Period Differences Between Segmental Oscillators Produce Intersegmental Phase Differences in the Leech Heartbeat Timing Network

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INTRODUCTION

Segmental oscillators are coordinated to produce constant phase relationships that are independent of cycle period between segments in isolated nerve cord preparations in many segmentally distributed motor pattern generating networks, including lamprey swimming (Cohen 1987a; Grillner et al. 1991, 1995) and crayfish swimmeret (Murchison et al. 1993) networks. Considerable experimental and theoretical effort has been exerted to understand how these constant phase relationships are produced (crayfish, Braun and Mulloney 1995; Mulloney 1997; Skinner and Mulloney 1998; Stein 1971; lamprey, Cohen 1987a; Grillner et al. 1993; Kottak et al. 1999a,b; Sigvardt 1993; Wadden et al. 1997; Wallén et al. 1992). An emerging consensus is that asymmetries in synaptic coupling between oscillators give rise to the phase differences between segments (Skinner and Mulloney 1998; Wadden et al. 1997). An alternative hypothesis (Excitability-Gradient), which states that differences in inherent cycle periods between symmetrically coupled segmental oscillators produce the observed phase differences, received some early support (Grillner et al. 1993). However, this hypothesis is now widely discounted in several systems (crayfish, Mulloney 1997; Skinner and Mulloney 1998; Skinner et al. 1997; lamprey, Cohen 1987a; Sigvardt 1993; Sigvardt and Williams 1996).

In contrast to these systems, the first paper of this series (Masino and Calabrese 2002) described the wide range of phase relationships observed in isolated ganglionic chains [G3 to G4 (G3-G4) or headbrain to G4 (HB-G4)] between the two segmental oscillators that form the timing network of the leech heartbeat central pattern generator. We also showed that, although the connections between the coordinating heart interneurons [in G1 and G2 (G1,2)] and the oscillator heart interneurons (in G3 and G4) are complex and anatomically asymmetric, the system functions mainly in a symmetric mode (Masino and Calabrese 2002). Most of the action potentials in the coordinating interneurons arise at an initiation site in G4 that is inhibited by the oscillator interneurons located in both G3 and G4. The modeling studies of the previous paper (Hill et al. 2002) predict that when the system functions in this symmetric mode the phase differences arise from differences in the inherent periods of the half-center oscillators that constitute the core of the two segmental oscillators. Moreover, these studies predict that the period of the timing network should be the period of the faster of the two independent segmental oscillators.

In this study, we developed methods for reversibly uncoupling the segmental oscillators (sucrose knife) and pharmacological manipulation of the individual oscillators (split bath) to test these predictions experimentally. In these experiments, the segmental oscillators were uncoupled, their independent periods assessed and in some cases altered with pharmacological manipulations, and then the timing network reconstituted. Thus the flexibility in phase relationship between segmental oscillators in the leech heartbeat timing network affords an opportunity to explore the adaptability of networks that produce coordinated segmental motor output and to uncover potentially novel mechanisms for intersegmental phase regulation.

METHODS

The methods for dissection, extracellular recording, data acquisition, and data analysis are as described in Masino and Calabrese (2002). All split-bath experiments were carried out on isolated G3–G4 chains, while concentration response experiments were carried out on isolated, single G3 and G4 ganglia.

Sucrose knife technique

We developed a method (sucrose knife technique) to reversibly uncouple segmental ganglia by blocking the propagation of action potentials in the interganglionic connective with a sucrose solution (260 mM) similar in osmotic concentration to normal leech saline. Small diameter tubing (1/16-in. ID, 1/8-in. OD) with a notch cut in its middle was placed across the center of the silicone elastomer (Sylgard)–lined preparation dish. The G3 to G4 (G3/G4) interganglionic connective was placed in the notch so that it traversed the tube (Fig. 1A), and petroleum jelly (Vaseline) was applied with a syringe to seal the notch in the tubing. Two different solutions could be gravity fed through the sucrose knife tubing. Normal leech saline was fed into the tubing under control conditions (coupled or recoupled states), while the sucrose solution was fed into the tubing to uncouple the ganglia. Reversible uncoupling and recoupling of the ganglia were used to assess period differences between segmental oscillators of the heartbeat timing network and their effects on the recoupled phase relationships.

