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Dynamic Analysis of a Rhythmic Neural Circuit in the Leech *Hirudo medicinalis*

ERIC L. PETERSON AND RONALD L. CALABRESE

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

SUMMARY AND CONCLUSIONS

1. The results of perturbation experiments demonstrate the functional diversity of the interneurons (HN cells) that generate heartbeat in the medicinal leech.

2. HN cells were individually stimulated by single current pulses. The induced activity of HN cells in the first four ganglia (cell pairs HN(1)–HN(4)) reset the rhythm of the interneuron network; induced activity of those in the fifth through seventh ganglia (cell pairs HN(5)–HN(7)) did not.

3. Cells HN(1)–HN(4) can entrain every other interneuron of the network; cells HN(5)–HN(7) cannot.

4. Thus the HN interneuron network includes two distinct subsets: cells HN(1)–HN(4) form the network's timing oscillator; cells HN(5)–HN(7), driven by the timing oscillator, force one of the two coordination states on the heart motor neurons.

5. In general the dynamic behavior of the heart interneuron network was predictable given the web of identified synapses between HN cells. Nevertheless, the unexpected capacity of cells HN(3) and HN(4) to entrain the network shows that there are functional connections still to be found. Burst termination experiments suggest that cells HN(3) and HN(4) inhibit directly the more rostral HN cells.

6. The timing oscillation seems to arise from a balance between the endogenous polarization rhythms of interneurons HN(1)–HN(4) and selective reciprocal inhibition between these same cells.

INTRODUCTION

The central nervous system (CNS) of the leech *Hirudo medicinalis* consists of a head-brain, a chain of 21 segmental ganglia, and a tail-brain, linked in series by connectives. In each segmental ganglion (except the first two and the last two) there is a bilateral pair of motor neurons, the HE cells. The coordinated cyclic activity of this set of HE motor neurons drives, by direct phasic excitation, the constriction of the two lateral heart tubes (5). The heartbeat motor pattern persists in the isolated CNS, sustained by the interaction of a set of rhythmically active, intersegmental interneurons, the HN cells, of which a bilateral pair is found in each of the first seven segmental ganglia.

Although every HN interneuron has its own distinctive structure (17), each sends a single axon caudally out the ipsilateral connective. Cells HN(3)–HN(7) (the HN cells originating in the third through seventh segmental ganglia) normally initiate impulses and receive inhibition in their ganglion of origin, although each has secondary initiation sites in more caudal ganglia that become operational if the primary site is inactivated (7). By contrast, cells HN(1) and HN(2) have active initiation sites only in the third or fourth ganglion (4, 23). With the exception of the HN(5) cells, each heart interneuron continues to produce rhythmic bursts when freed from synaptic inhibition in low-Cl^- saline (6).

Stent et al. (19) present a model that draws on the known intrinsic properties and synaptic connections (Fig. 1*A*) of the HN cells to explain the collective functioning of the network. The proposed circuit shows a clear hierarchy: phasic inhibition flows rearward, cascading from cells HN(1) and HN(2), to cells HN(3) and HN(4), then to cells HN(5), and finally to cells HN(6) and HN(7). In fact, the only forward-going in-

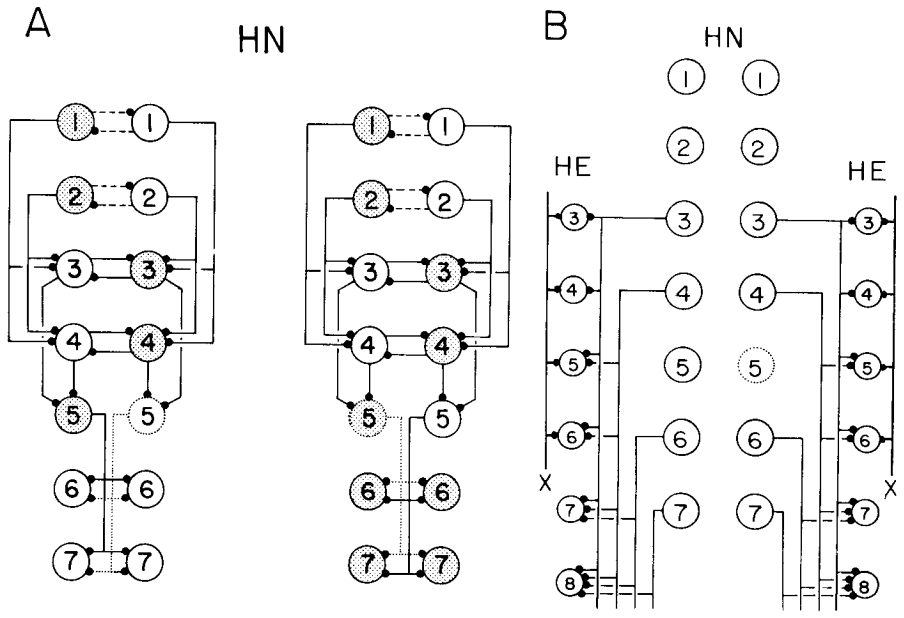

FIG. 1. Model of the heartbeat pattern generator (after Stent et al., Ref. 19). *A:* the two coordination states of the heart interneuron network. In each diagram the 14 circles represent left and right cells HN(1)–HN(7). A solid line leaving one HN cell and ending with a solid circle on another is an identified inhibitory synapse (4, 23). Cells with matching shading (white or stippled) are roughly in phase; those with contrasting shading are in antiphase. As shown the network oscillates in one of two metastable coordination states; in each state one HN(5) cell is totally inactive (dotted outline and connections). The agency that permits only one HN(5) cell to fire is unknown and appears not to involve any of the identified heart interneurons (4). The crucial features of the model are: *1*) All interneurons except cells HN(5) have endogenous polarization rhythms (6). *2*) Cells HN(1) and HN(2) are each linked with contralateral homologues by reciprocally inhibitory synapses (dashed lines), which coordinate these cells to generate an oscillation driving the entire system (19). (These connections were postulated to account for the observed phase relationships between these cells and have not been demonstrated directly.) *3*) There is reciprocal inhibition between HN(3) cells and between HN(4) cells. In the model this reinforces the pattern imposed by cells HN(1) and HN(2). *4*) Otherwise the pattern of inhibition is strictly front to rear, with no cell inhibiting a more rostral cell. The rhythm of the more rostral cells is imposed on cells HN(6) and HN(7) via the active HN(5) cell. *B:* the inhibitory synapses between HN interneurons and HE motor neurons in the first eight segmental ganglia. In the absence of inhibitory input the HE cells fire tonically (6). For future reference note that of the identified interneurons cell HN(3) alone inhibits motor neurons HE(3) and HE(4) and that all HN cells with connections to motor neurons inhibit the ipsilateral cell HE(8). An HN(X) unit (see text) on each side of the network is activated by the ipsilateral HN(5) cell and produces IPSPs in ipsilateral motor neurons HE(3)–HE(6) and excitatory postsynaptic potentials (EPSPs) in ipsilateral interneurons HN(3) and HN(4).

fluence of any sort is the activation by cell HN(5) of HN(X), an unidentified unit that excites ipsilateral cells HN(3) and HN(4) (4, 23). As we shall show, this unit has negligible influence over the timing of bursts in the identified HN cells.

Thus the model makes testable predictions regarding the dynamic relationships between HN cells, the most general being that whereas the activity of the given HN interneuron might affect other HN cells in the same or a more caudal ganglion, it should have no effect on more rostral cells. In particular, following any perturbation in the timing of bursts, the network should be entrained to the ongoing cycle of the most rostral interneurons, cells HN(1) and HN(2).

