Two interconnected kernels of reciprocally inhibitory interneurons underlie alternating left-right swim motor pattern generation in the mollusk *Melibe leonina*

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Sakurai A, Gunaratne CA, Katz PS. Two interconnected kernels of reciprocally inhibitory interneurons underlie alternating left-right swim motor pattern generation in the mollusk *Melibe leonina*. *J Neurophysiol* 112: 1317–1328, 2014. First published June 11, 2014; doi:10.1152/jn.00261.2014.—The central pattern generator (CPG) underlyng the rhythmic swimming behavior of the nudibranch *Melibe leonina* (Mollusca, Gastropoda, Heterobranchia) has been described as a simple half-center oscillator consisting of two reciprocally inhibitory pairs of interneurons called swim interneuron 1 (Si1) and swim interneuron 2 (Si2). In this study, we identified two additional pairs of interneurons that are part of the swim CPG: swim interneuron 3 (Si3) and swim interneuron 4 (Si4). These neurons fulfilled the criteria for inclusion as members of the swim CPG: 1) they fired at a fixed phase in relation to Si1 and Si2, 2) brief changes in their activity reset the motor pattern, 3) prolonged changes in their activity altered the periodicity of the motor pattern, 4) they had monosynaptic connections with each other and with Si1 and Si2, and 5) their synaptic actions helped explain the phasing of the motor pattern. The results of this study show that the motor pattern has more complex internal dynamics than a simple left/right alternation of firing; the CPG circuit appears to be composed of two kernels of reciprocally inhibitory neurons, one consisting of Si1, Si2, and the contralateral Si4 and the other consisting of Si3. These two kernels interact with each other to produce a stable rhythmic motor pattern.

The half-center oscillator, consisting of two populations of reciprocally inhibitory neurons, was first postulated more than a century ago to be a mechanism underlying alternating rhythmic activity (Brown 1911). The central pattern generator (CPG) underlying the swimming behavior of the nudibranch mollusk *Melibe leonina* was previously thought to be a very simple half-center oscillator consisting of only two pairs of mutually inhibitory neurons (Thompson and Watson 2005). The left and right halves of this oscillator cause the animal to flex its body rhythmically to the left and right. In this study, we found two additional pairs of neurons that are members of the CPG. These neurons have changed our understanding of the fundamental organization of the circuit as a classical half-center oscillator. Rather, a complex network of synaptic interactions produces a bursting pattern with phase progression involving four pairs of swim interneurons.

MATERIALS AND METHODS

Animal collection, maintenance, and dissection. *Melibe leonina* (3–15 cm in body length) were collected as adults by Monterey Abalone (Monterey, CA) and Living Elements (Delta, BC, Canada). Animals were kept in artificial seawater tanks at 10 –12°C with a 12:12-h light-dark cycle.

To isolate the brain, animals were anesthetized by injection of 0.33 M magnesium chloride into the body cavity. A cut was made in the body wall near the esophagus. The brain, consisting of the cerebral, pleural, and pedal ganglia, was removed by cutting all nerve roots. The brain was transferred to a Sylgard-lined dish, where it was superfused, at a rate of 0.5 ml/min, with normal saline (in mM: 420 NaCl, 10 KCl, 10 CaCl₂, 50 MgCl₂, 11 glucose, and 10 HEPES, pH 7.6) or with artificial seawater (Instant Ocean, Mentor, OH). Connective tissue surrounding the brain was manually removed with forceps and fine scissors while the brain was maintained at 4°C to reduce neuronal activity. The temperature was raised to 10°C for electrophys-
The previously published central pattern generator (CPG) underlying the swimming behavior of *Melibe leonina*. A: the swim CPG contains bilaterally represented pairs of swim interneuron 1 (Si1) and swim interneuron 2 (Si2). Lines terminating in filled circles indicate inhibitory synapses. Resistor symbols indicate electrical connections. The thickness of the line indicates the strength of connection. B: the swim motor pattern recorded intracellularly from all 4 swim interneurons. Si1 and Si2 produce alternating bursts of activity on the left and right sides.

