Local and Intersegmental Interactions of Coordinating Neurons and Local Circuits in the Swimmeret System

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Mulloney B, Hall WM. Local and intersegmental interactions of coordinating neurons and local circuits in the swimmeret system. J Neurophysiol 98: 405–413, 2007. First published May 16, 2007; doi:10.1152/jn.00345.2007. During forward swimming, periodic movements of swimmerets on different segments of the crayfish abdomen progress from back to front with the same period. Information encoded as bursts of spikes by coordinating neurons in each segmental ganglion is necessary for this coherent organization. This information is conducted to targets in other ganglia. When an individual coordinating neuron is stimulated at different phases in the system’s cycle of activity, the timing of motor output from other ganglia may be altered. In models of this coordinating circuit, we assumed that each coordinating neuron encodes information about the state of the local pattern-generating circuit in its home ganglion but is not part of that local circuit. We tested this assumption by stimulating individual coordinating neurons of two kinds—ASCE and DSC—at different phases under two conditions: with the target ganglion functional, and with the target ganglion silenced. Blocking a DSC neuron’s target ganglion did not alter its negligible influence on the output from its own ganglion; the phase-response curves (PRC) remained flat. Blocking an ASCE neuron’s target ganglion significantly affected its influence on the output from its home ganglion. We had predicted that ASCE’s modest phase-dependent influence would disappear with the target silenced, but instead the amplitude of the PRCs increased significantly. Thus we have two different results: DSC neurons conformed to prediction based on the models’ assumptions, but ASCE neurons showed an unexpected property, one that is partially masked when the bidirectional flow of information between neighboring ganglia is operating normally.

INTRODUCTION

The central nervous systems of most animals are able to coordinate the relative movements of different limbs so the mechanical forces these limbs produce are timed to permit effective behaviors. In many kinds of animals, the isolated CNS can produce fictive motor patterns like those driving normal behaviors. These fictive patterns are proof that neural circuits within the CNS coordinate limbs on different segments of the animal’s body (Kiehn 2006; Skinner and Mulloney 1998a; but see Friesen and Cang 2001). How these circuits work remains for the most part obscure because they can be studied only when the CNS is actively producing behaviorally relevant motor activity. One system in which such studies have been possible is the motor control of crayfish swimmerets (Hughes and Wiersma 1960; Ikeda and Wiersma 1964). When the crayfish swims forward, four pairs of swimmerets move in cycles of alternating power-strokes and return-strokes. These movements are driven by coordinated firing of modular pools of motor neurons located in each segment (Fig. 1A) (Mulloney and Hall 2000). Within each module, a local circuit constructs alternating bursts of spikes in the power-stroke (PS) and return-stroke (RS) motor neurons that then drive the swimmeret’s periodic movements (Murchison et al. 1993). Although these modules are anatomically separated, PS motor neurons in different segments fire bursts of spikes with the same period, and with a significant posterior-to-anterior phase progression that is maintained even when period changes (Mulloney et al. 2006).

Because the crayfish CNS will express the swimmeret motor pattern in vitro and has anatomical features that recommend it for analysis of intersegmental impulse traffic, it has proven to be an excellent system in which to analyze coordination. We have described its neural components in detail, including the motor neurons (Mulloney and Hall 2000), local interneurons (Mulloney 2003; Paul and Mulloney 1985), commissural interneurons (Mulloney and Hall 2003), and intersegmental coordinating neurons (Namba and Mulloney 1999). Two coordinating neurons called ASC_E and ASC_I project anteriorly from each module. A third coordinating neuron called DSC projects posteriorly (Namba and Mulloney 1999; Tschuluun et al. 2001). There is a tight correlation between bursts of spikes in ASC_E and DSC and expression of alternating bursts of spikes in PS and RS motor neurons (Mulloney et al. 2006).

