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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 leonine*.

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 (R_e=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 $CaCl_2$, 50 $MgCl_2$, 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’ (*Rsint1* and *Lsint1*; referred to as SiI 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 SiI 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.

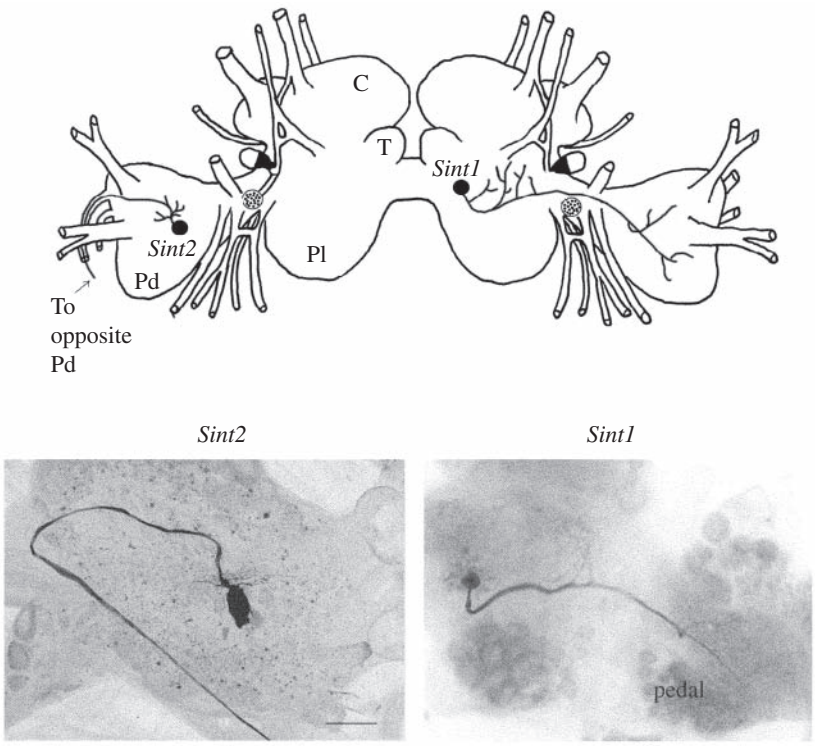

Fig. 1. Axon distributions of *sint1* and *sint2*. (Top) A diagram of the dorsal aspect of the *Melibe* (rostral at the top), illustrating the three major subdivisions of the CNS: the cerebral ganglia (C), pleural ganglia (Pl) and pedal ganglia (Pd). The cell bodies and major projections of *sint1* and *sint2* are shown diagrammatically, based on interpretation of 24 successful Lucifer Yellow dye fills. Examples of Lucifer Yellow stained interneurons are shown below. *Sint1* branches near its cell body located just caudal to the tentacular lobe (T) in the pleural ganglion and sends a process to the ipsilateral pedal ganglion that arborizes in the pedal ganglion neuropil. It does not project across the midline *via* the central commissure or beyond the CNS. *Sint2* branches in the pedal ganglion and sends a process *via* the pedal-pedal connective to the opposite pedal ganglion. No processes were seen to project to the pleural ganglia or leave the CNS. The dye fills illustrated in this figure were done in isolated ganglion preparations.

*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

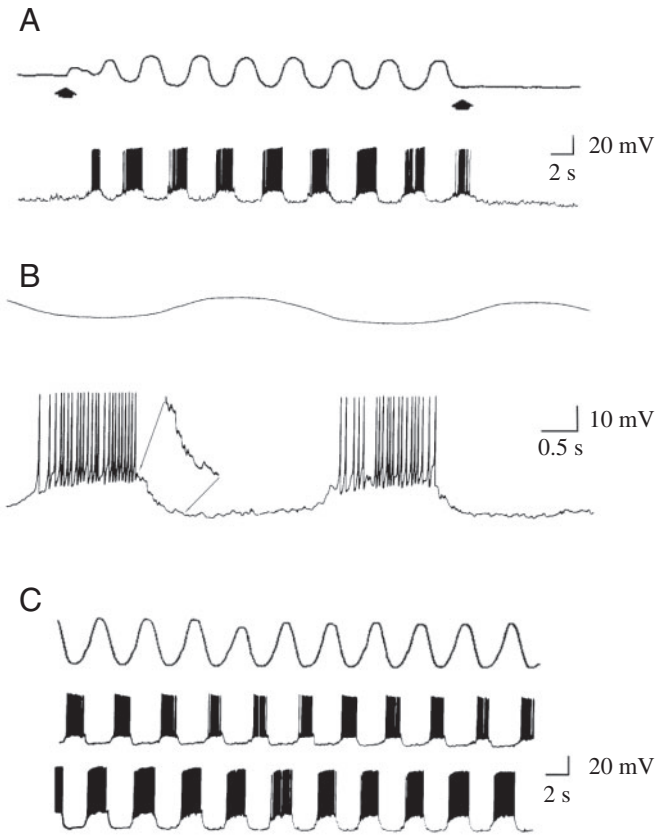

Fig. 2. Firing pattern in swim interneuron 1. (A) Lower trace: intracellular recording from *Rsint1* during a short episode of swimming. Swimming was initiated by separating the animal's foot from a surface at the first arrow and terminated by returning the surface at the second arrow. Upper trace: a 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.