METHODS

Preparation, recording, and stimulating techniques

Leeches (*Hirudo medicinalis*) obtained from commercial suppliers were maintained at 15°C in aquaria for up to 6 mo prior to use. Unless otherwise specified, we used an isolated CNS preparation including the head-brain and at least the first eight segmental ganglia, bathed in leech physiological saline (10). Each neuron of the heartbeat network could be identified unambig-

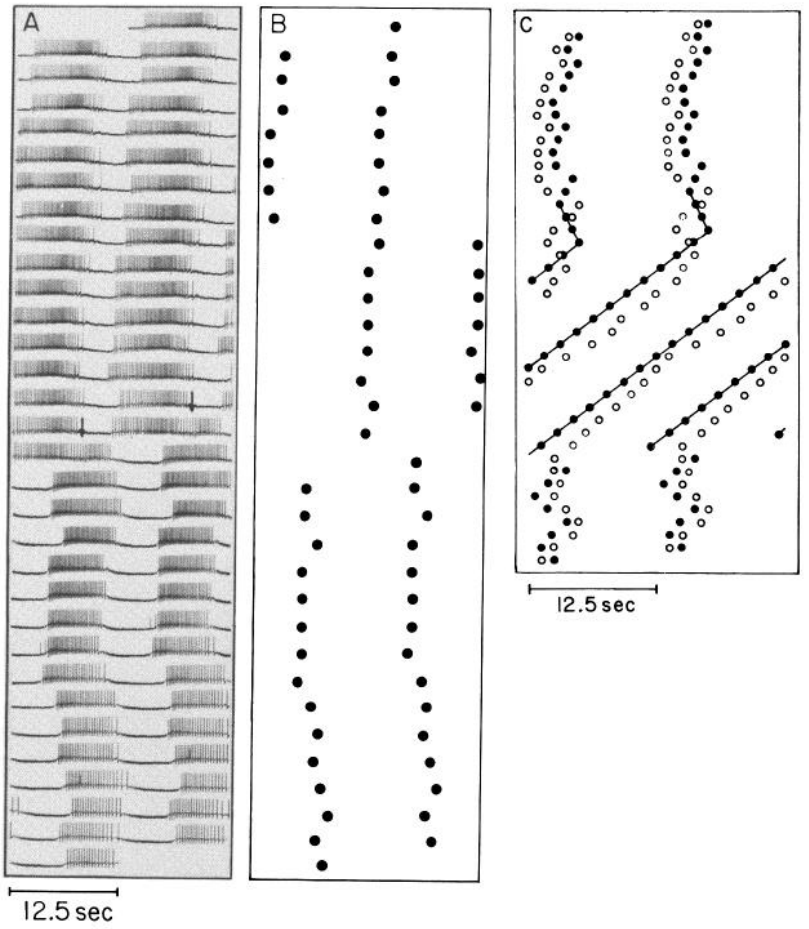

FIG. 2. Explanation of the plotting method for entrainment experiments. *A:* the left portion of the panel consists of consecutive 12.5-s segments of a continuous intracellular record of an HN(7) cell, aligned one under the other. The right portion is a duplicate, shifted up one cycle, added to preserve visual continuity. At the indicated time (arrows) a change of coordination state occurs and, as described in Fig. 1*A*, the phase relationship between cell HN(7) and interneurons HN(1)–HN(4) is thereby inverted. Because the absolute phase of the rostral cells is invariant with state transitions (4), cell HN(7) shows a 180° phase shift. *B:* each dot records the position of the first impulse in each burst of the record of panel *A*. The horizontal distance between dots is the cycle period. *C:* two records like that of *B*, obtained simultaneously from different cells, are superimposed. In the top third of the figure the impulse bursts of one cell (HN(L,4), filled circles) are nearly in phase with those of another cell (HN(L,3), open circles). (The phase angle between cells is the horizontally measured time interval between dots divided by the burst cycle period.) In the central third of the figure cell HN(4) is driven by a train of current pulses (solid line). Both cells are entrained to the stimulus as slopes of both sets of dots shift to match that of the stimulus. On release from entrainment the cells revert to the prestimulus period and phase relationship. (The intracellular records of a portion of this experiment are presented later (Fig. 11*A*).

uously by its characteristic soma position and physiology (4, 21–23).

Intracellular recording techniques were standard (21); current pulses were injected through the recording electrode via a bridge circuit. In resetting experiments we depolarized a given HN cell, causing it to fire at or above the intraburst frequency even during the interburst barrage of synaptic inhibition. In entrainment experiments, an individual HN cell was driven with repeated depolarizing pulses comprising 25–50% of the driving cycle. In each case that we report a negative result for entrainment, the experiment was repeated, with a range of entrainment frequencies slightly faster than the free-running rate, in at least three different preparations.

Presentation of entrainment experiments

To illustrate the response of the heart interneuron network to entrainment, we adopt a raster presentation often used to display circadian activity rhythms (e.g., Ref. 16). First we scan the chart record of the experiment and estimate the approximate heartbeat period. Using this estimate as the "reference cycle," we then cut the continuous record into consecutive intervals of that length and align these successive cycles one under the other at the left. Finally, to help the eye bridge the gap between the end of one reference cycle and the beginning of the next, we duplicate the entire record, shift the second copy up one cycle, and place it to the right of the first, as shown in Fig. 2*A*. In the reduced presentation of Fig. 2*B* only the burst onset times are marked. The value of this latter presentation is that one can superimpose such diagrams for different interneurons and thus display graphically their functional correlation (Fig. 2*C*). Note that if cell and reference cycle periods are identical, successive burst markers fall in a vertical column. If the column drifts to the left, the cell's period is shorter than that of the reference cycle; if, as near the bottom of Fig. 2*B*, the column drifts to the right, the cell's period is longer. As Fig. 2*A* shows, a given preparation at a fixed temperature cycles at a fairly constant rate (the coefficient of variation in period for successive cycles being less than 10%) (7).

In order to minimize damage to the HN cells, their impulses were often monitored indirectly via the corresponding inhibitory postsynaptic potentials (IPSPs) in an appropriate HE cell (Fig. 1*B*).

RESULTS

Functional diversity of heart interneurons revealed by resetting experiments

We assessed the contribution of each heart interneuron to the generation of the motor pattern by testing that cell's capacity to reset