Pot electrodes were used for extracellular recording and stimulation of the connective nerves in tests of the efficacy of the sucrose knife on G3–G4 chains. A Vaseline pot was formed around the cut end of the

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**FIG. 1.** A: the split bath–sucrose knife technique. The sucrose knife tubing separated the preparation dish into 2 separate compartments (anterior and posterior baths). The G3–G4 chain preparation is shown pinned (ventral surface up) in the dish with the G3–G4 interganglionic connective placed in the notch so that it traverses the tube. See text for details of operation. Under normal conditions, the ganglion in each bath was superfused with normal saline. To modify the cycle period of one oscillator, however, saline containing either myomodulin or Cs⁺ was applied to its bath. The electrical activity of the oscillator heart interneurons was recorded with extracellular suction electrodes. B: the sucrose knife technique reversibly blocked the propagation of action potentials in the G3–G4 interganglionic connective. Pot electrodes were formed around the cut ends of the connective anterior to G3 (stimulation) and of the connective posterior to G4 (recording). Coupled: prior to applying sucrose solution to the G3/G4 connective, a complex compound action potential generated by an extracellular electrical stimulus applied to the connective anterior to G3 was observed in an extracellular recording of the connective posterior to G4. Uncoupled: when the sucrose knife was applied to the G3/G4 connective, a complex compound action potential generated by an extracellular electrical stimulus applied to the connective anterior to G3 was observed in an extracellular recording of the connective posterior to G4. Recoupled: when the sucrose knife was removed by replacing sucrose solution in the knife tubing with normal saline, the activity generated by the stimulus was once again observed in the connective posterior to G4. The stimulus artifact (SA) was clipped in each panel. The extracellular records were scaled to the same size before and after the sucrose knife.
connective, and recording/stimulating silver wire was inserted into the Sylgard inside the pot. Recordings and stimuli were then referenced to a second silver wire in the general bath, and standard extracellular stimulating and recording techniques were applied. Electrical stimuli (1 ms) applied to the connective at one end of the coupled chain (with normal saline in the “knife”) evoked a complex compound action potential recorded at the other end (Fig. 1B, Coupled). This response was blocked when the ganglia were uncoupled (with sucrose solution in the knife; Fig. 1B, Uncoupled) by the sucrose knife and returned when the ganglia were recoupled (with normal saline in the knife; Fig. 1B, Recoupled).

**Concentration response curves**

Concentration response curves for leech myomodulin (provided by C. Sahley, Purdue University), molluscan myomodulin (Peninsula Labs, San Carlos, CA), and Cs⁺ (Cesium Chloride, Sigma, St. Louis, MO) on cycle period were measured in isolated G3 and G4 segmental oscillators and in the intact heartbeat timing network (HB–G4). Myomodulin and Cs⁺ solutions were made fresh in normal leech saline before each experiment. Increasing concentrations of myomodulin (10⁻⁹ to 5 × 10⁻⁵ M) or Cs⁺ (10⁻⁴ to 5 × 10⁻³ M) solutions were applied to the bath with 3- to 5-min normal saline washes between applications. The mean oscillator cycle period at each concentration was measured from at least 12 consecutive bursts and normalized to the cycle period of the timing network at the beginning of the experiment. These normalized periods were then averaged at each concentration across preparations.

**Split bath experiments**

Split bath preparations were created by forming a Vaseline partition between the G3 and G4 segmental ganglia across the G3/G4 connective, such that G3 was located in the anterior bath and G4 was located in the posterior bath (Fig. 1A). The segmental oscillators remained coupled for the duration of these experiments since the sucrose knife technique was not used. We applied saline containing myomodulin (10⁻⁶ M) to one bath, either anterior or posterior, while normal saline was applied to the other. The effects on network cycle period and the G3 to G4 phase relationship were assessed.

**Split bath–sucrose knife experiments**

The sucrose knife tubing separated the preparation dish into two compartments (split bath), such that G3 was in the anterior bath and G4 in the posterior bath (Fig. 1A). In the split bath–sucrose knife experiments, we created large period differences or reversed the period differences between segmental oscillators, which had been uncoupled, by applying saline containing myomodulin or Cs⁺ to one bath while normal saline was applied to the other. The effects of these period differences on the phase relationships of the segmental oscillators could then be assessed by recoupling the ganglia.

**RESULTS**

Do inherent cycle period differences between segmental oscillators determine their phase relationships?

We attempted to determine whether the different inherent cycle periods (T) of the G3 and G4 segmental oscillators determined their phase (φ) relationships, as predicted by the previous modeling studies (Hill et al. 2002), by reversibly uncoupling these oscillators and correlating their different inherent periods with their coupled and recoupled phase differences.