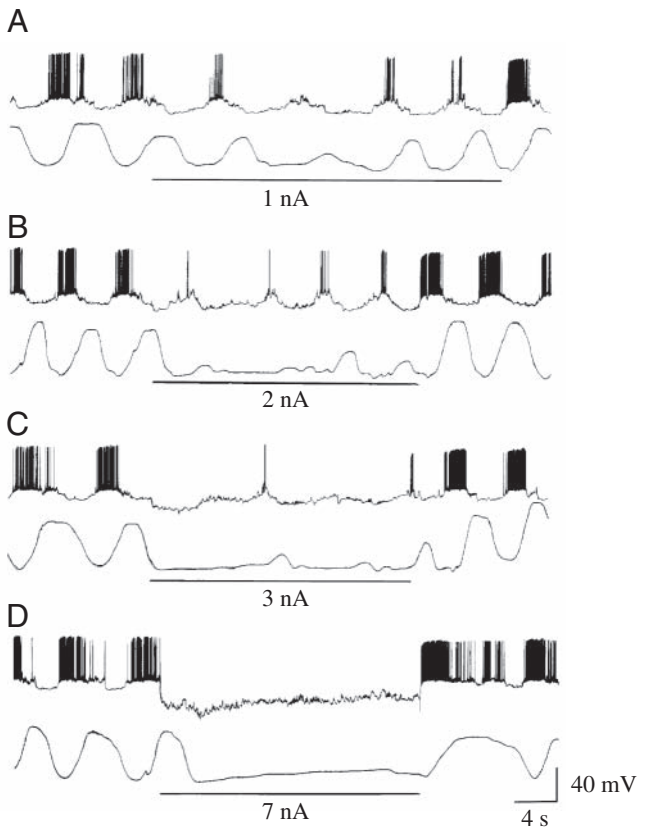

Fig. 3. Graded interruption of swimming by increasing hyperpolarization of *sint1*. (A–D) The upper traces in A–D show activity in *Rsint1* during a continuous episode of swimming. The lower traces show the animal's swimming movements. Hyperpolarizing currents were applied *via* the recording electrode using a bridge circuit. The timing of current pulses is indicated by solid bars drawn under the 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.

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.

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 *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 *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 *sint1*. All of these observations were consistently made in each of 32 separate whole animal preparations. Our interpretation of these results is that *sint1* is directly involved in pattern generation.

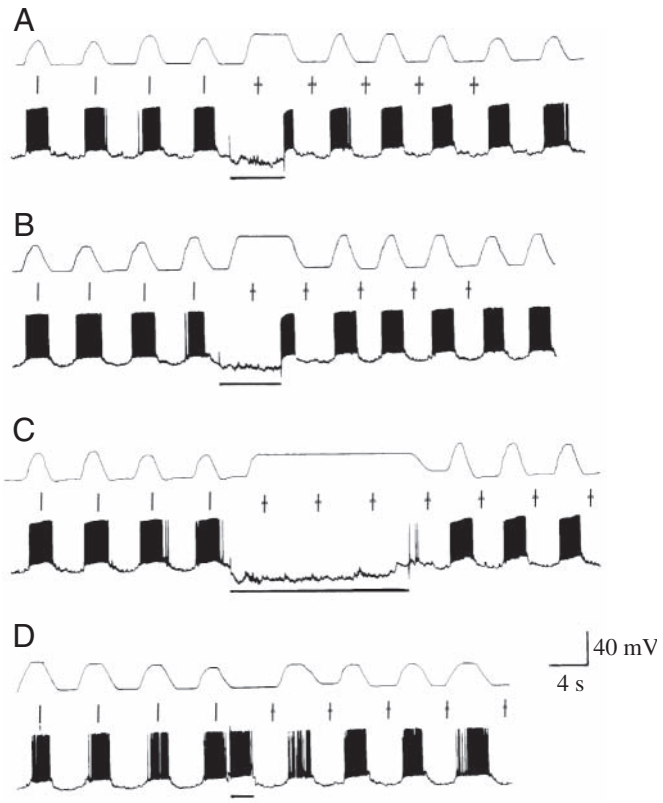

Fig. 4. Resetting the phase of swimming by stimulating *sint1*. The firing pattern in *Lsint1* was recorded along with a record of behavior (upper trace in each panel) during a continuous episode of swimming. (A–C) Hyperpolarizing current pulses sufficient to prevent soma and axon spikes in *Lsint1* 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 *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 ± S.D. of the swim period, which were then used to predict the time of occurrence of the *Lsint1* burst projected forward in time beyond the period of stimulation, based on the assumption that stimulation of *Lint1* has no effect on pattern generation. The predicted times of the center of *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 *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.

Stimulation of 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 *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, *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 *Lsint1*, was measured during ten cycles of behavior immediately preceding the stimulus and the means ± S.D. of the period calculated. These values were used to predict the expected time of occurrence of the *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 *Lsint1* caused the output of the CPG to experience a phase shaft. 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 *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, *Lsint1* was hyperpolarized for a time equal to the swimming period (T) by a current pulse beginning $0.47T$ 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 $0.46T$, the delay

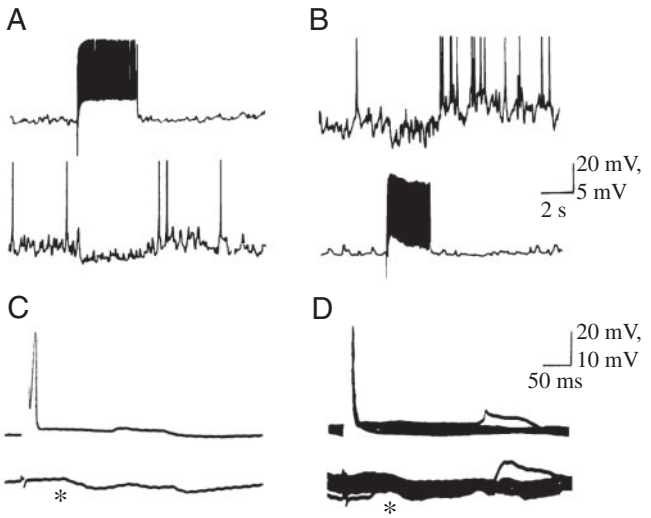

Fig. 5. Reciprocal inhibition between *sint1* neurons. (A,B). Simultaneous recordings from *Rsint1* (upper trace) and *Lsint1* (lower trace) in a quiescent whole animal preparation. In A, *Rsint1* was driven to fire a burst of action potentials while the voltage in *Lsint1* was recorded at 4× higher gain. The same procedure was followed in B, where *Lsint1* was stimulated. (C,D). Unitary ipsp in *Lsint1* during stimulation of *Rsint1*. *Rsint1* was driven to fire either a single action potential (C) or a series of seven action potentials at a rate of one per second (D, sweeps superimposed). Asterisks indicate the time of occurrence of the ipsp. This experiment was repeated in six different whole animal preparations with consistent results.