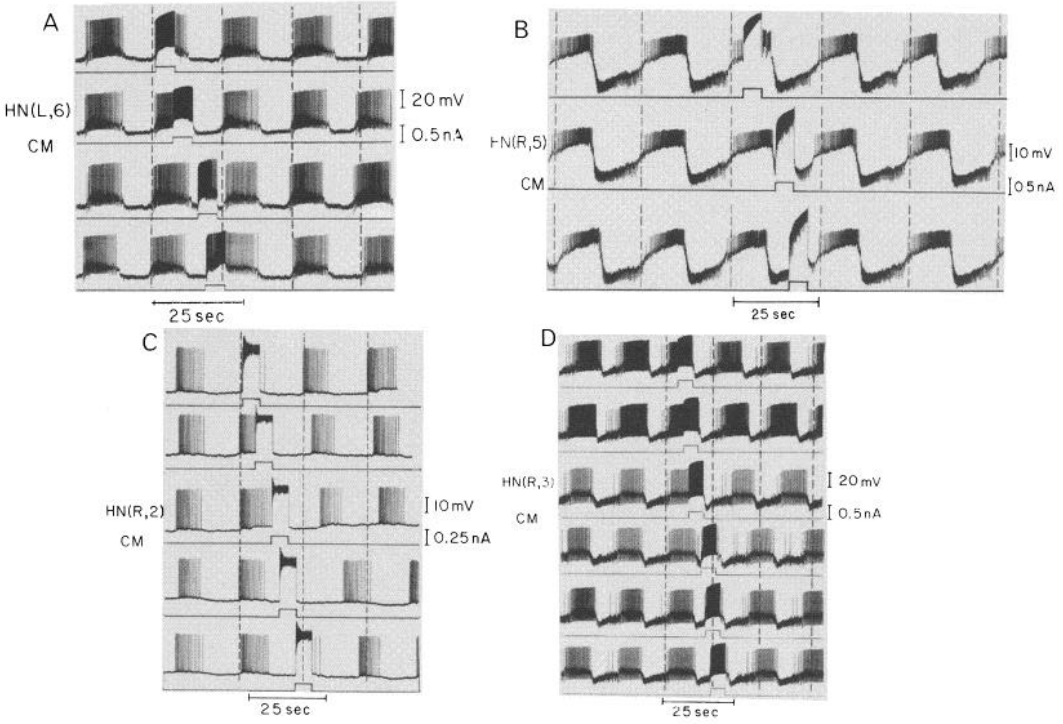

FIG. 3. Resetting the heart interneurons with injected current pulses. Each frame comprises a set of trials in which a single depolarizing current pulse timed to fall at selected phases of the burst cycle was injected via the recording electrode into an HN cell. Each record is aligned to bring the prestimulus bursts into register; the dashed vertical lines mark bursts predicted in the absence of a stimulus pulse. (In this figure and Figs. 5 and 6, trials were not necessarily conducted in the sequence presented.) *A:* HN(6); and *B:* HN(5) cells. Pulses elicit vigorous spike volleys but cause no phase shifts. *C:* HN(2), and *D:* HN(3) cell. The current pulses cause strong resetting. (Comparable experiments for cells HN(1), HN(4), and HN(7) are reported schematically in Figs. 5 and 6.)

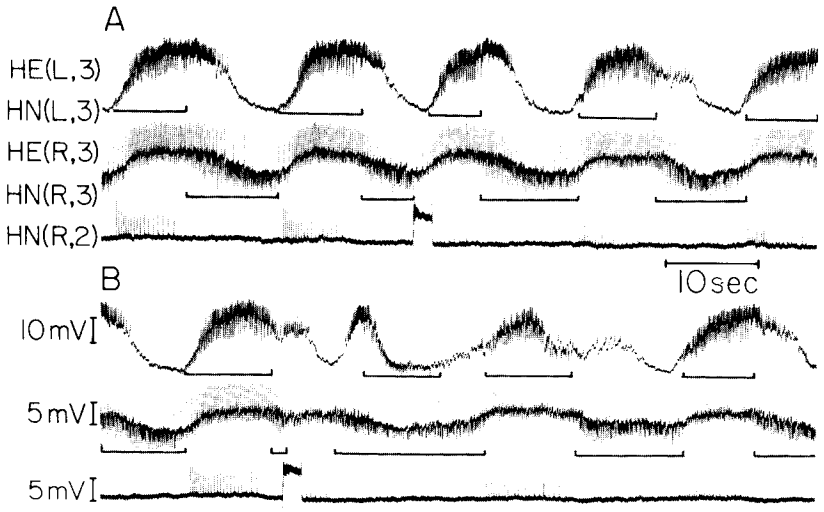

FIG. 4. Phase shifts of single HN cells reflect shifts of the entire network. *A* and *B* form a continuous record. *A:* horizontal bars bracket bursts of the respective HN(3) cells, deduced from their IPSPs in the ipsilateral HE(3) cells. A 2-s depolarizing current pulse given to cell HN(2) late in its cycle excited a volley of spikes that truncated bursts of the ipsilateral HN(3) cell and thereby activated the contralateral HN(3) cell. The net result was a stable phase advance. In all cases studied, whenever one component of the network reset the entire system shifted uniformly. *B:* a 2-s pulse given near phase 0.6 caused severe dislocation. The volley from cell HN(2) interrupted cell HN(R,3)'s burst and for a 4-s hiatus neither HN(3) cell fired. Cell HN(R,3) then resumed its burst, only to be joined a few seconds later by cell HN(L,3). Out of the ensuing confusion the normal oscillation emerged, then stabilized by the end of the record.

the entire heartbeat rhythm when stimulated with a pulse of depolarizing current. (In no case was a cell's capacity to reset contingent on the network being in a particular coordination state. Often the coordination state changed spontaneously during a resetting experiment and in such cases identical results were obtained in each state.)

As expected, current pulses given to cells HN(6) (Fig. 3*A*) and HN(7), the interneurons at the bottom of the hierarchy, directly stimulate a burst of action potentials and sometimes suppress firing for a short time thereafter but cause no phase shift. Similarly, pulses delivered to cell HN(5), the interneuron one rung up the hierarchy, fail to reset its phase (Fig. 3*B*). Ultimately the failure of these cells to reset reflects their inability to shift the phase of their synaptic input.

According to the model, the interneurons at the top of the hierarchy, cells HN(1) and HN(2), pace the rest of the network and receive no feedback from it. In keeping with this hypothesis, transient depolarization of either cell HN(1) or HN(2) resets that cell's activity cycle to a stable new phase (Fig. 3*C*). (Despite previous uncertainty (4), cells HN(1) and HN(2) do fire impulses if depolarized sufficiently.) The phase of the entire network shifts to match the reset of cells HN(1) and HN(2) (Fig. 4*A*). Normal phase relationships are always restored within a cycle after perturbation and hence the phase of the network can be inferred from the phase of a single interneuron. (Because of this tight synaptic linkage among the HN cells one cannot distinguish direct resetting effects of depolarization from indirect effects of feedback from postsynaptic cells shifted by the induced bursts of PSPs.)

Single pulses delivered to either of the remaining interneurons, HN(3) or HN(4), yield a puzzling result. Recall that via the web of identified connections, cell HN(3) or HN(4) might drive its contralateral homologue plus all more caudal heart interneurons, and thus shift the entire back section of the network. Nevertheless, since there is no known pathway forward to cells HN(1) and HN(2), the induced firing of cell HN(3) or HN(4) should cause no phase shifts. Yet, confounding prediction, single-current pulses can shift the phase of either cell HN(3) (Fig.

3*D*) or cell HN(4) (Fig. 6*C*). Moreover, the phase shift seems to extend to cells HN(1) and HN(2) and the rest of the network, since all inhibition and excitation to cells HN(3) and HN(4) shift to match their new phase. (The capacity of cells HN(3) and HN(4) to shift the other elements of the network is shown in Figs. 10 and 11.)

Dynamics of resetting heart interneurons

In general the nature of the reset of the network produced by the stimulation of a particular HN cell depended on both the strength and phase of the stimulus. In Fig. 5*A* a depolarization of cell HN(2) inducing only 3–4 impulses had a clear phase-dependent resetting effect. (Note that in Fig. 5*A* and *B*, as in Fig. 3, which displays equivalent experiments, the magnitude of the perturbation caused by a resetting pulse corresponds to the horizontal displacement of the burst markers from the dashed vertical lines. Phase-response curves for the first poststimulus cycle are shown at the right for comparison. During the cell's burst phase the pulse caused no phase shift; during the interval between bursts the pulse produced stable phase advances of the network, similar to those depicted in Fig. 4*A*. (Synaptic excitation or direct injection of depolarizing

