Network interneurons underlying ciliary locomotion in *Hermissenda*

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Crow T, Jin NG, Tian LM. Network interneurons underlying ciliary locomotion in *Hermissenda*. *J Neurophysiol* 109: 640–648, 2013. First published November 14, 2012; doi:10.1152/jn.00803.2012.—In the nudibranch mollusk *Hermissenda*, ciliary locomotion contributes to the generation of two tactic behaviors. Light elicits a positive phototaxis, and graviceptive stimulation evokes a negative gravitaxis. Two classes of light-responsive premotor interneurons in the network contributing to ciliary locomotion have been recently identified in the cerebropleural ganglia. Aggregates of type I interneurons receive monosynaptic excitatory (Ie) or inhibitory (Ii) input from identified photoreceptors. Type II interneurons receive polysynaptic excitatory (IIe) or inhibitory (IIi) input from photoreceptors. The ciliary network also includes type III inhibitory (IIIi) interneurons, which form monosynaptic inhibitory connections with ciliary efferent neurons (CENs). Illumination of the eyes evokes a complex inhibitory postsynaptic potential, a decrease of Ii spike activity, a complex excitatory postsynaptic potential, and an increase of Ie spike activity. Here, we characterized the contribution of identified I, II, and III interneurons to the neural network supporting visually guided locomotion. In dark-adapted preparations, light elicited an increase in the tonic spike activity of IIi interneurons and a decrease in the tonic spike activity of IIe interneurons. Fluorescent dye-labeled type II interneurons exhibited diverse projections within the circumesophageal nervous system. However, a subclass of type II interneurons, II ecp and II scp, were shown to terminate within the ipsilateral cerebropleural ganglia and indirectly modulate the activity of CENs. Type II interneurons form monosynaptic or polysynaptic connections with previously identified components of the ciliary network. The identification of a monosynaptic connection between Ie and IIIi interneurons shown here suggest that they provide a major role in the light-dependent modulation of CEN spike activity underlying ciliary locomotion.

CENs; photoreceptors; network synaptic interactions; premotor interneurons; gravitaxis

THE CONTRIBUTION OF NEURAL NETWORKS to the generation of motor patterns underlying various behaviors has been examined in a number of diverse invertebrates (Arshavsky et al. 1985; Briggman and Kristan 2006; Calabrese et al. 1995; Friesen 1989; Getting 1977; Getting and Dekin 1985; Gillette et al. 1982; Hening et al. 1979; Jing et al. 2004; Katz et al. 1994; Kupfermann and Weiss 2001; Lennard et al. 1980; Marder and Calabrese 1996; Marder et al. 2005; Morgan et al. 2002; Panchin et al. 1995a, 1995b; Rosen et al. 1991; Shaw and Kristan 1997; Susswein and Byrne 1988; Weiss et al. 1978; Yeoman et al. 1996). With regard to movement, the pattern of spike activity in efferent neurons that underlies different forms of locomotion, such as walking, crawling, or swimming, result from the synaptic connections of premotor interneurons that form complex networks supporting the generation of both rhythmic and nonrhythmic neural activity. While many forms of movement are rhythmic, ciliary locomotion or crawling is a nonmuscular, nonrhythmic gliding form of movement found in a number of mollusks (Audesirk 1978a, 1978b; Baltzley et al. 2011; Copeland 1919, 1922; Crow and Tian 2003; Deliagina and Orlovsky 1990; Gofen 1976; Jékely 2011; Syed and Winlow 1989; Willows et al. 1997). Visual, cutaneous, graviceptive, and chemosensory stimuli can initiate and modulate ciliary locomotion. Since many neurons in locomotor networks are polysensory, it is not surprising that ciliary locomotion may be elicited by different sensory stimuli in similar or quite different environmental contexts. In species where the same network is involved with both rhythmic escape swimming and nonrhythmic ciliary locomotion, the patterns of connectivity between premotor interneurons and ciliary efferent neurons (CENs) is complex, involving excitatory, inhibitory, direct, and indirect synaptic input (Audesirk 1978a; Jing and Gillette 1999, 2000; Popescu and Frost 2002). Multifunctional network control over muscular escape swimming and nonmuscular ciliary locomotion is well documented (Jing and Gillette 2000; Popescu and Frost 2002). However, the neural circuitry that supports the generation of ciliary locomotion in different behavioral contexts, and its activation and modulation by different sensory stimuli, is poorly understood.

