Central Pattern Generator for Escape Swimming in the Notaspid Sea Slug *Pleurobranchaea californica*

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**Jing, Jian and Rhanor Gillette.** Central pattern generator for escape swimming in the notaspid sea slug *Pleurobranchaea californica*. *J. Neurophysiol.* 81: 654–667, 1999. Escape swimming in the notaspid opisthobranch *Pleurobranchaea* is an episode of alternating dorsal and ventral body flexions that overrides all other behaviors. We have explored the structure of the central pattern generator (CPG) in the cerebropleural ganglion as part of a study of neural network interactions underlying decision making in normal behavior. The CPG comprises at least eight bilaterally paired interneurons, each of which contributes and is phase-locked to the swim rhythm. Dorsal flexion is mediated by hemiganglion ensembles of four serotonin-immunoreactive neurons, the As1, As2, As3, and As4, and an electrically coupled pair, the A1 and A10 cells. When stimulated, A10 commands fictive swimming in the isolated CNS and actual swimming behavior in whole animals. As1–4 provide prolonged, neuromodulatory excitation enhancing dorsal flexion bursts and swim cycle number. Ventral flexion is mediated by the A3 cell and a ventral swim interneuron, lv3, the soma of which is yet unlocated. Initiation of a swim episode begins with persistent firing in A10, followed by recruitment of As1–4 and A1 into dorsal flexion. Recurrent excitation within the As1–4 ensemble and with A1/A10 may reinforce coactivity. Synchrony among swim interneuron partners and bilateral coordination is promoted by electrical coupling among the A1/A10 and As4 pairs, and among unilateral As2–4, and reciprocal chemical excitation between contralateral As1–4 groups. The switch from dorsal to ventral flexion coincides with delayed recruitment of A3, which is coupled electrically to A1, and with recurrent inhibition from As1/v3 to A1/A10. The alternating phase relation may be reinforced by reciprocal inhibition between As1–4 and lv3. *Pleurobranchaea*’s swim resembles that of the nudibranch *Tritonia*; we find that the CPGs are similar in many details, suggesting that the behavior and network are primitive characters derived from a common pleurobranchid ancestor.

**METHODS**

Specimens (180) of *Pleurobranchaea californica* (3–600 g) were obtained from Sea-Life Supply (Sand City, CA) and Pacific BioMarine (Santa Monica, CA) and maintained in circulated artificial seawater at 14°C until use. All dissections were done under cold anesthesia at 4°C.

Whole animal preparations were made by accessing the cerebropleural ganglion through a 2-cm dorsal incision and pinning it to a wax platform (Jing and Gillette 1995a). Hooks retracting the incision partially supported and restrained the animal for stable intracellular recordings but left it capable of considerable movement, including vigorous swimming and feeding behavior. The preparation chamber was perfused constantly with fresh artificial seawater (14°C). Isolated CNS preparations included cerebropleural and pedal ganglia, occasionally with buccal ganglion attached, and were pinned to silicone elastomer (Sylgard) under saline (cooled to 13–14°C) of composition (in mM) 420 NaCl, 10 KCl, 25 MgCl2, 25 MgSO4, 10 CaCl2, and 10 3-N-morpholino)propanesulfonic acid (MOPS) buffer, adjusted to pH 7.5 with NaOH.

Intracellular and extracellular recordings were done with conventional KCl-filled glass micropipettes and polyethylene suction electrodes as previously described (Jing and Gillette 1995a). Data were recorded on chart recorder (Gould TA11; sampling rate: 250 kHz) and...
digitized on video tape for later measurements. Spike height measurements were taken when cells were spontaneously active at only low rates to avoid use-dependent attenuation.

To study fictive swimming, we selected animals that reliably swim in response to a mild electric shock applied to the dorsal mantle or tail; >90% of isolated CNS preparations showed fictive swim activity. When swimming episodes were induced repeatedly, trials were separated by ≥10 min to minimize habituation of swimming responses. Swimming in whole animal preparations and isolated CNS was usually elicited by shocks (monopolar, 2-ms duration, 3–15 V, 15 Hz, for 2–2.5 s) to the body wall nerve (BWN) of the cerebropleural ganglion, which innervates the dorsolateral mantle (Lee and Liegeois 1974); the effects of its stimulation are best analogous to noxious stimulation of the back and/or the tail. In those animals where shock was ineffective in eliciting a swim, withdrawal of the posterior part of the body occurred sometimes followed by a single ventral flexion. Postshock, whether or not a swim intervened, animals typically showed accelerated creeping locomotion accompanied by frequent turning. In tests of premotor neuron abilities to affect the swimming rhythm, experimental trials were sandwiched between two control trials. Experimental results were accepted only when both control trials were closely similar.

Functional synaptic connections were examined in normal saline for postsynaptic potential (PSP) ability to follow presynaptic spikes one for one as a criterion of probable monosynapticity. Assays of probable mono- or polysynapticity also were conducted in high-divalent saline (which contained (in mM) 240 NaCl, 10 KCl, 125 MgCl₂, 25 MgSO₄, 30 CaCl₂, and 10 MOPS) to elevate spike thresholds and curtail polysynaptic activation (London and Gillette 1984).

Electrical coupling was assayed by passing hyperpolarizing current into one cell and measuring steady-state polarization in its partner. The steady-state coupling coefficient was taken as the ratio of the post- to presynaptic voltage change. No appreciable differences in coupling were observed in normal versus high-divalent saline (n = 8).

