Network Interneurons Underlying Ciliary Locomotion in *Hermissenda*

Terry Crow, Nan Ge Jin*, and Lian-Ming Tian

Department of Neurobiology and Anatomy
University of Texas Medical School
6431 Fannin Street
Houston, TX  77030

INTERNEURONS IN THE LOCOMOTOR CIRCUIT OF *HERMISSEND*A

Correspondence to:  T. Crow, Department of Neurobiology and Anatomy, University of Texas Medical School, 6431 Fannin Street, Houston, TX  77030

Email:  terry.crow@uth.tmc.edu

Phone: 713-500-5613

FAX: 713-500-0621

* Present address: Department of Ophthalmology and Visual Science
University of Texas Medical School, Houston , TX
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 (I_e) or inhibitory (I_i) input from identified photoreceptors. Type II interneurons receive polysynaptic excitatory (II_e) or inhibitory (II_i) input from photoreceptors. The ciliary network also includes III_i interneurons that form monosynaptic inhibitory connections with ciliary efferent neurons (CENs). Illumination of the eyes evokes a complex IPSP and decrease of I_i spike activity, a complex EPSP and increase of I_e spike activity. Here we characterize the contribution of identified I, II, and III_i interneurons to the neural network supporting visually guided locomotion. In dark-adapted preparations light elicited an increase in the tonic spike activity of II_e interneurons and a decrease in the tonic spike activity of II_i interneurons. Fluorescent dye labeled type II interneurons exhibited diverse projections within the circumesophageal nervous system. However a subclass of type II interneurons, II_e(cp) and II_i(cp) 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 I_e and III_i interneurons shown here suggest that they provide a major role in the light-dependent modulation of CEN spike activity underlying ciliary locomotion.
INTRODUCTION

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,b; Rosen et al. 1991; Shaw and Kristan 1997; Susswein and Byrne 1988; Weiss et al. 1978; Yeoman et al. 1996). With regards 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,b; Baltzley et al. 2011; Copeland 1919, 1922; Crow and Tian 2003; Deliagina and Orlovsky 1990; Gainey 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 I<sub>b</sub> 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, 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 I<sub>c</sub>, I<sub>i</sub>, II<sub>c</sub>, II<sub>i</sub>, and III<sub>i</sub> interneurons that regulate the firing of CENs (Crow and Tian 2003). The light-adapted activity of I<sub>c</sub> and I<sub>i</sub> interneurons has been examined in detail, and the synaptic interactions between identified photoreceptors and I<sub>c</sub>-I<sub>i</sub> interneurons, III<sub>i</sub> 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 IIe interneurons and a decrease in the tonic spike activity of IIi interneurons. In addition, we found that type Ie interneurons form monosynaptic connections with IIIi interneurons and 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 that results in disinhibition of CENs.

METHODS

Animals

Adult *Hermissenda crassicornis* were used in the experiments. The animals obtained from Sea Life Supply, Sand City, CA or Monterey Abalone Co., Monterey, CA were maintained in closed artificial seawater aquaria at 14 ± 1°C on a 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 Ie,IIe, IIi, IIIi interneurons, or IIe, IIi, Ie, 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 IPSPs recorded from CENs that follow one-for-one current-evoked spikes in IIIi interneurons. In isolated circumesophageal nervous systems, CENs were identified based upon 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.

The partially desheathed circumesophageal nervous systems were pinned to a SYLGARD (Dow Chemical) stage in a recording chamber filled with artificial seawater (ASW) of the following composition (mM): 460 NaCl, 10 KCl, 10 CaCl₂, 55 MgCl₂, buffered with 10 mM HEPES and brought to pH 7.46 with dilute NaOH. The ASW in the recording chamber was monitored by a thermistor and held at 15 ± 0.5°C. Illumination of the eyes following 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 headstages of an Axoclamp 2A (Axon Instruments, Foster City, CA). Standard intracellular recording and stimulation techniques were employed. 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 PSPs with short and relatively constant latencies and a one-for-one relationship between action potentials and PSPs recorded in normal ASW and ASW containing high-divalent cations (3 x Ca$^{2+}$ and 3 x Mg$^{2+}$). Synaptic connections between interneurons that did not initially exhibit a one-for-one relationship between spikes and PSPs were not tested further in high-divalent cation ASW.

**Cell labeling**

For some experiments, following electrophysiological identification, interneurons were penetrated with microelectrodes containing filtered 4% Lucifer yellow in 0.2 M LiCl or 5% 5(6)-carboxyfluorescein in 0.1 M KAc to determine their axonal projections within the circumesophageal nervous system. In some cases the interneurons were initially penetrated with dye containing microelectrodes to eliminate repenetration following physiological identification. The electrode tips were filled with Lucifer yellow in LiCl and backfilled from the shank with 0.2 M LiCl. Type III 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 hr or 0.5 nA for 30 min with carboxyfluorescein labeling. After an additional 1 hr to allow for diffusion of the Lucifer yellow, the nervous systems were fixed overnight with 4% paraformaldehyde in 0.2 M cacodylate buffer (pH 7.4) followed by three rinses at 10-min intervals in 0.1 M phosphate-buffered saline, dehydrated in an ascending ETOH series and cleared with methyl salicylate. The Lucifer labeled interneurons were viewed under a fluorescent microscope and images were
collected using a laser scanning confocal microscope (BioRad Radiance 2100). The sampling steps were set at 2 µM and the z-stack of 65 sections was merged to generate the final image of Lucifer labeled interneurons. Carboxyfluorescein labeled interneurons were visualized with a fluorescent microscope in normal ASW and drawings were made to determine their anatomical projections.