Phototaxis and gravitaxis in *Hermissenda* involve ciliary activity mediated by interneurons that contribute to both muscular foot contractions and ciliary locomotion (Crow and Tian 2009). Polysensory type Ie interneurons are excited by graviceptive input sufficient to generate foot contractions and ciliary activity, but are not typically activated by visual input (Crow and Tian 2004, 2009). In contrast, the network supporting visually guided locomotion involves two classes of light-responsive interneurons in the cerebropleural ganglia that are weakly excited by mechanical stimulation of the statocysts (Akaike and Alkon 1980; Goh and Alkon 1984; Crow and Tian 2000, 2002, 2003, 2004, 2008). The first class of light-responsive premotor interneurons, designated as type I, receive monosynaptic input from identified photoreceptors (Crow and Tian 2000, 2002, 2003, 2004, 2008). Type II interneurons are a second group of light-responsive interneurons that receive polysynaptic input from photoreceptors (Crow and Tian 2002). The visual system contributes to ciliary locomotion by light-dependent modulation of the spike activity of type I excitatory (Ie), type I inhibitory (Ii), type II excitatory (IIe), type II inhibitory (IIi), and type III inhibitory (IIIi) interneurons that regulate the firing of CENs (Crow and Tian 2003). The light-adapted activity of Ie and Ii interneurons has been examined in detail, and the synaptic interactions between identified photoreceptors and Ie-Ii interneurons, IIIi interneurons, and CENs are well documented (Crow and Tian 2000, 2002, 2003, 2008). However, little is known about light-adapted activity of type II...
interneurons, their synaptic connections within the network supporting light-elicited ciliary activity, or potential direct excitatory/inhibitory connections with CENs. Using dye labeling in conjunction with electrophysiological studies, we have identified a subclass of type II interneurons that terminate within the ipsilateral cerebropleural ganglia and form monosynaptic connections with type I interneurons. Illumination of the eyes produced an increase in the tonic spike activity of IIc interneurons and a decrease in the tonic spike activity of IIi interneurons. In addition, we found that type Ic interneurons form monosynaptic connections with IIIi interneurons and that labeled IIIi interneurons project to the contralateral pedal ganglia and through the pedal commissure to the ipsilateral pedal ganglia. The present results and previously published work indicate that the premotor network supporting visual and graviceptive modulation of ciliary locomotion is both polymodal and modality specific. Previous work has shown that graviceptive stimulation elicits foot contractions and concomitant ciliary activity by direct excitation of CENs. In contrast, the regulation of light-elicited ciliary activity is through inhibition of IIIi interneuron spike activity, which results in disinhibition of CENs.

METHODS

Animals. Adult *Hermissenda crassicornis* were used in the experiments. Animals [Sea Life Supply (Sand City, CA) or Monterey Abalone (Monterey, CA)] were maintained in closed artificial seawater (ASW) aquaria at 14 ± 1°C on a 12:12-h light-dark cycle. All electrophysiological procedures were conducted during the light phase of the light-dark cycle.

Intracellular recordings. Simultaneous intracellular recordings from ipsilateral pairs of identified Ic, Ii, IIc, IIi, and IIIi interneurons or Ic, IIc, Ii, and IIIi interneurons and contralateral CENs were collected from isolated nervous systems. As previously reported (Crow and Tian 2000), the criteria for identifying type I and II interneurons consisted of soma size, cell layer, location in the cerebropleural ganglion, and the magnitude and latency of electrophysiological responses to illumination of the eyes. The identification of IIIi interneurons required simultaneous intracellular recordings from CENs and IIIi interneurons to verify a monosynaptic connection. The monosynaptic connection is characterized by short-latency inhibitory postsynaptic potentials (IPSPs) recorded from CENs that follow one-for-one current-evoked spikes in IIIi interneurons. In isolated circumesophageal nervous systems, CENs were identified based on soma size, position along the anterior-ventral edge of the pedal ganglion, electrophysiological responses to light stimulation of the photoreceptors, and current stimulation of type IIIi interneurons (Crow and Tian 2003). Surgical desheathing of a small area of the cerebropleural ganglion was conducted to expose cell bodies of the interneurons and a small area of the ventral pedal ganglion to expose cell bodies of CENs.

Partially desheathed circumesophageal nervous systems were pinned to a SYLGARD (Dow Chemical) stage in a recording chamber filled with ASW of the following composition (in mM): 460 NaCl, 10 KCl, 10 CaCl₂, and 55 MgCl₂, buffered with 10 mM HEPES, and brought to pH 7.46 with dilute NaOH. ASW in the recording chamber was monitored by a thermistor and held at 15 ± 0.5°C. Illumination of the eyes after the appropriate periods of dark adaptation was provided by a tungsten halogen incandescent lamp attached to a fiber optic bundle mounted underneath the recording chamber. Maximum light intensity (≈10⁻⁴ W/cm²) was attenuated with neutral density filters expressed in negative log units. Pairs of interneurons or interneurons and CENs were penetrated with microelectrodes filled with 4M KAc. Microelectrodes were connected to the two heads of an Axoclamp 2A (Axon Instruments, Foster City, CA). Standard intracellular recording and stimulation techniques were used. Electrophysiological data were digitized with a CED power 1401 (Cambridge Electronic Design) and stored on a computer hard drive. Digitized data were analyzed and plotted using Spike 2 software (Cambridge Electronic Design). Single spikes and trains of action potentials were elicited by depolarizing current steps applied in the dark through a bridge circuit. Evidence for monosynaptic connections between pairs of interneurons was provided by postsynaptic potentials with short and relatively constant latencies and a one-for-one relationship between action potentials and postsynaptic potentials recorded in normal ASW and ASW containing high-divalent cations (3× Ca²⁺ and 3× Mg²⁺). Synaptic connections between interneurons that did not initially exhibit a one-for-one relationship between spikes and postsynaptic potentials were not tested further in high-divalent cation ASW.