Nerve backfills and intracellular staining

Neurons with axons in specific nerves or connectives were backfilled via axons in the cut nerves with biocytin (Sigma). Neuron morphology was studied by intracellular injection of biocytin or neurobiotin (Vector, Burlingame, CA) from the recording electrode (Jing and Gillette 1995a). After an incubation of varying periods, tissues were fixed, and stain was developed and viewed in cleared whole mounts. Pressure injection of somata allowed staining of axon processes ≤2 cm from injection site after ganglia were incubated overnight at 8°C.

Immunocytochemistry and double labeling

Serotonin immunoreactivity was studied in whole mounts with the avidin-biotin peroxidase (ABC peroxidase) technique (Beltz and Burd 1989) as used previously (Sudlow et al. 1998). After fixation, immunoreaction with rabbit anti-serotonin (5-HT) primary antibody (Incstar, Stillwater, MN) and stain development, tissues were cleared and viewed as whole mounts.

For double labeling with 5-HT antibody and neurobiotin, cells were injected with neurobiotin after identification and processed as above, except that the primary antibody (1:5,000 to 1:10,000 dilution, reacted at 4°C for 72 h) was visualized with rhodamine conjugated goat anti-rabbit secondary antibody (Cappel, Durham, NC) and neurobiotin was visualized by fluorescein-conjugated Avidin D (Vector) under confocal fluorescence microscopy. Images were stored as digitized image files (gray scale, 8 bit, 512 × 512 pixel) and processed with Adobe Photoshop software.
rhinophore nerve up to the central commissure (Fig. 1A). This
group comprises most cells of the dorsal ganglion sending
descending axons to the pedal ganglia via the anterior cerebro-
pedal connective (aCPC; Fig. 1B); most A cluster cells send
their axons contralaterally. The aCPC corresponds to the cere-
bropedal connectives, and the posterior CPC (pCPC) corre-
sponds to the pleuropedal connectives, of more primitive gas-
tropods in which cerebral and pleural ganglia are not fused.

Identified neurons of the swim pattern generator are embed-
ded in the A cluster. Most A cluster cells are either part of
the swimming CPG or can modulate its output or receive inputs
from it. Identified neurons are named alphanumerically, with
‘‘s’’ added to designate members of a 5-HT-immunoreactive
population. For A cluster cells with no CPC axons, the term
‘‘rh’’ indicates a rhinophore nerve axon and ‘‘ci’’ designates
interneurons with a commissural axon. In earlier preliminary
reports (Jing and Gillette 1995b, 1996; Jing et al. 1997), a
slightly different naming procedure was used, where As1–3
were called A3a–c, As4 was A8b, A3 was A3d, and A4 was
A3e. Because As2 and As3 were indistinguishable (see also
Table 1), the term As2/3 is used to refer to a single one of the
pair, and As2–3 refers to them collectively. Only the swim
CPG neurons are discussed here, but all identified neurons are
shown in Fig. 1 for accuracy and future reference.

The swim pattern generator is composed of at least three
bilaterally paired groups of interneurons: four 5-HT-immuno-
reactive cells (As1, As2, As3, and As4) coactive throughout
the dorsal flexion phase, two cells (A1 and A10) strongly
electrically coupled and also most active in dorsal flexion, and
two cells [A3 and lV3 (ventral swim interneuron)] active in
ventral flexion and that inhibit the dorsal swim interneurons.

Neuron somata found in backfills of CPCs (Fig. 1B) were
probed with microelectrodes in isolated ganglia, and their axon
paths were confirmed by intracellular recording and dye inject-
ion. Most somata were visually identifiable as individuals or a
subcluster (As1–4, A3, A4). In each bilateral A cluster, there
are only two distinctly white somata, the larger and more
anterolateral of which is A1 (Jing and Gillette 1995a). The
second white soma, A2 (diam: 45–65 μm), is postero-
medial from A1, separated by one to two orange somata, one of
which is A7. Just postero medial from A2 is a subcluster of seven
cells with similar appearance: A-ci1, A-ci2, As1, As2, As3, A3,
and A4. The relative positions of As1–3, A3 and A4 (Fig. 1A)
are slightly among preparations. These neurons are somewhat
distinguishable by size; As1 and A3 tended to be larger, 60–90
μm diam, whereas others ranged 40–65 μm. In two prepara-
tions, all As1–3, A3, and A4 were identified and dye injected,
thus confirming the existence of all.

Posteromedial to the As1–3 lie A10, As-rh, A8, and As4. The
soma of A10 is medium-sized and translucent (65–85 μm
diam) and sometimes separated from the As1–3 by another
cell. The soma of A10 often is overlain partly by another cell
and thus appears smaller than its actual size. As4 is postero-
medial of A10 and is one of the largest somata (75–95 μm)
of the A cluster. As-rh has a single axon in the ipsilateral rhino-
phore nerve. A8 has a single axon in contralateral aCPC
(c-aCPC).