**RESULTS**

**Identification of swim interneurons 3 and 4.** It was previously demonstrated that the CPG for the swimming behavior of the nudibranch mollusk *M. leonina* contains two pairs of mutually inhibitory interneurons (Si1 and Si2; Fig. 1) (Thompson and Watson 2005). In this study, we identified two additional bilaterally represented neurons that are involved in the swim motor pattern generation. We named these neurons swim interneuron 3 (Si3) and swim interneuron 4 (Si4). Examples of biocytin-labeled Si3 and Si4 are shown in Fig. 2, A and B. The cell bodies of Si3 and Si4, which were located near the Si2 cell body on the dorsal surface of the pedal ganglion (Fig. 2, C and D), were 30–45 µm in diameter (Si3, n = 7; Si4, n = 4), which is smaller than Si2 (~40–60 µm; Sakurai et al. 2011; Thompson and Watson 2005). The input resistances of Si3 and Si4 were 30.7 ± 15.9 MΩ (n = 3) and 32.2 ± 2.2 MΩ (n = 5), respectively, approximately twice those of Si1 (15.7 ± 2.5 MΩ, n = 8) and Si2 (14.3 ± 2.8 MΩ, n = 6).

Similar to Si2, both Si3 and Si4 had primary axonal processes projecting toward the contralateral pedal ganglion through the pedal commissure [PP2; nomenclature according to Newcomb (Newcomb et al. 2006); Fig. 2, Ai and Bi]. Both neurons had dense branching in the ipsilateral and contralateral pedal ganglia (Fig. 2, A, ii and iii, and B, ii and iii). Unlike Si2,
Fig. 2. The newly found swim interneurons 3 and 4 (Si3, Si4) labeled by biocytin. A: Si3 had its cell body on the dorsal surface of pedal ganglion (black arrow). It projected its main axon to the contralateral pedal ganglion through the pedal commissure (PP2) (Ai). Extensive branching was seen near the terminal in the contralateral pedal ganglion (Aii). Many branches were also seen along the main axon near the soma (black arrow, Aiii). B: Si4 also had its cell body on the dorsal surface of the pedal ganglion (black arrow) and projected its axon through PP2 (Bi). Compared with Si3, dendritic branching was constrained to the terminal of the process (Bii) and near the cell body (black arrow, Biii). There were characteristic bends in the pedal ganglia of both sides (white arrows in Bii, Biii). C: schematic drawing of the cell body locations and the axonal projections of Si1 (pink), Si2 (blue), Si3 (red), and Si4 (green) in the Melibe brain, showing cerebral, pleural and pedal ganglia, and two pedal-pedal commissures (PP1, PP2). Si1 has the cell body in the cerebro-pleural ganglion; its axon projects toward the ipsilateral pedal ganglion and into PP2. Si2, Si3, and Si4 have their cell bodies in the same vicinity of the pedal ganglion. They each project an axon to the contralateral pedal ganglion through PP2. D: Si2, Si3, and Si4 labeled by intracellular iontophoresis of biocytin (red). The preparation was double-labeled with GABA immunohistochemistry, showing that swim interneurons are distinct from the dPd/H9253 neurons (green).

Fig. 3. Phase relationships of the swim interneurons. A: simultaneous recordings from all of the swim interneurons on the left side. The order of bursting was L-Si4, L-Si3, and then L-Si1 and L-Si2. Gray bar shows duration of a single period. B: phase diagram of the bursts for all 8 neurons (4 pairs). The median spike of R-Si2 was set to be the phase zero. Bars run from the average phase for the first spike to the average phase of the last spike for each burst. The central vertical bar is the averaged phase of the median spike in each burst. The error bars are SD. Sample sizes are listed in Table 1. Neurons on the right and left side of the brain are indicated by filled and open bars, respectively. C: the bilateral pair of Si3 neurons burst in alternation. Gray bar shows duration of the L-Si3 burst. Note that the bursts of L-Si3 and R-Si3 overlapped. Asterisk indicates the phase delay from R-Si2 to L-Si3. D: the bilaterally paired Si4 neurons burst in alternation. Gray bar shows duration of the L-Si4 burst, which fires with a slight delay from the contralateral Si1. There is no overlap between right and left Si4 bursts.
which has only a few branches near the cell body, Si3 and Si4 had extensive arborizations near their somata.

The branching patterns differed between Si3 and Si4; Si3 had many branches that came directly off the primary neurite in both the axon terminal (Fig. 2Ai) and near the soma (Fig. 2Aii). In contrast, Si4 had branches that projected orthogonally from the primary axon in the terminal (Fig. 2Bi) and near the soma (Fig. 2Bii). The Si4 axon showed characteristic sharp turns in both pedal ganglia (Fig. 2B, ii and iii; n = 5) that were not observed in Si2 or Si3.