Cell labeling. For some experiments, after electrophysiological identification, interneurons were penetrated with microelectrodes containing filtered 4% Lucifer yellow in 0.2 M LiCl or 5% 5(6)-carboxyfluorescein in 0.1M KAc to determine their axonal projections within the circumesophageal nervous system. In some cases, interneurons were initially penetrated with dye-containing microelectrodes to eliminate repenetration after physiological identification. Electrode tips were filled with Lucifer yellow in LiCl and backfilled from the shank with 0.2 M LiCl. Type IIIi interneurons in isolated circumesophageal nervous systems with both intact and transected pedal commissures were labeled with Lucifer yellow as described. Iontophoresis was conducted for Lucifer yellow using constant negative current (0.5–1.0 nA) for 1 h or 0.5 nA for 30 min with carboxyfluorescein labeling. After an additional 1 h to allow for the diffusion of Lucifer yellow, nervous systems were fixed overnight with 4% paraformaldehyde in 0.2 M caccodylate buffer (pH 7.4) followed by three rinses at 10-min intervals in 0.1 M PBS, dehydrated in an ascending ethanol series, and cleared with methyl salicylate. The Lucifer yellow-labeled interneurons were viewed under a fluorescent microscope, and images were collected using a laser scanning confocal microscope (Radiance 2100, Bio-Rad). The sampling steps were set at 2 μM, and the z-stack of 65 sections was merged to generate the final image of Lucifer yellow-labeled interneurons. Carboxyfluorescein-labeled interneurons were visualized with a fluorescent microscope in normal ASW, and drawings were made to determine their anatomic projections.

RESULTS

The network diagram representing sensory neurons, premotor interneurons, and CENs that support ciliary locomotion is shown in Fig. 1. The network diagram shows previously established synaptic connections and the newly described synaptic connections presented in this report.

Light responses of type II interneurons. Spike activity was examined in IIc (n = 10) and IIi (n = 10) interneurons before (dark adapted) and during 5 min of illumination of the eyes (light adapted) to characterize light responses under conditions of illumination supporting phototactic behavior. Previous results have shown that the spike activity of dark-adapted type IIi and IIc interneurons is characterized by a change in tonic firing with the generation of occasional irregular burst activity that is not repetitive or rhythmic (Crow and Tian 2008). Group summary data showing the mean spike frequency measured at consecutive 1-min periods, 5 min before light onset, during 5 min of light, and 5 min after light offset are shown in Fig. 2. The results of ANOVA revealed a significant overall effect of light on the spike activity of IIc and IIi interneurons (F₁₄,₂₅₂ = 2.85, P < 0.01). The interaction between interneuron type and light was also statistically significant (F₁₄,₂₅₂ = 18.84, P <
interneurons were identified by their stereotyped light response followed by intracellular labeling with either Lucifer yellow (n = 7) or carboxyfluorescein (n = 39). Consistent with an earlier report conducted with a smaller sample of cells (Crow and Tian 2002), we observed that type II interneurons projected to different regions of the circumesophageal nervous system. However, one subclass of light-responsive interneurons not previously identified were found to project within the ipsilateral cerebropleural ganglion. A total of 17 labeled type II interneurons were identified as terminating within the ipsilateral cerebropleural ganglia and were designated as IIe(cp) (n = 9) or IIi(cp) (n = 8). A sample of interneurons (n = 7) classified as type IIe(cp) and IIi(cp) were selected to examine potential synaptic connections with CENs. Depolarization of IIe(cp) and IIi(cp) interneurons with 2-s current pulses significantly changed IPSP frequency recorded from identified CENs. A paired t-test analysis of difference scores comparing the number of IPSPs in CENs occurring during the current pulse with an identical period immediately before current stimulation revealed a statistically significant increase produced by IIi(cp) stimulation (n = 4) (t = 4.2, P < 0.02). Current-evoked depolarization of IIe(cp) interneurons (n = 3) resulted in a significant decrease in the number of IPSPs recorded from CENs (t = 5.3, P < 0.03). In contrast, current-evoked depolarization of IIi (n = 8) and IIi (n = 7) interneurons that projected to the pedal ganglia did not significantly change IPSP activity of CENs (t = 1.3, P = not significant; t = 0.24, P = not significant), respectively. Examples of Lucifer yellow-labeled IIe(cp) and IIi(cp) interneurons are shown in Fig. 3. The results of the cell labeling experiments indicated that, collectively, type II interneurons represent different functional subclasses that support diverse light-dependent processes. However, interneurons that projected to different regions of the circumesophageal nervous system could not be differentiated based on their response to illumination.

