receptors for such feedback be present. The pattern of discharge of the selected motor neurons in these innervated preparations matches the fictive motor pattern well, and we aligned this innervated motor pattern to the fictive motor pattern by aligning the median spikes of the reference heart motor neurons (segment 10; Fig. 10; dark gray bars). To align the motor patterns to the constriction pattern required a decision because each of the selected segments led to a different alignment. The differences in alignment arise because the latency from the start of motor neuron discharge to the beginning of constriction varied along the body in the segments selected (Wenning et al. 2004). We chose to make this alignment in segment 4 because that alignment led to the greatest congruence between the patterns. This alignment emphasizes the similarities but is by no means definitive because should we have chosen to align the patterns in segment 10, then this congruence would be destroyed but the similarity in structure would still remain. To align these patterns by congruence while convenient may not be correct because the pattern of motor discharge and movement are not always congruent in intact animals due to biomechanical factors. For example, in the intact swimming lamprey, the motor pattern traverses about one wavelength along the body (100% of phase) but the body traverses only about three-fourths of a wavelength (75% of phase) (Williams et al. 1989). To fully understand the relation of the motor pattern to the constriction pattern will require simultaneous recordings of motor neuron discharge and monitoring heart performance in minimally dissected leeches. Nevertheless, our results show that the fictive motor pattern comprises all the elements necessary for the proper timing, coordination, and switching in the beating of the two lateral hearts.

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References