RESULTS

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

Light responses of type II interneurons

Spike activity was examined in IIe (n=10) and Ii (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 showed that spike activity of dark-adapted type Ie and Ii 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 depicting 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 the ANOVA revealed a significant overall effect of light on the spike activity of IIe and Ii interneurons (F_{14,252}=2.85; p<0.01). The interaction between interneuron type and light was also statistically significant (F_{14,252}=18.84; p<0.01). Therefore statistical tests were conducted on the simple main effects (Winer, 1962). The dark-adapted spontaneous spike activity of IIe interneurons was significantly less than the spontaneous
activity of dark-adapted IIi interneurons (Fig.2). Compared with IIi spike activity in the dark, IIc
interneurons exhibited a significantly lower spike frequency at the one min (F_{1,18}=5.58; p<0.05),
two min (F_{1,18}=11.92; p<0.01), three min (F_{1,18}=7.29; p<0.05), four min (F_{1,18}=9.48; p<0.01), and
5 min test (F_{1,18}=15.93; p<0.01) immediately before the onset of light. The analysis of the effect
of light on spike activity of IIc interneurons showed a significant increase compared with dark-
adapted baseline activity (F_{14,252}=8.66; p<0.01). Compared to dark-adapted baseline activity,
light produced a significant decrease in spike activity of IIi interneurons (F_{14,252}=7.97; p<0.01).
In addition, during illumination the spike discharge frequency of IIc interneurons decreased over
time, which may reflect spike frequency accommodation or a change in synaptic input from a
presynaptic source or sources. The analysis of IIc mean spike frequency during the 5 min light
period using the Newmann-Keuls procedure showed that spike frequency assessed one min after
the onset of light was significantly higher than the spike frequency at min two (p<0.05), min
three (p<0.05), min four (p<0.01) and min five (p<0.01). In contrast, the decrease in spike
frequency of IIi interneurons was constant during the 5 min period of illumination (Fig.2). At
light offset both classes of type II interneurons returned to their respective baseline dark-adapted
spike frequency. Rhythmic or patterned burst activity was not detected in type II interneurons,
either in the dark or during illumination.

Type II interneuron anatomical projections

To assist in the initial identification and characterization of type II interneurons contributing to
the network supporting light-dependent behavior, the 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 IIf(cp) (n=8). A sample of interneurons (n=7) classified as type IIe(cp) and IIf(cp) were selected to examine potential synaptic connections with CENs. Depolarization of IIe(cp) and IIf(cp) interneurons with 2 sec current pulses significantly changed IPSP frequency recorded from identified CENs. 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 IIf(cp) stimulation (n=4) (t3=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 (t2=5.3; p<0.03). In contrast, current-evoked depolarization of IIe (n=8) and IIf (n=7) interneurons that projected to the pedal ganglia did not significantly change IPSP activity of CENs (t7=1.3; NS), (t6=.24; NS) respectively. Examples of Lucifer yellow labeled IIe(cp) and IIf(cp) interneurons are shown in Fig. 3. The results of the cell labeling experiments indicate 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 upon their response to illumination.

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 Ie and IIe interneurons (n=11) to determine if the synaptic
connection was monosynaptic or polysynaptic. An example of a simultaneous recording from a pair of I_ε and II_ε interneurons is shown in Fig. 4. The interneurons were identified by their stereotyped depolarizing response to illumination shown in Fig. 4A. Current-evoked depolarization of the I_ε interneuron elicited a complex EPSP recorded from the II_ε interneuron (Fig. 4B) and depolarization of a II_ε interneuron evoked a complex EPSP recorded in the I_ε interneuron (Fig. 4C). The reciprocal synaptic connection between I_ε and II_ε interneurons is likely polysynaptic since PSPs and spikes recorded from II_ε interneurons did not follow spikes in the I_ε interneuron one-for-one.