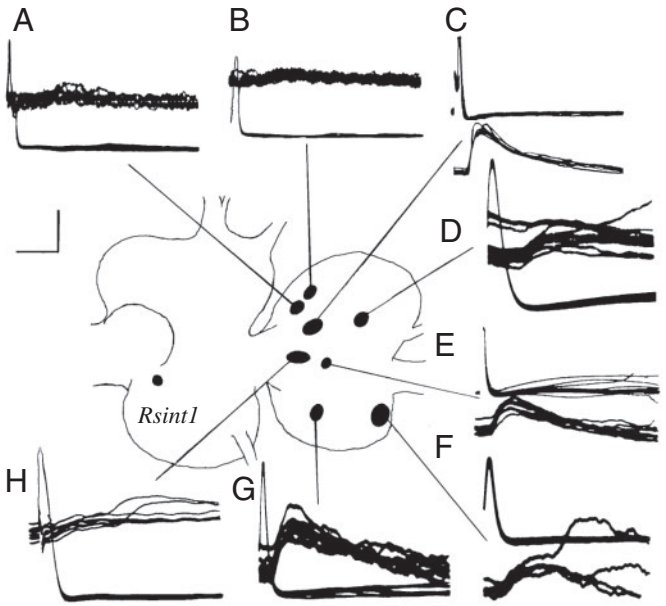

Fig. 6. Excitatory synaptic output from *sint1* to synergistic motoneurons. The dorsal aspect of the right half of the CNS, including the right pleural and pedal ganglia, is shown diagrammatically (rostral direction upward). The diagram also shows the relative positions of the soma of *Rsint1* in the pleural ganglion and of eight individual motoneurons in the ipsilateral pedal ganglion that are known to participate in swimming behavior. Single spikes were driven in *Rsint1* at a rate of one per second in a quiescent whole animal preparation while recording membrane voltage at higher gain in the motoneurons. The results from 5–10 repetitions are superimposed. In each case the spike in *Rsint1* elicits an epsp in the motoneuron. The time calibration bar (near A) corresponds to 40 ms (A–C,G) and 20 ms (D–F,H). For the postsynaptic potentials, the vertical calibration corresponds to 2 mV (A,B), 4 mV (F–H), and 20 mV in the other recordings. The epsps in A and B occur with latencies of 20–40 ms, while those in C–H occur with latencies of 2–5 ms. These results are characteristic of those obtained in 30 separate whole animal preparations involving >150 paired recordings.

expected (0.47*T*) 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 1.25*T* by a current pulse that began 0.38*T* after the peak of the contralateral movement. The hyperpolarization delayed the swimming rhythm by a factor of 0.62*T*, the delay expected (0.63*T*) if the pattern generator was halted by the hyperpolarization. In the third example (Fig. 4C), the cell was hyperpolarized for 3.2*T* beginning 0.38*T* after the peak bending movement. This caused a phase shift of 0.6*T*, again close to the expected value of 0.58*T*. 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 ipsp 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

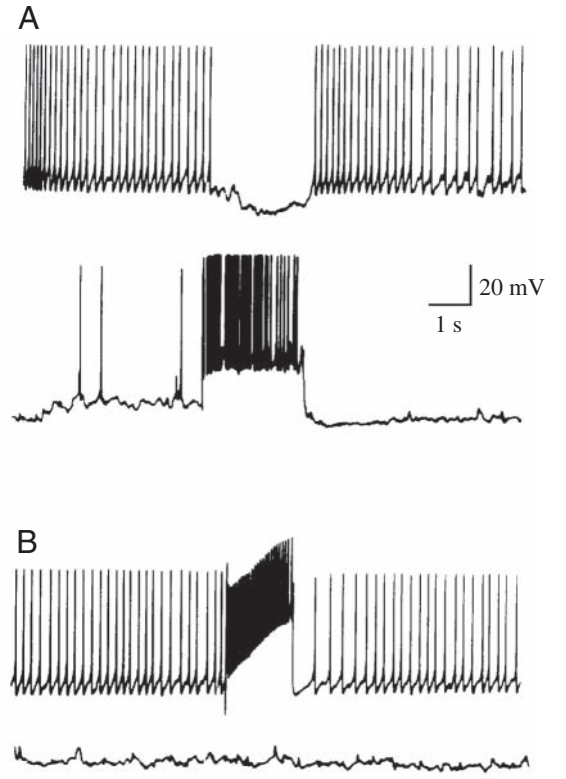

Fig. 7. Inhibitory synaptic output from *sint1* to an antagonistic motoneuron recorded in a quiescent whole animal preparation. (A) A driven burst of action potentials in *Rsint1* (lower trace) causes hyperpolarization of an identified antagonistic motoneuron located in the opposite pedal ganglion. (B) A driven burst in the motoneuron has no effect on *Rsint1*.

