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Central pattern generator for swimming in *Melibe*

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Summary

The nudibranch mollusc *Melibe leonina* swims by bending from side to side. We have identified a network of neurons that appears to constitute the central pattern generator (CPG) for this locomotor behavior, one of only a few such networks to be described in cellular detail. The network consists of two pairs of interneurons, termed ‘swim interneuron 1’ (*sint1*) and ‘swim interneuron 2’ (*sint2*), arranged around a plane of bilateral symmetry. Interneurons on one side of the brain, which includes the paired cerebral, pleural and pedal ganglia, coordinate bending movements toward the same side and communicate via non-rectifying electrical synapses. Interneurons on opposite sides of the brain coordinate antagonistic movements and communicate over mutually inhibitory synaptic pathways. Several criteria were used to identify members of the swim CPG, the most important being the ability to shift the phase of swimming behavior in a quantitative fashion by briefly altering the firing pattern of an individual neuron. Strong depolarization of any of the interneurons produces an ipsilateral swimming movement during which the several components of the motor act occur in sequence. Strong hyperpolarization causes swimming to stop and leaves the animal contracted to the opposite side for the duration of the hyperpolarization. The four swim interneurons make appropriate synaptic connections with motoneurons, exciting synergists and inhibiting antagonists. Finally, these are the only neurons that were found to have this set of properties in spite of concerted efforts to sample widely in the *Melibe* CNS. This led us to conclude that these four cells constitute the CPG for swimming. While *sint1* and *sint2* work together during swimming, they play different roles in the generation of other behaviors. *Sint1* is normally silent when the animal is crawling on a surface but it depolarizes and begins to fire in strong bursts once the foot is dislodged and the animal begins to swim. *Sint2* also fires in bursts during swimming, but it is not silent in non-swimming animals. Instead activity in *sint2* is correlated with turning movements as the animal crawls on a surface. This suggests that the *Melibe* motor system is organized in a hierarchy and that the alternating movements characteristic of swimming emerge when activity in *sint1* and *sint2* is bound together.

Key words: pattern generator, locomotor system, nudibranch, *Melibe leonina*.

Introduction

The orchestration of animal locomotion involves networks of neurons in the central nervous system that function as the central pattern generator (CPG) for the behavior and are responsible for coordinating locomotor movements (Stent et al., 1978; Delcomyn, 1980; Grillner and Wallen, 1985; Grillner et al., 1989; Getting, 1988, 1989b). While this concept is widely accepted (Friesen, 1994), few examples of locomotor CPG networks are known in detail. The nudibranch mollusc *Melibe leonina* swims by bending from side to side in a behavior that can continue for hours in freely swimming animals (Hurst, 1968; Watson et al., 2001, 2002; Lawrence and Watson, 2002). We studied the neural network responsible for generating the swimming rhythm in *Melibe* using microelectrode techniques applied to whole animal preparations, in which cellular activity and behavior could be recorded simultaneously. We also studied specific features of the *Melibe* swim CPG in isolated ganglion preparations that continue to express the swimming motor program *ex vivo*. Using these two approaches, we were able to identify a network of interneurons in the central nervous system that is responsible for determining the form, frequency and amplitude of swimming movements. We believe this network represents the core and possibly the entirety of the central pattern generator for *Melibe* swimming.

We propose that the swim CPG consists of two pairs of interneurons. Interneurons on the same side of the brain function as synergists and are electrically coupled, while interneurons on opposite sides of the brain are antagonists and communicate over mutually inhibitory synaptic pathways. Mutual inhibition appears to be critically important for the expression of alternating activity in the network. Variations on this theme appear again and again in the analysis of oscillatory
networks in central nervous system structures at every level of complexity, from molluscan ganglia to mammalian cortex. It is one of the core motifs in neuronal architecture. In concept, the _Melibe_ swim CPG resembles the paired half-centers model introduced by Graham Brown (1911) that provided an early model for the stepping pattern generator in mammalian locomotion. The relative simplicity of the _Melibe_ system has allowed us to study some of the properties of half-center networks in physiological rather than computational experiments. The interneurons thought to constitute the central pattern generator for swimming, their synaptic interactions, and their output to motoneurons are described here.

**Materials and methods**

Specimens of _Melibe leonina_ Gould were collected near the Hopkins Marine Station, Pacific Grove, CA, USA and near Friday Harbor Laboratories, Friday Harbor, WA, USA and kept in flowing seawater aquaria at ambient temperature (~15°C; Schivell et al., 1997). Most electrophysiological recordings were made using the whole animal preparation developed by Dennis Willows and colleagues (Dorsett et al., 1969; Getting, 1989a; Willows, 1991). This report is based on the results of 73 successful whole-animal experiments in which the animal was able to swim normally throughout the period of intracellular recording.

The brain, which consists of the fused cerebral, pleural and pedal ganglia, was exposed by a small dorsal incision and stabilized against a rigid platform while the animal was suspended in a tank of cooled re-circulating seawater at 15°C (Watson et al., 2002). _Melibe_ can be induced to swim when prepared in this way by depriving the animal of a surface to stand on. Swimming movements were monitored by tying a suture through the posterior tip of the body and connecting it to a lever that partially shielded a photocell. This device produces an oscillating output during swimming with maximum voltage at the peak of the movement to the right and minimum voltage at the peak of the movement to the left. It allowed us to monitor the timing of swimming movements, but occasionally the detector restricted the movement and did not accurately record the maximum excursion.