The swimmeret system can be viewed as having four pairs of local modules that separately produce local motor output (Fig. 1A) but are organized by an intersegmental coordinating circuit within the CNS. Intersegmental neurons named ASC_E and DSC arise in each module (Fig. 1B). They are essential components of the coordinating circuit (Namba and Mulloney 1999; Stein 1971; Tschuluun et al. 2001). This circuit imposes one period on all the modules and constructs a posterior-to-anterior progression of power-stroke movements that is unaffected by changes in period (Mulloney and Hall 2007; Mulloney et al. 2006). To analyze the system’s performance, we constructed a set of computational models of the coordinating circuit (Skinner and Mulloney 1998b; Skinner et al. 1997) that are based on the system’s cellular organization. These models effectively capture the key features of the system’s dynamics. In particular, they couple local circuits corresponding to swimmeret modules (Fig. 1A) so that these circuits have the same robust phase progression. Analysis of these models has given new insight into the mechanisms that stabilize these interseg-
FIG. 1. A–C: diagrams that illustrate the organization of the swimmeret system. A: in each of 4 ganglia, a pair of local circuits (→) coordinate the firing of the power-stroke (PS) and return-stroke (RS) motor neurons that innervate 1 pair of swimmerets. These pairs of local circuits and these pools of motor neurons lie separated on opposite sides of the ganglion. B: interganglionic pathways (arrows) that connect local swimmeret circuits in neighboring ganglia, in this case 3 and 4. From each local circuit, ASCcant axons conduct information to more anterior ganglia. DSC axons conduct information to more posterior ganglia. Currents injected into an ASC or a DSC neuron affect the firing of the neuron’s target. D: example of 1 current pulse (stimulus) injected into an ASC neuron in A4 (ASC4) recorded simultaneously with bursts of spikes in power-stroke motor neurons in ganglia A3 and A4 (PS3, PS4). The 2 rows of daggers mark the beginning of each PS burst. The double daggers above the PS3 and PS4 bursts that occurred during the stimulus show when these PS3 and PS4 bursts were predicted to start if the stimulus had no effect on their timing. The period of the PS3 cycle was 24% shorter than predicted, and the period of the PS4 cycle was 9% shorter than predicted.

METH0DS

We first anesthetized a crayfish, Pacifastacus leniusculus, by chilling it on ice and then exsanguinated it by transfusion with chilled saline. To expose the abdominal CNS for recording, we removed the abdominal nerve cord, a chain of six ganglia (Huxley 1880; Mulloney et al. 2003), to a dish lined with transparent silicone elastomer (Sylgard, Dow-Corning) and pinned it out linearly dorsal-side up with small amount of Vaseline. Around a neuron’s target ganglia and superfuse these ganglia separately without interrupting impulse traffic across the barrier.

Electrophysiological recordings

In this species, there are four pairs of swimmerets used for swimming, located on abdominal segments 2–5. Each pair of swimmerets is innervated by a pair of nerves (N1) that projects from the segment’s ganglion directly to the swimmerets. Each N1 contains the motor and sensory axons that control one swimmeret. The axons of PS and RS motor neurons that project to each swimmeret are separated, respectively, into its N1’s posterior and anterior branches (Mulloney and Hall 2000). To record firing of PS and RS motor neurons from each ganglion, we separated the anterior and posterior branches of each N1 and placed extracellular stainless-steel pin electrodes in contact with these branches. We insulated each electrode from the bathing saline with a small amount of Vaseline. Extracellular recordings were amplified and filtered using A-M Systems Model 1700 amplifiers. Microelectrode recordings were made using an Axon Instruments Axoclamp 2B amplifier. Signals from both sources were digitized using an Axon Instruments Digidata 1322A digitizer and PC clamp software and recorded as computer files for later analysis. Microelectrodes contained 1% Dextran Texas Red (Dextran Texas Red MW 3000; Molecular Probes) dissolved in a solution of 1 M KCl +10

KCl, 52 MgCl2, 2.6 CaCl2, and 118 NaCl, buffered with 10 mM Tris maleate at pH 7.4. This low-Ca2+ saline reversibly suppressed chemical synaptic transmission (Nakagawa and Mulloney 2001; Sherff and Mulloney 1996). TTX saline was composed of 0.5 μM tetrodotoxin (Sankyo) dissolved in normal saline. This TTX saline stopped axonal impulse conduction reversibly. We elicited stable expression of the swimmeret motor pattern by bath application of the cholinergic agonist carbachol (1.5–4 μM in different preparations) dissolved in normal saline (RBI, Sigma). The ED50 for carbachol’s excitation of the swimmeret system is 7.8 μM (Mulloney 1997).

The segmental ganglia in the abdominal nerve cord are separated by intersegmental connectives, bundles of axons that do not contain neuronal cell bodies or synaptic processes (Mulloney et al. 2003). This separation allowed us to place a petroleum jelly (Vaseline) well around a neuron’s target ganglia and superfuse these ganglia separately without interrupting impulse traffic across the barrier.

mental phase differences (Jones et al. 2003), and how local parameters can affect them (Jones and Kopell 2006).

In modeling this coordinating circuit, we assumed that each coordinating axon encodes information about activity in its home module but does not directly influence that activity (Jones et al. 2003; Skinner and Mulloney 1998b). We interpreted the minor effects of these stimuli on the neuron’s home module as the work of a polysynaptic intersegmental loop (Fig. 1B): the stimulated axon projected to the target, altered its activity, and, within the same cycle information, returned from that target to affect the home module. Is this correct?