The morphologies of the swim interneurons are shown in

<table>
<thead>
<tr>
<th>Cell Name</th>
<th>Axon Path</th>
<th>Cell Size, μm; Color, Serotonin Immunoreactivity</th>
<th>Electrophysiology</th>
<th>Spontaneous Spike and PSPs Activity</th>
<th>Activity When No Swim Occurs Upon BWN Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>c-aCPC + PC</td>
<td>70–100, white</td>
<td>RP, mV</td>
<td>−48.6 ± 4.3</td>
<td>SH, mV</td>
</tr>
<tr>
<td>As1</td>
<td>c-aCPC + PC</td>
<td>60–90, translucent, 5-HT positive</td>
<td></td>
<td>−49.7 ± 4.3</td>
<td>87.9 ± 5.3</td>
</tr>
<tr>
<td>As2 and As3</td>
<td>c-aCPC + PC</td>
<td>40–65, translucent, 5-HT positive</td>
<td></td>
<td>−48.1 ± 3.7</td>
<td>77.1 ± 6.3</td>
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<tr>
<td>A3</td>
<td>c-aCPC</td>
<td>60–90, translucent</td>
<td>RP, mV</td>
<td>−47.5 ± 3.8</td>
<td>89.6 ± 4.9</td>
</tr>
<tr>
<td>As4</td>
<td>c-aCPC + c-pCPC + peripheral nerves</td>
<td>75–95, translucent, 5-HT positive</td>
<td>RP, mV</td>
<td>−46.0 ± 3.9</td>
<td>82.8 ± 4.7</td>
</tr>
<tr>
<td>A10</td>
<td>i-pCPC + PC</td>
<td>65–85, translucent</td>
<td>RP, mV</td>
<td>−47.6 ± 3.4</td>
<td>85.3 ± 4.6</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. RP, resting potential; SH, spike height; SD, spike duration at half amplitude. PSP, postsynaptic potentials; c-aCPC, contralateral anterior cerebropedal connective; IPSP, inhibitory PSP; 5-HT, serotonin; EPSP, excitatory PSP; c-pCPC, contralateral posterior CPC; PC, pedal commissure. * Percent of preparations observed. † Slow bursting: in some preparations, As2/3 spontaneous activity followed a very slow, cyclic bursting form, with peak spike frequencies near 3 Hz, burst durations of 16–40 s, and cycle periods of 80–100 s. This slow bursting activity occurred frequently after body wall nerve (BWN) stimulation whether or not a swim episode was initiated.
Fig. 2 and summarized in Table 1. Of them, the neurons A1 (Jing and Gillette 1995a), As1–4, A3 each have at least one axon crossing the central commissure to exit in the c-aCPC. As4 also has an axon branch going to the contralateral pCPC and additional fine branches going to the periphery in the contralateral tentacle, rhinophore, and body wall nerves. The A10 axon exits in the ipsilateral pCPC (i-pCPC) and crosses from one pedal ganglion to the next in the pedal commissure.

A study of 5-HT immunoreactivity in the *Pleurobranchaea* CNS (Sudlow et al. 1998) located five immunoreactive neurons in the A cluster region. These cells were identified with intracellular electrodes, injected with neurobiotin, processed for 5-HT immunocytochemistry and found to be As1–4, and As-rh (Fig. 3). Other A cluster neurons tested in double-labeling experiments were immunonegative (A10, A-ci1, A-ci2, A3, A4, and A8).

Central pattern generator

**PHASIC ACTIVITY OF THE PATTERN-GENERATING ELEMENTS.** The activities of the various CPG neurons during fictive swims were related to the rhythmic firing recorded in the A1, aCPC, and/or the anterior lateral body wall nerve (alBWN) of the pedal ganglion. Previously we showed that the A1 burst during the swim in the whole animal occurred just before and during the dorsal flexion phase (Jing and Gillette 1995a). We also established that escape swimming in the whole animal and fictive swimming in the isolated CNS are indistinguishable with respect to both A1 and other premotor activity recorded in the aCPC and that spike activity in the alBWN is a useful monitor of the motor output of escape swimming (Jing and Gillette 1995a).

After nerve stimulation, a swim episode began with the induction of sustained activity in A10. The dorsal flexion phase of each swim cycle began with the onset of bursts in the putatively serotonergic As1–4 neurons, followed by recruitment of the burst of A1 (Fig. 4). During dorsal flexion, a decline in the initially high As1–4 activity was reversed as A1/A10 spike rate accelerated, causing two distinct peaks in As1–4 activity during this dorsal swim burst (Fig. 5). During swims, burst activities of A1 and A10 were closely synchronized in both phase and duration (Figs. 4, 7B, and 8), an apparent consequence of the strong electrical coupling described later. However, it was notable that A10 activity differed from all other swim interneurons in that it continued spiking at a low rate (2–4 Hz) during the hyperpolarized interburst interval without complete spike inhibition. The spiking frequency during A10 bursts was typically higher than for A1 (peak and mean frequency ranges 40–60 and 15–20 Hz vs. 10–30 and 5–12 Hz, respectively).
The putatively serotonergic As1–4 were coactive in the same phase of dorsal flexion (cf. Fig. 9). The burst durations of the ensemble members were similar except that when the spike activity in bursts declined late in a swim episode, As1 and As4 bursts ceased somewhat before As2–3. Spike rates in the bursts of the two neurons As2 and As3 were virtually identical, whereas those for As1 and As4 were lower (peak and average frequency ranges 10–25 and 6–15 Hz vs. 5–12 and 3–8 Hz, respectively). Spike rates for As1–4 varied with cycle period, such that more intense spiking occurred in the earlier, shorter cycle periods (Fig. 5).

The transition from the dorsal to the ventral flexion phase was attended by bursting in A3. A3 activity began when A1 spike activity was highest and ended with or shortly after the A1 bursts (Figs. 4, 7B, and 11). The burst duration of A3 (0.7–1.6 s) was shorter than for the other identified CPG members. A3 burst intensity (6–17 Hz) tended to fade over successive swim cycles as the cycle period lengthened, especially as A1 burst intensity waned.