The sucrose knife, which blocked the conduction of action potentials in the G3/G4 connective (METHODS), was used to reversibly uncouple the G3 and G4 segmental oscillators, and their inherent periods were measured. An example demonstrating the efficacy of the sucrose knife technique is shown in Fig. 2. As expected, the period of the timing network was regular cycle-by-cycle, and the burst activities in ipsilateral G3 and G4 oscillator interneurons were phase locked in the coupled state (Fig. 2, Coupled). When sucrose solution was fed into the sucrose knife tubing, there was a brief interval during which network behavior was somewhat chaotic (Fig. 3). This interval lasted <3 min, after which the G3 and G4 oscillator interneurons began to burst independently (Fig. 2, Uncoupled). In this case, the G3 oscillator interneuron slowed, while the G4 oscillator interneuron sped with respect to the coupled period. In
many preparations, the period of the G3 oscillator interneuron was more regular than that of the G4 oscillator interneuron when uncoupled. When saline was fed back into the sucrose knife tubing (Recoupled), the oscillator interneurons quickly (<3 min) attained the same cycle period and became phase locked close to their original G3 to G4 phase relationship (Fig. 2, Recoupled). In some preparations, during the transition period from the coupled to uncoupled or the uncoupled to recoupled states, regular bursting broke down and was replaced by either intense firing [Fig. 3, HN(L,3) in Transition to Recoupled State] or by sporadic bursting for a brief period (~30 s). This disorganized behavior was expressed by the oscillator interneuron in G3 or G4, or by both concurrently, and was followed by a normal bursting pattern.

Similar results were obtained in all 26 preparations uncoupled with the sucrose knife in this phase of the study. The recoupled period and the coupled period were strongly correlated \((r = 0.9, P < 0.001)\) in these preparations, but there was some tendency for preparations with long periods to have shorter periods when recoupled (Fig. 4A). Moreover, the recoupled G3 to G4 phase relationship was strongly correlated to the coupled G3 to G4 phase relationship (Fig. 4B; \(r = 0.9, P < 0.001\)). The coefficient of variation \((CV)\) was used to compare the variability of the uncoupled G3 and G4 cycle periods. We calculated \(CV\) for the mean cycle period of the uncoupled oscillators in each preparation \((n = 26)\). The mean \(CV\) for each group \((CV_{G3} \text{ or } CV_{G4})\) was calculated from the individual \(CVs\) across all preparations in that group. The periods of the uncoupled G3 oscillators were more regular (indicated by a low \(CV_{G3} = 4.5 \pm 1.7\%\), mean \(\pm SD\)) than were the periods of the G4 oscillators (indicated by a high mean \(CV_{G4} = 10.1 \pm 5.3\%\)) across preparations. Variability of cycle period in the G3 oscillators was significantly lower than in the G4 oscillators (paired \(t = -5.7, P < 0.001, n = 26\)). Similar results were obtained with isolated ganglia used in the concentration response analyses of Fig. 5, B and C (mean \(CV_{G3} = 4.0 \pm 1.8\%\) and mean \(CV_{G4} = 9.0 \pm 6.0\%\); paired \(t = -2.8, P = 0.03, n = 6\)).

These results indicate that the sucrose knife can be used to rapidly and reversibly uncouple the G3 and G4 segmental oscillators without fundamentally altering their original period and phase when recoupled. The uncoupled segmental oscillators cycle independently and appear to express their own inherent periods; however, the G3 oscillator is more regular than the G4 oscillator (Fig. 2). The bases of this difference are not known but may reflect the greater complexity of the G4 oscillator. The axonal processes of the G3 oscillator interneurons remain active in isolated ganglia, but their activity is not well coordinated with the other elements of the network (Peterson 1983a). They thus represent a potential source of sporadic input to the coordinating interneurons in isolated or uncoupled G4.

The G3 and G4 segmental oscillators expressed different inherent periods in the majority (20 of 26) of the preparations, when uncoupled with the sucrose knife. There were a number of preparations in which the G4 oscillator was faster (9 of 26) and a number in which it was slower (11 of 26). In those preparations where their periods were similar, the oscillators nevertheless cycled independently as judged by the variability in their phase relationships. We correlated both the coupled and the recoupled G3 to G4 phase difference \((\phi_{G3} - \phi_{G4})\) with the inherent period difference \((T_{3} - T_{4})\) of the segmental oscillators (Fig. 4C) and found a strong and significant correlation for each (coupled, \(r = 0.7, P < 0.001\); recoupled, \(r = 0.7, P < 0.001\)). These results indicate that the period difference between the uncoupled oscillators is a good predictor of the
recoupled phase relationship of the G3 and G4 oscillators, regardless of which oscillator is faster or which is slower.

How is the cycle period of the timing network determined?

The modeling studies of the previous paper (Hill et al. 2002) predict that the period of the coupled timing network should be the period of the faster segmental oscillator. To determine whether the cycle period of the recoupled timing network was established by one or the other, or both of the segmental oscillators, the recoupled period was plotted against the period of the faster oscillator and of the slower oscillator (Fig. 4D). The plotted points for the faster oscillators fell along and on either
side of the unity line, while all but one of the points for the slower oscillators fell below the unity line. Thus the inherently faster uncoupled segmental oscillator, regardless of its ganglion of origin, established the cycle period of the recoupled network.