To test this assumption, we stimulated bursts of spikes in individual ASC or DSC neurons at different phases under two conditions: with the whole system active and with the neuron’s target ganglia silenced. We plotted phase-response curves (PRC) for PS motor output from each ganglion in each condition. By comparing the home ganglion’s PRCs obtained under normal conditions and with the target ganglia blocked, we found that the assumption is correct for DSC neurons but not for ASC neurons.
mM K$_2$HPO$_4$-KH$_2$PO$_4$ buffer (pH 7.4), and had resistance of 30–50 MΩ. Individual interneurons were penetrated in the lateral Neurupil of ganglion A4. Depolarizing current pulses were injected using the amplifier’s bridge circuit.

Coordinating neurons were penetrated in the lateral Neurupil, LN (Skinner 1985), of the ganglion in which they originated. Each neuron was first identified physiologically by the criteria given in Namba and Mulloney (1999). These identifications were confirmed independently by filling the neuron with dextran Texas Red using 1.0-nA current pulses, 250 ms long at 2 Hz. After fixing the preparation overnight in 4% paraformaldehyde, the neuron’s structure was examined in the cleared whole-mount using a confocal microscope.

Commissural interneurons 1 (ComInt 1) were penetrated in the LN of ganglion A4, on the side to which they projected, and identified by physiological criteria given in Mulloney and Hall (2003). In each case reported here, these identifications were confirmed anatomically by filling the ComInt 1 using the same methods described in the preceding text.

Our experimental design required that we first locate a neuron with a microelectrode and stimulate it to generate responses for the normal (before) PRC, then block its target ganglia and repeat the stimulation for the blocked PRC. With that accomplished, we then filled the neuron while washing out the blocking solution and repeated the stimulation for the third (recovery) PRC. This paper is the result of 186 preparations that yielded 13 experiments that are included here. We completed this protocol in 11 experiments and included data from two more experiments in which the filled neuron’s anatomy confirmed the physiological identification but recovery was not achieved. Data from experiments during which we failed to fill the cell or in which the anatomy of the filled cell did not confirm the physiological identification were excluded.

Generating PRCs

Once an individual coordinating neuron had been identified, pulses of depolarizing current were injected periodically to perturb the timing of the neuron’s firing. The durations of these current pulses were approximately the duration of a normal burst of spikes in that neuron (Fig. 1C), and their frequency ranged from 0.07 to 0.45 Hz (1/9 to 1/15 the frequency of the ongoing swimmeret activity), long enough for transient effects to die out before the next stimulus. Their frequency was adjusted in each experiment so that their phases drifted relative to the cycle of PS bursts. By recording the motor output from the neuron’s home ganglion and its target ganglion continuously during a series of more than fifty pulses, we collected the responses of their local circuits to these perturbations.

The start and stop times of bursts of spikes in PS recordings from the neuron’s home ganglion and its target ganglion were measured using Dataview (http://www.st-andrews.ac.uk/~wjh/) and used to describe the temporal structure of the motor output. Each burst’s duration was the difference between its start and stop times. The period of each cycle was the interval from the start of one PS burst to the start of the next PS burst (Fig. 1C). The latency of a stimulus occurring in a cycle was measured as the time interval between the start of the stimulus and the start of the preceding PS burst that marked the start of the cycle. The phases of these events then were defined as the ratio of these latencies to that cycle’s period. Therefore phase could range from 0 to 1.0. Because there is usually a phase difference between the home ganglion and the target ganglion (e.g., Fig. 1C), this definition of phase required that the phase of each current pulse relative to PS firing in the home ganglion and in the target ganglion be calculated separately.

To describe changes in the cycle periods caused by each pulse of injected current, we measured the periods of the four cycles that immediately preceded the start of the pulse and the period of the cycle in which the pulse occurred. For current pulse, i, and each ganglion, j, the mean of these four preceding periods, $\overline{X}_{ij}$, made a good predictor of the expected period after the start of the experimental burst (Fig. 1C). This method of calculating the expected period reduced errors that otherwise might be introduced by slow fluctuations in excitation or rundown of the preparation. For each stimulus, we calculated the normalized period difference, $D_i$, between each experimental period, $\overline{X}_{ij}$, and the mean period just preceding it, $\overline{X}_o$, as $D_i = (X_{ij} - \overline{X}_o)/\overline{X}_o$. Plotting these $D_i$ as functions of the phase of the stimulus gives the PRC. Note that this definition of $D_i$ is common among biologists and consistent with our prior work but opposite in sign to that common in mathematics (Canavier 2006; Izhikevich 2007).