_Melibe_ neurons are not distinctly pigmented (Cohen et al., 1991) and their locations are somewhat variable between preparations. We used the whole animal preparation in most of our experiments, which allowed individual neurons to be tested for functional equivalence on the basis of the movement produced when stimulated, the phase relationship between activity and behavior, the pattern of synaptic input received during swimming and during rest, and the response to tactile stimulation. These criteria for the identification of equivalent neurons were applied in every experiment. Intracellular recordings were made from neuron cell bodies using glass microelectrodes filled with 3 mol l⁻¹ KCl (Rₑ=20–40 MΩ). Stimulating currents were applied _via_ the recording electrode using a constant current source and a bridge-circuit to null the voltage drop across the electrode resistance. The motor program for swimming continues to be expressed in the _Melibe_ central nervous system after cutting all of the nerve trunks exiting the brain, taking care to leave the circumesophageal connectives intact (Watson et al., 2002). We refer to expression of the motor program in the isolated CNS as _fictive swimming_.

The isolated nervous system was removed to a Plexiglass chamber and bathed in filtered natural seawater or in physiological saline containing (mmol l⁻¹): 470 NaCl, 10 KCl, 10 CaCl₂, 50 MgCl₂, 10 Hepes (pH 8) at 15°C. Data from five isolated nervous system preparations contributed to this report. In order to view the axonal projections of interneurons, cell bodies was injected with Lucifer Yellow (LY) by electrophoresis from an intracellular electrode containing a 5% solution of LY in 0.15 mol l⁻¹ LiCl (N=24) using 500 ms, 10 nA current pulses applied at 1 Hz for 20 min. Preparations were fixed in 4% paraformaldehyde overnight and viewed using an epifluorescence microscope. Where appropriate, values in the text are specified as mean ± S.D.

**Results**

_Melibe_ swimming results from alternating activity in two motoneuron pools responsible for producing left and right bending of the body (Watson et al., 2002). Changing the firing pattern of single motoneurons or pairs of motoneurons does not change the frequency or phase of swimming, indicating that the motoneurons are not part of the central pattern generator for the behavior and do not directly influence pattern generation (S. H. Thompson, unpublished observations). Instead, motoneurons appear to function as a common path for behaviors that involve the same sets of body wall musculature. The question that arises is what binds multipurpose motoneurons together in order to generate the coordinated movements involved in swimming? Watson et al. (2002) showed that motoneurons that function as synergists during swimming fire in-phase because of shared excitatory synaptic drive, while those that function as antagonists fire out-of-phase because of alternating drive. The convergent synaptic drive onto motoneurons suggests that there must be pre-motor neurons that feed-forward to excite synergists and inhibit antagonists. A search for the sources of synaptic drive onto motoneurons during swimming led to the identification of two important classes of central nervous system interneurons.

One interneuron in each of the two pleural ganglia fires strong bursts of action potentials in-phase with swimming and makes synaptic connections with motoneurons that drive the movement. These two cells have been named the right and left ‘swim interneuron 1’ (R_{sint1} and L_{sint1}; referred to as SI by Watson et al., 2001). Their cell bodies are located on the medial dorsal surface just caudal to the prominent tentacular lobe that rises from the center of the pleural ganglion (Fig. 1). The cell body of _sint1_ is unpigmented and 30–50 µm in diameter. It is surrounded by similar looking cells but can be identified on functional grounds because it is the only neuron in the region that fires bursts of action potentials phase-locked to swimming movements. The axon distribution of _sint1_ was
examined after injecting LY into the soma (Fig. 1). Sint1 branches in the pleural ganglion neuropil near the base of the optic lobe and projects to the ipsilateral pedal ganglion via the dorsal pleural-pedal connective, where it forms a series of arborizations. We did not observe processes projecting to the contralateral side via the central commissure or circumesophageal connectives and no processes were seen to exit the central nervous system.

A second pair of interneurons with cell bodies located in the pedal ganglia shares many of the properties of sint1. These two cells are termed the right and left ‘swim interneuron 2’ (Rsint2 and Lsint2; referred to as SiII by Watson et al., 2001). A single sint2 is found near the dorsal midline of each pedal ganglion (Fig. 1). Like the motoneurons in the pedal ganglia (Watson et al., 2002), sint2 fires strong bursts of action potentials during swimming but can be easily distinguished from motoneurons on the basis of the synaptic connections it makes with sint1, its influence on the timing of swimming movements, and its synaptic output to motoneurons. LY staining shows that sint2 branches in the pedal ganglion neuropil and sends a major process to the opposite pedal ganglion via the circumesophageal, pedal-pedal connective. No processes were seen to travel directly to the pleural ganglia or exit the central nervous system. The detailed properties of sint1 and sint2 are described below.

Swim interneuron 1 (sint1)

The pattern of activity in Rsint1 during a brief episode of swimming is shown in Fig. 2A along with a record of swimming behavior. Swimming was initiated by removing a surface from the animal’s foot at the first arrow (movement at this time is due to the physical intervention) and terminated by returning the surface at the second arrow. Sint1 is silent in non-swimming animals and rarely fires even in response to tactile stimulation. Once the animal is dislodged, Rsint1 immediately depolarizes and begins to fire in a bursting mode coincident with the beginning of swimming. In contrast to spike bursts in motoneurons (Watson et al., 2002), the bursts in sint1 ride on a depolarized plateau such that the membrane voltage during the intervals between bursts is 5–10 mV more positive than the voltage recorded when the animal is quiescent or crawling on a surface. The implication is that sint1 is tonically inhibited when the foot is in contact with a surface, but once contact is broken and inhibition is removed, sint1 depolarizes to a level sufficient to maintain spiking activity.