Type II interneuron anatomic projections. To assist in the initial identification and characterization of type II interneurons contributing to the network supporting light-dependent behavior, interneurons were identified by their stereotyped light response followed by intracellular labeling with either Lucifer yellow (n = 7) or carboxyfluorescein (n = 39). Consistent with an earlier report conducted with a smaller sample of cells (Crow and Tian 2002), we observed that type II interneurons projected to different regions of the circumesophageal nervous system. However, one subclass of light-responsive interneurons not previously identified were found to project within the ipsilateral cerebropleural ganglion. A total of 17 labeled type II interneurons were identified as terminating within the ipsilateral cerebropleural ganglia and were designated as IIe(cp) (n = 9) or IIi(cp) (n = 8). A sample of interneurons (n = 7) classified as type IIe(cp) and IIi(cp) were selected to examine potential synaptic connections with CENs. Depolarization of IIe(cp) and IIi(cp) interneurons with 2-s current pulses significantly changed IPSP frequency recorded from identified CENs. A paired t-test analysis of difference scores comparing the number of IPSPs in CENs occurring during the current pulse with an identical period immediately before current stimulation revealed a statistically significant increase produced by IIi(cp) stimulation (n = 4) (t = 4.2, P < 0.02). Current-evoked depolarization of IIe(cp) interneurons (n = 3) resulted in a significant decrease in the number of IPSPs recorded from CENs (t = 5.3, P < 0.03). In contrast, current-evoked depolarization of IIi (n = 8) and IIi (n = 7) interneurons that projected to the pedal ganglia did not significantly change IPSP activity of CENs (t = 1.3, P = not significant; t = 0.24, P = not significant), respectively. Examples of Lucifer yellow-labeled IIe(cp) and IIi(cp) interneurons are shown in Fig. 3. The results of the cell labeling experiments indicated that, collectively, type II interneurons represent different functional subclasses that support diverse light-dependent processes. However, interneurons that projected to different regions of the circumesophageal nervous system could not be differentiated based on their response to illumination.

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Fig. 1. Diagram of previously established and newly described synaptic connections between sensory neurons, premotor interneurons, and ciliary efferent neurons in the network supporting ciliary locomotion. LB, lateral type B photoreceptor; HC, statocyst hair cell; Ie, type I excitatory interneuron; Ib, type Ib interneuron; IIe, type II excitatory interneuron; IIi, type II inhibitory interneuron; IIIi, type III inhibitory interneuron; CEN, ciliary efferent neuron. Excitatory synapses are indicated by bars, and inhibitory synapses are indicated by solid circles. Solid lines indicate monosynaptic connections, and dashed lines indicate polysynaptic connections.

Fig. 2. Light-evoked changes in the tonic spike activity of IIe and IIi interneurons. A graph of mean spike activity (±SE) of IIe and IIi interneurons plotted by 10.220.32.247 on September 29, 2016 http://jn.physiology.org/ Downloaded from
In contrast to the monosynaptic connection between $I_i$ and $II_{e(cp)}$ interneurons, the connection between $I_i-II_e$ ($n = 8$) and $I_i-II_i$ ($n = 7$) interneurons is polysynaptic. Current-evoked depolarization of an identified $I_i$ interneuron elicited spikes recorded from an identified $II_e$ interneuron (Fig. 6A). The evoked spikes and postsynaptic potentials in the $II_e$ interneurons did not follow $I_i$ spikes one for one. Current-evoked depolarization of identified $I_i$ interneurons inhibited $II_i$ interneurons through a polysynaptic pathway (Fig. 6B). The synaptic connections between pairs of $II_e$ and $II_i$ interneurons were also examined. As shown in Fig. 6C, interneurons were identified by their characteristic light responses. Current-evoked depolarization of $II_i$ interneurons resulted in a depolarization of $II_e$ interneurons ($n = 7$; Fig. 6D), and current-evoked depolarization of $II_e$ interneurons produced inhibition of spike activity in $II_i$ interneurons ($n = 4$; Fig. 6E). Postsynaptic potentials did not follow spikes one for one, indicating that the connection between $II_e$ and $II_i$ interneurons is polysynaptic.

**Monosynaptic connection between $I_e$ and $III_i$ interneurons.** Simultaneous recordings from pairs of $I_e$ and $III_i$ interneurons ($n = 5$) revealed that $I_e$ interneurons form monosynaptic excitatory connections with $III_i$ interneurons. As shown in Fig. 7A,

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**Type II interneuron synaptic connections within the ciliary network.** Synaptic connections between identified type II interneurons and neurons that have been shown previously to be part of the network supporting visually guided ciliary locomotion were examined. We recorded from pairs of $I_i$ and $II_e$ interneurons ($n = 11$) to determine if the synaptic connection was monosynaptic or polysynaptic. An example of a simultaneous recording from a pair of $I_i$ and $II_e$ interneurons is shown in Fig. 4. Interneurons were identified by their stereotyped depolarizing response to illumination, as shown in Fig. 4A. Current-evoked depolarization of the $I_i$ interneuron elicited a complex excitatory postsynaptic potential (EPSP) recorded from the $II_e$ interneuron (Fig. 4B), and depolarization of a $II_e$ interneuron evoked a complex EPSP recorded in the $I_i$ interneuron (Fig. 4C). The reciprocal synaptic connection between $I_i$ and $II_e$ interneurons is likely polysynaptic, since postsynaptic potentials and spikes recorded from $II_e$ interneurons did not follow spikes in the $I_i$ interneuron one for one.