For each PRC, we fitted a regression of the normalized differences on the phase of the stimulus using SigmaStat (SysStat Software, Point Richmond CA). We chose linear or cosine functions for different PRCs, as was appropriate. We also used SigmaStat for Paired t-test and repeated-measure ANOVA (RM ANOVA). We used our own software for descriptive statistics (Mulloney and Hall 1987). Statistics that describe each parameter are given as means ± SD.

**RESULTS**

The goal of these experiments was to test the idea that ASC$_E$ and DSC neurons had no direct effects on the local circuits in their home modules. We begin by considering whether there is enough time in each cycle for a polysynaptic intersegmental loop to affect events in a given module. We continue with the PRCs for ASC$_E$ and DSC neurons under different experimental conditions. Tschuluun et al. (2001) demonstrated that if an individual swimmeret ganglion was bathed in a low-Ca$_2^+$–high-Mg$_2^+$ saline, swimmeret motor neurons in that ganglion were silenced, and as they fell silent the coordinating axons from that ganglion also fell silent. We used this technique again in this paper to silence a coordinating neuron’s target ganglion.

*There is enough time for an intersegmental loop to operate in each cycle*

Coordinating axons originate in the lateral neuropil, LN, of each swimmeret ganglion—A2–A5—and run in the intersegmental connectives to targets in other ganglia (Mulloney and Hall 2003; Tschuluun et al. 2001). The conduction velocities of each type of coordinating axon have been measured (Tschuluun et al. 2001); the slowest is ASC$_E$, whose velocity is 0.6 mm/ms. The LNs are located near the base of each swimmeret nerve, N1. We used an ocular micrometer in a wild M5 stereo microscope to measure the distances between swimmeret nerves, N1, in each segment both before the nerve cord was detached from the abdomen and then when it had been laid out for recording using our standard methods (Tschuluun et al. 2001). Taking the longest distance from one N1 to the next posterior N1, 7.3 mm, as the distance between home and target to be traveled by spikes in the ascending and descending coordinating axons, and the velocity of the slowest coordinating axon, the time required from conduction alone to make a round trip would be $2 \times 7.3$ mm/0.6 mm/ms, or $\sim 25$ ms. In the crayfish CNS at these temperatures, delays at chemical synapses are $\sim 2.5$ ms (Mulloney 2003; Nagayama and Sato 1993; Nagayama et al. 1997). The shortest cycle periods we have recorded are $\sim 200$ ms, so within even the shortest cycle, there is time available for a polysynaptic intersegmental exchange of information (Fig. 1B). Therefore the models’ assumption that coordinating neurons do not directly affect their home ganglion cannot be dismissed on that basis.
Silencing the target ganglion decreases the numbers of EPSPs recorded in ComInt 1

Within each swimmeret ganglion, ASC_E axons from posterior modules and DSC axons from anterior modules converge onto a local interneuron called ComInt 1 (Mulloney and Hall 2003). ComInt 1 integrates the EPSPs that result from bursts of spikes in ASC_E and DSC axons arriving from other ganglia, and transmits this information to the pattern-generating kernel of the local circuit (Fig. 2A). ComInt 1 therefore might be a route through which information in an interganglionic loop could return to affect the output from a coordinating neuron’s home ganglion. To test this idea, we recorded intracellularly from ComInt 1 in A4 under normal conditions and when ganglia anterior to A4 were silenced by low-Ca\(^{2+}\)-high-Mg\(^{2+}\) saline (Fig. 2B). This block of A5–A6 would silence ASC_E\(^5\) (TsChuluun et al. 2001). In four experiments, the number of EPSPs observed in ComInt 1 during each cycle of activity under normal conditions (23.6 ± 3.1 SD, n = 10 cycles per experiment) was greater than when A5 was silenced (13.9 ± 3.5, n = 10 cycles; Paired t-test \(P < 0.001, n = 4\) experiments). From this evidence, it is plausible that a closed loop including the pathway through ComInt 1 could couple modules in neighboring ganglia under normal conditions but be opened when one ganglion is blocked.

Silencing the target ganglion had no effect on DSC’s influence on its own home ganglion

Stimulating a DSC neuron normally has negligible effects on activity in its home ganglion. This might be because DSCs do not synapse onto neurons in their home module or because they have two simultaneous but opposite effects, one direct due to local synaptic connections and one indirect due to an intersegmental loop, and these two effects normally cancel each other. In the first case, blocking the target ganglia would have no effect on DSC’s local influence. In the second case, blocking the target ganglion would unbalance the system and reveal the local effect. In four experiments, we stimulated a DSC neuron in A4 when A5, its target ganglion, was active and when A5–A6 had been silenced with low-Ca\(^{2+}\)-high-Mg\(^{2+}\) saline (Fig. 3A). Under normal conditions, both before bathing the target ganglion in low-Ca\(^{2+}\) saline and after restoring normal saline (Fig. 3C), stimulating DSC with periodic current pulses had no phase-dependent effect on its home ganglion even though the same stimuli caused a clear, phase-dependent change in the timing of the period of PS bursts in A5, the DSC neuron’s target ganglion (Fig. 3C). The PRCs from the DSC’s home ganglion before blocking the target ganglion were essentially flat (Table 1), and were indistinguishable from the PRCs obtained once the blocks had been removed.