Testing the period difference hypothesis

Both the sucrose knife experiments described above and the modeling studies described in the previous paper (Hill et al. 2002) lead to the hypothesis that inherent period differences between segmental oscillators produced the phase relation of the heartbeat timing network and that the faster of the two segmental oscillators determined its period. To further test this hypothesis, we markedly altered the period of one of the oscillators in the uncoupled state and assessed the effects on the recoupled phase difference and period.

First, we sought exogenous substances that could alter the period of an isolated oscillator in a concentration-dependent fashion. The neuropeptide myomodulin has been shown to modulate ion channels and motor activity in a variety of invertebrate systems including Aplysia (Cropper et al. 1987a,b) and Lymnaea (Santama et al. 1994a,b). Recently, a novel myomodulin-like peptide was identified and characterized in the CNS of the leech (Wang et al. 1998). Synthetic leech and molluscan (Aplysia) myomodulins showed identical neuronal modulatory effects on the Retzius cell in the leech (Wang et al. 1998), thus we assessed whether leech and molluscan myomodulins affected the cycle period of the heartbeat segmental oscillators in isolated ganglia. Leech myomodulin in normal saline produced a concentration-dependent decrease in cycle period for the isolated G3 segmental oscillator (Fig. 5A). The mechanism by which myomodulin decreased cycle period is not known, but it is likely that it may modulate the intrinsic membrane properties of the heart interneurons, as does the peptide FMRFamide (Nadim and Calabrese 1997). We used molluscan myomodulin instead of the leech form for the following experiments, however, because it produced similar effects on cycle period (Fig. 5B) as did the leech form and because it was more readily available.

The mean cycle period of the oscillators at each myomodulin concentration was normalized to the mean cycle period prior to any myomodulin application (control). At low concentrations (≤10⁻⁸ M) the cycle period of the segmental oscillators remained close to the control period. The cycle period of the segmental oscillators, however, decreased when 5 × 10⁻⁸ M myomodulin was present in the normal saline and continued to decrease as the myomodulin concentrations increased. At a concentration of 10⁻⁵ M myomodulin, the G3 and G4 oscillator cycle periods were nearly twice as fast (~0.5; normalized) as their original periods. We did not measure responses at concentrations >10⁻³ M because the robust response at concentrations between 10⁻³ and 10⁻⁵ M was sufficient for our needs. In subsequent split bath and split bath–sucrose knife

FIG. 5. Concentration response curves for the effect of myomodulin and Cs⁺ on the cycle period of the oscillators in the heartbeat timing network. A: plot of the normalized cycle period for isolated G3 segmental oscillators at each concentration of leech myomodulin. Leech myomodulin had a similar effect to molluscan myomodulin on cycle period in the isolated G3 oscillator (compare plots A and B). B: plot of the normalized cycle period for isolated G3 and G4 segmental oscillators at each concentration of myomodulin. Molluscan myomodulin produced a similar concentration-dependent decrease in cycle period for the 2 segmental oscillators. C: plot of normalized cycle period for isolated G3 and G4 segmental oscillators at each concentration of Cs⁺. Cs⁺ produced a concentration-dependent change in cycle period that was similar for the 2 segmental oscillators. For each concentration response curve, the data were pooled from 6 preparations. Error bars indicate SD.
experiments, we typically used $10^{-6}$ M molluscan myomodulin to speed one segmental oscillator.

In addition, we assessed the concentration response of myomodulin in cycle period for G3–G4 chains over a limited range ($5 \times 10^{-8}$ to $5 \times 10^{-6}$ M) of the most effective concentrations. The chain preparations produced a concentration response relationship that was similar to the relationship observed for the isolated ganglia, however, as the cycle period decreased with increased concentrations of myomodulin, the G3 to G4 phase relationship usually shifted toward zero (data not shown).

Low concentrations of Cs+ in normal saline produced a concentration-dependent increase in cycle period that was similar for the isolated G3 and G4 segmental oscillators (Fig. 5C), presumably by blocking the hyperpolarization-activated inward current ($I_h$) (Angstadt and Calabrese 1989). At higher concentrations, however, this effect was reversed. The mean cycle period of the oscillators at each Cs+ concentration ($10^{-4}$ to $2 \times 10^{-3}$ M) was normalized to the mean cycle period prior to any Cs+ application (control). At the lowest ($10^{-4}$ M) concentration, the normalized cycle periods of the oscillators were similar to the control period. The period of the segmental oscillators increased, however, as the Cs+ concentrations ($10^{-4} < 2 \times 10^{-3}$ M) increased. The period peaked at $2 \times 10^{-3}$ M, and at higher ($3 \times 10^{-3} - 5 \times 10^{-3}$ M) concentrations the period of the segmental oscillators decreased toward the control period (Fig. 5C). At concentrations above $2 \times 10^{-3}$ M, it is possible that nonspecific effects masked the effects of $I_h$ blockade. In subsequent split bath–sucrose knife experiments, we typically used $10^{-3}$ to $2 \times 10^{-3}$ M Cs+ to slow one segmental oscillator.