The action potential burst in sint1 precedes the movement to the ipsilateral side and occupies about 40% (39.1±6.2%; N=27) of the swim period. It begins before the movement toward the opposite side reaches its peak and continues into the beginning of the movement to the same side, ending before the peak of the ipsilateral movement. Using the time of maximum ipsilateral bending as a reference, the burst begins 250.1±24.7° (N=27) before the peak and ends 114.2±28.6° (N=27) before the peak. In each individual preparation, the phase relationship is maintained throughout long episodes of swimming but the number of action potentials in the burst (the burst size) can be more variable. In one example, burst size varied between 11 and 33 spikes per burst over 50 cycles of continuous swimming. Variability in burst size in sint1 may explain the observation that the amplitudes of swimming movements wax and wane during long bouts of swimming while the frequency remains more constant. Swimming ends abruptly when the foot contacts a surface (Fig. 2A). As contact is made, sint1 is immediately hyperpolarized and becomes silent, again suggesting that sint1 may receive tonic inhibition as a direct result of foot contact. The bursting activity in Rsint1 during swimming is shown at higher gain and on an expanded
time scale in Fig. 2B. This record shows that action potential bursts in *sint1* are shaped by strong synaptic input. It appears that burst termination results from both cessation of excitatory drive and the appearance of ipsp input that continues into the interburst interval (see insert). Fig. 2C shows a simultaneous recording of activity in *Rsint1* and *Lsint1* during swimming. The two antagonistic interneurons fire in antiphase and there is no overlap between the bursts in these two cells. The interval between the last spike in the burst in one neuron and the first spike in its homologue is fairly constant (519±60 ms; *N*=20). This is a defining feature of the swim CPG and suggests that the network includes mechanisms for maintaining a nearly constant latency between bursts in the two *sint1* values.

**Fig. 2.** Firing pattern in swim interneuron 1. (A) Lower trace: intracellular recording from *Rsint1* during a short episode of swimming. Swimming was initiated by separating the animal’s foot from a surface at the first arrow and terminated by returning the surface at the second arrow. Upper trace: a record of the animal’s side-to-side swimming movements (upward deflection indicates bending toward the right). (B) Lower trace: bursting activity in *Rsint1* at higher gain and on an expanded time base. Insert: the trajectory of membrane voltage at the end of a burst on an expanded scale. Action potentials were truncated by the recording device in this example. Upper trace: a record of the animal’s movement. (C) Simultaneous recording of activity in *Rsint1* (middle) and *Lsint1* (bottom) during a long swimming episode along with the record of swimming movements (top). Note that the spike bursts in these two cells do not overlap.

**Fig. 3.** Graded interruption of swimming by increasing hyperpolarization of *sint1*. (A–D) The upper traces in A–D show activity in *Rsint1* during a continuous episode of swimming. The lower traces show the animal’s swimming movements. Hyperpolarizing currents were applied via the recording electrode using a bridge circuit. The timing of current pulses is indicated by solid bars drawn under behavioral record, and the strength of the hyperpolarizing current is indicated under each bar. Changes in absolute membrane voltage during stimulation are inaccurate because of errors in bridge balance.

**Influence of sint1 on behavior**

Stimulation of *sint1* to fire a burst of action potentials in a quiescent animal causes a bending movement toward the ipsilateral side and the animal remains in that posture for the duration of the stimulus. The driven movement appears to include all of the components that occur during swimming and closely resembles the swimming movement. Lawrence and Watson (2002) described *Melibe* swimming in detail. Swimming movements result from contractions of the longitudinal and diagonal body wall musculature. Contraction of longitudinal muscles pulls the anterior and posterior of the animal together to form a C-shaped bend. Muscles that course dorsally over the oral hood pull the dorsum of the hood to the contracting side and this imparts a corkscrew twist to the body. Stimulation of a single *sint1* results in the same pattern of contractions. Similarly, when *sint1* is stimulated to fire a prolonged burst in a swimming animal, side-to-side movements cease and the animal remains maximally...
contracted to the stimulated side. Fig. 3 shows the effect of applying hyperpolarizing currents of increasing strength to a single \textit{sint1} during swimming. As the current is increased, swimming becomes progressively disorganized. Small currents cause the period of the oscillation to increase and cause asymmetrical contractions (Fig. 3A–C). Strong currents sufficient to prevent firing in the soma and axon branches of \textit{sint1} (judged by the absence of axon spikes) interrupt swimming altogether and cause the animal to remain contracted to the opposite side throughout the hyperpolarization (Fig. 3D). It is particularly interesting that as the hyperpolarizing current is increased, the phasic synaptic input normally seen in the interneuron during swimming becomes progressively diminished until it completely vanishes (Fig. 3D). This demonstrates that alternating activity in the entire swim CPG, as evidenced by cyclic synaptic drive, is brought to a halt by hyperpolarizing a single \textit{sint1}. All of these observations were consistently made in each of 32 separate whole animal preparations. Our interpretation of these results is that \textit{sint1} is directly involved in pattern generation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Resetting the phase of swimming by stimulating \textit{sint1}. The firing pattern in \textit{Lsint1} was recorded along with a record of behavior (upper trace in each panel) during a continuous episode of swimming. (A–C) Hyperpolarizing current pulses sufficient to prevent soma and axon spikes in \textit{Lsint1} were applied via the recording electrode. The timing and duration of pulses were varied (pulse timing indicated by solid lines below the voltage traces). In each experiment, the interval between the centers of \textit{Lsint1} bursts was measured during the 10 cycles preceding the onset of the stimulus (indicated by vertical strokes above the voltage recording). These measurements were used to calculate the means \( \pm \) s.d. of the swim period, which were then used to predict the time of occurrence of the \textit{Lsint1} burst projected forward in time beyond the period of stimulation, based on the assumption that stimulation of \textit{Lint1} has no effect on pattern generation. The predicted times of the center of \textit{Lsint1} bursts are shown as vertical lines above the voltage recording, with the s.d. represented by horizontal tics through the lines. The results show that the assumption fails and that the phase of swimming behavior is reset by the stimulus. The difference between the predicted and actual time of occurrence of the burst provides a quantitative measure of phase resetting (see details in the text). (D) The experiment was repeated with an extra burst driven in \textit{Lsint1}. The extra burst also reset the phase of swimming in a quantitative fashion (see text). The bridge circuit used to deliver stimulating currents was imperfectly balanced and, therefore, the absolute membrane voltage is not accurately represented during periods of stimulation. This experiment was conducted 28 times in four different whole animal preparations with consistent results.}
\end{figure}