When the target ganglion was blocked, the effects of stimulating DSC4 on ganglion A4, its home ganglion, were indistinguishable from the effects recorded under normal conditions (Fig. 3, B and C). In each experiment, the populations of normalized period differences recorded with the target ganglion functional or blocked were not significantly different (RM ANOVA, \(P \geq 0.192, n = 4\) experiments). The slopes of the linear regressions fitted to the before PRCs were not significantly different from the slopes of blocked PRCs, and the intercepts of these regressions were not significantly different (Table 1). These results are consistent with the assumptions made in our models (Jones et al. 2003; Skinner and Mulloney 1998b) that DSC neurons do not directly affect the motor output from their home ganglion.

Silencing target ganglia increased ASC_E’s influence on timing of PS bursts in its own ganglion

If it is correct that coordinating neurons function only as reporters of activity in their home ganglion, then any responses of the home ganglion to stimulating a coordinating neuron must be due to information returning back from the neuron’s targets in other ganglia. These responses therefore should disappear if the neuron’s target ganglia are blocked. In five experiments, we compared the effects of stimulating individual ASC_E neurons with their more anterior target ganglia func-

FIG. 2. Excitatory postsynaptic potentials (EPSPs) recorded in a ComInt 1 neuron in ganglion A4 (C1A4) that is a target of ascending and descending coordinating axons. From A3 and A5, ComInt 1 integrates this information and modulates the timing and strength of the local circuit’s activity. A: diagram shows how this neuron is thought to connect with the kernel of the local circuit in A4. The pattern-generating kernel of the circuit is composed of 2 sets of reciprocally inhibitory local interneurons (1, 2). When the system is active, these neurons drive ASC_E4 and DSC4 axons that fire bursts of spikes at particular phases in each cycle. ComInt 1 affects the local inhibitory circuit directly. Solid unlabeled circles symbolize inhibitory synapses, triangles symbolize excitatory synapses, and the colors identify the segments in which axons originate (cf. Fig. 1A). Arrows mark the direction of orthodromic impulse conduction. B: 2 sections of a continuous recording from a C1A4 made under normal conditions and later when the next posterior ganglion, A5, had been silenced with low Ca\(^{2+}\)-high Mg\(^{2+}\) saline (A5 Blocked). When A5 was blocked, the numbers of EPSPs recorded in each cycle was significantly less than during the normal condition. These recordings were digitally filtered using a low-pass Gaussian procedure with a 250 Hz, −3dB cut.
tional and with these targets blocked. In four experiments, we blocked anterior ganglia with TTX saline; in one more, we used low-Ca\(^{2+}\)/H11001–high-Mg\(^{2+}\)/H11001 saline. Although in two of the TTX experiments we did not get recovery, in other respects the results were the same.

Under normal conditions, stimulating an ASC\(_E\) neuron altered the timing of the PS burst in the ASC\(_E\)’s target ganglion in a phase-dependent manner (Fig. 4C) as we have previously reported (Jones et al. 2003; Namba and Mulloney 1999). The same stimuli also had a lesser effect on the timing of output from the neuron’s home ganglion (Fig. 4C). The PRCs constructed from these data were well-fitted by a cosine function,

\[
y = a \times \cos(2\pi x + c)
\]

where \(x\) is the phase of the stimulus, \(a\) is the amplitude of the PRC, \(c\) positions the peak of the curve, and \(y\) is the predicted difference in period caused by the stimulus. Here, amplitude corresponds to normalized period difference and can range from 0 to 1.0. For each experiment, a

![Diagram](http://example.com/diagram.png)