In split bath preparations, the cycle period of the intact timing network (no sucrose knife) decreased when myomodulin ($10^{-6}$ M) was applied to one of the baths, regardless of whether it was applied to the G3 (anterior bath) or G4 (posterior bath) segmental oscillator. The cycle period of the modulated oscillator sped up (data not shown) in a manner consistent with the myomodulin concentration response curve (Fig. 5, A and B). During modulation, the unmodulated oscillator assumed the same cycle period as the modulated oscillator, and the two oscillators remained phase locked. Once myomodulin was washed out, the timing network returned to a period close to the original cycle period. In five of six preparations, the modulated oscillator led in phase, regardless of its original phase relationship (leading or lagging). In one preparation, where the original G3 to G4 phase difference was moderately large (~9%), the modulated G3 oscillator did not assume a phase lead, but instead lagged the unmodulated G4 oscillator in phase. However, the original phase difference was reduced from ~9% to a modulated phase of ~4.

Combining the split bath–sucrose knife technique with the ability to alter cycle period with myomodulin and Cs+ allowed us to assess how artificially large and/or reversed period differences between the uncoupled segmental oscillators shaped the phase relationship and period in the recoupled timing network. The four examples illustrated in Figs. 6–9 are typical results from various experiments in which we altered the period of one oscillator, when G3 and G4 were uncoupled, with the application of molluscan myomodulin or Cs+. The basic paradigm was to uncouple the G3 and G4 segmental oscillators and either speed the slower one or slow the faster one, as illustrated in Figs. 6–9. In some preparations, however, the faster oscillator was sped or the slower one slowed. Regardless of which oscillator was altered or whether it was sped or slowed, when recoupled the faster oscillator led in phase and the period of the coupled system was close to that of the faster uncoupled oscillator. In 23 of 24 such split bath–sucrose knife experiments, the faster oscillator led in phase when the oscillators were recoupled. In some cases, these manipulations caused an oscillator that had led in the coupled state to lag in the recoupled state, and an oscillator that had lagged in the coupled state to lead in the recoupled state (Figs. 6, 7, and 9).

We correlated the recoupled G3 to G4 phase difference ($\phi_a - \phi_d$) with the inherent period difference ($T_a - T_d$) of the uncoupled segmental oscillators (Fig. 10A) for both the split bath–sucrose knife experiments by themselves (open and gray symbols) and in conjunction with the sucrose knife experiments of Fig. 4C (control; indicated by black triangles in Fig. 10A). There was a strong and significant correlation in both cases ($r = 0.8, P < 0.001$ and $r = 0.8, P < 0.001$, respectively). Period differences, both naturally occurring and altered by myomodulin or Cs+, between the segmental oscillators determined the recoupled G3 to G4 phase difference.

We also lumped the data from these split bath–sucrose knife and control sucrose knife (Fig. 4C, control) experiments to assess the relation of the recoupled period to the period of the uncoupled segmental oscillators. As in Fig. 4D, the recoupled period was plotted against the period of the faster (●) and of the slower (○) of the two uncoupled segmental oscillators for each preparation (Fig. 10B). The plotted points for the faster oscillators fell along and nearly evenly on either side of the unity line, while all but one of the points for the slower oscillators fell below the unity line. Thus the inherently or artificially faster uncoupled segmental oscillator established the cycle period of the recoupled network.

**DISCUSSION**

The leech heartbeat pattern generating network is an attractive model to study the intersegmental coordination of coupled segmental oscillators because of its relatively simple design and well-described circuitry (Calabrese and Peterson 1983; Calabrese et al. 1995; Peterson 1983a,b; Schmidt and Calabrese 1992). In the first paper of this series (Masino and Williams 1984; leech swimmeret, Friesen and Peterson 1993, 1998; Murchison et al. 1993; Stein 1971) or the swimming networks in leech (Friesen and Pearce 1993; Hocker et al. 2000) and lamprey (Cohen 1987a; Cohen et al. 1982; Grillner et al. 1995). In these other systems, oscillators in adjacent segments produce a constant intersegmental phase lag that is independent of cycle period in both the intact animal and in the isolated nerve cord (either intact or reduced) (crayfish swimmeret, Braun and Mulloney 1993; lamprey swimming, Cohen and Wallén 1980; Wallén and Williams 1984; leech swimming, Friesen and Pearce 1993; Kristan et al. 1974). In contrast, the phase lag between coupled oscillators in the isolated leech heartbeat
timing network, although quite regular within individual preparations, varies considerably among different preparations. In effect, there is not a constant phase relationship between the segmental oscillators in the isolated timing network.