CONTRIBUTIONS OF THE PATTERN-GENERATING ELEMENTS TO INITIATION, PATTERNING, AND MAINTENANCE OF THE SWIM. A10. Among the swim interneurons, only A10 could consistently drive swim motor output (Figs. 6 and 7A). Stimulation of a single A10 at spike rates of 10–25 Hz drove cyclic activity appropriate to the swim rhythm in 14 of 19 isolated CNS preparations. In the four cases where A10 activity alone was unable to drive the cyclic activity of the swim, BWN stimulation did not initiate the fictive swim episode either. However,
in these four cases, when A10 activity was driven shortly after BWN stimulation, the swim rhythm could be activated successfully. In four of five whole animal preparations, driving a single A10 induced swimming behavior similar to that caused by BWN stimulation save that the ventral body flexion of the cycle was not as strong. The ability of A10 to drive patterned swimming output contrasted with the much weaker ability of A1, which was shown previously to be effectual in only a small fraction of isolated CNS preparations and never so in whole animal preparations (Jing and Gillette 1995a). Where we examined both A10 and A1 in six isolated CNS preparations, only A10 was able to drive coordinated swim activity.

However, the fictive swim driven by A10 activity was incomplete compared with swim episodes induced by BWN stimulation in that the swim episode showed no signs of being self-sustained and halted immediately when depolarizing stimulation of A10 was halted. Moreover, A10 activity did not effectively recruit burst activity in As1–3 (Fig. 7). In five preparations, As1–3 burst activity driven by A10 in As1–3 was either quite weak \((n = 3)\) or absent \((n = 2)\). This is consistent with the observation that A1 and A10 are only weakly synaptically coupled to As1–4 (see Connectivity). Recruitment of As1–3 by BWN stimulation may be necessary to the normal swim episode, for when a fictive swim was induced by A10 depolarization after BWN stimulation (as described earlier), As1–3 bursts were obviously strengthened \((n = 3)\).

Hyperpolarization of a single A10 interrupted the swim induced by nerve stimulation for the duration of current injection \((n = 4,\) Fig. 8), pointing to an obligatory role in pattern generation. This also directly hyperpolarized A1, to which A10 is coupled electrically (see Electrical coupling between A1 and A10). Hyperpolarization of A1 was itself previously shown to effectively block expression of swim motor activity (Jing and Gillette 1995a). The strong electrical coupling between A1 and A10 made it difficult to differentiate the functions of the two cells, and we consider them both necessary to pattern generation.

As1–4. Through their synchronous activity during swims, shared 5-HT immunoreactivity and mutual excitatory connections (see Connectivity), the As1–4 cells appeared to act collectively in the pattern generator as a functional unit. Individually their effects were relatively weak: in only one of seven cases did hyperpolarization of a single As1–3 affect the fictive swim pattern. However, in three of three cases where we hyperpolarized two cells at a time, the ensuing burst cycle was delayed for 0.5–2.7 s, and the fictive swim episode was terminated early relative to pre- and postcontrol measures (Fig. 9), results with a random probability of \(<0.002\) (Bernoulli distribution).

The effects of driving As1–4 on the fictive swim were tested in 14 preparations. In eight of these, driving a single As1–4 \((As1, n = 3; As2/3, n = 3; As4, n = 2)\) prolonged the swim
episode by one to two extra cycles relative to both pre- and postcontrol measures (Fig. 10). For the 14 cases, the increased number of cycles on the second test was significant ($P < 0.002$; Fisher’s exact test). For the eight cases, the random probability of this exact result occurring was $<6 \times 10^{-8}$. In the six cases where the number of swim cycles was not increased, the mean spike frequency of the A1 bursts was enhanced on average 87% ($P < 0.02$; 2-tailed $t$-test). By themselves, As1–4 were not effective in inducing a swim episode. The distinctly suppressive effects of hyperpolarizing two of the eight As1–4, and the stimulatory effects of depolarization of single cells, indicate that collective As1–4 activity significantly contributes to excitation of the swim CPG.

A3. Driving A3 tonically during the swim episode hyperpolarized A1 and suppressed the swim episode for the duration of A3 activity (Fig. 11; $n = 7$ observations). In two cases, a complete swim cycle rebounded after release of A3 depolarization. Hyperpolarization of single A3 neurons during fictive swim episodes had no discernible effects ($n = 5$). The inhibitory effects of A3 on the swimming pattern generator and the cyclic activity of the neuron during the swim are consistent with a role in terminating the dorsal flexion phase of the burst cycle, in particular the A1/A10 bursting, shared with another interneuron(s), IVS.

I VS. Activity in A1/A10 activates inhibitory feedback from this interneuron (Jing and Gillette 1995a), the soma of which remains unidentified. Inhibition from I VS is distributed among all swim interneurons active during dorsal flexion (A1/A10, As1–4) and is presumed to make the major contribution to termination of dorsal flexion and to the duration of the ventral flexion phase. The onset of activity in the I VS pathway as observed in the feedback inhibition of A1 is coincident with inhibition of the feeding network, and the cell potentially has widespread effects in the CNS.

Connectivity

In summary to this point, the cells A1, A10, As1–4, and A3 are part of the CPG for escape swimming. Of these, A1, As1–4, and A10 drive the dorsal flexion phase of the swim cycle. They compose two functional ensembles with distinct firing patterns: A1/A10 and As1–4. Activity in A3 leads up to ventral flexion, can contribute to the dorsal/ventral flexion phase transition, and in conjunction with I VS may mediate inhibition to A1/A10. Inhibition to As1–4 is provided only by I VS. The synaptic coupling among the neurons described in the following sections is consistent with these roles.