**FIG. 3.** Results of stimulating DSC neurons in A4. A: in this diagram, which illustrates the logic of the experiment, arrows show the paths of intersegmental information. Firing of PS motor neurons in the DSC’s home ganglion and target ganglion were recorded simultaneously, and pulses of depolarizing current were injected periodically into a DSC4 neuron with the target ganglion functional or blocked. A cartoon shows the positions of the electrodes and the well used to silence the target ganglion. Each ellipse symbolizes 1 abdominal ganglion. The rectangle shows the position of the well surrounding A5 and A6 used to bathe these ganglia in normal or low-Ca\(^{2+}\) saline. DSC4, microelectrode recording from DSC neuron in A4; A4, A5, ganglia A4 and A5; PS4, PS5, extracellular recordings of firing in power-stroke motor axons from ganglia A4 and A5. B: distributions of normalized period differences in the DSC neuron’s home ganglion under normal and blocked conditions. C: phase-response curves (PRC) for normalized period differences of PS bursts in the DSC neuron’s home ganglion, A4, and target ganglion, A5, under normal (before, recovery) and experimental conditions (target blocked). The solid lines in the home ganglion’s PRCs are linear regressions of normalized period differences on phase for which the before and recovery data were combined. The solid line in the target ganglion’s PRC is a cubic polynomial regression, again with the before and recovery data combined.

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**TABLE 1.** Parameters of linear regressions on PRCs of normalized period differences of PS bursts in DSC neurons’ home ganglia before and during a block of the DSCs’ target ganglia

PRC, phase response curve; PS, power stroke; \(R^2\), coefficient of determination = regression SS/total SS, where SS is sum of squared differences from the mean; df, residual degrees of freedom, which defines the critical values for \(t\); \(t\), Student’s \(t\)-value resulting from comparing the two slopes from each experiment (Zar 1996, pp. 353–355). For each experiment, the probability that the slopes are the same is \(>0.5\).
plot of the residuals of regressions of the data on this function were flat (see supplementary data1).

Stimulating the same ASCe neuron in the same way when its targets were blocked caused a larger change in the timing of PS bursts in its home ganglion than did stimulation before the block or later once the block had been removed (Fig. 4C). The populations of normalized period differences expanded relative to those recorded under normal conditions and with the target ganglion blocked. C: phase-response curves (PRCs) for normalized period differences of PS bursts in the ASCe neuron’s home ganglion, A4, and target ganglion, A3, under normal (before, recovery) and experimental conditions (blocked). The solid lines in the normal and target blocked PRCs are cosine regressions of normalized period differences on phase with before and recovery data combined.

The PRCs from the blocked condition could be fitted by a cosine regression. In all five experiments (Table 2), the amplitudes of the cosine regressions to the blocked PRCs were larger than the amplitudes of their normal controls. Omitting experiment 5’s exceptional increase because it includes an offset that is absent in the other regressions (see supplementary data), a paired t-test of the different amplitudes in experiments 1–4 yielded $P = 0.05$ that the normal and blocked amplitudes are the same ($t = -3.130, df = 3$). For the two experiments with the largest differences between normal and blocked PRCs, the plots of the residuals of these fits were not flat (see supplementary data). The coefficients of determination of these two regressions were $\approx 0.566$, but there remained a distinct discon-

$y = a \times \cos(2\pi \times x + c)$.

<table>
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1 The online version of this article contains supplemental data.
tivity near phases of 0.4–0.5. This discontinuity is apparent in the middle PRC of Fig. 4C.

In addition to the analysis of changes in period, we plotted changes in PS burst duration as functions of stimulus phase. For the same five ASC_E experiments, we observed no phase-dependent change in PS burst durations.

**DISCUSSION**

In constructing models of the swimmeret coordinating circuit, we assumed that each coordinating axon encodes information about activity in its home module [Fig. 1B, but does not directly influence that activity (Skinner and Mulloney 1998b; Jones et al. 2003)]. From this perspective, coordinating axons may encode essential information, but they are reporters of local activity, not direct participants in generating that activity. The goal of the these experiments was to test this assumption. The first most direct test was to see if there was enough time in each cycle of the motor pattern for an intersegmental loop to operate. The dimensions of the ventral nerve cord and the measured conduction velocities show that less than fifteen percent of each period would be consumed by intersegmental impulse conduction, so the assumption cannot be dismissed because an intersegmental loop would be too slow.

Consider the prediction that follows from the assumption that these neurons encode information about their home modules but do not contribute directly to these modules’ dynamics: any effect of stimulating a coordinating neuron on its home module must be a reflection of its effects on target modules. Therefore silencing a neuron’s targets should eliminate any observed effects on the home module. The results from experiments on DSC neurons (Fig. 3, Table 1) are consistent with this assumption. Stimulating a DSC neuron normally had a negligible effect, and this was not changed by silencing the neuron’s target ganglion. Attempts to fit cosine functions to these DSC PRCs yielded curves that were almost flat or sometimes truly flat; there were no phase-dependent responses. These results strengthen our confidence in this feature of our models.