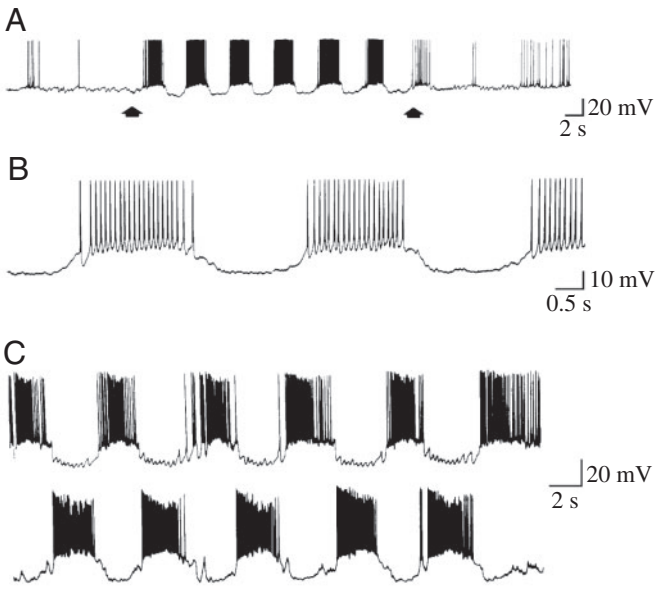

Fig. 8. Firing pattern in swim interneuron 2 (*sint2*). (A) Activity in *Lsint2* during a brief episode of behavior. Swimming was initiated by removing a surface from the animal's foot at the first arrow and terminated by replacing the surface at the second arrow. (B) Action potential bursts in *Lsint2* shown at higher gain and on an expanded time scale. (C) Alternating bursts in the two antagonistic *sint2* neurons recorded simultaneously during a long episode of swimming.

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.

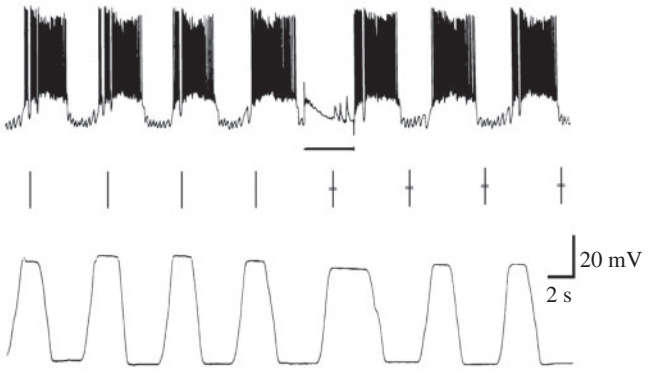

Fig. 9. Resetting the phase of swimming by stimulating *sint2*. (Top) An intracellular recording from *Lsint2* during swimming; (bottom) a record of the animal's movements. The movement detector saturated and did not report the full range of side-to-side movement in this example. Hyperpolarizing current sufficient to silence the cell was applied *via* the recording electrode during the time indicated by the bar under the voltage recording. The time of maximal right flexion (shown by vertical lines above the behavior record) was measured during the 10 cycles preceding the onset of the stimulus in order to calculate the mean ± S.D. of the swimming period. These values were projected forward in time to predict the expected time of occurrence of maximal right flexion The bars and vertical lines above the behavioral record show the predicted time of occurrence of peak right flexion after the stimulus ends. Horizontal tics show the S.D. The difference between the predicted time of occurrence and the actual occurrence of peak flexion provides a measure of phase resetting. This experiment was repeated 15 times in three different whole animal preparations with consistent results.

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-

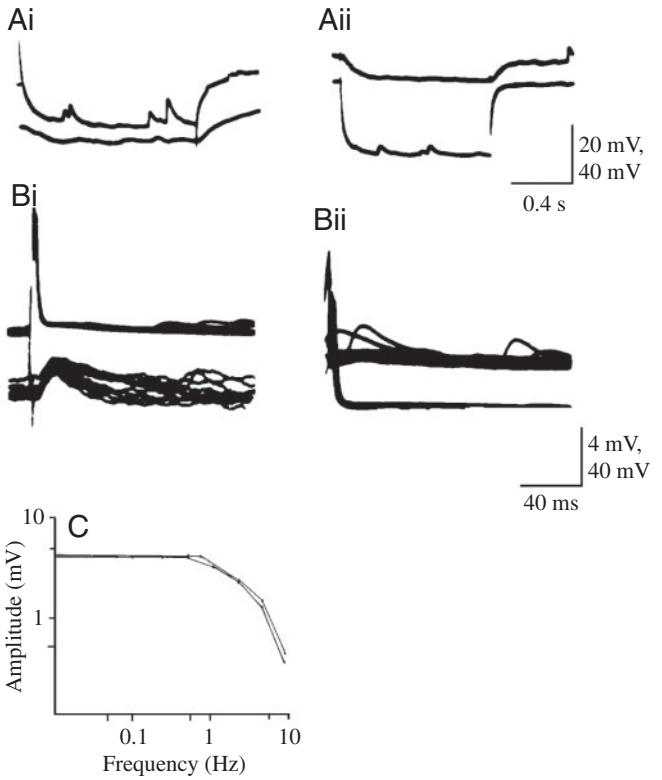

Fig. 10. Electrical coupling between *sint1* and the synergistic *sint2*. (A,B) Simultaneous recordings from *Rsint1* (upper traces) and *Rsint2* (lower traces) in a quiescent whole animal preparation. Hyperpolarizing current pulses were applied to one of the cells *via* the recording electrode while membrane voltage was recorded in the other. Bridge balance was checked using short hyperpolarizing pulses of the same amplitude before and after the recording. (Ai) A current pulse applied to *Rsint1* causes hyperpolarization of *Rsint2* due to current flow across the electrical junction. (Aii) The reciprocal connection. (Bi) The electrically coupled epsp in *Rsint2* (lower trace; recorded at higher gain) in response to driven action potentials in *Rsint1* (eight superimposed traces, stimulus rate one per second). (Bii) The strongly attenuated electrically coupled epsp recorded in *Rsint1* (upper trace; recorded at higher gain) in response to single action potentials driven in *Rsint2* (seven superimposed traces, stimulus rate one per second). (C) Bode plot showing conduction of sinusoidal currents across the electrical junction in both directions. *Rsint1* and *Rsint2* were stimulated, one at a time, with subthreshold currents of fixed amplitude but varying frequencies while recording the coupled sine wave in the other cell. The electrical junction passes current symmetrically in both directions and has the characteristics of a low-pass filter with a corner frequency of 1.5 Hz and final slope of 6 dB per octave in frequency. These results were confirmed in each of six separate whole animal preparations.