To address what mechanisms might establish the intersegmental phase differences observed in the isolated timing network, we used the split bath–sucrose knife technique as a tool to reversibly uncouple the segmental oscillators, and measure and manipulate their inherent cycle periods. We then recoupled the timing network and related period differences to the phase relationships in the network. Differences in the inherent cycle periods between oscillators correlated to the coupled and recoupled phase relationships in the timing network (Fig. 4C). The same relationship between cycle period difference and phase was observed when larger than normal period differences were induced or when period differences were reversed between the segmental oscillators by markedly altering the period of one of the oscillators in the uncoupled state (Figs. 6–9 and 10A). These experiments indicated that the uncoupled period difference (inherent and altered) between the segmental oscillators was a good predictor of the recoupled phase relationship (Fig. 10A). Regardless of which oscillator was faster or which was slower, the magnitude of the phase was directly related to the magnitude of the period difference (Fig. 10A), and the cycle period of the recoupled timing network was established by the inherently faster segmental oscillator (Fig. 10B). These data are consistent with the excitability-gradient hypothesis, which states that differences in the inherent cycle periods between symmetrically coupled segmental oscillators generate the intersegmental phase differences in rhythmic activity among oscillators (Grillner et al. 1993; Matsushima and Grillner 1990, 1992). Moreover, modeling studies of the leech heartbeat timing network presented in the second paper of this series (Hill et al. 2002) indicate that inherent period differences

FIG. 6. Speeding of the G3 segmental oscillator caused it to lead in phase. In Figs. 6–9 the layout is as follows: the left column shows extracellular recordings from a single preparation before (Coupled), during (Uncoupled; 1 oscillator sped/slowed with myomodulin/Cs+), and after (Recoupled) the segmental oscillators are uncoupled with the sucrose knife. Actograms in the middle column demonstrate the timing relationships of the G3 and G4 oscillator interneurons. In each panel, the actogram’s reference cycle is defined by the mean cycle period ($T_C$) of the phase marker cell from the coupled state. This convention was chosen so that the actograms would indicate the period differences, both natural and altered, from the original coupled state as well as the phase relation slope in the recoupled state. The cartoons in the right column illustrate the state of coupling in the G3–G4 chain and to which bath (anterior or posterior) myomodulin (MM) or Cs+ was applied. Coupled State: the cycle period of the timing network ($T_C = 13.6$ s) and the phase relationship between the ipsilateral oscillator interneurons were regular when the oscillators were coupled. Notice the G4 oscillator interneuron had a slight phase lead. Uncoupled State: the segmental oscillators were uncoupled by applying the sucrose knife to the G3/G4 interganglionic connective. Once uncoupled, applying $10^{-6}$ M myomodulin (MM) to the anterior bath sped the G3 segmental oscillator. The electrical traces and actogram show that the oscillators cycled independently at different periods. Notice the independent cycle period of the unmodulated G4 oscillator was similar to the original (coupled) cycle period. The modulated cycle period of the G3 oscillator, however, was considerably faster than the cycle period of both the originally coupled timing network and the uncoupled G4 segmental oscillator; the G3 symbols drift sharply to the left. Recoupled State: the segmental oscillators were recoupled by removing the sucrose knife. Myomodulin was still present in the anterior bath. The faster oscillator (G3) led in phase and established the cycle period of the recoupled network; both the G3 and G4 symbols drift sharply to the left.
between the segmental oscillators determine the phase relationships in both the symmetric and asymmetric versions of the network. Asymmetries in synaptic connectivity incorporated in these models are insufficient to generate phase differences, which are produced only when period differences between the segmental oscillators are present.