In contrast, the results from experiments on ASC_E neurons (Fig. 4) not only contradict the assumption, they also reveal that ASC_E’s contribution is normally muted by some mechanism that is inactivated when the ASC_E neuron’s target ganglia are silenced (Fig. 4C). These results were unexpected. Because we observed the same result using TTX saline and low-Ca^2+–high-Mg^2+ saline, the difference between ASC_E and DSC (Figs. 3 and 4) cannot be ascribed to differences in experimental technique. Instead, ASC_E neurons must have direct access to elements in the pattern-generating kernel of their home module, access that is in some way minimized when the target ganglia are not blocked.

What neural mechanisms produce the ASC_E results? Under our normal experimental conditions, ASC_E neurons in each module fire a burst of spikes simultaneously with the PS motor neurons in their home module (Mulloney et al. 2006). Intracellular recordings from ASC_E neurons and PS neurons look quite similar, enough so that we cannot distinguish them by inspection alone. In our models, we have assumed that both ASC_E and PS neurons are driven by synaptic currents from local nonspiking neurons (Fig. 2A). If an ASC_E neuron has a direct synaptic feedback onto neurons in the pattern-generating kernel, it would normally be but one of several sources of synaptic current in these kernel neurons. If this is correct, then the effects of altered timing of an ASC_E burst would normally be opposed by other synaptic currents, for example, from ComInt 1 and the other nonspiking local interneurons (Fig. 2A). Silencing the target ganglion would reduce the strength of synaptic currents evoked in ComInt 1 and so reduce the strength of currents caused by transmitter release from this neuron (Fig. 2A). This reduction would increase the relative influence of currents caused by ASC_E’s untimely depolarization. This is essentially a postsynaptic integration mechanism that might account for changes in ASC_E’s local influence on timing of PS bursts.

The ability of ASCE and DSC neurons to encode information about the activities of the pattern-generating modules in which they arise is similar to that of corollary discharge interneurons that modulate the sensitivity of sensory systems during an animal’s own movements. Corollary discharge neurons also encode motor information; they are thought to differ from coordinating neurons because their axons project to sensory afferent terminals and sensory-processing circuits and inhibit these targets while the animal is moving (Poulet and Hedwig 2007). Thus their function is to prevent desensitization of these circuits. Despite these functional differences, coordinating neurons and corollary discharge neurons seem to perform exactly the same encoding tasks.

Several corollary discharge neurons are known to protect sensory afferents from depression by presynaptic inhibition. Under normal circumstances, presynaptic inhibition might also reduce ASC_E’s influence on the local pattern-generating circuit by inhibiting release from ASC_E’s synapses within its home module. However, this inhibition must normally be regulated by information from the target ganglion. To distinguish between these alternatives, we need new results from experiments mapping the internal synaptic organization of swimmeret modules.

Coordinating neurons also occur in other segmental nervous systems (Büsches 2005; Hill et al. 2003). The most thoroughly analyzed example is the heart coordinating circuit in the first seven segmental ganglia of the leech. Four modular premotor rhythm-generating circuits located in separate ganglia are coupled by two pairs of coordinating interneurons and a pair of switch interneurons that arise in the other three ganglia (Masino and Calabrese 2002). The intersegmental phase relations among these circuits and their output to heart motor neurons are more flexible than those we see in the swimmeret system, but the apparent separation of functions—rhythm-generation and coordination (Iezzini et al. 2004; Norris et al. 2006)—is the same as we have proposed for the swimmeret system (Jones et al. 2003; Skinner and Mulloney 1998b).

The leech swim circuit is strikingly different. There some neurons that participate in generating the motor output from each segmental circuit send axon collaterals to neighboring segments where they make the same pattern of synaptic connections as they do at home (Cang and Friesen 2002; Friesen and Pearce 1993). These more distant connections are weaker, and proprioceptive feedback is required to reach the range of intersegmental phase differences observed in the intact swimming animal, but the same neurons seem to participate both in local rhythm production and in intersegmental coordination.
The thoracic nervous systems that control walking in crustaceans and insects are closely akin to the swimmeret system in both developmental and evolutionary terms, but the central mechanisms that coordinate walking legs have been more difficult to analyze. Like the swimmeret coordinating neurons, intersegmental axons that fire bursts during each step cycle occur in the cockroach CNS (Pearson and Iles 1973). However, the isolated thoracic nerve cord preparations that would open the way to cellular analysis have been difficult to achieve with insects. In stick insects, locusts, and crayfish preparations, the local pattern-generating modules can also be activated with cholinergic agonists, but unless some proprioceptive feedback is preserved, the motor output from different segments is usually uncoordinated ( Büschges et al. 1995; Chrachri and Clarac 1990; Ryckebusch and Laurent 1994; Sillar et al. 1987). It is tempting to think this apparent requirement for proprioception is due to the complexity of accurate foot placement and body support ( Ludwar et al. 2005), but there is a counter example: the three thoracic ganglia of the hawk moth can generate coordinated fictive motor patterns equivalent to the animal’s tripod gait ( Johnston and Levine 2002). Therefore we do not dismiss the possibility that the failures of other arthropod preparations to produce coordinated multisegmental fictive walking is due to experimental factors not to fundamental differences in the organization of these nervous systems.