We next recorded from pairs of identified $I_e$ and $II_{e(cp)}$ interneurons ($n = 10$). Interneurons were identified based on stereotyped light responses, as noted previously (Crow and Tian 2000, 2002), and the evidence for a monosynaptic connection between $I_e$ and $II_{e(cp)}$ interneurons. As shown in Fig. 5A, light elicited a hyperpolarization and inhibition of spike activity in the $I_e$ interneuron and a small depolarization and increase in spike activity in the $II_{e(cp)}$ interneuron. Current-elicited depolarization of the $I_e$ interneuron inhibited spike activity in the $II_{e(cp)}$ interneuron (Fig. 5B), and depolarization of the $II_{e(cp)}$ interneuron inhibited spike activity in the $I_e$ interneuron (Fig. 5C), indicating a reciprocal synaptic connection between $I_e$ and $II_{e(cp)}$ interneurons. Consistent with a monosynaptic connection between $I_e$ and $II_{e(cp)}$ interneurons, IPSPs recorded from $II_{e(cp)}$ interneurons followed $I_e$ spikes with a short and relatively constant latency in ASW and high-divalent cation ASW (Fig. 5, D–F).

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**Fig. 3.** Electrophysiological identification followed by Lucifer yellow labeling revealed that a subclass of $II_i$ and $II_e$ interneurons, designated as $II_{e(cp)}$, terminate within the ipsilateral cerebropleural ganglion. A: Lucifer yellow-labeled $II_{e(cp)}$ interneuron exhibiting two axonal branches (white arrows). One axonal process formed a loop near the soma that terminated in a series of secondary processes (right white arrow). The second axonal projection terminated in a cluster of secondary processes within the ipsilateral cerebropleural ganglion (left white arrow). B: Lucifer yellow-labeled $II_{e(cp)}$ interneuron exhibiting a cluster of secondary processes near the soma (right white arrow) and processes near the termination of the axon in the ipsilateral cerebropleural ganglion (left white arrow). The outline of the right cerebropleural ganglion and tentacular nerve is indicated by the white lines in A and B. Anterior, posterior, medial, and lateral orientation are shown in the diagram in the inset. Scale bar = 100 μm for both images (A and B).

**Fig. 4.** Type $I_i$ and $II_e$ interneurons exhibit reciprocal excitatory synaptic connections. A: simultaneous recording from a pair of identified type $I_e$ and $II_e$ interneurons before light onset (dark adapted), during light (light adapted), and after light offset. The onset and offset of illumination are indicated by the arrows above the recordings in A. Light evoked a depolarization and increased spike activity in both $I_e$ and $II_e$ interneurons. The dashed line indicates a break in the continuous recordings during the 5-min light period. B: simultaneous recording from an identified pair of $I_e$ and $II_e$ interneurons. A current pulse applied to the $I_e$ interneuron evoked a depolarization (complex excitatory postsynaptic potential (EPSP)) recorded from the $II_e$ interneuron. C: simultaneous recording from an identified pair of $I_i$ and $II_i$ interneurons. A current pulse applied to the $II_i$ interneuron evoked a depolarization (complex EPSP) recorded from the $I_i$ interneuron. Light attenuated $-1.0$ log unit.
spikes from the Ii interneuron and two short-latency IPSPs recorded in the short-latency EPSP recorded from the IIIi interneuron in normal ASW (Fig. 7C). As shown in Fig. 7D, spikes evoked in the Ie interneuron elicited one-for-one EPSPs recorded from the IIIi interneuron in high-divalent cation ASW.

IIIi interneuron projections. Projections of IIIi interneurons within the circumesophageal nervous system were examined in labeled preparations (n = 5). Lucifer yellow labeling of identified IIIi interneurons revealed that the primary axonal process projected to the contralateral pedal ganglion. In preparations with an intact pedal commissure, IIIi interneurons were found to project to both the ipsilateral and contralateral pedal ganglia. Figure 8A shows a preparation with an intact pedal commissure. A secondary axonal process entered the pedal commissure and projected to the contralateral pedal ganglion. The Lucifer yellow-labeled IIIi interneuron shown in Fig. 8A exhibited a bifurcation of the primary axonal process in the pedal ganglion (arrows), with one labeled process entering the pedal commissure (Fig. 8B, right arrow). Near the cell body of the IIIi interneuron are numerous Lucifer yellow-labeled secondary processes (Fig. 8C).

We have previously shown that IIIi interneurons form monosynaptic inhibitory synaptic connections with identified CENs (Crow and Tian 2003). Consistent with the monosynaptic excitatory synaptic connection between Ie and IIIi interneurons

![Diagrams showing neural activity](https://via.placeholder.com/150)

Fig. 5. Identified Ii and IIe(cp) interneurons exhibit reciprocal inhibitory synaptic connections. A: identification of Ii and IIe(cp) interneurons was based on stereotyped light responses and synaptic connection. Simultaneous recordings from a pair of identified Ii and IIe(cp) interneurons during illumination. The onset and offset of illumination are indicated by the arrows above the recording in A. B: a depolarizing current pulse applied to a type Ii interneuron inhibited spike activity recorded from the type IIe(cp) interneuron. C: a depolarizing current pulse applied to the type IIe(cp) interneuron inhibited spike activity in the type Ii interneuron. D: a current-evoked spike in the Ii interneuron elicited a short-latency inhibitory postsynaptic potential (IPSP) recorded from the IIe(cp) interneuron in normal artificial seawater (ASW). E: superimposed spikes and IPSPs recorded in high-divalent cation (Hi Di ASW) solution (3× Ca²⁺ and 3× Mg²⁺). F: in high-divalent cation ASW, a current pulse elicited two spikes from the Ii interneuron and two short-latency IPSPs recorded in the IIe(cp) interneuron. Light attenuated −1.0 log unit in A.