Filling all three swim interneurons (Si2, Si3, Si4) in the same pedal ganglion with biocytin showed that they were located in the same vicinity (Fig. 2, C and D; n = 3). There are three GABA-immunoreactive neurons (dPd) on the dorsal side of the pedal ganglion near Si2 (Gunaratne et al. 2014). Double labeling with biocytin and GABA immunohistochemistry revealed that neither Si3 (n = 5) nor Si4 (n = 5) was GABA immunoreactive, although they were located near the dPd neurons (Fig. 2D).

Si3 and Si4 are rhythmically active in a constant phase relationship with the other members of the swim CPG. During the swim motor pattern, Si3 and Si4 were rhythmically active, firing bursts of action potentials with fixed phase relations to Si1 and Si2 (Fig. 3). The relative phases and duty cycles of each neuron are listed in Table 1 and diagrammed in Fig. 3B. The periods of the swim motor patterns in these recordings ranged from 3.0 s to 13.8 s, with an average period of 6.5 ± 2.7 s and median of 5.8 s (n = 25).

There was a progression of firing of ipsilateral neurons, with Si4 firing first followed by Si3 (Fig. 3A). Si1 and Si2 began firing after the ipsilateral Si3. With the phases arranged relative to the median spike of the right Si2 (Fig. 3B), it can be seen that the left Si3 followed the right Si2 with a phase of ~0.2 (Fig. 3C), whereas the left Si4 fired in phase with the right Si1 (Fig. 3D). Si3 (Fig. 3C) and Si4 (Fig. 3D) each fired 50% out of phase with their contralateral counterparts.

The neurons differed in their duty cycles (Table 1 and Fig. 3B). Si3 had the longest duty cycle (51%). Its bursts frequently overlapped with those of its contralateral counterpart (Fig. 3C). Si4 had about a 40% duty cycle, and like Si1 and Si2 (Thompson and Watson 2005), its bursts did not overlap at all with those of its contralateral counterpart (Fig. 3D).

Si3 and Si4 can reset the swim motor pattern. Both Si3 and Si4 were able to perturb the swim motor pattern. Injecting a brief depolarizing or hyperpolarizing current pulse into Si3 caused a phase shift in Si1 and Si2 bursting (Fig. 4A). In the example shown, depolarization of the right Si3 had the effect of extending the burst of the right Si2 and thereby causing a phase delay (Fig. 4A). When the right Si3 was hyperpolarized, the burst duration of the left Si2 increased, which also caused a phase delay (Fig. 4B).

Similarly, a brief current pulse injected into Si4 also reset the rhythm of Si1/Si2 bursts (Fig. 4, C and D). Depolarization of the left Si4 extended the burst of the right Si1 and Si2, causing a phase delay of the following cycle (Fig. 4C). Hyperpolarization of the left Si4 caused a premature end to the bursts in Si1 and Si2 and phase advanced the next cycle (Fig. 4D).

The effect of the current pulse depended upon the phase at which it was applied.