\textbf{Stimulation of \textit{sint1} shifts the phase of swimming}

One of the strongest criteria one can use to determine whether a particular neuron is a member of the CPG for a rhythmic behavior is a demonstration that the phase of the behavior can be shifted in a predictable manner by altering the firing pattern in the neuron (Friesen, 1994). We designed an experiment to test this as follows. A single microelectrode was used to record from \textit{sint1} and to apply a constant current pulse of sufficient amplitude to control its activity in a swimming animal. The time of onset of the current pulse relative to the swimming cycle and the duration of the pulse were varied. In the experiments illustrated in Fig. 4, \textit{Lsint1} was current-clamped while cellular activity and swimming behavior were recorded. The period of the normal swimming cycle, defined as the interval between the midpoints of adjacent spike bursts in \textit{Lsint1}, was measured during ten cycles of behavior immediately preceding the stimulus and the means \( \pm \) s.d. of the period calculated. These values were used to predict the expected time of occurrence of the \textit{Lsint1} burst projected forward in time beyond the period of stimulation. This allowed us to compare the actual time of occurrence of succeeding bursts after the stimulus with the expected time of occurrence calculated from the activity pattern prior to the stimulus. Using this comparison, we could determine whether stimulation of \textit{Lsint1} caused the output of the CPG to experience a phase shift. Predictions from the experiment are quantitative because we can ask whether the phase shift is accurately predicted simply from knowledge of the stimulus duration and its time of onset. In addition, we can ask whether phase shifts produced by stimulating \textit{sint1} are permanent or whether the behavior relaxes back into the original phase relationship over time. A permanent phase shift is only expected if the stimulus resets the swim CPG.

In Fig. 4A, \textit{Lsint1} was hyperpolarized for a time equal to the swimming period \( T \) by a current pulse beginning 0.47\( T \) after the peak contralateral bending movement. The current was sufficient to prevent action potentials in both the soma and axon. The result was that the behavior, measured two cycles after the stimulus, was delayed by a factor of 0.46\( T \), the delay
expected (0.47T) if the swim CPG had stopped for the duration of the hyperpolarization and then re-started when the hyperpolarization ended. In Fig. 4B, the cell was hyperpolarized for 1.25T by a current pulse that began 0.38T after the peak of the contralateral movement. The hyperpolarization delayed the swimming rhythm by a factor of 0.62T, the delay expected (0.63T) if the pattern generator was halted by the hyperpolarization. In the third example (Fig. 4C), the cell was hyperpolarized for 3.27T beginning 0.38T after the peak bending movement. This caused a phase shift of 0.6T, again close to the expected value of 0.58T. Similar phase shifts occur when sint1 is depolarized to drive an out-of-phase burst (Fig. 4D). Because the phase shift on stimulation of sint1 is predicted exactly from the stimulus timing and duration, occurs in response to a single stimulus pulse, and because the phase shift is maintained after the stimulus ends, we conclude that sint1 is an integral member of the Melibe swim CPG.

**Mutual inhibition between sint1 interneurons**

The two sint1 neurons on opposite sides of the brain interact over mutually inhibitory synaptic pathways. Fig. 5A,B shows that spike bursts driven in sint1 result in hyperpolarization of the homologous cell on the opposite side of the brain. Fig. 5C,D illustrates what appears to be a unitary ipsps recorded in Lsint1 in response to either a single action potential or a series of seven action potentials in Rsint1. The apparent synaptic delay is 40 ms, which suggests either a polysynaptic pathway or a monosynaptic connection that involves substantial conduction time. We can gain some insight into the nature of this pathway from LY dye-fills, which show that sint1 projects to the ipsilateral pedal ganglion but does not project directly to the opposite pleural ganglion via the dorsal commissure. It appears, therefore, that if the inhibitory connections between the two sint1 values are monosynaptic, the synaptic contact would have to be made in the pedal ganglion neuropil and the axons of sint1 interneurons would have to course through the ipsilateral pedal ganglion and continue to the opposite side via the circumesophageal connective. This long path would involve considerable conduction time and might explain a synaptic delay of 40 ms. The evidence from LY dye-fills is inconclusive on this point. We did not see a process of sint1 that projects all the way to the opposite pedal ganglion, although this would be a difficult result to achieve since it is unlikely that the dye could travel over the entire distance in a thin axonal process. Another
possibility is that the inhibitory connection is polysynaptic and involves other interneurons. We describe a second class of interneurons, termed sint2, which could in principle fill this role but caution that there may be still others that have not yet been identified.