Excitability-gradient hypothesis and phase generation

The excitability-gradient hypothesis has been tested and rejected in a variety of different pattern generating systems (crayfish, Braun and Mulloney 1995; Mulloney 1997; lamprey swimming, Cohen 1987a; Sigvardt 1993; Sigvardt and Williams 1996; leech swimming, Pearce and Friesen 1985). For example, since the crayfish swimmeret rhythmic motor activity originates in the most posterior abdominal ganglion, the excitability-gradient hypothesis predicts that this oscillator should cycle faster than the oscillators in more anterior ganglia (Mulloney 1997). No evidence for an excitability-gradient was found along the nerve cord, however. When anterior and posterior segments of the cord were isolated by blocking information flow in the connective between the third and fourth segmental ganglia by applying TTX or sucrose solution to the connective or by severing the connective, Mulloney and co-workers found that the anterior ganglia cycled faster than the posterior ganglia (Braun and Mulloney 1995; Mulloney 1997). Further, they showed that the rear-to-front phase relationship of the system was not reversed when a gradient of excitation along the nerve cord (anterior ganglia were more strongly excited with modulators than the posterior ganglia), which should oppose this phase relationship, was created. To assess whether asymmetry in the network connectivity might generate phase differences, Skinner and Mulloney (1998) modeled several alternative swimmeret circuits, each with a different connectivity between the oscillator modules in the four adjacent segmental ganglia, and examined their activity. One of these
circuits effectively reproduced the invariant intersegmental phase relationships and relative durations of activity that characterize the crayfish swimmeret network in the absence of an excitability gradient. These modeling studies support the alternative hypothesis that asymmetry in the pattern of intersegmental synaptic connections, not an excitability gradient along the nerve cord, may account for the generation of the observed intersegmental phase relationships (Skinner and Mulloney 1998).

The excitability-gradient hypothesis has also been tested in the lamprey swim pattern generating network, which produces a front-to-rear progression of motor activity. Swimming in lamprey, which is characterized by a lateral undulation of the body, is governed by a spinal locomotor pattern generating network that consists of ~100 coupled oscillators distributed segmentally along the length of the spinal cord (Grillner et al. 1983). The phase lag between adjacent segments is typically ~1% and is independent of cycle period, which ensures the production of a constant wavelength of activity along the body axis (Cohen 1987a; Grillner et al. 1993; Sig vardt 1993).

To ascertain whether different regions of the lamprey nerve cord produced a gradient of swim cycle periods, Cohen (1987b) cut the spinal cord into numerous small pieces and measured their inherent cycle periods. No evidence was found for any consistent differences in cycle period among the segments. However, indications of asymmetry in the intersegmental coordinating system of the swim network were seen in forcing experiments where rhythmic bending applied to one end of the nerve cord entrained the activity of the entire network (Williams et al. 1990). Activity was entrained over a broader range when the cord was forced from the caudal end than when it was forced from the rostral end, indicating that ascending coupling dominates in the network.

In another series of experiments, the split-bath technique was used to examine intersegmental phase lags produced when one-half of the spinal cord was more excited than the other half.

**FIG. 8.** Slowing the G3 segmental oscillator caused it to lag in phase. Figure layout as in Fig. 6. Coupled State: the cycle period of the timing network ($T_\text{C} = 9.6$ s) and the phase relationship between the ipsilateral oscillator interneurons were regular when the oscillators were coupled. Notice that there was no phase difference between the G3 and G4 segmental oscillators. Uncoupled State: the segmental oscillators were uncoupled by applying the sucrose knife to the G3/G4 interganglionic connective. Once uncoupled, application of Cs⁺ ($10^{-3}$ M) to the anterior bath slowed the G3 oscillator. The electrical traces and actogram show that the segmental oscillators cycled independently at different cycle periods. Notice the independent cycle period of the unmodulated G4 oscillator was slower than the original (coupled) cycle period. The altered cycle period of the G3 oscillator, however, was considerably slower than the cycle period of both the originally coupled timing network and the uncoupled G4 segmental oscillator. Recoupled State: the segmental oscillators were recoupled by removal of the sucrose knife. Note that Cs⁺ was still present in the anterior bath. The slower oscillator (G3) lagged in phase, while the faster oscillator (G4) established the cycle period of the recoupled timing network.
Theoretical analyses of split bath experiments indicate that, in an asymmetrically coupled network, the dominant coupling determines the intersegmental phase lag in that half of the spinal cord from which the dominant coupling pathway originates and forces the phase lag in the other half to change (Kopell and Ermentrout 1990). For example, if ascending coupling dominates in the lamprey swim network then bathing the two halves of the spinal cord in different concentrations of excitatory amino acid would cause the phase lag in the rostral half of the cord to change, while phase in the caudal half would remain unchanged (changes in phase were determined relative to the control phase lags). Anterior and posterior sections of the spinal cord were differentially excited by applying different [high (0.8 mM) or low (0.2 mM)] concentrations of excitatory amino acid to each bath of the split bath preparation (Sigvardt and Williams 1996). Regardless of which concentration (high or low) of excitatory amino acid was applied to each bath, phase in the posterior half of the cord did not change, while phase in the anterior half of the cord did change (phase decreased when the concentration was low and increased when the concentration was high). These experiments corroborate the result of the forcing experiments and are consistent with the suggestion that an asymmetry in the network, rather than an excitability gradient, generates the phase relationship between the segmental oscillators.