Table 1. Phases and duty cycles of CPG neurons

<table>
<thead>
<tr>
<th>Neuron</th>
<th>Phase ± SD</th>
<th>Duty Cycle</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>R-Si1</td>
<td>−0.04 ± 0.03</td>
<td>0.32 ± 0.10</td>
<td>11</td>
</tr>
<tr>
<td>L-Si1</td>
<td>0.45 ± 0.05</td>
<td>0.31 ± 0.12</td>
<td>10</td>
</tr>
<tr>
<td>R-Si2</td>
<td>0</td>
<td>0.41 ± 0.08</td>
<td>21</td>
</tr>
<tr>
<td>L-Si2</td>
<td>0.49 ± 0.04</td>
<td>0.39 ± 0.10</td>
<td>9</td>
</tr>
<tr>
<td>R-Si3</td>
<td>0.69 ± 0.08</td>
<td>0.52 ± 0.14</td>
<td>9</td>
</tr>
<tr>
<td>L-Si3</td>
<td>0.19 ± 0.08</td>
<td>0.51 ± 0.06</td>
<td>6</td>
</tr>
<tr>
<td>R-Si4</td>
<td>0.52 ± 0.05</td>
<td>0.47 ± 0.09</td>
<td>7</td>
</tr>
<tr>
<td>L-Si4</td>
<td>0.04 ± 0.02</td>
<td>0.40 ± 0.07</td>
<td>6</td>
</tr>
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CPG, central pattern generator.
potentials (IPSPs) in the other Si3 (Fig. 5, A and B). The reversal potential of the Si3-to-Si3 synapse was relatively close to the resting potential of the postsynaptic neuron and thus easily reversed by negative current injection (Fig. 5B). This is unlike the Si2-to-Si2 synapse, in which the IPSPs were difficult to reverse with strong hyperpolarization (Fig. 5C). The Si4 pair was also mutually inhibitory, producing one-for-one constant-latency synaptic potentials (Fig. 6). However, the synaptic potentials often appeared as depolarizing potentials when Si4 became quiescent in Hi-Di saline (Fig. 6A). In normal saline, the Si4-evoked synaptic potentials appeared as hyperpolarizing IPSPs during the swim motor pattern (Fig. 6B). However, the synaptic potentials became large and positive-going when the postsynaptic Si4 was hyperpolarized slightly, whereas a slight depolarization made them appear as IPSPs (Fig. 6, B and C). The reversal potential for the Si4-to-Si4 synapse was on, or very close to, the resting potential of the postsynaptic neuron (Fig. 6C).

Synaptic connections of Si3 with Si1 and Si2. The Si3 pair exhibited complex synaptic interactions with Si1 and Si2 neurons (Fig. 7). Si1 and Si2 make excitatory and inhibitory synapses onto both Si3 neurons (Fig. 7A). Simultaneous excitation of Si1 and Si2 on the same side caused a transient excitation of the ipsilateral Si3 and an initial inhibition and a subsequent excitation of the contralateral Si3 (Fig. 7B). Stimulation of Si1 alone elicited IPSPs and a slow depolarization in the contralateral Si3 (Fig. 7C). The fast IPSPs were one-for-one with contralateral Si1 spikes (Fig. 7Ci) but rapidly decreased in amplitude (Fig. 7Ci). Si1 also evoked large, rapidly depressing excitatory postsynaptic potentials (EPSPs) in the ipsilateral Si3 (Fig. 7Di), which were one-for-one with Si1 spikes (Fig. 7Di). A train of action potentials in Si2 caused a slow depolarization in the contralateral Si3 (Fig. 7Ei); there were no discrete synaptic potentials corresponding one-to-one to the Si2 spikes (Fig. 7Eii). In return, Si3 inhibited the contralateral Si1 and Si2 (Fig. 7, F and G). Although a train of Si3 action potentials produced a hyperpolarization in both Si1 and Si2 (Fig. 7Gi), only Si1 received discrete one-for-one IPSPs (Fig. 7Gii). In Si2, the Si3-evoked synaptic potential

Fig. 5. Mutual inhibition between the Si3 pair. A: a train of spikes evoked by injecting a current pulse (1 nA, 4 s) into 1 Si3 produced a train of inhibitory postsynaptic potentials (IPSPs) in the contralateral Si3. Recordings were made in high-divalent cation (Hi-Di) saline. B: reversal of Si3-to-Si3 synaptic potentials. Action potentials were evoked by injecting steady 1 nA current into L-Si3 through the bridge-balanced recording electrodes. Traces were triggered at the peak of action potentials and overlaid. The membrane potential of the postsynaptic neuron (R-Si3) was changed by injecting steady current (1 nA and −1 nA) via the recording electrode under discontinuous current-clamp (DCC) mode as indicated by arrows during an ongoing swim motor pattern. C: overlaid action potentials and the synaptic potentials recorded from the Si4 pair. The synaptic potentials in R-Si4 were time-locked to L-Si4 spikes. They were easily reversed when hyperpolarized. The membrane potential of R-Si4 was changed by injecting steady current (1 nA and −1 nA) via the recording electrode under DCC mode.

Fig. 6. Mutually inhibitory synapses between the Si4 pair. A: a spike train evoked in one Si4 by a current pulse produced a train of depolarizing IPSPs in the contralateral Si4. Spontaneous activity was suppressed by Hi-Di saline. B: the polarity of the Si4-evoked synaptic potentials is strongly affected by changes in membrane potential level of the postsynaptic Si4. Brief current pulses (1 nA and −1 nA) were injected through the recording electrode under discontinuous current-clamp (DCC) mode as indicated by arrows during an ongoing swim motor pattern. C: overlaid action potentials and the synaptic potentials recorded from the Si4 pair.
was complex, with fast EPSPs corresponding one-to-one with Si3 spikes (Fig. 7Gii) and a slow overall hyperpolarization (Fig. 7Gi).