**Output from sint1 to motoneurons**

Sint1 makes excitatory synaptic connections with the motoneurons in the ipsilateral pedal ganglion that are responsible for contraction of the ipsilateral body musculature during swimming. Fig. 6 shows epsps in eight different right pedal ganglion motoneurons in response to action potentials driven in Rsint1. The epsps in Fig. 6C–H appear to be monosynaptic because they follow sint1 action potentials at frequencies in excess of 10 Hz and occur with latencies of 2–5 ms (measured from the peak of the presynaptic action potential recorded in the soma to the foot of the epsp). The epsps in Fig. 6A,B occur with longer latencies (20 and 40 ms, respectively) and may represent excitatory input to motoneurons over polysynaptic pathways. Even in these examples, however, synaptic transmission did not fail during repetitive stimulation at frequencies like those seen during swimming. All of the synaptic potentials illustrated in Fig. 6 appear to be chemically mediated because in each case the amplitude of the epsp increased when the postsynaptic cell was hyperpolarized. There is considerable variability in the rise time, amplitude, and duration of epsps recorded in different motoneurons. It is not known whether this involves presynaptic or postsynaptic mechanisms but it is apparent that action potentials in sint1 give rise to postsynaptic potentials in follower cells that differ in amplitude and time course. These differences undoubtedly contribute to the characteristic differences in the firing patterns of individual motoneurons during swimming.

Sint1 inhibits antagonistic swim motoneurons in the opposite pedal ganglion. Fig. 7A shows a simultaneous recording from Rsint1 and a motoneuron in the left pedal ganglion in a quiescent whole animal preparation. When Rsint1 was driven to fire a burst, the motoneuron was strongly hyperpolarized. Similar results were obtained in isolated ganglion preparations (N=5). We found no evidence for direct or indirect synaptic feedback from synergistic or antagonistic motoneurons to sint1 in any of 32 experiments employing simultaneous intracellular recording. Single action potentials or sustained bursts driven in synergistic or antagonistic motoneurons did not produce discernible psps or change the voltage recorded in sint1 (example in Fig. 7B). When sint1 was depolarized with constant current sufficient to drive low frequency repetitive firing in a quiescent animal, the firing frequency was not altered by driving strong bursts in motoneurons. The conclusion we draw is that while sint1 is presynaptic to many of the motoneurons involved in
swimming, its activity is not influenced by synaptic feedback from motoneurons.

Swim interneuron 2 (sint2)

A second type of interneuron, termed swim interneuron 2 (sint2), shares many of the properties of sint1. Two cells of this type have been identified, one on the dorsal surface of each pedal ganglion (see Fig. 1). The pattern of activity in Lsint2 during a brief episode of swimming is shown in Fig. 8A. When swimming was initiated by withdrawing a surface from the foot at the first arrow, Lsint2 began to fire in bursts that begin before and continue throughout most of the bending movement to the ipsilateral side. When swimming was terminated by returning the surface at the second arrow, bursting activity ceased and sint2 resumed irregular firing. The action potential burst in sint2 occupies 49.7±8.1% (N=9) of the swim period. Using the time of maximum ipsilateral bending as a reference, the action potential burst in sint2 begins 206.8±6.6° (N=5) before the peak of the ipsilateral movement and ends 4.5±17.4° (N=5) before the peak, a phase relationship that is maintained during long episodes of swimming. From these measurements it is clear that the beginning of the burst in sint2 lags the beginning of the burst in the synergistic sint1 by about 43° and that sint2 continues to fire for a greater fraction of the period (see Fig. 15). The latency between the beginning of the burst in sint1 and the beginning of the burst in the synergistic sint2 was measured in dual microelectrode experiments and found to be 460±124 ms (N=3).

The structure of action potential bursts in sint2 is shown on an expanded scale in Fig. 8B. During swimming, bursts appear to be driven by strong excitatory synaptic input while the intervals between bursts are characterized by prominent ipsps. A simultaneous recording from both sint2 neurons during an episode of sustained swimming is shown in Fig. 8C. The two cells fire in antiphase and the end of the burst in one sint2 either does or does not overlap the beginning of the burst in the contralateral homologue. The bursts in sint2 have a complicated substructure, but these recordings suggest that the two cells may share synaptic inputs from some of the same sources. For example, when there is a pause in the burst in one sint2, its homologue experiences an abrupt depolarization. This suggests that there may be presynaptic neurons that simultaneously excite one sint2 and inhibit the other. We demonstrate below that sint1 has precisely these properties.

Effect of sint2 on swimming behavior

Sint2 resembles sint1 in its influence on swimming behavior. When a single sint2 is driven to fire a burst while the animal is crawling on a surface, the animal bends toward the ipsilateral side for the duration of the stimulus. The driven movement resembles the normal ipsilateral swim movement. Similarly, when sint2 is driven to fire a sustained burst while the animal is swimming, the behavior is interrupted and the animal remains contracted to the stimulated side. Hyperpolarizing sint2 to prevent firing during swimming also interrupts the behavior, causing the animal to remain contracted to the opposite side.

Stimulation of sint2 also resets the phase of swimming. We performed an experiment identical to the one used to show phase resetting on stimulation of sint1. In the example in Fig. 9, Lsint2 was hyperpolarized with current sufficient to block soma and axon spikes for 3.8 s (equivalent to 0.51 times the period in the freely swimming animal) while simultaneously recording intracellular activity and swimming behavior. The current pulse began 622·ms after the end of a sint2 burst and caused the animal to spend more time contracted to the unstimulated side, lengthening the interburst interval by 2.1 s. The result was a delay in the swimming rhythm (measured two cycles after the stimulus) of 2.3 s, very close to the delay expected if the CPG for swimming had been halted as long as sint2 was hyperpolarized and then resumed its activity immediately after the hyperpolarization ended. Phase resetting was also observed when sint2 was driven to produce a novel burst of action potentials (not shown). Similar results were obtained in 3 separate whole animal preparations. Our interpretation of these results is that sint2 is also an integral member of the CPG for swimming.