In a continuous network computer model of the lamprey swim system the cells of the rhythmic generating network are dispersed along the length of the nerve cord such that there are no segmental boundaries (Wadden et al. 1997). This network is composed of a column of 420 excitatory interneurons and 300 inhibitory interneurons on each half of the nerve cord. The excitatory interneurons project equal distances in the rostral and caudal directions, while the inhibitory interneurons have longer caudal than rostral projections, thereby introducing asymmetry to the network. This model produces stable, front-to-rear phase relationships (~0.5–2.5% per segment) that in-
crease slightly as the cycle period decreases. Nonetheless, these phase relationships are similar to those observed in both isolated spinal cord and in intact preparations (Wallén and Williams 1984). Thus Wadden et al. (1997) suggest that the asymmetric coupling present in the network generates the intersegmental phase lags. This model abandons the traditional thinking about the lamprey swim system as a series of coupled segmental oscillators and views the swim central pattern generator as a distributed network of concatenated interneurons that produce a temporal wave of activity and drives segmental motor outflow.

What of asymmetries in the leech heartbeat timing network?

Asymmetry in the leech heartbeat timing network is evident in the connections of the timing network; the G3 oscillator interneurons inhibit the coordinating interneurons in both G3 and G4, whereas the G4 oscillator interneurons only inhibit the coordinating interneurons in G4 (Fig. 1A, Masino and Calabrese 2002). Asymmetry is also indicated by the observation that the phase of the coordinating interneurons is more tightly tied to the G3 than the G4 oscillator interneurons (Masino and Calabrese 2002, Fig. 11C). Presently, the functional significance of this apparent asymmetry in the timing network is not understood. To examine the mechanisms underlying intersegmental phase generation in the heartbeat timing network, Hill et al. (2002) created two versions (symmetric or asymmetric coupling) of a simplified computer model. In the symmetric model where G3 and G4 oscillator interneurons both inhibit the G1,2 coordinating interneurons, when two segmental oscillators with different cycle periods are coupled, the faster one leads in phase and determines the period of the coupled system (see Hill et al. 2002, Figs. 3 and 4). In contrast, in the asymmetric model where only G3 oscillator interneurons inhibit the G1,2 coordinating interneurons, when two segmental oscillators with different cycle periods are coupled, the G3 oscillator interneurons, but not the G4 oscillator interneurons, can lead in phase and control the period of the coupled system (see Hill et al. 2002, Fig. 10). Clearly this later condition does not pertain to the isolated heartbeat timing network.

In the split bath–sucrose knife experiments (G3–G4 preparations) described here, where each segmental oscillator entrains the other over a similar range of period and phase relationships (Fig. 10A), the isolated timing network appears to function in a symmetric mode when tested under closed loop conditions. Under these experimental conditions (mutual entrainment between the 2 oscillators in the recoupled condition) there is a closed feedback loop between the two oscillators. A functional asymmetry may be expressed when the network is tested under open loop conditions, for example, in “driving” experiments like those described in the preceding paper (Hill et al. 2002, Figs. 8, 9, and 11), where one of the segmental oscillators is forced to assume a cycle period established by injecting current pulses into one of the paired oscillator interneurons in that ganglion. In such driving (open loop) experiments, feedback from the nondriven (“follower”) oscillator onto the driven oscillator will be substantially reduced and thus the follower oscillator should lose its ability to influence activity in the timing network. If so, we predict that the G3 oscillator will be more effective than the G4 oscillator at driving and entraining the timing network to various cycle periods because the G3 oscillator interneurons inhibit the coordinating interneurons at both their G3 and G4 spike initiation sites, while the G4 oscillator interneurons inhibit the coordinating interneurons only at their G4 spike initiation site. Future experimental and modeling studies will focus on exploring such potential asymmetries and their functional consequences.

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![Figure 10](http://jn.physiology.org/)

**Fig. 10.** Analysis of the split bath–sucrose knife experiments. A: plot of the recoupled G3 to G4 phase relationships vs. differences in cycle period between the G3 and G4 segmental oscillators. Period differences, both naturally occurring (control; ▲) and altered by myomodulin (MM) or Cs′ (open and gray symbols), between the segmental oscillators predict the recoupled G3 to G4 phase difference. The faster (inherent or altered) uncoupled oscillator led in phase. B: plot of the recoupled timing network cycle periods vs. the inherent cycle periods of the uncoupled segmental oscillators. For each preparation the faster and slower of the 2 segmental oscillators was determined, and they were plotted separately. Notice that the cycle period of the faster oscillators (●) fell more closely to the unity line than did the cycle period of the slower oscillators (○). The recoupled cycle period of the timing network was established by the cycle period of the faster (inherent or altered) uncoupled oscillator.
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