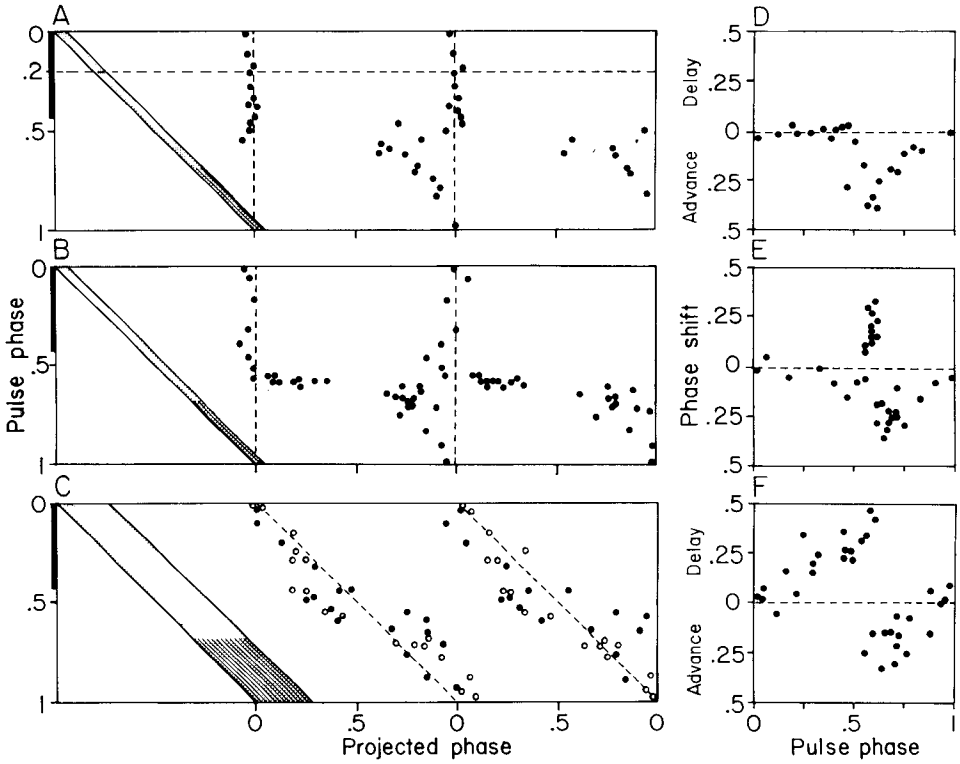

FIG. 5. Weak and strong resetting of interneuron HN(2). Each of frames *A*–*C* shows an experiment similar to those of Fig. 3. Here we plot the burst onsets only on the horizontal axis at a level corresponding to the phase of the cycle at which the stimulus began. The abscissa shows the phase projected if the stimulus caused no phase shift. Vertical dashed lines mark projected burst onsets; dashed diagonal lines, where drawn, are at fixed latency from the stimulus. For example, the horizontal dashed line of *A* shows a single trial in which the stimulus pulse began at phase 0.2 and the cell subsequently produced a burst at each point marked by a circle. The filled circles of frames *A*–*C* indicate observations made during a single penetration of an HN(2) cell. Observations of another HN(2) cell in a different preparation (open circles) supplement frame *C*. In this figure and Fig. 6, periods varied between 16 and 22 s; all data are normalized to a 20-s period. The heavy vertical bar on the ordinate marks the phase of the cell's spontaneous burst. Diagonal shading within the stimulus zone marks the approximate range of stimuli for which stable phase advances of the network were seen. Frames *D*–*F* show conventional phase-response curves (12). Advances and delays are interpreted, as outlined in the text.

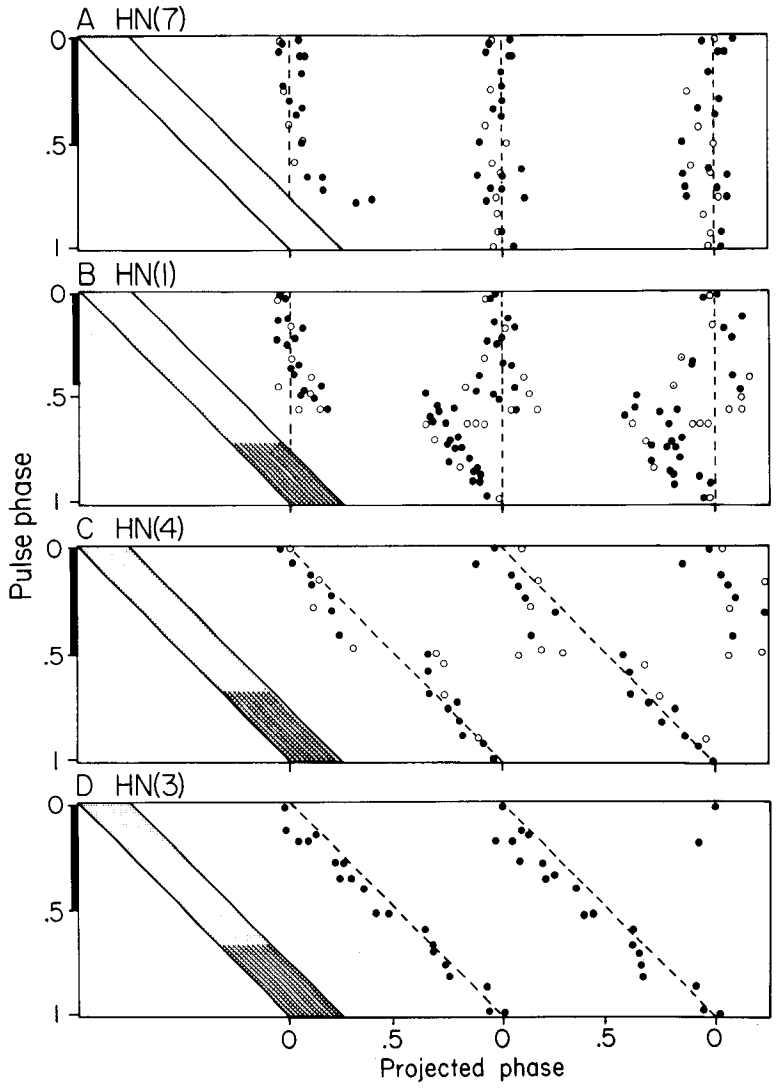

FIG. 6. Weak and strong resetting of heart interneurons. Each frame displays an experiment of the type shown in Fig. 3, presented as described in Fig. 5. The pulse duration is 5 s in each case. *A:* cell HN(7); *B:* cell HN(1); *C:* cell HN(4); and *D:* cell HN(3). In *A–C* the two types of symbol denote results obtained in two different preparations.

current late in the interval between bursts causes phase advances of neuronal oscillators in other preparations (2, 3).) Considering the resetting pattern as a whole, the burst markers following treatment fluctuate in an apparently continuous curve about the vertical dashed line. In oscillator theory this pattern is termed weak or type 1 resetting (12, 25).

An increase of the stimulus duration to 5 s (Fig. 5*C*) dramatically altered the resetting pattern. The striking feature of the data is the obvious clustering of the bursts at nearly fixed latency (modulo the cycle period) from the resetting pulse. This is an example of strong or type 0 resetting (25). Pulses given near phase 0.25 cause what appears to be phase delays of the network; those given near phase 0.75 elicit apparent advances of the network. The effect of pulses given near phase 0.6 cannot be characterized unambiguously as advances or delays: some

network cells appear to be advanced, others appear to be delayed. In the purely descriptive presentation of Fig. 5*C* this uncertainty presents no problem; in the interpretative Fig. 5*F* we adopted the convention that the absolute value of all phase shifts be less than 0.5. At phase 0.6 the phase-response curve (Fig. 5*F*) shows the discontinuity characteristic of strong resetting.

Figure 5*B* shows the result for a stimulus intermediate between those of Fig. 5*A* and *C*. (The duration of the pulse was 1 s, as in Fig. 5*A*, but the current was slightly greater, inducing 6–7 impulses versus 3–4 in Fig. 5*A*.) (Between phases 0.5 and 0.7 the curve of Fig. 5*B* is irregular, showing properties intermediate between those of Fig. 5*A* and *C*. Following pulses near phase 0.6 phase shifts were unpredictable with repeated trials producing widely scattered results. Figure 4*B* illustrates the responses of other cells in the network to such stimuli. As shown, in this region a pulse dislocates the rhythm, producing an interval of jumbled activity. In repeated trials the same stimulus generated similar confusion from which the rhythm recovered at an unpredictable phase.