Electrical coupling between synergistic interneurons sint2 and sint1

Sint2 is electrically coupled to the synergistic sint1 by a non-

![Image](https://via.placeholder.com/150)
rectifying electrical synapse. Fig. 10 illustrates simultaneous microelectrode recordings from Rsint1 and Rsint2. It shows that d.c. current is conducted symmetrically in both directions and that the junction is characterized by a d.c. coupling coefficient of 0.1 at voltages near the resting potential (Fig. 10A). Action potentials are not coupled symmetrically, however, and conduction in the direction sint1 to sint2 is much stronger (Fig. 10B). This asymmetry can be expressed in terms of a spike-coupling coefficient, defined as the maximum amplitude of the electrical psp divided by the amplitude of the presynaptic action potential. With this definition, the spike-coupling coefficient for conduction from sint1 to sint2 is 0.03 while the coupling coefficient in the opposite direction, from sint2 to sint1 is nearly ten times less (0.005).

Asymmetrical spike coupling could result from several causes. Because the d.c. coupling coefficient is the same in both directions, it would appear that the asymmetry reflects a capacitative term, possibly due to the physical location of the junction relative to the stimulating and recording sites. To test this idea, transfer functions characterizing the electrical junction were measured in both directions. One cell was driven with constant amplitude, subthreshold, sinusoidal current of varying frequency via a somatic microelectrode, while the electrically coupled sine wave was recorded in the soma of the other cell. The experiment was then repeated after switching the current source. The normalized amplitudes of coupled sine...
waves for transmission in both directions are plotted in Fig. 10C. The transfer functions are identical and show that the junction has the characteristics of a low-pass filter with a cut-off frequency of 1.5 Hz and final slope of 6 dB per octave in frequency. From these measurements we can conclude that asymmetrical spike coupling does not result from differences in cell input capacitance or junctional capacitance. The most likely explanation is that the asymmetry has an anatomical basis. Results from LY dye-fills show that sint1 sends an axonal projection to the ipsilateral pedal ganglion but sint2 does not project to the pleural ganglion, indicating that the electrical junction must be formed in the pedal ganglion neuropil. With this arrangement a spike originating near the cell body of sint1 is expected to propagate actively over much of the pathway and produce a relative large electrical psp in the soma of sint2. A spike originating near the cell body of sint2, however, would propagate actively over only a fraction of the distance, spreading passively the rest of the way, and is expected to produce a more attenuated psp in the soma of sint1. An important consequence is that the electrically coupled psp is expected to have a significant effect on the excitability of sint2 because it occurs close to the site of spike initiation. In contrast, an electrical junction located far from the spike initiation zone of sint1 would be expected to have little effect on its excitability. This anatomical arrangement may allow sint1 and sint2 to function independently under conditions in when sint2 receives excitatory synaptic input that is not shared with the synergistic sint1.

Mutual inhibition between sint2 neurons

The two sint2 neurons form mutually inhibitory synaptic connections. When either cell is driven to fire a burst of action potentials (Fig. 11), its homologue in the opposite pedal ganglion receives strong inhibitory input that begins after a delay (42.8±15.1 ms; N=5). The summed inhibitory potential has a prolonged time course, decaying with a half time of 342±23.9 ms (N=5) after the stimulus ends. This slow decay could be explained either by the presence of interposed interneurons or by prolonged transmitter action. In addition, the inhibitory pathway is somewhat labile. When sint2 is driven to fire a burst of action potentials at a frequency like that seen during a swimming burst (e.g. 10–16 Hz) the amplitude of the summed ipsp in the contralateral sint2
Fig. 14. Inhibitory output from sint2 to an antagonistic motoneuron. (A) Single action potentials were driven in sint2 at a rate of one per second while recording from an antagonistic motoneuron in the opposite pedal ganglion in a quiescent whole animal preparation. Two traces are superimposed. The ipsp occurs with a delay of about 35 ms. (B) A driven burst in sint2 causes sustained hyperpolarization in the motoneuron due to summation of ipsps. These results are characteristic of 7 separate experiments.

The temporal relationships between activity in the interneurons during swimming

The antagonistic Sint2 projects to the same pedal ganglion neuropil via the pedal-pedal connective. This means that if the mutually inhibitory connections between sint2 and the contralateral sint1 are monosynaptic, they are likely to be made in the neuropil of the pedal ganglion on the same side as the sint1 cell body. In this case, conduction through the sub-esophageal connective might explain the long synaptic delay.

**Output from sint2 to motoneurons**

Sint2 makes excitatory synaptic connections with synergistic motoneurons and inhibitory connections with antagonists. Fig. 13 shows results of experiments using an isolated brain preparation. In Fig. 13A, sint2 (lower trace) was driven to fire a burst of action potentials while recording from a synergistic motoneuron in the same pedal ganglion. There is one-for-one correspondence between spikes in sint2 and epsps in the motoneuron. Fig. 13B shows the relationship between activity in sint2 (lower trace) and an antagonistic motoneuron located in the opposite pedal ganglion during fictive swimming. There is close correspondence between action potentials in the interneuron and individual ipsps in the motoneuron. Similar results were obtained in whole animal preparations. Fig. 14A illustrates what appear to be unitary ipsps in a pedal ganglion motoneuron in response to driven spikes in the contralateral sint2. The postsynaptic potential begins with a delay of 35–45 ms after the peak of the spike in the interneuron. A significant fraction of the delay must be the result of conduction time since the only known pathway between sint2 and the neuropil of the opposite pedal ganglion is via the subesophageal pedal-pedal connective. Fig. 14B illustrates summation of ipsps in the motoneuron when sint2 was driven to fire a burst of action potentials. The inhibitory connection is not reciprocal because a driven burst in the motoneuron had no effect on sint2 (not shown).