ELECTRICAL COUPLING BETWEEN A1 AND A10. A most prominent feature of the connections among the A cluster neurons was abundant electrical coupling. Among the swim interneurons, appreciable coupling occurred between A1 and A10 and among As2, As3, and As4. Within a unilateral A cluster, the highest electrical coupling ratio was found for A1 and A10 (Table 2A, Fig. 12A). This coupling was asymmetric: the coupling coefficient for steady-state voltage change with current passage from A10 to A1 (0.41) was
1.4 times that for A1–A10 (0.29). The strong A1/A10 coupling was expressed in frequent simultaneous spiking in the cells and in the subthreshold spike-like potentials in A1 synchronous with A10 spikes (Figs. 4, 7, and 8).

Electrical coupling between the bilateral homologues of A1 was also strong (Fig. 12B) with an average coupling ratio of 0.16. A1 appeared to be directly coupled to contralateral A10 because the coupling ratio between contralateral A1 and A10 cells was higher than between bilateral A1s (Table 2B, Fig. 12B). Reciprocal coupling between bilateral A10s must be effected entirely through the A1s instead of directly because the A10 axons do not cross the commissure (Table 2B). The strong electrical connections among the ipsilateral and contralateral A1/A10 ensembles can explain the observations that hyperpolarization of only one of these cells is sufficient to suppress swimming (Jing and Gillette 1995a) (Fig. 8).

Reciprocal excitation with in A1–4: Electrical coupling and long-lasting compound EPSPs. Electrical coupling was found among ipsilateral A2, A3, and A4 (Fig. 13A, I and 2). The coupling between A2 and A3 was symmetric (Table 2A). The A2–3 pair also had mutually excitatory chemical connections; firing one cell induced a long-lasting excitatory PSP (EPSP) in the other, superimposed on the electrically mediated depolarization (Fig. 13A4).

Lacking electrical connections with other neurons in this ensemble, A1 made reciprocal, excitatory, and monosynaptic chemical connections with A2–4 demonstrable in high-divalent cation saline (Fig. 13A, 3–5), causing long-lasting, slow EPSPs. The amplitude of the compound EPSPs induced by a train of 10–20 spikes ranged from 1 to 4 mV (4–9 observations were made for each neuron pair). Timing of compound EPSPs in the different neurons was comparable with time to peak of 3–4 s and decay occurring during 10–20 s.

Contralateral connections among A1–4 were similar to their ipsilateral connections. Contralateral A2–4 were electrically coupled (Table 2B, Fig. 13B2); the coupling ratios were low relative to A1s and A10s, except for the contralateral A4s with a high coupling ratio of 0.38. A1 also was coupled weakly electrically to its contralateral counterpart (Fig. 13B1) but not to the contralateral A2–4. Generally coupling coefficients among this population ipsilaterally and contralaterally were smaller than those for A1/A10 populations.

Reciprocal excitatory connections mediating slow EPSPs were found between contralateral A1 neurons and between A1 and contralateral A2/3. The contralateral connections between A1 were strong enough to drive postsynaptic spiking even in high-divalent saline. Characteristics of timing and amplitude were similar to ipsilateral connections, reaching amplitudes of 1–3.5 mV and enduring to 16 s (Fig. 13B3, n = 7 pairs). The compound EPSPs from A1 to contralateral A2/3 (1.6–2.4 mV; Fig. 13B4, n = 4) and from A2/3 to A1 (2.2–5.5 mV; Fig. 13B5, n = 4) were similar.

Reciprocal excitation and inhibition between A1/A10 and A1–4. A1 and A10 made mixed chemical synaptic connections with the ipsilateral A1–4 (Fig. 14A). In normal saline, a train of spikes in A1 evoked early excitation followed by

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**FIG. 11.** Induced tonic firing in A3 during the swim episode hyperpolarized A1 and suppressed the swim episode. A and C: control swim episodes initiated by BWN stimulation (bar). B: tonic depolarization of A3 after the initial burst cycle of the swim inhibited A1 spiking and prevented a 2nd burst until the release of A3. As2/3 remained active during A3 firing although at somewhat lower frequency.

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**FIG. 12.** Electrical coupling between ipsilateral and contralateral A1 and A10 recorded in high-divalent saline. Top: current-injected cell. A: ipsilateral A1 and A10. B: coupling from left A1 (IA1) to right A10 (rA10) was stronger or similar to that from IA1 to rA1, suggesting that IA1 and rA10 coupling is direct and not simply via a IA1-rA1-rA10 route. Calibration bar: vertical, A, 20 mV (top) and 4 mV (bottom); B, 40 mV (top) and 8 mV (bottom); horizontal, 4 s.
inhibition in As1–4 \((n = 34\) of 39; in 5 cases the connection was quite weak). The connection from As1–3 to A1 was excitatory; PSPs from As1 to A1 were observed more frequently \((n = 10\) of 14) than for those from As2/3 to A1 \((n = 12\) of 24), and the amplitude was typically larger. The connection from As4 to A1 is similar but weaker. Where connections were not observed, synaptic potentials may have been buried in the synaptic noise. High-divalent cation saline significantly suppressed the connection strength from A1 to As1–3 and from As1–3 to A1, suggesting that some components were polysynaptic. For the connections from A1 to As1–3, both the early excitatory and late inhibitory components were suppressed.