This transition from weak to strong resetting with increasing stimulus strength is a common and profound property of biological oscillators (25). Similarly, the result that appropriately timed pulses of interme-

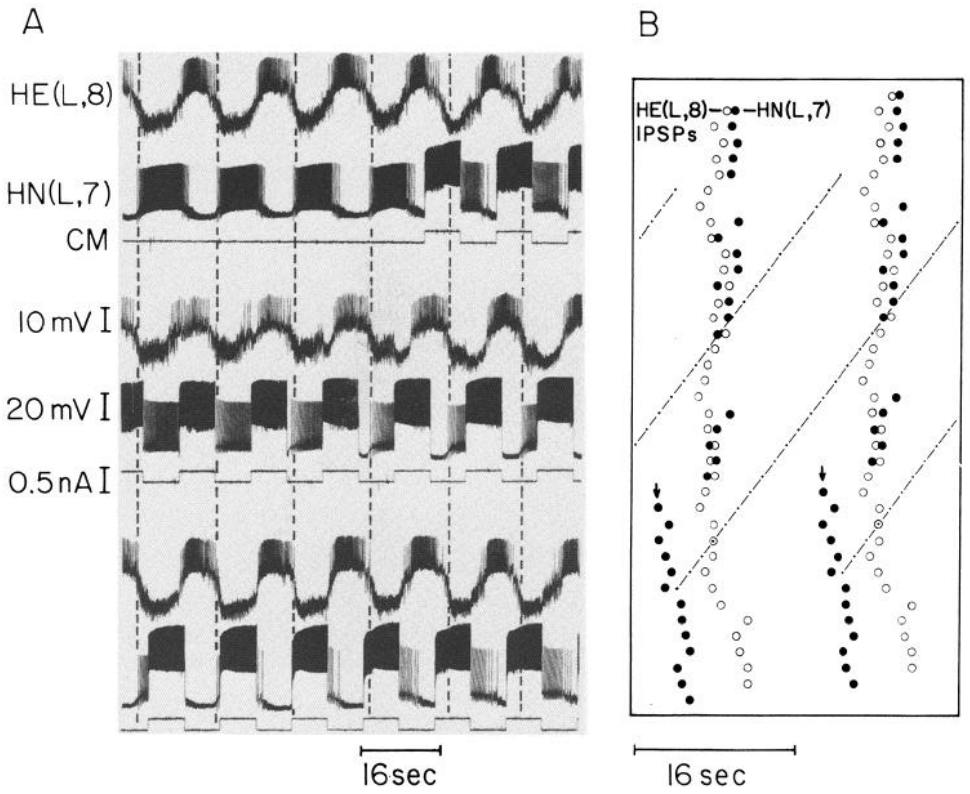

FIG. 7. Entrainment fails with cell HN(7). *A:* continuous intracellular record of cell HN(7). A train of depolarizing current pulses, with frequency slightly higher than that of the free-running system, has no effect on the ongoing burst rhythm of cell HN(7). Note that when not obscured by an injected current pulse, a burst begins precisely on the schedule projected from the free-running portion of the record (dashed lines). *B:* entrainment diagram. Superimposed in this figure and those like it that follow are two diagrams of the form described in Fig. 2. In one are marked the spontaneous bursts of cell HN(7) (filled circles). The open circles mark the beginning of each composite burst of IPSPs from ipsilateral cells HN(3), HN(4), and HN(6) in the ipsilateral motor neuron HE(8). The long train of current pulses (dots joined by straight line) has no effect on the ongoing cycles of any cell. At the arrow there is a change of coordination state, thus showing that cell HN(7) entrains the network in neither state.

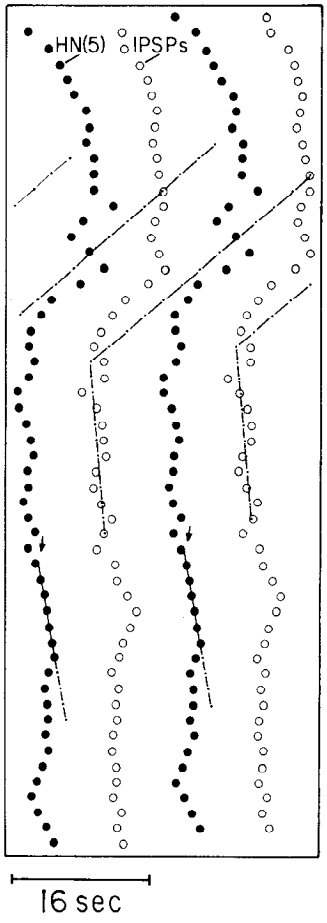

FIG. 8. No clear entrainment with cell HN(5). *A*: entrainment diagram. Filled circles mark the spontaneous impulse bursts of cell HN(5). Open circles mark the IPSP bursts in cell HN(5) and hence correspond to the action-potential bursts of ipsilateral cells HN(3) and HN(4) (Fig. 1*B*). In several replicates of the experiment, trains of current pulses (connected dots) injected into cell HN(5) had no effect on the more rostral heart interneurons. In other cases, including that shown, there was a slight change of frequency in response to a pulse train (the upper portion of the figure). Nevertheless, even in such cases there was no true entrainment. For example, in the lower half of the figure the pulse frequency is adjusted to match that of the free-running system, and one might think that the network is phase locked to the entrainment schedule. Yet when the schedule is shifted 180° (↓) the heart interneurons fail to follow, continuing instead to free run.

diate strength cause rhythm disruption and unpredictable phase shifts has precedents in other systems (13, 26).

Figure 6 summarizes data from similar resetting experiments with other HN cells. Three types of results were obtained. First, as typified in Fig. 6*A*, cells HN(5), HN(6), and HN(7) showed no phase shift in response to a 5-s pulse, and hence the burst markers fall along the vertical dashed lines with only random variations. Second, like cell HN(2) (Fig. 5*C*), cells HN(4) (Fig. 6*C*) and HN(3) (Fig. 6*D*) typically reset strongly when given a 5-s pulse. (In some experiments (e.g., Fig. 6*C*, open circles) cell HN(4)'s resetting deviated slightly from the idealized strong pattern.) Third, by contrast, for the same pulse deviation cell HN(1) reset weakly (Fig. 6*B*) much as cell HN(2) did for very weak stimuli (Fig. 5*A*).

The resetting test segregates the HN cells into two classes: those that cause resets of the network (cells HN(1)–HN(4)) and those that do not (cells HN(5)–HN(7)).

The experimental conditions were sufficiently artificial that exact prediction of a cell's timing strength within the network from its resetting strength is difficult. Nevertheless the striking discrepancy between the resetting capacities of cells HN(1) and HN(2) deserves note. These cells have similar physiology and identical identified synaptic connections (4, 23), yet with 5-s pulses cell HN(2) consistently showed strong resetting (four preparations; range of induced spike frequency, 4–10 Hz), whereas cell HN(1) consistently showed weak resetting (four preparations; range of induced spike frequency, 4–8 Hz). Similar resetting patterns were obtained with a 1-s depolarization of cell HN(2) producing 3–4 impulses (Fig. 5*A*) and a 5-s pulse producing a continuous 6- to 8-Hz discharge of cell HN(1) (Fig. 6*B*, filled circles).