The vertebrate CNS also contains modular segmental circuits coupled together by coordinating neurons. In the spinal cords of lamprey and tadpoles, hemisegmental circuits that produce bursts of spikes in pools of motor neurons are coupled bilaterally by commissural neurons that coordinate left-right alternation ( Grillner 2003; Tunstall et al. 2002). Some of these commissural neurons project axons contralaterally to other segments, synapse with components of those hemisegmental pattern-generator circuits and contribute to stable intersegmental phase delays ( Kotaaleski et al. 1999a). Excitatory interneurons that are components of the hemisegmental burst-generating circuits also can project ipsilateral axons to neighboring segments ( Kotaaleski et al. 1999b). Less cellular detail is known about the spinal cord circuitry of amniote vertebrates, but intersegmental neurons that fire bursts at particular phases in walking and scratching and project to other spinal segments have been demonstrated in turtle and mouse preparations ( Berkowitz 2005; Berkowitz et al. 2006; Zhong et al. 2006).

Genetic targeting of specific neuronal phenotypes in mice ( Kiehn 2006) have identified classes of commissural neurons that are components of the hemisegmental burst-generating circuits coupled together by coordinating neurons. In the spinal cords of lamprey and tadpoles, hemisegmental circuits that produce bursts of spikes in pools of motor neurons are coupled bilaterally by commissural neurons that coordinate left-right alternation ( Grillner 2003; Tunstall et al. 2002). Some of these commissural neurons project axons contralaterally to other segments, synapse with components of those hemisegmental pattern-generator circuits and contribute to stable intersegmental phase delays ( Kotaaleski et al. 1999a). Excitatory interneurons that are components of the hemisegmental burst-generating circuits also can project ipsilateral axons to neighboring segments ( Kotaaleski et al. 1999b). Less cellular detail is known about the spinal cord circuitry of amniote vertebrates, but intersegmental neurons that fire bursts at particular phases in walking and scratching and project to other spinal segments have been demonstrated in turtle and mouse preparations ( Berkowitz 2005; Berkowitz et al. 2006; Zhong et al. 2006).

Genetic targeting of specific neuronal phenotypes in mice ( Kiehn 2006) have identified classes of commissural neurons that affect bilateral coordination during walking. These methods are likely to identify intersegmental coordinating neurons required for walking and flying in terrestrial vertebrates.

Our experiments and analyses have been done on isolated nervous systems, and the stimuli used to perturb the coordinating neurons were selected for analytical convenience, not for relevance to sensory modulation of the system. In the intact animal, proprioceptive, statocyst, and tactile information would actively modulate the system’s performance, probably on a cycle-by-cycle scale (Pearson 1993; Rossignol et al. 2006; Wilson 1968). Early work on sensory modulation of swimmeret activity ( Davis 1968; Heitler 1982, 1986; Killian and Page 1992a,b; Takahata and Hisada 1982; West et al. 1979) has not yet been integrated with more recent findings on the system’s central organization. ASC_E neurons, in particular, encode information not only about the timing and duration of PS bursts in their home modules but also about the strengths of each PS burst (Mulloney et al. 2006). We predict that sensory modulation of the swimmeret system will affect the firing of coordinating neurons, and the integrated sensorimotor performance of the system depends on these neurons.

ASC_E and DSC neurons differ in many ways: the direction of their axonal projections and the phases in which they fire during normal periodic activity ( Namba and Mulloney 1999), their distributions in the chain of four active swimmeret ganglia ( Tschuluun et al. 2001), the information they encode about the motor output from their home modules ( Mulloney et al. 2006), and their responses to differences in excitation of local modules ( Mulloney and Hall 2007). These new results add a significant new difference to this list: ASC_E neurons are able to affect directly the operations of the local circuit that constructs the alternating bursts of spikes in PS and RS neurons. These well-timed bursts of spikes in motor neurons are the essential product of the local module ( Murchison et al. 1993). Since ASC_E neurons can alter this timing, they are candidates for components of the module’s pattern-generating kernel (Fig. 2).

DSC neurons lack these properties (Fig. 3), and do seem to be reporters of events in their home modules, not participants in shaping those events.

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