Consistently, in high-divalent saline, connections from A1 to As1–3 remained as biphasic excitatory-inhibitory connections of attenuated amplitude \((n = 9\), Fig. 14A3), whereas the connection from A1 to As4 was purely inhibition \((n = 5\), Fig. 14A3), of 0.7-

### Table 2. Electrical coupling ratios (steady state) among swim interneurons

<table>
<thead>
<tr>
<th></th>
<th>A1</th>
<th>A10</th>
<th>A3</th>
<th>As2/3</th>
<th>As4</th>
<th>As1</th>
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<tr>
<td><strong>A. Coupling between ipsilateral neurons</strong></td>
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<td>Post/Pre</td>
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<tr>
<td>A1</td>
<td>—</td>
<td>0.41 ± 0.018 (26)</td>
<td>0.01 ± 0.001 (3)</td>
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<tr>
<td>A10</td>
<td>0.288 ± 0.012 (23)</td>
<td>—</td>
<td>0.01 ± 0.002 (3)</td>
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<tr>
<td>A3</td>
<td>0.0174 ± 0.003 (5)</td>
<td>0.021 ± 0.002 (3)</td>
<td>—</td>
<td>0.088 ± 0.005 (41)</td>
<td>0.086 ± 0.009 (10)</td>
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<tr>
<td>As4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.079 ± 0.008 (10)</td>
<td>—</td>
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<tr>
<td><strong>B. Coupling between contralateral neurons</strong></td>
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<tr>
<td>Contra-Ipsilateral</td>
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<tr>
<td>A1</td>
<td>0.156 ± 0.014 (13)</td>
<td>0.166 ± 0.015 (6)</td>
<td>—</td>
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<td>—</td>
<td>—</td>
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<tr>
<td>A10</td>
<td>0.157 ± 0.01 (7)</td>
<td>0.109 ± 0.011 (2)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>As2/3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.060 ± 0.007 (12)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>As4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.376 ± 0.025 (4)</td>
<td>—</td>
</tr>
<tr>
<td>As1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.024 ± 0.004 (7)</td>
</tr>
<tr>
<td>A3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.120 ± 0.016 (7)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE, number in parentheses indicates the number of measurements. *Electrical coupling between As2/3 and contralateral As4 was observed in three preparations, but accurate measurements of coupling were not obtained.

**FIG. 13.** Ipsi- and bilateral connections in As1–4 groups. A: ipsilateral connections. Electrical coupling was observed between As2 and As3 but not with them and As1 (A1) and among As2, As3, and As4 (A2). A, 3–5: slow, monosynaptic and reciprocal excitation between As1 and As2–4. B: contralateral As1–3 connections. Electrical coupling was found between contralateral As1 (B1) and among the contralateral As2/3, but not with contralateral As1 (B2). B, 3–5: monosynaptic excitations between contralateral As1 (B3) and between As1 and contralateral As2/3 (B, 4 and 5). Calibration bar: vertical, 40 mV for all top traces and 4 mV for all bottom traces; horizontal, 4 s. All recordings were made in high-divalent saline.
2-mV amplitude, 6-s duration, and time to peak of 2.4 s. Connections between A10 and As1–3 were similar to that between A1 and As1–3 but were somewhat weaker. Unlike A1, A10 still weakly excited As4 in high-divalent saline.

RECURRENT INHIBITION FROM A3 TO A1/A10. A3 received excitation from both A1/A10 and As1. A1/A10 and A3 were connected weakly electrically (Table 2A), a connection observable only in high-divalent cation saline where background synaptic noise was suppressed. A3 also was connected electrically to its contralateral homologue (Table 2B). In normal saline, excitatory connections from A1 and A10 to A3 (Fig. 14B1) were variable and dependent on presynaptic firing rate. At higher discharge rates, the initially small EPSP in A3 facilitated and reached spike threshold. In tests following closely on BWN stimulation, EPSPs showed apparent heterosynaptic facilitation. This connection disappeared in high-divalent saline, leaving behind only the weak electrical coupling (n = 9) and suggesting its polysynaptic origin. Also, a one-way monosynaptic excitatory connection from As1 to A3 was found, of amplitude 0.9–1.6 mV, average duration of 7.2 s, and time to peak of 1.2 s (n = 4; Fig. 14B2).

A3 sent phasic and facilitating unitary IPSPs to A1 with amplitudes of 0.15–0.5 mV and duration of ~0.6 s (Fig. 14B3) and to A10 with smaller amplitudes that were resistant to high-divalent cation saline (n = 9), but not to As1–4. The summed IPSP from a driven burst of A3 spikes (10–20 Hz) to A1 had an amplitude of 3–5 mV, duration of 3–10 s, time to peak of 0.9 s; that from A3 to A10 was similar but smaller (not shown). This inhibitory connection may account for the suppressive effects of A3 on swim pattern generation when it is driven tonically (Fig. 11).

RECIprocal inhibition BETWEEN AS1–3 AND IVS. One source of inhibition from A1 to As1–3 could come from the IVS neuron, which was excited by A1 activity to mediate strong feedback inhibition (Jing and Gillette 1995a). The onset of feedback inhibition in A1 coincided with the late inhibition in As1–3 (see Fig. 15C); suggesting that IVS inhibited As1–3 as well. Moreover, As1–3 appeared to make effective reciprocal inhibitory connections with IVS; the feedback inhibition in A1 from IVS was suppressed when As1–3 were coactive with A1 (n = 3; Fig. 15).
that proposed for Tritonia
prominence of feedback inhibition in the pattern generating mechanisms (see
omitted for clarity. Model in B

classic transitions of dorsal to ventral flexion in part accomplished by A1 recurrent inhibition from As1–4 and VS. Mechanism is generally in accord with that proposed for Tritonia (Getting 1989b). However, the transition from dorsal to ventral flexion in Pleurobranchaea also is aided by feedback inhibition from A3 solely to A1/A10 but not to As1–4, suggesting a greater prominence of feedback inhibition in the pattern generating mechanisms (see DISCUSSION and Table 3).