![Diagrams showing neural activity](https://via.placeholder.com/150)

Fig. 6. Simultaneous recording from a pair of identified Ii and IIi interneurons and a pair of Ii and IIe interneurons. A: a current pulse evoked a depolarization of the Ii interneuron and spikes recorded from the IIe interneuron. B: current-evoked depolarization of the Ii interneuron elicited an inhibition of spike activity recorded from the IIe interneuron. Identification of type II interneurons is shown. C: light evoked a stereotyped depolarization of the IIe interneuron and hyperpolarization of the IIi interneuron. Light initially blocked the EPSPs, as indicated by the arrowheads shown in the recording from the IIi interneuron. The onset of light is indicated by the arrow above the IIi recording. Simultaneous recordings from pairs of identified II interneurons exhibited both excitation and inhibition. D: current-evoked depolarization of a IIi interneuron elicited a depolarization and spike activity recorded from the IIi interneuron. E: current-evoked depolarization of a IIi interneuron elicited a hyperpolarization and inhibition of spike activity of the IIe interneuron. Light attenuated −1.0 log unit in the recording of light-elicited activity shown in C.
their contribution to the network supporting ciliary locomotion, type II interneurons are also involved in other light-dependent processes based on their anatomic projections and not differences in light responses. The spike activity of dark-adapted and light-adapted type II interneurons is tonic, which is consistent with the nonrhythmic spike activity of Ie and Ii interneurons recorded in the dark and during illumination (Crow and Tian 2008).

Ciliary locomotor network. In this report, we examined synaptic interactions between identified premotor interneurons that contribute to the network controlling light-elicited ciliary activity in *Hermissenda*. The network may also contribute to a graviceptive-elicited reflex since some interneurons exhibit weak synaptic connections with statocyst hair cells (Akaiake and Alkon 1980). Graviceptive stimulation of *Hermissenda* in a horizontal orientation produces a shortening of the foot and an inhibition of forward ciliary locomotion (Lederhendler et al. 1986; Matzel et al. 1990). A similar defensive response is elicited in the pond snail *Planorbis*, where the activation of statocyst receptor cells by rapid tilting evokes foot shortening and inhibition of ciliary locomotion (Arshavsky et al. 1994). In contrast, *Hermissenda* placed in a vertical orientation exhibit a gravitactic response that involves the initiation of ciliary locomotion in the direction opposite the gravitational force vector. Ciliary locomotion elicited in two quite different behavioral contexts involves different components of the premotor network. Foot shortening and graviceptive-elicited increased ciliary activity involve premotor interneurons that are different from interneurons contributing to light-modulated ciliary locomotion (Crow and Tian 2004, 2009). The network supporting visually modulated ciliary locomotion consists of photoreceptors and hair cells, primary (type I), secondary (type II), tertiary

shown in Fig. 7, current-evoked depolarization of an identified Ie interneuron elicited an increase in IPSP frequency recorded from an identified CEN (Fig. 9A). The increase in Ie-evoked IPSPs recorded in CENs can be accounted for by IIIi interneuron synaptic inhibition of CENs. As shown in Fig. 9B, IPSPs recorded from an identified CEN followed spontaneous spikes recorded from the IIIi interneuron one for one. The inset in Fig. 9B shows an example of an evoked spike in a IIIi interneuron and a short-latency IPSP recorded from the CEN. In the example shown in Fig. 9C, light produced an increase in the spike discharge of the IIIi interneuron, an increase in IPSP frequency, and inhibition of spike activity recorded from the CEN. Hyperpolarizing the IIIi interneuron below the threshold for spike generation revealed a complex EPSP evoked by light detected in the IIIi interneuron and an absence of IPSPs recorded from the CEN (Fig. 9D).

DISCUSSION

Anatomic diversity of type II interneuron projections. In the present study, we found that type II interneurons project to different regions of the circumesophageal nervous system. However, a subclass of interneurons, I_{I_e(cp)} and I_{I_i(cp)}, terminated within the ipsilateral cerebropleural ganglia. Differences in the light responses of type II interneurons that terminated within the cerebropleural ganglia and type II interneurons that projected to other regions of the circumesophageal nervous system were not observed. This indicates that in addition to

Fig. 7. Ie interneurons form monosynaptic connections with IIIi interneurons. A: simultaneous recording from Ie and IIIi interneurons revealed that current-evoked depolarization of the Ie interneuron elicited a depolarization and spike activity recorded from the IIIi interneuron. B: short-latency EPSPs recorded in the IIIi interneuron followed spikes evoked from the Ie interneuron one for one. C: a current evoked spike in a Ie interneuron in normal ASW elicited a short-latency EPSP recorded in the IIIi interneuron. D: recording in high-divalent cation ASW showing superimposed current-evoked spikes in a Ie interneuron and superimposed short-latency EPSPs recorded from the IIIi interneuron.