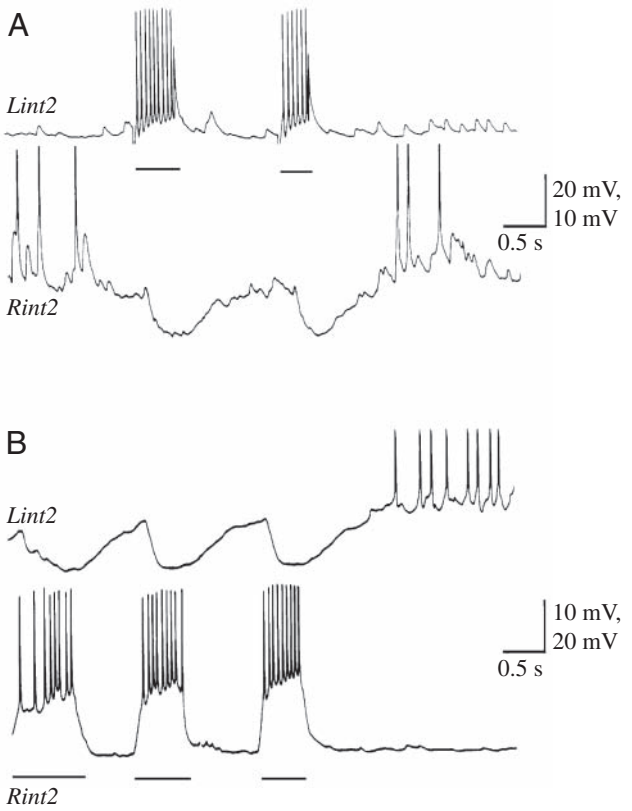

Fig. 11. Mutual inhibition between antagonistic *sint2* neurons. Simultaneous recordings from *Lsint2* and *Rsint2* in a quiescent whole animal preparation. (A) *Lsint2* (upper trace) was driven to fire two bursts of action potentials while recording membrane voltage in *Rsint2* (lower trace) at higher gain (time of stimulus marked by bar). (B) Stimulation of *Rsint2* to fire three bursts while recording from *Lsint2*. Action potentials were truncated by the recording device in the high gain records. The firing frequency during driven bursts was similar to what is seen during normal swimming (see Fig. 8). Similar results were obtained in each of five whole animal experiments.

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

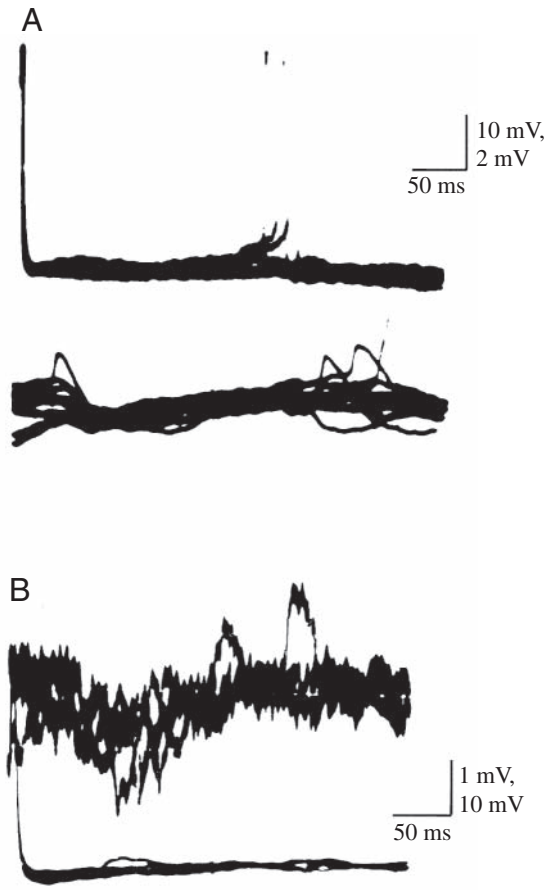

Fig. 12. Mutual inhibition between the antagonistic *sint1* and *sint2*. Simultaneous recordings from *Lsint2* (upper traces) and *Rsint1* (lower traces) in a quiescent whole animal preparation. (A) Single action potentials were driven in *Lsint2* at a rate of one per second while recording from *Rsint1* at higher gain (7 superimposed traces). (B) The reciprocal ipsp in *Lsint2* during stimulation of single action potentials in *Rsint1* at 1 Hz (6 superimposed traces). Similar results were obtained in each of 12 separate whole animal experiments.

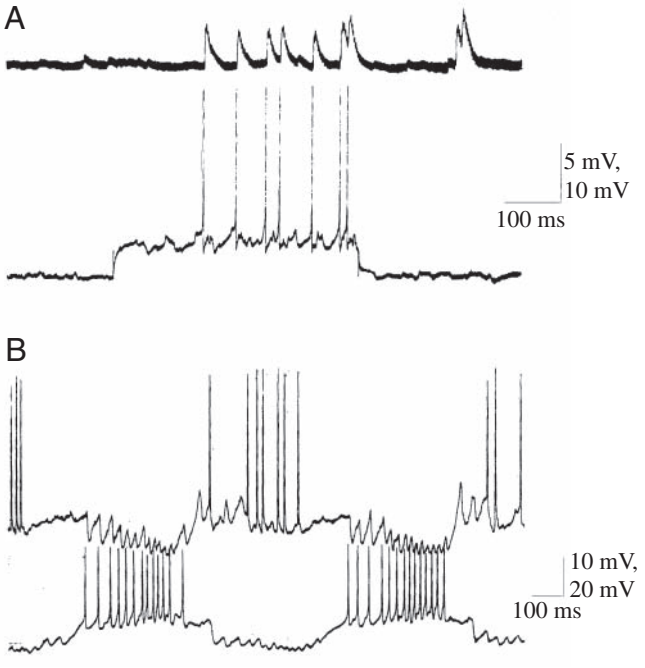

Fig. 13. Synaptic output from *sint2* to motoneurons. (A) Excitatory output from *sint2* to a synergistic motoneuron recorded in a quiescent isolated brain preparation. *Sint2* (lower trace) was driven to fire a burst of action potentials by direct stimulation while recording membrane voltage in the motoneuron (upper trace). Individual spikes in *sint2* correspond with unitary epsps in the motoneuron. (B) Ipsps in an antagonistic motoneuron coincident with action potentials in *sint2* during fictive swimming in an isolated brain preparation.