DISCUSSION
Mechanisms of pattern generation of escape swimming

Seven neurons, about a third of the A cluster, take part in escape swimming pattern generation. Each fires cyclically in phase with the swim rhythm, and all seven are coupled by electrical and/or chemical connections. Moreover each as an individual or part of an ensemble can perturb the ongoing rhythm by its discharge or hyperpolarization. At least one other element, VS, remains to be located. The CPG interneurons mediate either the dorsal flexion phase of the swim (A1/A10, and As1–4), or ventral flexion (A3 and VS). The pattern of electrical and chemical connections is summarized in the diagram of Fig. 16A. The pattern of connectivity is simplified further in 16B, where the hypothetical CPG structure is shown in terms of functional ensembles. The synaptic mechanisms...
inhibition between IVS and As1–4 may depend on physical cation of the activation of the CPG and the nature of reciprocal interest and significance for future study. In particular, clarifi-
an adenosine 3
and Gillette 1995) or variously potentiates (Huang et al. 1998) and the nudibranch branchaea
mechanisms in a variety of molluscan cells. In other seroto-
(Lennard et al. 1980). 5-HT depolarizes and activates bursting
swimming and locomotion in opisthobranchs
Comparative neurobiology of the premotor networks for
circuits of the animals, as summarized in Table 3, points to
numerous possible homologies in neuron identities and con-
nection patterns. Moreover, the likely pattern-generating mechan-
isms appear to be well conserved in both networks (cf. Getting 1989b).

We previously reported similarity of Pleurobranchaea’s
A1 neuron to C2 of Tritonia (Jing and Gillette 1995a). We
now show that the interneurons As1–3 resemble the Tritonia
dorsal swim interneurons DSI-A-C, sharing morphological characters, functional roles (Getting et al. 1980; Lennard et
al. 1980), and 5-HT immunoreactivity (Katz et al. 1994;
McClellan et al. 1994) and show similarities down to con-

### TABLE 3. Comparison of the escape swimming premotor networks of Pleurobranchaea and Tritonia

<table>
<thead>
<tr>
<th>Network elements</th>
<th>Pleurobranchaea</th>
<th>Tritonia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mediating dorsal flexion</strong></td>
<td>A1</td>
<td>C2*</td>
</tr>
<tr>
<td></td>
<td>Serotonergic As1–3</td>
<td>Serotonergic DSI-A-C*</td>
</tr>
<tr>
<td></td>
<td>Serotonergic As4</td>
<td>Not reported (see DISCUSSION)</td>
</tr>
<tr>
<td><strong>Mediating ventral flexion</strong></td>
<td>IVS (inferred from network connectivity) A3</td>
<td>VSI-B*</td>
</tr>
<tr>
<td><strong>Commandlike</strong></td>
<td>A10: providing electrical excitation to A1</td>
<td>DRI: providing monosynaptic excitation to DSIs; not a likely homologue of A10</td>
</tr>
<tr>
<td><strong>Pattern of connectivity and proposed pattern-generating mechanisms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Recurrent excitation</strong></td>
<td>Lasting compound EPSPs among ipsilateral As1–3 (As4)</td>
<td>Lasting compound EPSPs among ipsilateral DSIs*</td>
</tr>
<tr>
<td></td>
<td>Lasting compound EPSPs among contralateral As1–3 (As4)</td>
<td>Not present among contralateral DSIs</td>
</tr>
<tr>
<td></td>
<td>Electrical coupling between ipsilateral As2–3 (As4), and contralateral A1, and As1–3</td>
<td>Electrical coupling between ipsilateral DSI-B-C, and contralateral C2, and DSI-A-C*</td>
</tr>
<tr>
<td></td>
<td>Between A1 and As1–3 (As4)</td>
<td>Between C2 and DSIs*</td>
</tr>
<tr>
<td><strong>Recurrent inhibition</strong></td>
<td>From A1 to IVS</td>
<td>From C2 to VSI-B (appears weaker, because of virtual absence of feedback inhibition in C2)*</td>
</tr>
<tr>
<td><strong>Reciprocal inhibition</strong></td>
<td>From A1–A10 to A3</td>
<td>Between DSIs and VSI-B*</td>
</tr>
<tr>
<td><strong>Strength of electrical coupling</strong></td>
<td>Left A1–right A1 (0.16)</td>
<td>Left C2-right C2 (0.02, weaker)</td>
</tr>
<tr>
<td></td>
<td>A1–A10</td>
<td>— (see DISCUSSION)</td>
</tr>
<tr>
<td></td>
<td>Among As2–3 (ipsilateral: 0.09, contralateral: 0.06, weaker)</td>
<td>Among DSI-B-C (ipsilateral: 0.19, contralateral: 0.14)</td>
</tr>
</tbody>
</table>

VSI, ventral swim interneuron. * Possible homology.

bly augmented the number of cycles expressed in a given swim episode (Fig. 9), similar to the action of the likely homologous dorsal swim interneurons (DSI) in the swim CPG of Tritonia (Lennard et al. 1980). 5-HT depolarizes and activates bursting mechanisms in a variety of molluscan cells. In other serotonergic neurons of Pleurobranchaea, 5-HT activates (Sudlow and Gillette 1995) or variously potentiates (Huang et al. 1998) an adenosine 3’,5’-cyclic monophosphate-gated Na+ current; a similar current is present in the swim CPG neurons (Jing et al. 1997) that could underlie their prolonged recurrent excitation and bursting. This work has provided a partial characterization of the swim CPG in Pleurobranchaea and leaves several issues of interest and significance for future study. In particular, clarification of the activation of the CPG and the nature of reciprocal inhibition between IVS and As1–4 may depend on physical identification of the sensory inputs to the swim interneurons and of IVS.