Fig. 8. IIIi interneurons project to the contralateral pedal ganglion and through the pedal commissure to the ipsilateral pedal ganglion. A: photograph of the circumesophageal nervous system with an intact pedal commissure showing a Lucifer yellow-filled IIIi interneuron in the cerebropleural ganglion. White arrows indicate the axonal branch that terminates in the contralateral pedal ganglion and an axonal branch that projected to the contralateral pedal ganglion through the pedal commissure. B: high-magnification image of the bifurcation (white arrows) of the primary IIIi axonal process shown in A. C: high-magnification image of the secondary processes (white arrows) projecting from the IIIi interneuron cell body. E, eye; S, statocyst.
Interneurons in the locomotor circuit of *Hermissenda*

![Diagram](http://jn.physiology.org/)

**Fig. 9.** Ie interneurons regulate CEN spike activity through monosynaptic excitatory connections with IIIi interneurons. A: simultaneous recording from a Ie interneuron and CEN. Current-evoked depolarization of the Ie interneuron produced an increase in IPSPs recorded in the CEN. IIIi interneurons inhibited CENs. B: simultaneous recording from a IIIi interneuron and an identified CEN in a preparation with a transected pedal commissure. IPSPs recorded in the CEN followed spontaneous spikes in the IIIi interneuron one for one, consistent with a previous report showing a monosynaptic inhibitory connection. *Inset,* a single current-evoked spike in a IIIi interneuron evoked a monosynaptic IPSP in the CEN. C: in this example from a depolarized IIIi interneuron [membrane potential (MP) = −40 mV], light evoked an increase in spike activity recorded from the IIIi interneuron and inhibition of the CEN expressed by an increase in IPSP frequency. D: hyperpolarizing the IIIi interneuron (MP = −47 mV) blocked spike activity elicited by light, revealing a complex EPSP and elimination of IPSPs typically recorded from CENs during illumination. The onset and offset of illumination in C and D are indicated by the arrows above the recordings. Light attenuated −1.0 log unit for the recordings shown in C and D.

(type IIIi) interneurons, and CENs. Modulation of activity in the network is also provided by synaptic input from the somatosensory and chemosensory systems (Akaike and Alkon 1980; Alkon et al. 1978; Crow and Tian 2000, 2008). The synaptic interactions within the sensory systems and between the visual and graviceptive sensory neurons and type I interneurons are well characterized (Alkon and Fuortes 1972; Alkon 1973a, 1973b; Akaike and Alkon 1980; Alkon et al. 1978; Crow and Tian 2003). Each identified photoreceptor forms a monosynaptic connection with different aggregates of electrically coupled type Ie and electrically coupled type Ii interneurons (Crow and Tian 2000, 2008). Electrical coupling contributes to the synchronous firing of pairs of Ie interneurons and pairs of Ii interneurons under both dark-adapted and light-adapted conditions (Crow and Tian 2008). Here, we showed that Ie and Ii interneurons express monosynaptic and polysynaptic interactions with identified second-order type II interneurons. Ie and IIe interneurons exhibit reciprocal excitatory polysynaptic connections, and Ie and IIe(cp) interneurons form reciprocal monosynaptic inhibitory connections. In addition, IIe and IIIi interneurons are reciprocally connected through an excitatory/inhibitory polysynaptic connection. The complexity of the synaptic interactions within the network provides for both feedback and feedforward excitation and inhibition. Ie interneurons form monosynaptic connections with IIIi interneurons. Synaptic input from the visual system to type I interneurons regulates the spike activity of type IIIi interneurons through both monosynaptic and polysynaptic pathways. The synaptic connections between network components shown in the present study indicate that the primary determinant of IIIi interneuron spike activity is provided by the monosynaptic input from Ie interneurons and polysynaptic input from Ii interneurons. The spike activity of CENs is regulated by the monosynaptic inhibitory connection with IIIi interneurons. In semi-intact preparations, illumination of the eyes produces a complex IPSP in Ie interneurons, inhibition of Ii spike activity, decreased spike activity of IIIi interneurons, an increase in spike activity of CENs, and movement of the cilia on the foot (Crow and Tian 2003). The complex EPSP and increase in Ii spike activity evoked by light are less effective in exciting IIIi interneurons than the disinhibition of IIIi interneurons produced by light inhibition of Ii interneurons. The regulation of ciliary activity by IIIi interneuron spike activity is an efficient means of modulating spike activity in CENs, since their membrane potential under baseline conditions is near the threshold for spike generation. Therefore, presynaptic input from network components that result in the excitation or inhibition of spike activity in IIIi interneurons can efficiently modulate spike activity of CENs.