Certain heart interneurons can entrain entire heartbeat system

A sensitive assay for the phasic influence of one component of a network on another is the entrainment test, used previously by Wendler (24) to show the phasic contribution of proprioceptive feedback to the locust flight rhythm. In our work the entrainment test took this form: we drove a selected interneuron with a train of current pulses at a rate slightly different from the system's unstimulated frequency and noted if the activity of a second interneuron assumed the period of the stimulated neuron, locking to

it with a characteristic phase. Such entrainment of one cell by another necessarily implies a synaptic pathway, perhaps indirect, between the two.

Entrainment studies verified the functional subservience of cells HN(6) and HN(7) (Fig. 7) to the other heart interneurons. An entraining regime applied to either of these cells not only cannot entrain the other interneurons (Fig. 7*B*), but even fails to control the bursting of the cell directly injected with current (Fig. 7*A*). Thus by all criteria cells HN(6) and HN(7) are passively driven by the more rostral heart interneurons. Although both appear to be capable of endogenous oscillation when freed from inhibition (6), this property is submerged when the cells are locked into the network.

In some cases trains of pulses given to cell HN(5) appeared to cause slight changes in the period of the heartbeat system, although entrainment was never observed (Fig. 8). A possible pathway for this weak influence of cell HN(5) is via the HN(X) excitatory synaptic potentials in the ipsilateral cells HN(3) and HN(4), which are driven by the impulses of cell HN(5) (4). Figure 9 verifies that HN(X) impulses are induced by the stimulation of cell HN(5) but fail to entrain the more rostral HN cells.

On the other hand, results from entrainment experiments underline the pervasive influence of cell HN(3). When an HN(3) cell is driven by a sequence of depolarizing current pulses, the activity of each HN cell in the third through seventh ganglion is entrained, as monitored by the IPSP bursts in cell HE(8) (Fig. 10*A*). Furthermore, cell HN(3) can entrain both ipsilateral and contralateral cells HN(1) and HN(2) (Fig. 10*B*). This entrainment verifies the feedback phenomenon suggested by the single pulse results.

One of the virtues of the leech heartbeat system is that often one can simplify the network by excising ganglia. For example, Fig. 10*C* and *D* show that the feedback interaction between cell HN(3) and the ipsilateral cells HN(1) and HN(2) probably occurs in the third segmental ganglion, since it persists when the ganglia caudal to the third are eliminated.

When the impulse bursts of interneuron HN(4) are driven slightly faster than the system's normal frequency, cell HN(3) becomes phase locked to the higher frequency (Fig. 11*A*). In fact, as one might suspect given the results with cell HN(3) (Fig. 10), the entire heartbeat network is entrained. One can verify this by monitoring cell HN(1) or HN(2) (Fig. 11*C*), or the synaptic input to HE motor neurons in the midbody. Again the site of feedback interaction of cell HN(4) with more rostral cells can be crudely located by selective ablation of ganglia. The persistence of the effect when ganglia caudal to the fourth are removed (Fig. 11*D*) shows that cell HN(4) drives the more rostral HN cells without the participation of the more caudal cells of the network. A direct means by which cell HN(4) might entrain the ipsilateral cell HN(3) is by inducing antidromic impulses from the latter cell's secondary initiation site in the fourth ganglion (7). Figure 11*B* shows that during the clear entrainment bout of Fig. 11*A* there were no antidromic HN(3) impulses and hence no support for this direct mechanism.

Finally, as predicted from the connection diagram (Fig. 1*A*), a train of pulses applied to cell HN(1) or HN(2) can entrain inter-

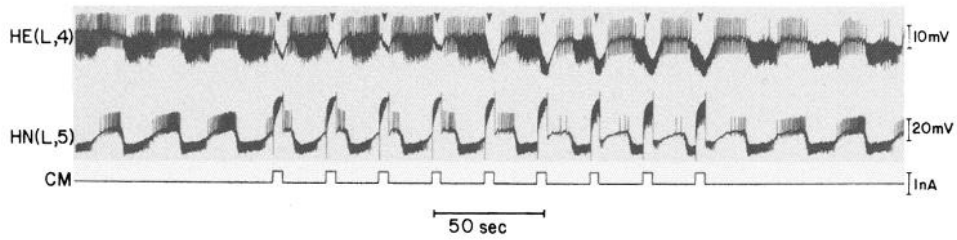

FIG. 9. The failure of cell HN(5) to entrain the more rostral HN cells is not due to its inability to drive the HN(X) unit. Clusters of HN(X) IPSPs (↓) match induced bursts in cell HN(5). The activity of the ipsilateral HN(3) cell, monitored here as the main IPSP burst in cell HE(4), continues without perturbation, drifting slowly relative to the induced activity.

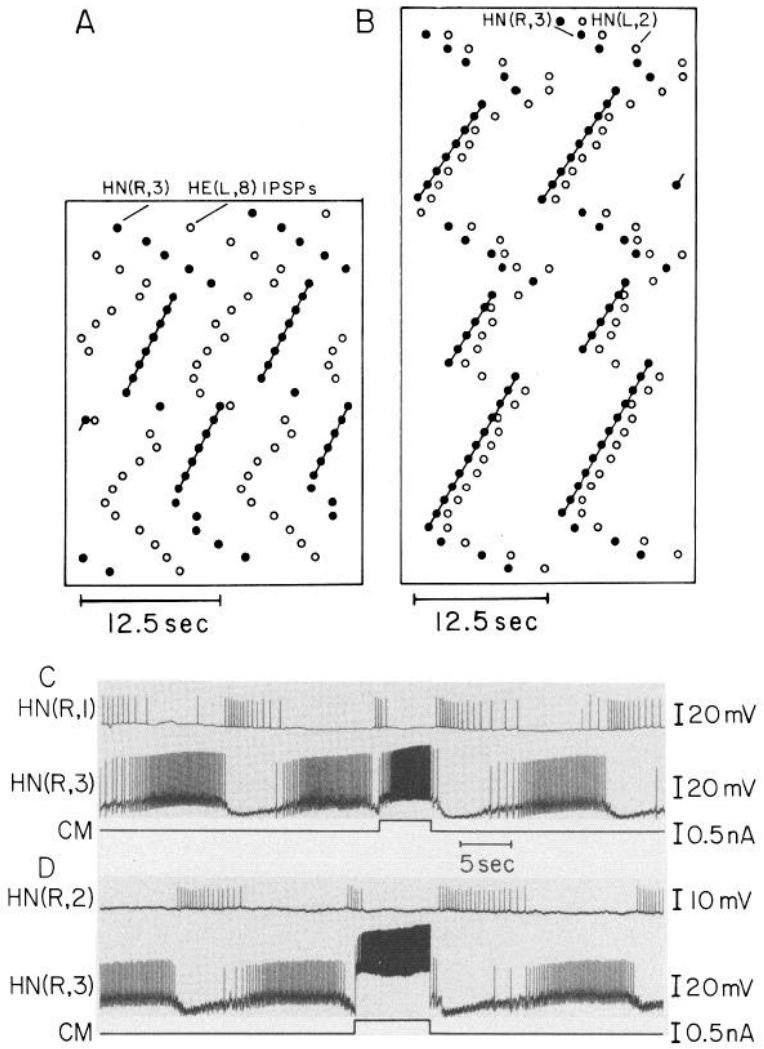

FIG. 10. Cell HN(3) entrains the other heart interneurons. *A:* this example shows that the activity of cell HN(3) (filled circles) entrains that of the contralateral HN cells, as monitored by the homogeneous burst of IPSP in motor neuron HE(8) (open circles). That is, when cell HN(3) is driven by trains of current pulses (connected portion of the record), the trend of the open circles shifts to remain approximately parallel with that of the filled circles. Note that during the two entrainment bouts the phase relationship between driving and driven cells is the same. *B:* through three bouts cell HN(3) (filled circles) entrains the activity of the contralateral cell HN(2) (open circles). *C, D:* paired intracellular records show that a burst induced in cell HN(3) immediately shuts off a burst in the ipsilateral HN(1) and HN(2) cells and resets the activity cycles of both cells. Preparations included the head-brain and the first three segmental ganglia only.

neuron HN(3) (Fig. 12) and hence the entire network.