### Comparative neurobiology of the premotor networks for swimming and locomotion in opisthobranchs

The escape swimming behaviors of the notaspidean Pleurobranchaea and the nudibranch Tritonia are similar in their patterning and episodic natures. Comparison of the CPG circuits of the animals, as summarized in Table 3, points to numerous possible homologies in neuron identities and connection patterns. Moreover, the likely pattern-generating mechanisms appear to be well conserved in both networks (cf. Getting 1989b).

Extrapolating from the extensive similarities, we expect that the differences in reported composition of the swim CPGs of Pleurobranchaea and Tritonia largely reflect incompleteness of description of both networks. Notably, quite different command-like elements, able to drive the motor program, have been found in both species: A10 of Pleurobranchaea and the ‘‘dorsal ramp interneuron’’ (DRI) of Tritonia (Frost and Katz 1996). These cells are functionally distinct and not apparently homologous; it is possible that their counterparts still will be found in the two species. If so, the contrasting functions of the two command-like neurons will be of interest to compare.
An apparent interspecific difference between the escape swim CPGs is in the strengths of the electrical connections (Table 3). This might extend to coupling of the C2 cell of Tritonia and a possible A10 homologue because there is no indication in the published C2 records or our occasional recordings in Tritonia C2 of the frequent attenuated spike potentials of the A10 homologue. The possible difference in electrical coupling could explain why hyperpolarization of only a single A1 completely suppresses the swim in Pleurobranchaea (Jing and Gillette 1995a) but hyperpolarization of both C2s is required to just phase shift the cycle in Tritonia (Getting et al. 1980; Taghert and Willows 1978).

Weaker coupling of As2–3 neurons is observed than for the DSI-B-C (Getting 1981), consistent with a less intense spiking during the swim. Also when we compared records in high-divalent saline, chemical connections between A1 and As1–3 appeared weaker than for C2 and the DSI. As the spike activity of both As1–4/DSIs populations is correlated with cycle period and number of cycles in an episode, this observation is consistent with our impression that the swim cycle of Pleurobranchaea averages fewer cycles and shows a broader range of cycle periods (2.4–8.8 s).

The apparent conservation of the escape swim and its CPG circuitry in Pleurobranchaea and Tritonia has interesting implications for molluscan evolution. Anatomic and developmental evidence suggests that nudibranch snails evolved from pleurobranchomorph ancestors with the loss or translocation of the gill and changes in other characters. Reconstruction of a hypothetical ancestor of Nudibranchia and Pleurobranchomorpha could appear quite similar to a living pleurobranchid (Schmekel 1985). The action pattern of escape swimming behavior is found in multiple Pleurobranchaea species of the family Pleurobranchaeinae, which lack an internal shell and is not found in the only other pleurobranchomorph family, the Pleurobranchidae (cf. Gillette et al. 1991). Thus by extension our data suggest that members of the genus Pleurobranchia most closely resemble the ancestor(s) of the nudibranch radiation from which the tritonids conserve a primitive escape swimming behavior and CPG. Although we presently cannot exclude the possibility that the animals independently elaborated a similar swim network from homologous cells, such an hypothesis requires more assumptions and so is less likely.

Comparative studies of identifiable neurons and circuits help to understand the evolution of the nervous system (Arbas et al. 1991; Bulloch and Ridgway 1995; Gillette 1991; Katz and Tazaki 1992). In this light, it is of interest to speculate on the evolution of the escape swimming CPG of Pleurobranchaea and Tritonia. This CPG differs from other molluscan swimming CPGs in being located in the cerebropleural ganglion complex rather than in the paired pedal ganglia. Swimming pattern generation in other opisthobranchs so far investigated emerges from interactions among pedal interneurons mediated via pedal commissural axons, including animals that swim with symmetrical and simultaneous “clap and fling” movements of the parapodia such as Clione limacina and Aplysia brasiliana, and those that swim with lateral undulations of the body as Melibe (Arshavsky et al. 1985a–c; Lawrence 1997; McPherson and Blankenship 1991; Parsons and Pin斯基 1988; Satterlie 1985; Thompson 1974). In Pleurobranchaea and Tritonia, the cerebropleural location of the oscillator CPG suggests that it is derived from premotor neurons that mediate the motor decisions for pedal locomotion, body withdrawal, and turning movements. On the basis of their relative location and axon paths, these neurons are possibly homologous to premotor neurons identified in Clione (Panchin et al. 1995; Satterlie and Norekian 1995) and Aplysia (Freedman and Jahan-Parwar 1983; Gamkrelidze et al. 1995) that initiate swimming or pedal creeping or modulate ongoing swimming movements.

The present description of the CPG for the escape swim in Pleurobranchaea provides a fuller circuitry context in which to probe the alternative expression of feeding and avoidance behaviors at the neural network level. Getting (1989a) suggested that neurons of the swim CPG function outside of the escape swim in reflex withdrawal. Other of our data suggest that the swim neurons are specifically sensitive to noxious stimuli and contribute to performance of avoidance turns when the escape swim CPG is not active (Jing and Gillette 1995a, 1996; unpublished data). Future research may test the possibility that some CPG neurons and others of the A cluster compose a multifunctional network specifically devoted to mediating a range of avoidance and defensive behaviors.

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