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*

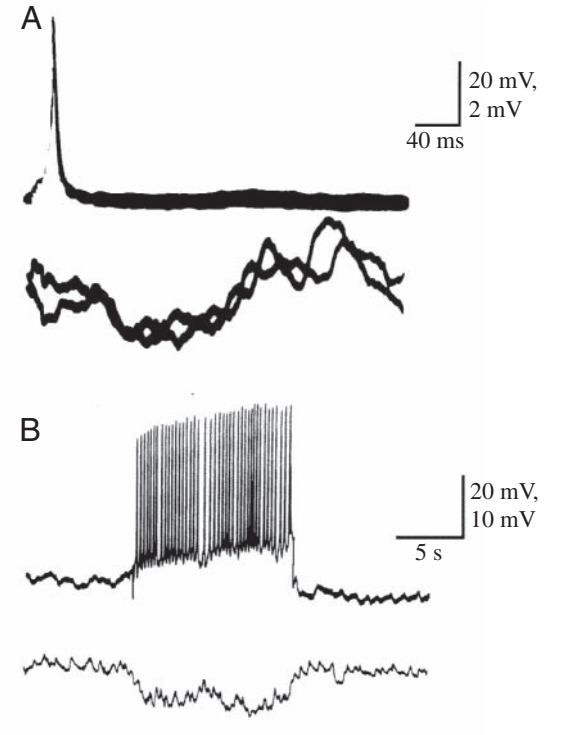

Fig. 14. Inhibitory output from *sint2* to an antagonistic motoneuron. (A) Single action potentials were driven in *sint2* at a rate of one per second while recording from an antagonistic motoneuron in the opposite pedal ganglion in a quiescent whole animal preparation. Two traces are superimposed. The 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.

declines to 70% of its peak amplitude with a half-time of 4.5 s. This decline suggests a synaptic fatigue process that may play a role in timing oscillation in the network.

Mutual inhibition between sint2 *and the contralateral* sint1

Sint2 also forms mutually inhibitory synaptic connections with the antagonistic *sint1* located in the pleural ganglion on the opposite side of the brain. Fig. 12A shows the ipsp recorded in *Rsint1* when *Lsint2* was driven to fire single action potentials. The synaptic connection in the opposite direction is illustrated in Fig. 12B. The ipsps are similar in amplitude and duration at a given postsynaptic voltage and the inhibitory pathways do not fail at stimulus rates as high as 10 Hz. When the presynaptic cell is stimulated to fire a burst that matches the frequency and duration of the swimming burst, the summed ipsp does not decline, indicating that the pathway is resistant to fatigue. Ipsps follow presynaptic spikes with latencies of 35 to 45 ms, suggesting either a polysynaptic pathway or significant conduction time. LY dye-fills show that *sint1* sends a process to the ipsilateral pedal ganglion where it arborizes. 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

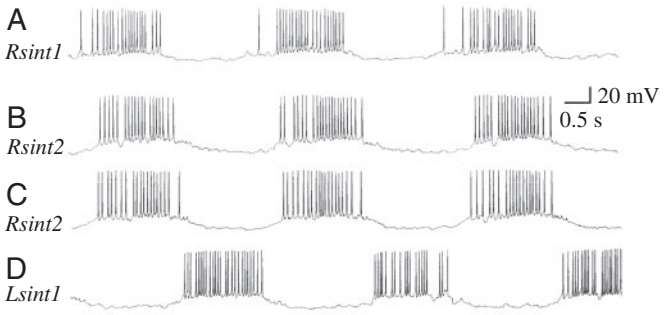

Fig. 15. The timing of bursts in *sint1* and *sint2* during swimming. (A,B) Simultaneous recording from *Rsint1* (A) and *Rsint2* (B) during an episode of swimming in a whole animal preparation. After this recording was taken, the electrode was removed from *Rsint1* and *Lsint1* was impaled. (C,D) Simultaneous recording from *Rsint2* (C) and *Lsint1* (D) during the same swimming episode. The two sets of records were aligned to the midpoint of the center burst in *Rsint2* so that the timing of bursts could be compared. All recordings are from the same whole animal preparation. Similar results were obtained in three separate whole animal experiments.

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 subesophageal 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. Again, 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 subesophegeal 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

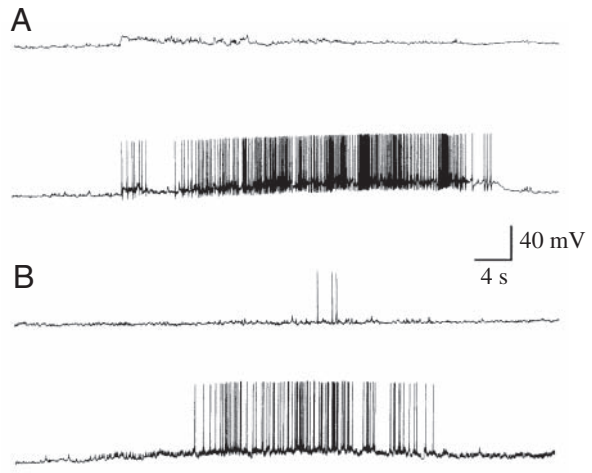

Fig. 16. Recordings from *sint1* and *sint2* during turning movements. (A) A turning movement to the right was initiated by a tactile stimulus applied to the left side of the body while recording from *Rsint1* (upper trace) and *Rsint2* (lower trace). (B) Activity in the same two cells during a spontaneous 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.

figure shows pair-wise recordings from *Rsint1* and *Rsint2* (two upper traces) and from *Rsint2* and *Lsint1* (two lower traces). 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.