The inhibitory action of Si3 onto the contralateral Si1 and Si2 appears to play a role in regulating the duration of the Si1/2 burst (Fig. 8). When Si3 was depolarized with current injection to fire earlier than its original phase, the duration of the contralateral Si1 burst was shortened (Fig. 8A). In contrast, when the Si3 burst was delayed by injecting a hyperpolarizing current pulse, the Si1 burst was extended (Fig. 8B).

Taken together with previously determined synaptic connectivity (Sakurai et al. 2011; Thompson and Watson 2005), both Si1 and Si2 receive inhibitory synaptic input from the contralateral Si1, Si2, and Si3. Si3 receives excitation from the ipsilateral Si1 and the contralateral Si2 and mixed inhibition/excitation from the contralateral Si1.

Synaptic connections of Si4 with Si1, Si2, and Si3. Strong electrical coupling was detected between Si1 and the contralateral Si4 \( (n = 5) \) and between Si2 and the contralateral Si4 \( (n = 6) \) (Fig. 9, A and B). In one preparation, in which the strength of the electrical connection was compared among Si1, Si2, and Si4, we found that electrical coupling was the strongest between Si1 and the contralateral Si4 and the weakest between Si2 and Si4 (Fig. 9B). The coupling coefficient measured from Si1 to Si4 with two electrodes in Si1 was \( 0.21 \pm 0.04 \).
We previously showed that altered the swim motor pattern. From Si4 to the contralateral Si3 was inhibitory. Depolarization of Si3 could halt bursting (Fig. 11). In contrast, firing D tended to be more effective (Fig. 11). Similarly, hyperpolarization of Si2 halted the swim motor pattern, but depolarization (Fig. 11) or depolarization (Fig. 11) of Si1 halted the swim motor pattern, but depolarization (Fig. 11) or depolarization (Fig. 11) of Si1 also had profound effects on the motor pattern (Fig. 11).

Thus, despite the rapid EPSPs, the net synaptic action potentials corresponding one-to-one with Si4 spikes (Fig. 9). However, there were also discrete depolarizing potentials corresponding one-to-one with Si4 spikes (Fig. 9). Thus, despite the rapid EPSPs, the net synaptic action from Si4 to the contralateral Si3 was inhibitory.

Prolonged perturbation of one swim interneuron strongly altered the swim motor pattern. We previously showed that either depolarization or hyperpolarization of one of the Si1 pair lengthened the period of the swim motor pattern, halting it altogether when upwards of ±2 nA was injected (Sakurai et al. 2011). Here we tested the other neurons and found that each of them also had profound effects on the motor pattern (Fig. 11).

Either hyperpolarization (Fig. 11A) or depolarization (Fig. 11B) of Si2 halted the swim motor pattern, but depolarization tended to be more effective (Fig. 11C). Similarly, depolarization of Si3 had complicated effects on the swim motor pattern; depolarization of Si3 transiently halted bursting, but the rhythmic bursting of other neurons recovered after ~5 s (Fig. 11, E and F). Finally, current injection into Si4 generally lengthened the period but never halted the motor pattern (Fig. 11, G–I).

DISCUSSION

The Melibe swim CPG was first suggested to be an example of a half-center oscillator (Thompson and Watson 2005) as conceived by Graham Brown (1911) with two sets of mutually

![Fig. 8](image_url)

**Fig. 8.** Si3 alters the burst duration of Si1 during a swim motor pattern. A: injection of a depolarizing current pulse (2 nA, 2.5 s) into the left Si3 foreshortened (asterisk) the burst of the right Si1. B: injection of hyperpolarizing current in the left Si3 (~2 nA, 2.5 s) elongated (asterisk) the burst of the right Si1. Traces in A and B were obtained from the same preparation.

0.10 (n = 3), which is similar to the previously reported Si1-Si2 coupling (0.19 ± 0.05, n = 6; Sakurai et al. 2011).