Comment on hyperpolarizing pulses

Hyperpolarizing current pulses given to cells HN(3) or HN(4) can reset the network but because of two complicating features of the heartbeat system we did not examine their effects in detail. First, hyperpolarizing pulses injected into the soma of an HN cell exert no direct control of impulse initiation sites located in more caudal ganglia (7). Pulses are thus only partially effective in regulating the activity of cells HN(3) and HN(4), and completely ineffective in controlling cells HN(1) and HN(2), which initiate all impulses in the third or fourth ganglia (4, 23). Second, hyperpolarizing pulses

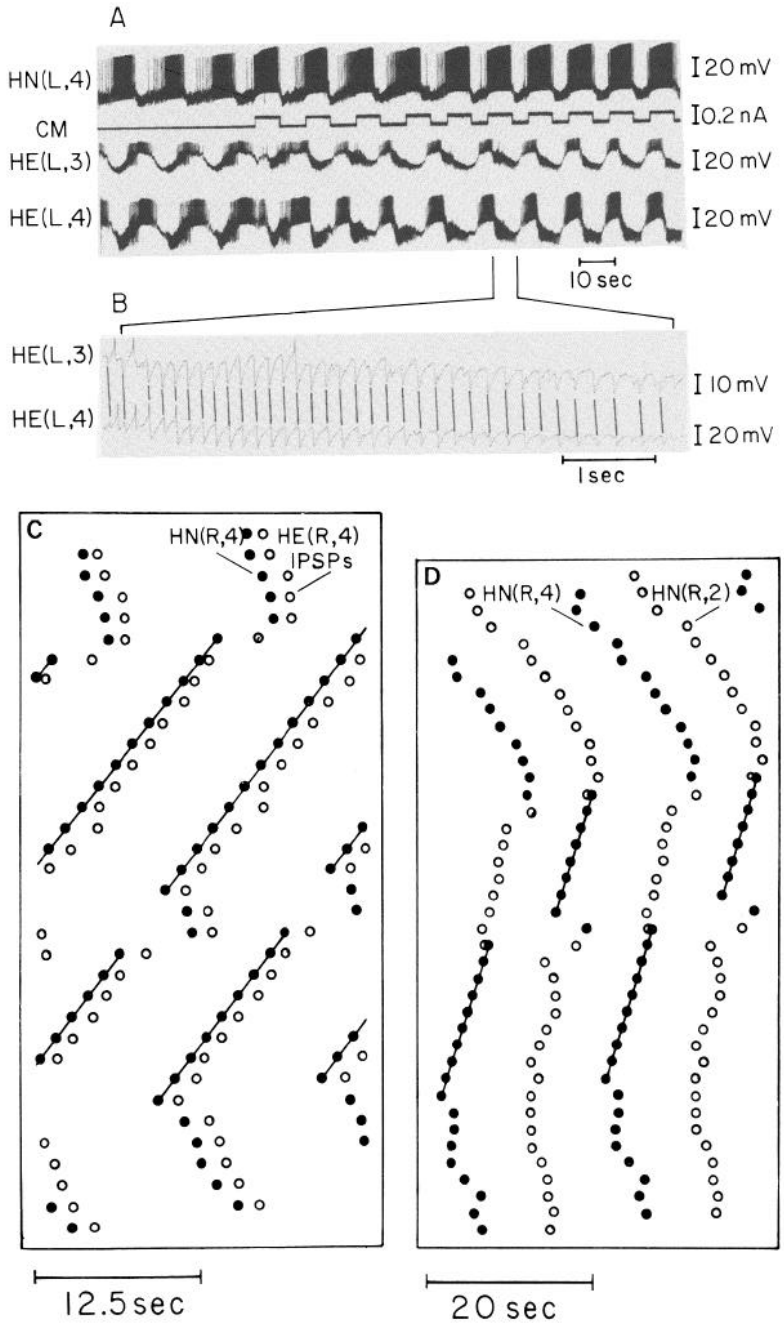

FIG. 11. Cell HN(4) entrains the other heart interneurons. *A:* simultaneous intracellular records taken from cell HN(4) and the ipsilateral HE(3) and HE(4) motor neurons show that pulses applied to cell HN(4) entrain that cell and the ipsilateral HN(3) cell (which produces the major class of IPSPs in these motor neurons (Fig. 1*B*)). *B:* cell HN(3) impulses travel orthodromically during entrainment by the ipsilateral HN(4) cell. IPSPs from HN(3) can be easily identified in the ipsilateral HE(3) motor neuron. The matching IPSPs in the HE(4) motor neuron, coming about 30 ms later, show that the HN(3) cell impulses originate in the third ganglion. All such IPSPs from 20 entrainment cycles of the experiment shown in Fig. 11*A* were analyzed, and no case was found in which the HN(3) cell impulse traveled antidromically. *C:* cell HN(4) filled circles) also entrains the ipsilateral HN(2) cell (open circles), holding it in antiphase. *D:* cell HN(4) (filled circles) can entrain cell HN(3) (open circles) when the more caudal ganglia are eliminated. The preparation includes only the head-brain and the first four segmental ganglia.

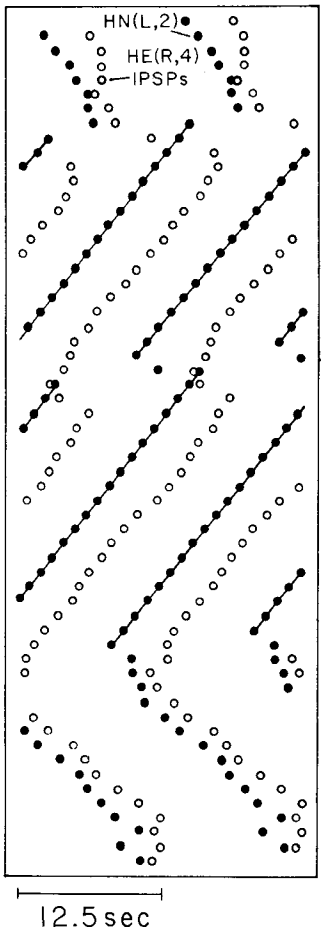

FIG. 12. Cell HN(2) entrains the other heart interneurons. Cell HN(2) (filled circles) entrains the contralateral HN(3) cell (as monitored by its IPSPs) (open circles).

induce vigorous postinhibitory rebound firing of cells HN(3) and HN(4). The relative contribution of hyperpolarization and rebound firing to resetting is difficult to assess in such cases.

DISCUSSION

Interneurons HN(1)–HN(4) constitute timing oscillator of heartbeat system

Four pairs of heart interneurons are distinguished from the rest by their capacity to reset and entrain the rhythms of all other cells in the heartbeat system, and hence these interneurons, HN(1)–HN(4), constitute the timing oscillator of the system or its "central pattern generator" (9). There must be a synaptic pathway linking each of these oscillator interneurons to every other one, as the activity of any one can dictate that of the entire set. Numerous linking pathways are already documented (Fig. 1*A*). For example, cells HN(1) and HN(2) inhibit ipsilateral cells HN(3) and HN(4); HN(3) cells excite ipsilateral HN(4) cells via rectifying electrical synapses (23), and both cells inhibit their contralateral homologues. Although these interactions explain to a great extent the dynamic relationships between the cells of the oscillator, this report shows that the known web of synaptic connections is incomplete.