The graviceptive system modulates spike activity of CENs by excitation of Ie interneurons. In contrast to the monosynaptic inhibitory connection between IIIi interneurons and CENs, Ie interneurons form monosynaptic excitatory connections with both dorsal and ventral CENs. Ie interneurons exhibit strong synaptic activation by stimulation of statocyst hair cells and are weakly excited by light-elicited synaptic input from photoreceptors (Crow and Tian 2004, 2009). In dark-adapted conditions, Ie interneurons exhibit low spontaneous spike activity, which is typically not increased by light stimulation of the photoreceptors unless the interneurons are depolarized by extrinsic current (Crow and Tian 2009). These characteristics suggest that Ie interneurons are not part of the network controlling visually guided ciliary locomotion. However, graviceptive synaptic input that results in the depolarization of Ie...
interneurons during illumination of the eyes may enhance the efficacy of excitatory synaptic input from the visual system. This represents an interesting example of a modality-specific switch that results in the modulation of a multifunctional circuit by the inclusion of an additional sensory input. The dual use of a neural pathway in different conditions of illumination (light or dark) has been reported to underlie approach sensitivity in the retina (Münch et al. 2009). The ciliary locomotor network in Hermissenda may engage different components depending on the environmental requirements provided by the different contexts eliciting phototaxis or gravitaxis.

Cross-species comparisons of ciliary locomotor networks. The identification of homologous neurons across species can be helpful in the analysis of neural networks that support similar behaviors. In Pleurobranchaea and Tritonia, interneurons in the central pattern generators (CPGs) exhibit rhythmic neural activity during escape swimming and tonic firing during nonrhythmic ciliary locomotion (Jing and Gillette 1999, 2000, 2003; Popescu and Frost 2002). Dorsal swim interneurons (DSIs) in Tritonia and As1–As4 neurons in Pleurobranchaea fire rhythmically during swimming and tonically during crawling. While there are similarities in the regulation and generation of this form of locomotion in related mollusks, the network supporting visually guided ciliary locomotion in Hermissenda does not appear to be homologous to the multifunctional swim and ciliary locomotor network in either Tritonia or Pleurobranchaea. CENs in Tritonia and locomotor G neurons in Pleurobranchaea are excited by input from interneurons in the swim CPG, whereas in Hermissenda, the light-dependent activity of CENs is regulated by excitation or inhibition of IIIi interneurons. In addition, DSI s and As1–As4 neurons are serotonin (5-HT) immunoreactive (Jing and Gillette 1999; Katz et al. 1994; McClellan et al. 1994), in contrast to Ie, Ii, and Ih interneurons in Hermissenda (Tian et al. 2006). Moreover, 5-HT-immunoreactive cerebropleural ganglion triplet interneurons in Hermissenda do not project to CENs and are inhibited by Ii interneurons (Tian et al. 2006). In Tritonia, type A ventral swim interneurons (VSIs) and type B VSIs form inhibitory connections with ciliary neurons and have been proposed to mediate touch-evoked inhibition of ciliary crawling. In addition, current-elicted depolarization of C2 interneurons directly excite Pd21 CENs (Audesirk 1972). However, excitatory synaptic input from DSI s appears to provide the primary regulation of increased ciliary neuron spike activity, since C2 and VSI-B interneurons are not active after the termination of the swim motor program (Popescu and Frost 2002).

The premotor interneuronal regulation of ciliary locomotion in Hermissenda has a modality-specific preference, although locomotion may be influenced by the stimulation of all sensory systems. Phototaxis is supported by synaptic excitation and inhibition of IIIi interneurons that inhibit the spike activity of CENs and graviceptor-dependent excitation of Ii interneurons results in the direct excitation of CENs. The absence of a network homology underlying ciliary locomotion in related species may be the result of the development of a light-modulated network supporting a positive phototaxis in Hermissenda. Light modulation of ciliary activity is a requirement in Hermissenda that may not be found in other species that exhibit ciliary locomotion. To our knowledge, a positive phototaxis has not been reported for either Tritonia or Pleurobranchaea.

The polysensory premotor network is multifunctional, supporting the generation of both ciliary activity and foot contraction that is regulated by visual and/or graviceptive stimulation. However, components of the network support ciliary locomotion in different environmental contexts where the primary pathway supporting visually guided ciliary locomotion has marginal overlap with the pathway supporting graviceptive-elicited ciliary locomotion and muscular foot contractions. The network architecture also provides for the modification of the modality-specific preference under different physiological conditions. As an example, Pavlovian conditioning changes sensory-interneuron excitability and synaptic strength to amplify the effect of Ii synaptic input to IIIi interneurons, resulting in an inhibition of light-elicited forward locomotion (Crow and Tian 2006). In addition, conditioning may result in a change in the strength of the synaptic connection between photoreceptors and type Ii interneurons. This relatively simple neural network expresses a great deal of functional complexity and flexibility that is regulated by different environmental conditions. The network contributes to the generation of a number of sensory-elicited behaviors, such as dark-adapted ciliary locomotion, inhibition of forward locomotion produced by graviceptive stimulation in a horizontal orientation, gravitaxis in a vertical orientation, light-elicited inhibition of ciliary locomotion produced by Pavlovian conditioning, phototaxis, chemotaxis, and the generation of muscular foot movements.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: T.C. conception and design of research; T.C., N.G.J., and L.-M.T. analyzed data; T.C., N.G.J., and L.-M.T. interpreted results of experiments; T.C. drafted manuscript; T.C. edited and revised manuscript; T.C., N.G.J., and L.-M.T. approved final version of manuscript; N.G.J. and L.-M.T. performed experiments.

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