Si4 received spike-mediated EPSPs from the contralateral Si1 and Si2 (Fig. 9, C and D). The large EPSPs recorded in Si4 were one-for-one and at a constant latency with spikes in Si1 (Fig. 9Ei) and Si2 (Fig. 9Fi). These EPSPs were largely eliminated by low-calcium saline (Fig. 9, Eii and Fii), suggesting that Si1 and Si2 make both chemical and electrical synapses in the contralateral Si4. With strong electrical coupling and excitatory synapses, these three neurons tend to fire together by forming a functional unit or “kernel” in the circuit.

We could not detect chemical synaptic action from Si4 onto Si1 or Si2, but Si4 had a complex inhibitory effect on the contralateral Si3, with excitatory and inhibitory components (Fig. 10A). In normal saline, a train of Si4 action potentials inhibited the contralateral Si3, suppressing its spontaneous spiking activity (Fig. 10B). In Hi-Di saline, an Si4 spike train caused an overall hyperpolarization in the contralateral Si3 (Fig. 10Ci). However, there were also discrete depolarizing potentials corresponding one-to-one with Si4 spikes (Fig. 10Cii). Thus, despite the rapid EPSPs, the net synaptic action from Si4 to the contralateral Si3 was inhibitory.

![Fig. 9](image_url)

**Fig. 9.** Synaptic connectivity of Si4 with Si1 and Si2. A: schematic diagram showing Si1 and Si2 having both electrotonic and chemical excitatory synaptic connections to the contralateral Si4. B: hyperpolarizing current pulses (~2 nA, 4 s) were injected into L-Si1, L-Si2, or R-Si4 as indicated by arrows through bridge-balanced recording electrodes. In all cases current injection produced hyperpolarization in the other neurons. Note that the connection between Si1 and Si4 was stronger than between Si1 and Si2 in this animal. C and D: spiking in Si1 (C) and Si2 (D) spikes produced large EPSPs in the contralateral Si4. Action potentials in L-Si1 (C) or L-Si2 (D) were evoked by injecting a steady current (2 nA or 1 nA, respectively) through the bridge-balanced recording electrode as indicated by arrows. EPSPs in the L-Si4 corresponded one-to-one to spikes in the R-Si1 (Ei) or R-Si2 (Fi). In both cases, the EPSPs were largely reduced in amplitude when extracellular calcium was reduced (Eii and Fii).
excitation of Si4 by Si1/2 (cf. Fig. 9). Each of these neurons inhibits its contralateral counterpart (Fig. 12, A and B, synapses a and b), forming coupled reciprocally inhibitory pairs. Together, Si1, Si2, and the contralateral Si4 form a functional kernel with their contralateral partners (Fig. 12A).

Each Si3 also has reciprocal inhibitory synapses with its contralateral counterpart, and together the pair functions as the second kernel. The bursting activities of neurons in the first kernel (Si1, Si2, and Si4 pairs) and those in the second kernel (Si3 pair) showed fixed phase relationships due to complex synaptic interactions between them. The right Si3 receives slow excitatory input from left Si1/2 (Fig. 12B, synapses c and d), which counteracts and eventually overcomes the inhibitory input from the left Si3 (Fig. 12B, synapse e). While bursting, the right Si3 starts to receive excitatory and inhibitory synaptic inputs simultaneously from the right Si1 (Fig. 12C, synapse h) and the left Si4 (Fig. 12C, synapse j). The left Si4 burst was induced by the right Si1 (Fig. 12C, synapse i) and the right Si2 (not shown) via electrical connection and excitatory synapse. Similar concurrent actions of excitatory and inhibitory synapses have been shown to provide flexibility in the firing activity of postsynaptic neurons (Berg et al. 2007; Sasaki et al. 2009).

In the Melibe swim CPG, Si3 receives concurrent synaptic inputs from multiple neurons, but their timing and temporal properties are different. At the beginning of the Si3 burst, it receives fast IPSPs and slowly depolarizing EPSPs from Si1 and Si2. Later in the burst it receives large but depressing EPSPs from the ipsilateral Si1 (Fig. 7D). Finally, a slow inhibitory input from the left Si4 terminates the Si3 firing (Fig. 10B). Burst firing of Si3 returns the feedback inhibition to the left Si1/2 and terminates their bursting (Fig. 7F and Fig. 12, synapses f and g). Thus Si3 provides negative feedback to the contralateral Si1/2. Termination of the left Si2 burst induces firing of the right Si3, which further hyperpolarizes the left Si1/2 through inhibitory synaptic action (Fig. 12B, synapses a and b).