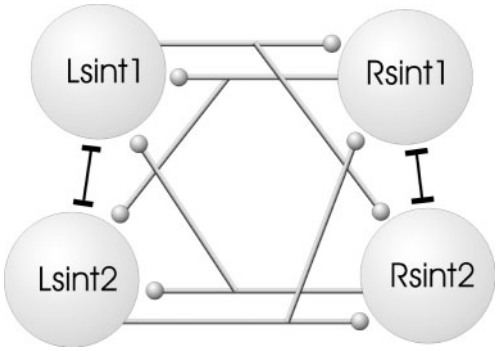

Fig. 17. Network model for the *Melibe* swim CPG. The mutually inhibitory connections linking the left (L) and right (R) *sint1* and *sint2* neurons are shown by lines terminating in circles. The electrical synapses between the synergistic *sint1* and *sint2* neurons are shown by lines terminating in bars.

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 *sint1* or *sint2* 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 *sint1* and *sint2* 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 Fetcho (1996) we take these findings as strong evidence that the two *sint1* and the two *sint2* neurons are integral members of the CPG for *Melibe* swimming. The experiment in which *sint1* was progressively hyperpolarized during swimming (Fig. 3) led to an additional important observation. It appears that strong hyperpolarization of a single *sint1* 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 *sint1* and *sint2* 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 *sint1* is silent, perhaps because it receives inhibitory input from sensory pathways signaling foot contact, but *sint2* is not silent and fires irregularly. Furthermore, *sint2* begins to fire during the initiation of turning movements toward the ipsilateral side and continues to fire throughout turning while the synergistic *sint1* remains silent. It would appear that *sint2* is multifunctional, participating in at least two locomotor behaviors (swimming and turning), while *sint1* participates only in swimming. Although the synergistic *sint1* and *sint2* are electrically coupled, they can work independently under certain conditions because the anatomical arrangement favors conduction of action potentials in only one direction, from *sint1* to *sint2*. 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 *sint2* 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, *sint1* immediately depolarizes to a level 5–10 mV above the voltage recorded in the quiescent animal. Spike bursts in *sint1* are superimposed on this depolarization (Fig. 2A). This feature is unique to *sint1* and is not seen in *sint2* or in the motoneurons. The sustained depolarization in *sint1* 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 *sint1*, 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 *sint1* and *sint2* both fire bursts during swimming, the timing of the bursts in the two classes of interneurons is characteristically different. The *sint1* burst always begins before the burst in the synergistic *sint2* and ends before the end of the *sint2* burst, a sequence that persists even though *sint1* and *sint2* are electrically coupled. Apparently the electrical synapse is not strong enough to fully synchronize their activity. Spike bursts in the two *sint1* neurons do not overlap while bursts in the two antagonistic *sint2* neurons may or may not overlap. Finally, termination of swimming is correlated with cessation of firing in *sint1* but not with silencing of *sint2*. These observations suggest that the two types of interneurons occupy different positions in a hierarchically arranged motor system. Because *sint1* 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.

Sint2 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 motoneuron cell body by an axonal process of *sint2* traveling to that neuropil *via* the pedal-pedal connective. If the axons responsible for mutual inhibition between *int2* values follow the same route, then a synaptic delay of 40–45 ms is not inconsistent with a monosynaptic connection.

The pathway responsible for mutual inhibition between the two *sint1* interneurons is less clear. In anatomical studies, *sint1* was never found to project to the opposite pleural ganglion *via* the central commissure. It is possible that *sint1* projects to the contralateral side through the sub-esophageal connective, although this too was not seen in dye-fills. An alternative suggestion is that the mutual inhibition between *sint1* neurons involves a polysynaptic pathway, and there are two possibilities. *Sint1* might first drive the ipsilateral *sint2,* which in turn makes inhibitory synapses with the antagonistic *sint1* and *sint2* in the neuropil of the opposite pedal ganglion. The second possibility is that another as-yet-unidentified interneuron mediates mutual inhibition between the two *sint1* neurons.

*Are s*int1 *and* sint2 *the only members of the swim CPG, or are there missing elements?*

We identified four interneurons as integral members of the CPG for *Melibe* swimming. The question remains, however, whether there are additional neurons that we have not yet identified that contribute in important ways to network function. Microelectrode sampling bias can never be completely eliminated. Although we tried to minimize the problem by sampling widely in the *Melibe* central nervous system, there may be other important units that are missing from our analysis because they were not observed or because their importance was not recognized. One observation in particular suggests that additional neurons may be involved in pattern generation. We are not able to explain the appearance of ipsps in *sint1* at burst termination (see Fig. 2B) because none of the interneurons demonstrated to have inhibitory interactions with *sint1* are active at this time. Recent studies suggest that another interneuron, anatomically distinct from *sint1* and *sint2*, may play a role in swim generation (J. Newcomb, personal communication). It is not known whether this newly described neuron inhibits *sint1* or *sint2,* but determining if it has a role in the swim CPG and defining its function will be important.

Comparison of the Melibe *swim CPG with that of other opisthobranchs*

Several opisthobranch species exhibit swimming behavior, some for escape and some for locomotion. In five species there is specific knowledge of the organization of the swim CPG (*Clione limacine, Aplysia brasiliana, Tritonia diomedea, Pleurobranchaea californica* and now *Melibe leonina).* 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

Arshavsky, Yu. I., Beloozerova, I. N., Orlovsky, G. N., Panchin, Yu. V. and Pavlova, G. A. (1985). Control of locomotion in marine mollusc *Clione limacina*. II. Rhythmic neurons of pedal ganglia. *Exp. Brain Res.* **58**, 263-272.