**Timing of bursts in the interneurons during swimming**

The temporal relationships between activity in the interneurons during swimming are illustrated in Fig. 15. The
Fig. 16. Recordings from sint1 and sint2 during turning movements. (A) A turning movement to the right was initiated by a tactile stimulus applied to the left side of the body while recording from Rsint1 (upper trace) and Rsint2 (lower trace). (B) Activity in the same two cells during a spontaneous turn to the right. A and B are from a whole animal preparation that was allowed to crawl on a blade of seagrass during the recording. Similar observations were made in each of 12 separate whole animal experiments.

The recordings were obtained from the same whole animal preparation during a single experiment and are aligned on the action potential bursts in Rsint2 (see Fig. 15 legend). There are three important timing relationships to note. (1) The burst in sint1 begins before the burst in the synergistic sint2 and ends before the sint2 burst ends. (2) In this example, activity in sint1 begins somewhat after the end of the burst in the antagonistic sint2 and there is no overlap. This is not always the case, however, and the burst in sint1 can begin during the last one or two spikes in the contralateral sint2 burst. (3) Bursts in the two antagonistic sint1 neurons alternate without overlap. This last point has an important consequence. It was inferred from Fig. 2 that burst termination in sint1 coincides with an increase in ipsp input yet the antagonistic sint1 and sint2, the two cells known to inhibit sint1, do not fire at this time. This raises the possibility that there may be additional neurons participating in swim generation that have not yet been identified.

Activity in Sint1 and Sint2 dissociates during other locomotor behaviors

Sint1 is normally silent while the animal is crawling on a surface and it is largely insensitive to sensory input, firing at most a few spikes in response to tactile stimulation of the body. In contrast, sint2 fires sporadically in crawling animals, exhibiting periods of sustained firing along with periods of silence. Sustained firing is always correlated with turning movements toward the ipsilateral side and sint2 receives prolonged inhibitory input during turning toward the opposite side. Fig. 16A shows simultaneous recordings from Rsint1 and Rsint2 during turning toward the right in response to a tactile stimulus applied to the left body wall of an animal that was crawling on a seaweed surface. Rsint2 (lower trace) fires throughout the turning movement and it is apparent that this is the result of a sustained increase in epsp input. The synergistic sint1 (upper trace) also receives excitatory synaptic input, especially at the beginning of the movement, but the input is subthreshold and sint1 does not fire. It would appear that even though sint1 is inhibited by foot contact, it nevertheless receives subthreshold excitation during turning movements toward the ipsilateral side. Activity in the same two cells during a spontaneous turning movement toward the right is shown in Fig. 16B. It appears that sint2 participates in both spontaneous and stimulated turning while sint1 fires weakly or not at all during turning. This suggests that the CPG for swimming is formed dynamically, when activity in the sint1 and sint2 cell pairs becomes bound together. When this does not occur, the same interneurons appear to function independently during the performance of other behaviors that involve the same or similar musculature, such as turning.

Discussion

The synaptic interactions between the four interneurons we identified as members of the Melibe swim CPG are summarized diagrammatically in Fig. 17. Interneurons on the same side of the midline are electrically coupled, while those on opposite sides are linked by mutually inhibitory connections. The major criterion we used to identify members of the swim CPG was the ability to permanently shift the phase of swimming by stimulating individual neurons. Both sint1 and sint2 meet this criterion and they share a number of other features that suggest that they work together to generate the behavior. Both types of interneurons fire in bursts that are phase-locked with swimming and precede the ipsilateral swimming movement. Both have a strong and lasting effect on behavior since a brief depolarizing or hyperpolarizing current applied to any of them is sufficient to cause a permanent phase shift. In addition, stimulation of any of the interneurons in a
resting animal causes the animal to bend to the ipsilateral side, producing a coordinated movement that resembles the swimming movement toward that side. When \( s1t1 \) or \( s1t2 \) is driven to fire a long burst in a swimming animal, the behavior is interrupted and the animal remains contracted to the stimulated side; conversely, when the cell is hyperpolarized to prevent firing during swimming the animal remains contracted to the opposite side. Finally, the output from both \( s1t1 \) and \( s1t2 \) is distributed in a manner appropriate for members of the swim CPG, exciting synergistic motoneurons and inhibiting antagonists, thus providing the reciprocal drive onto motoneurons necessary to generate alternating side-to-side movements. Following the reasoning of Friesen (1994) and Svoboda and F etcho (1996) we take these findings as strong evidence that the two \( s1t1 \) and the two \( s1t2 \) neurons are integral members of the CPG for Melibe swimming. The experiment in which \( s1t1 \) was progressively hyperpolarized during swimming (Fig. 3) led to an additional important observation. It appears that strong hyperpolarization of a single \( s1t1 \) abolishes the periodic synaptic drive that interneurons within the CPG normally receive during swimming. Apparently, hyperpolarization of a single member of the network can stop rhythmic activity in the entire network (see also Figs 4, 9). It appears, therefore, that the CPG for swimming functions when activity in all four interneurons is bound together and the electrical connections between synergistic interneurons may help to establish this grouping.