Some of the synapses to or from Si3 and Si4 evoked multicomponent potentials. For example, a train of Si3 spikes caused overall hyperpolarization in the contralateral Si2, but each Si3 spike evoked a transient depolarizing potential (Fig. 7G). Similarly, a single Si4 spike produced an EPSP-like discrete potential in Si3, but the Si4 spike train caused overall hyperpolarization (Fig. 10C). The multicomponent synaptic potentials might indicate that neurotransmitters from the presynaptic neuron act at multiple postsynaptic receptors with different time courses, which may involve multiple transmitters (Jacklet 1995; Karhunen et al. 2001; Li et al. 2004; Seal and Edwards 2006; Vilim et al. 1996, 2000). There are many reports of multicomponent synapses in molluscan systems (e.g., Getting 1981; Hume and Getting 1982), and postsynaptic mechanisms have been suggested (Clemens and Katz 2001; Magosi and Bullough 1999). In this system, however, the strong electrical connections among Si1, Si2, and Si4 made it difficult to determine whether such multicomponent synaptic potentials were a synaptic response of a single postsynaptic neuron or summed responses of multiple neurons that are electrically coupled. It has been also reported that pure electrical synapses can produce such biphasic potentials through their low-pass filter property (Bennett and Zukin 2004).

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1 Supplemental Material for this article is available online at the Journal website.
Overall, sequential actions of synapses with distinct properties and time courses play essential roles in setting the phase relationships between the first and second kernels (see Fig. 7B). We could not find any synaptic drive that triggered the onset of bursts in the first kernel. Rather, they tended to fire when not inhibited, because the suppression of Si3 firing allowed Si2 to fire tonically (cf. Fig. 11D). This may indicate that a tonic excitatory input or extrinsic neuromodulation onto Si1/2 may be involved for their excitability (Friesen 1994). Further studies are needed to determine the mechanism that underlies spontaneous depolarization of the Si1, Si2, and Si4 complex when released from the inhibition.

**Electrical coupling in other neural circuits.** The Si1, Si2, and Si4 neurons exhibit strong electrical coupling that makes them fire as a unit. It is generally acknowledged that common functions of electrical synapses are neuronal synchronization and enhancement of membrane potential oscillation (Bennett and Zukin 2004). Such functional units can be found in the pyloric CPG in the crustacean stomatogastric ganglion (STG), where the AB and PD neurons are electrically coupled and fire as a single pacemaker unit (Eisen and Marder 1982; Marder and Eisen 1984; Selverston and Miller 1980). In the Aplysia feeding CPG, it was shown that electrical connections in a CPG play a major role in promoting similar firing patterns of coupled neurons (Sasaki et al. 2013). In the mammalian spinal cord, coordination of membrane potential activity across a population of neurons through gap junctions plays important roles in the generation of the motor pattern for locomotion (Hinckley and Ziskind-Conhaim 2006; Tresch and Kiehn 2000).

In addition to the electrical connection, Si1 and Si2 form excitatory synapses onto Si4. Such functional units with both electrical connections and excitatory synapses have also been reported in the buccal ganglia of Aplysia, where B31/2 and B63 are coupled together as a unit (Cropper et al. 2004; Hurwitz et al. 1997, 2003; Susswein et al. 2002). In the spinal cord of the Xenopus embryo, motoneurons form electrical connections and excitatory synapses (Perrings and Roberts 1995). Electrical coupling and excitatory synapses in pre-Bötzinger complex are important for the generation of the breathing rhythm in mammals (Rekling et al. 2000). Coexistence of excitation and electrical coupling may increase the reliability of synchronous discharge during the motor activity.

**Comparisons to other CPGs with intrinsic phase delays.** The mechanisms and the roles of phase differences have been explored in other animals in which the CPG resides in a
segmented nerve cord and controls segmented motor organs (Friesen 1994, 2010; Grillner 2006; Kristan et al. 2005; Masino and Calabrese 2002; Skinner and Mulloney 1998). In such systems, a number of factors have been identified that determine intersegmental phase relationships. These include period gradients in unit oscillators, coupling strength, and asymmetry in intersegmental interactions (Calabrese 1998; Grillner 2003; Kristan et al. 2005). Muscular activity along segments directly reflects the phase delays between elemental CPG activities. Phase relationships between the elemental CPGs can be flexible and under control of neuromodulation (Kristan et al. 2005; Matsushima and Grillner 1992).