For example, the entrainment of cells HN(1) and HN(2) by cell HN(3) (Fig. 9*B*) is not explained by the existing circuit diagram. Cell HN(3)'s strong control of the ipsilateral cells HN(1) and HN(2) (Fig. 10*D*) suggests that cell HN(3) makes direct inhibitory synapses with more rostral interneurons at their impulse-initiation sites in the third ganglion. Because discrete IPSPs cannot be measured in the somata of cells HN(1) and HN(2) (Fig. 3*C*), this hypothesis is difficult to test. The observation that cell HN(3)'s powers of entrainment remained when ganglia caudal to the third were eliminated (Fig. 10*C*, *D*) supported this

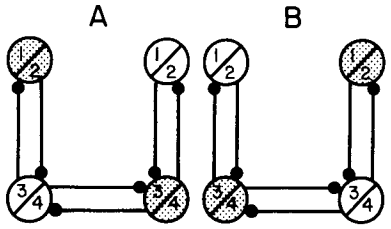

FIG. 13. Reciprocal inhibition between oscillator interneurons. Shown schematically are the identified and postulated inhibitory connections among the oscillator HN cells. Details of the reciprocal connections between HN(3) and HN(4) bilateral homologues and links from cells HN(1)/HN(2) to cells HN(3)/HN(4) are given in Fig. 1*A*. The resetting and entrainment experiments suggest a feedback connection from cells HN(3)/HN(4) to cell HN(1)/HN(2). In the two diagrams, shaded cells are depolarized and firing; unshaded cells are hyperpolarized and quiescent. If no special cellular properties are invoked, one predicts that the system will stabilize in one of the two configurations shown here. The reciprocal inhibition between HN(1) and HN(2) bilateral homologues, postulated in the original model (Fig. 1*A*), would reinforce this pattern, but is not necessary for its expression.

direct mechanism and ruled out others that required interganglionic connections.

Similarly, based on the known connections, one cannot account for cell HN(4)'s ability to entrain the other oscillator interneurons. We considered four possible mechanisms. The first hypothesis, that the interaction is mediated by the forward-going HN(X) unit via cell HN(5), was ruled out by simply removing all ganglia caudal to the fourth and showing that cell HN(4)'s ability to entrain persisted (Fig. 11*D*). Second, the possibility that cell HN(4) directly entrained the HN(X) unit, which in turn entrained the more rostral cells, was considered. The fact that cell HN(5) strongly entrains the HN(X) input to the more rostral cells, yet fails to entrain them (Fig. 9), makes this mechanism for the effect of cell HN(4) on those same cells improbable. A third explanation considered was that the interaction of cell HN(4) with the more rostral interneurons is mediated by stimulation of antidromic activity of the ipsilateral cell HN(3) via its secondary initiation site in the fourth ganglion. An experiment (Fig. 11*B*) showing that, during entrainment by cell HN(4), all impulses in the ipsilateral HN(3) cell travel orthodromically from the third to the fourth ganglion dismissed this possibility. The fourth hypothesis, still under investigation, is that cell HN(4) inhibits the ipsilateral cells HN(1) and HN(2) directly in the fourth segmental ganglion where these cells have initiation sites (4, 7).

Answers to these and other outstanding questions await a more detailed analysis of the interconnections of the eight oscillator interneurons. Our working hypothesis, shown in a deliberately flexible form in Fig. 13, is that within the central oscillator the major force holding cells in the proper phase relationship is reciprocal inhibition. First there is the well-characterized inhibition between the HN(3) and HN(4) bilateral homologues (11, 23). Based on the preliminary evidence reported here, we propose that the identified inhibitory connection from the HN(1)/HN(2) cells to the ipsilateral HN(3)/HN(4) cells is balanced by inhibition in the opposite direction, thus creating two additional inhibitory loops. These reciprocal connections account qualitatively for the observed phase relationships between the oscillator interneurons. The reciprocal connections between HN(1) and HN(2) bilateral homologues, postulated to explain the antiphasic firing of these cell pairs, are now unnecessary and are thus omitted from the scheme.

The network of Fig. 13 is not intrinsically oscillatory, and without additional hysteretic, restorative features it would freeze in one of the stable configurations shown (8). What could rescue the system from these end points are the endogenous polarization rhythms of each cell (6). For example, in time the depolarized cells of Fig. 13*A* spontaneously cease firing, freeing the hyperpolarized cells from inhibition, which allows them to proceed to the burst phase of their duty cycles. This creates the Fig. 13*B* configuration, which persists until it too decays back to that of Fig. 13*A* due to the same special cellular properties of the heart interneurons. (Equivalently, one can view the oscillator as a set of mutually entraining, endogenously bursting neurons that interact via reciprocal inhibition.)

Interneurons HN(5)–HN(7) elaborate heartbeat pattern in one of two coordination states

Although our results show that the subsection of the network including cells HN(5)–HN(7) plays no part in timing the oscillation of the interneuron network, it is nonetheless essential for the full expression of the heartbeat rhythm. The HN(5) cell pair receives two distinct types of input. First, some agency allows only one HN(5) cell to produce impulses, thus effectively removing the contralateral homologue from the circuit (4). Second, phasic IPSPs from the oscillator cells impinge on the active HN(5) cell, gating its impulse bursts and thereby pacing interneurons HN(6) and HN(7). As Fig. 1 shows, the inactivity of one HN(5) cell causes a crucial break in the bilateral symmetry of the network: cells HN(3)/HN(4) and HN(6)/HN(7)—those interneurons that drive the motor neurons—are in phase on one side of the animal and out of phase on the other. Ultimately this asymmetry leads to synchronous constrictions of the heart tube on one side of the animal and rear-to-front peristalsis on the other (4, 21). After 10–100 cycles, the active and inactive HN(5) cells trade roles to produce the other coor-

dination state (Fig. 1*A*). The elaboration of these distinct coordination states, perhaps the most interesting feature of the system, is thus exclusively the province of cells HN(5)–HN(7).

Resetting and entrainment as related phenomena

The relationship between resetting and entrainment of single endogenously bursting neurons was defined by Pinsker (14, 15). Consider a neuronal oscillator driven by a stimulus train having a period shorter than that of the free-running oscillator by an increment $\Delta\tau$. Stable entrainment of the oscillator to the stimulus schedule necessarily entails a cycle-by-cycle correcting phase advance of $\Delta\tau$. Given the resetting pattern for a single synaptic perturbation, Pinsker could predict the range of frequencies at which a neuron could be driven by a train of such stimuli. This concept, that resetting and entrainment are roughly equivalent properties, can be extended from single cell to neural networks and higher level oscillators (16, 18).

Our own study has been rudimentary in this regard, but we do note that heart interneurons entrain if and only if they reset. Moreover, each of interneurons HN(1)-HN(4) entrains to frequencies at least 10% faster than their free-running rates, and each shows phase shifts of at least 10% in comparable experiments.

Entrainment and modulation of oscillator by exogenous inputs

Ayers and Selverston (2) looked at the effect of exogenous synaptic input on the neuronal networks controlling the lobster stomach musculature. They showed that phasic stimulation could entrain the complex network that generates the pyloric rhythm. Moreover, the same input applied at a different frequency entrains the gastric mill network (1), a system in which many cells by their interactions produce and control oscillation. By analogy, in *Hirudo* the eight oscillator HN cells, each capable of entraining the entire heartbeat system, present eight potential targets for entrainment or modulation by outside agencies. Such pathways undoubtedly exist given the variety of stimuli, including connective shocks (20) and tactile stimulation (E. Arbas and C. Atkeson, unpublished results) that accelerate the heartbeat rate. More work in this latter vein should reveal how the heartbeat network, now so well understood in isolation, fits into the larger context of the animal's physiology.

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