Although \( s1t1 \) and \( s1t2 \) fire in a coordinated fashion during swimming, they have very different firing patterns and serve different roles in the generation of other behaviors. When the animal is crawling on a surface \( s1t1 \) is silent, perhaps because it receives inhibitory input from sensory pathways signaling foot contact, but \( s1t2 \) is not silent and fires irregularly. Furthermore, \( s1t2 \) begins to fire during the initiation of turning movements toward the ipsilateral side and continues to fire throughout turning while the synergistic \( s1t1 \) remains silent. It would appear that \( s1t2 \) is multifunctional, participating in at least two locomotor behaviors (swimming and turning), while \( s1t1 \) participates only in swimming. Although the synergistic \( s1t1 \) and \( s1t2 \) are electrically coupled, they can work independently under certain conditions because the anatomical arrangement favors conduction of action potentials in only one direction, from \( s1t1 \) to \( s1t2 \). This feature may allow the network to rearrange in a dynamic fashion, dictated by the nature of the synaptic input from other sources. In this way, part of the motor system responsible for generating side-to-side swimming movements can be used to orchestrate non-rhythmic, unilateral movements that involve the same musculature. Even during turning, however, the mutually inhibitory connections between \( s1t2 \) neurons ensure that when synergistic motoneurons are excited, antagonists will be inhibited. This organization is reminiscent of the situation in Tritonia where the CPG for escape swimming can act in different states of coordination to serve different functions (Katz et al., 1994, 2001).

We observed that when the foot loses contact with a surface, \( s1t1 \) immediately depolarizes to a level 5–10 mV above the voltage recorded in the quiescent animal. Spike bursts in \( s1t1 \) are superimposed on this depolarization (Fig. 2A). This feature is unique to \( s1t1 \) and is not seen in \( s1t2 \) or in the motoneurons. The sustained depolarization in \( s1t1 \) may provide an important clue into how the CPG is dynamically structured and it is possible that control over the initiation of swimming involves only removal of inhibition at the level of \( s1t1 \), a situation reminiscent of the role of tarsal inhibition in locust flight (Ritzmann et al., 1980). This has not yet been demonstrated by direct experiment, but it raises the interesting possibility that a change in sensory input might cause dynamic restructuring of the CPG network.

The swimming motor system appears to be hierarchically arranged. While \( s1t1 \) and \( s1t2 \) both fire bursts during swimming, the timing of the bursts in the two classes of interneurons is characteristically different. The \( s1t1 \) burst always begins before the burst in the synergistic \( s1t2 \) and ends before the end of the \( s1t2 \) burst, a sequence that persists even though \( s1t1 \) and \( s1t2 \) are electrically coupled. Apparently the electrical synapse is not strong enough to fully synchronize their activity. Spike bursts in the two \( s1t1 \) neurons do not overlap while bursts in the two antagonistic \( s1t2 \) neurons may or may not overlap. Finally, termination of swimming is correlated with cessation of firing in \( s1t1 \) but not with silencing of \( s1t2 \). These observations suggest that the two types of interneurons occupy different positions in a hierarchically arranged motor system. Because \( s1t1 \) appears to be active only during swimming, we think of it as a key element in the swim CPG and that without its participation, the network cannot function in the alternating mode characteristic of swimming. At still another level, the motoneurons appear to act as followers whose activity is determined by input from interneurons (Watson et al., 2002). There are between 14 and 21 motoneurons in each pedal ganglion that participate in swimming (S. H. Thompson, unpublished observations) but there is no evidence that any of the motoneurons feed back to interneurons, a finding consistent with the idea that motoneurons function as a final common path for locomotor behavior but do not participate in pattern generation.

**Where are the inhibitory connections between interneurons made?**

Swim interneurons on opposite sides of the brain make reciprocal inhibitory connections, an organization that provides a plausible mechanism for the production of alternating activity in the network (Perkel and Mulloney, 1974; Marder and Eisen, 1984; Getting, 1989a; Satterlie, 1985, 1989; Pearson, 1993; Friesen, 1994). There is uncertainty, however, about where the inhibitory connections are made and whether they are monosynaptic or polysynaptic. Resolving these issues will be important in order to fully understand which features of network architecture are responsible for determining its output frequency and stability.

\( s1t2 \) projects to the opposite pedal ganglion via a process that runs in the sub-esophageal pedal-pedal connective. We
consistently found that alternating activity in the swim CPG, and swimming behavior, ceases when this pathway is cut (N=5; W. Watson, unpublished observations). We interpret this as strong evidence that axons traveling in the sub-esophageal connectives are necessary for the expression of oscillating activity in the network, the most likely reason being that these axons are responsible for the mutually inhibitory interactions between interneurons. The apparent synaptic delay for the inhibitory interactions (35–45 ms) could be the result of conduction time or it could indicate that the pathways are polysynaptic. Some insight into conduction time over the sub-esophageal connectives can be gained from the following observation. A synaptic delay of 40–45 ms was measured for an apparently monosynaptic inhibitory connection between sint2 and an antagonistic motoneuron in the opposite pedal ganglion. Evidence from dye-fills indicates that this synapse is most likely to be made in the neuropil that contains the ganglion. Given the diversity of swimming modes and the wide phylogenetic divergence between these five species, it is not surprising to find more differences than similarities in swim CPGs (Katz et al., 2001). The Tritonia and Pleurobranchaea CPGs are the only two that have clear similarities, possibly because the mode of swimming is so similar. Both animals swim to escape from predators and their swimming activity is limited to short bouts. Many homologous neurons have been identified in these two species and the basic mechanisms underlying initiation, production and termination of the swim rhythm are similar (Getting, 1989a; Jing and Gillette, 1999: Gillette and Jing, 2001). The interneurons identified in Melibe have features in common with the A4 and A10 interneurons in Pleurobranchaea, which also project to the ipsilateral pedal ganglia and have major influence on the generation of swimming (Jing and Gillette, 2003). Clione swims for locomotion rather than primarily for escape. The swimming circuit in Clione resembles the swim CPG in Melibe in that reciprocal inhibition appears to be the dominant mechanism for pattern generation and key elements of the CPG appear to reside in the pedal ganglia in both animals (Satterlie, 1985; Arshavsky et al., 1985).

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References