Like other gastropods, but unlike the examples above, Melibe does not have a segmented nervous system. Therefore, phase progression in the Melibe swim CPG is not related to segmental identity. Phase differences have also been studied in the pyloric CPG of the crustacean STG, which is not a segmental oscillator (Marder and Bucher 2007; Nusbaum and Beenhakker 2002). In the STG, phase relations are maintained with respect to a pacemaking oscillator, which is lacking in Melibe. The phase relationships in the pyloric CPG seem to be primarily caused by differences in the active conductances of the neurons; however, there is a role for synaptic time course as well (Marder and Eisen 1984). The phase delays of the gastric mill CPG of the STG also rely heavily on the membrane properties of oscillatory neurons (Selverston et al. 2009).

Phase delays caused by synaptic properties seem to be a general feature of CPGs in gastropods. For example, in the Aplysia feeding CPG, slowly facilitating EPSPs produce delayed excitation of postsynaptic neurons (Hurwitz et al. 1997; Susswein et al. 2002). Delayed excitation caused by a rebound from inhibitory input is the major cause of phase delay in other
gastropod feeding and respiratory CPG circuits (Quinlan and Murphy 1996; Syed et al. 1990). In the nudibranch Tritonia, the escape swim CPG relies on delayed excitation from one neuron to another (Getting 1983). It is likely that the relatively long periods of these rhythms in gastropods makes them more likely to employ synaptic mechanisms for phasing.

Another nudibranch, Dendronotus iris, has neurons homologous to Si1 and Si2. However, only the Si2 neurons produce the alternating bursts; the Si1 neurons fire tonically (Sakurai et al. 2011). Preliminary evidence suggests that this CPG may also contain additional neurons. It will be interesting to see how they affect the stability of the oscillator.

Functional significance of a complex half-center network. That Si3 fires at a shifted phase might indicate that there are additional phases of body motion during swimming behavior. The lateral body flexions to either side appear as one motion, but it may also contain an additional subtle movement that produces somewhat undulating body motions rather than simple left-right body flexions. Indeed, Bornella anguilla (Bornellidae, Cladobranchia) swims with an eellike movement caused by waves of muscular contraction (Mills 1994). This may indicate that the undulating motion and the left-right flexions seen in species from two sister families in Cladobranchia might employ similar neural mechanisms. Si3 might provide rhythm-driveto additional sets of motor neurons, which could produce such a movement. At present, we have not been able to find nerve units that correspond to the Si3 burst phase (data not shown). Therefore, it is not clear whether Si3 bursts really contribute an additional phase to body flexion.

An alternative explanation for the phase difference of the Si3 burst from the Si1/Si2 burst is that it enhances the stability of the motor pattern. There is no evidence that any of the neurons are intrinsic bursters. Each neuron not only acts as a half-center in each kernel by mutually inhibiting its counterpart but also prompts switching of the alternating left-right activity in the other kernel. For example, coactivation of Si1 and Si2 caused sequential activation of Si3 (Fig. 7B). The excitation-to-inhibition in one Si3 was caused by a fast excitatory input from the ipsilateral Si1 (Fig. 7D) and a delayed inhibition from the contralateral Si4 (Fig. 10, B and C), while the inhibition-to-excitation in the other Si3 was caused by an initial inhibition from the contralateral Si1 (Fig. 7C) and a delayed excitation from the contralateral Si1/2 (Fig. 7, C and E). On the other hand, activation of Si3 by depolarizing current injections cut the Si1/2 burst short (Fig. 8A and Fig. 11B), while suppression of Si3 by hyperpolarizing current injection extended the Si1/2 burst (Fig. 8B and Fig. 11A). Thus two functional kernels appear to rely on each other’s activity and synaptic inputs for their smooth phasing. A mathematical model of the Melibe swim CPG was recently developed to examine the role of individual synaptic connections in phase-locking and the robustness in the swim motor pattern (Jalil et al. 2013). Their modeled circuit showed that the Melibe swim CPG can be interpreted as two half-center oscillators that are interconnected by synapses. They also found that well-tuned synaptic connections for contralateral feedforward inhibition (i.e., Si3-to-Si1/2 inhibition) and the ipsilateral excitation (Si1-to-Si3 excitation) are necessary to generate stable bursting activity. Their findings support our conclusion that the stable phase relationships among the swim interneurons originate from synaptic interactions between two oscillator kernels.

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