Cohen, L. B., Watson, W., Trimarchi, J., Falk, C. X. and Wuy, J. Y. (1991). Optical measurement of activity in the *Melibe leonina* buccal ganglion. *Soc. Neurosci. Abstr.* **17**, 1593.

Delcomyn, F. (1980). Neural basis of rhythmic behavior in animals. *Science* **210**, 492-498.

Dorsett, D. A., Willows, A. O. D. and Hoyle, G. (1969). Centrally generated nerve impulse sequences during swimming behavior in *Tritonia*. *Nature* **244**, 711-712.

Friesen, W. O. (1994). Reciprocal inhibition: A mechanism underlying oscillatory animal movements. *Neurosci. Biobehav. Rev.* **18**, 547-553.

Getting, P. A. (1988). Comparative analysis of invertebrate central pattern generators. In *Neural Control of Rhythmic Movements in Vertebrates* (ed. A. H. Choen, S. Rossignol and S. Grillner), pp. 101-128. New York: J. Wiley & Sons.

Getting, P. A. (1989a). A network oscillator underlying swimming in *Tritonia*. In *Cellular and Neuronal Oscillators* (ed. J. W. Jacklet), pp. 215-236. New York: Marcel Dekker.

Getting, P. A. (1989b). Emerging principles governing the operation of neural networks. *Ann. Rev. Neurosci.* **12**, 185-204.

Gillette, R. and Jing, J. (2001). The role of the escape swim motor network in the organization of behavioral hierarchy arousal in *Pleurobranchaea*. *Amer. Zool.* **41**, 983-992.

Graham Brown, T. (1911). The intrinsic factors in the act of progression in the mammal. *Proc. R. Soc. Lond. B* **84**, 308-319.

Grillner, S., Christenson, J., Drodin, L., Wallen, P., Lansner, A., Hill, R. H. and Ekeberg, O. (1989). Locomotor system in lamprey: Neuronal mechanisms controlling spinal rhythm generation. In *Cellular and Neuronal Oscillators* (ed. J. W. Jacklet), pp. 407-434. New York: Marcel Dekker.

Grillner, S. and Wallen, P. (1985). Central pattern generators, with special reference to vertebrates. *Ann. Rev. Neurosci.* **8**, 233-261.

Hurst, A. (1968). The feeding mechanism and behaviour of the Opisthobranch *Melibe leonina*. *Symp. Zool. Soc. Lond.* **22**, 151-166.

Jing, J. and Gillette, R. (1999). The central pattern generator for escape swimming in the sea slug *Pleurobranchaea californica*. *J. Neurophysiol*. **81**, 654-657.

Jing, J. and Gillette, R. (2003). Directional avoidance turns encoded by single interneurons and sustained by multifunctional serotonergic cells. *J. Neurosci*. **23**, 3039-3051.

Katz, P. S., Fickbohm, D. J. and Lynn-Bullock, C. P. (2001). Evidence that the central pattern generator for swimming in *Tritonia* arose from a non-rhythmic neuromodulatory arousal system: implications for the evolution of specialized behavior. *Amer. Zool*. **41**, 962-975.

Katz, P. S., Getting, P. A. and Frost, W. N. (1994). Dynamic neuromodulation of synaptic strength to a central pattern generator circuit. *Nature* **367**, 729-731.

Lawrence, K. A. and Watson, W. H., III (2002). Swimming behavior of the nudribranch *Melibe leonina*. *Biol. Bull*. **203**, 144-151.

Marder, E. and Eisen, J. S. (1984). A mechanism for the production of phase shift in a pattern generator. *J. Neurophysiol.* **51**, 1375-1393.

Pearson, K. G. (1993). Common principles of motor control in vertebrates and invertebrates. *Ann. Rev. Neurosci*. **16**, 265-297.

Perkel, D. H. and Mulloney, B. (1974). Motor pattern production in reciprocally inhibitory neurons exhibiting postinhibitory rebound. *Science* **185**, 181-183.

Ritzmann, R. E., Tobias, M. L. and Fourtner, C. R. (1980). Flight activity initiated via giant interneurons of the cockroach: Evidence for bifunctional trigger interneurons. *Science* **210**, 443-445.

Satterlie, R. A. (1985). Reciprocal inhibition and postinhibitory rebound produce reverberation in a locomotor pattern generator. *Science* **229**, 402-404.

Satterlie, R. A. (1989). Reciprocal inhibition and rhythmicity: Swimming in a pteropod mollusk. In *Cellular and Neuronal Oscillators* (ed. J. W. Jacklet), pp. 191-171. New York: Marcel Dekker.

Schivell, A. E., Wang, S. S.-H. and Thompson, S. H. (1997). Spontaneous behavior of the nudibranch mollusc *Melibe leonina* is a Markov process. *Biol. Bull.* **192**, 418-425.

Stent, G. S., Kristan, W. B., Jr, Friesen, W. O., Ort, C. A., Poon, M. and Calabrese, R. L. (1978). Neuronal generation of the leech swimming movement. *Science* **200**, 1348-1356.

Svoboda, K. R. and Fetcho, J. R. (1996). Interaction between the neural networks for escape and swimming in goldfish. *J. Neurosci.* **16**, 843-852.

Watson, W. H., Lawrence, K. D. and Newcomb, J. M. (2001). Neuroethology of *Melibe leonine* swimming behavior. *Am. Zool.* **41**, 1026-1035.

Watson, W. H., Newcomb, J. M. and Thompson, S. (2002). Neural correlates of swimming behavior in *Melibe leonina*. *Biol. Bull*. **203**, 152-160.

Willows, A. O. D. (1991). Giant nerve cells in mollusks. *Sci. Am.* **224**, 68-75.