We next recorded from pairs of identified I_ι and II_ε(cp) interneurons (n=10). The interneurons were identified based upon stereotyped light responses as noted previously (Crow and Tian 2000, 2002), and the evidence for a monosynaptic connection between the I_ι and II_ε(cp) interneurons. As shown in Fig. 5A, light elicited a hyperpolarization and inhibition of spike activity in the I_ι interneuron and a small depolarization and increase in spike activity in the II_ε(cp) interneuron. Current-elicited depolarization of the I_ι interneuron inhibited spike activity in the II_ε(cp) interneuron (Fig. 5B) and depolarization of the II_ε(cp) interneuron inhibited spike activity in the type I_ι interneuron (Fig. 5C) indicating a reciprocal synaptic connection between I_ι and II_ε(cp) interneurons. Consistent with a monosynaptic connection between I_ι and II_ε(cp) interneurons, IPSPs recorded from II_ε(cp) interneurons followed I_ι spikes with a short and relatively constant latency in ASW and high-divalent cation ASW (Fig5 D-F).

In contrast to the monosynaptic connection between I_ι and II_ε(cp) interneurons, the connection between I_ι-II_ι (n=8), and I_ε-II_ι (n=7) interneurons is polysynaptic. Current-evoked depolarization of an identified I_ι interneuron elicited spikes recorded from an identified II_ι interneuron (Fig. 6A). The evoked spikes and PSPs in the II_ι interneurons did not follow I_ι spikes one-for-one.
Current-evoked depolarization of identified Ie interneurons inhibited IIi interneurons through a polysynaptic pathway (Fig. 6B). The synaptic connections between pairs of IIe and IIi interneurons were also examined. As shown in Fig. 6C the interneurons were identified by their characteristic light responses. Current-evoked depolarization of IIe interneurons resulted in a depolarization of IIi interneurons (n=7) (Fig. 6D) and current-evoked depolarization of IIi interneurons produced inhibition of spike activity in IIe interneurons (n=4) (Fig. 6E). The PSPs did not follow spikes one-for-one, indicating that the connection between IIe and IIi interneurons is polysynaptic.

**Monosynaptic connection between Ie and IIIi interneurons**

Simultaneous recordings from pairs of Ie and IIIi interneurons (n=5) revealed that Ie interneurons form monosynaptic excitatory connections with IIIi interneurons. As shown in Fig. 7A, current-evoked depolarization of a Ie interneuron elicited spikes recorded from a IIIi interneuron, and short-latency EPSPs followed Ie spikes one-for-one (Fig. 7B). These results suggest that the synaptic connection between Ie and IIIi interneurons is monosynaptic, which is supported by high-divalent cation experiments. A spike evoked in a Ie interneuron elicited a 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**

The 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. Fig. 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 III_i 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
III_i interneuron are numerous Lucifer yellow labeled secondary processes (Fig. 8C).

We have previously shown that III_i interneurons form monosynaptic inhibitory synaptic
connections with identified CENs (Crow and Tian 2003). Consistent with the monosynaptic
excitatory synaptic connection between I_e and III_i interneurons shown in Fig. 7, current-evoked
depolarization of an identified I_e interneuron elicited an increase in IPSP frequency recorded
from an identified CEN (Fig. 9A). The increase in I_e evoked IPSPs recorded in CENs can be
accounted for by III_i interneuron synaptic inhibition of CENs. As shown in Fig. 9B, IPSPs
recorded from an identified CEN followed spontaneous spikes recorded from the III_i interneuron
one-for-one. The inset in Fig. 9B shows an example of an evoked spike in a III_i 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 III_i interneuron, an increase in IPSP frequency and
inhibition of spike activity recorded from the CEN. Hyperpolarizing the III_i interneuron below
the threshold for spike generation revealed a complex EPSP evoked by light detected in the III_i
interneuron and an absence of IPSPs recorded from the CEN (Fig. 9D).

DISCUSSION

Anatomical 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, II_e(cp) and II_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 their contribution to the network supporting ciliary locomotion, type II interneurons are also involved in other light-dependent processes based upon their anatomical 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 $I_e$ and $I_i$ interneurons recorded in the dark and during illumination (Crow and Tian 2008).

**Ciliary locomotor network**

In this report we have 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 (Akaike 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 activation of statocyst receptor cells by rapid tilting evokes foot shorting 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 (type III), 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, 2000, 2002, 2003, 2004, 2008, 2009). Each eye contains three type B photoreceptors and two type A photoreceptors (Alkon and Fuortes 1972). Photoreceptors and statocyst hair cells project to postsynaptic targets in the cerebropleural ganglia (Akaike and Alkon 1980; Crow et al. 1979; 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 and pairs of Ii interneurons under both dark-adapted and light-adapted conditions (Crow and Tian 2008). Here we show 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 Ii and IIe(cp) interneurons form reciprocal monosynaptic inhibitory connections. In addition, IIe and IIi interneurons are reciprocally connected through an excitatory/inhibitory polysynaptic connection. The complexity of the synaptic interactions within the network provides for both feedback and feed-forward 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 III 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 III interneurons. In semi-intact preparations illumination of the eyes produces a complex IPSP in II interneurons, inhibition of Ie spike activity, decreased spike activity of III 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 Ie spike activity evoked by light is less effective in exciting III interneurons than the disexcitation of III interneurons produced by light inhibition of II interneurons. The regulation of ciliary activity by III 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 excitation or inhibition of spike activity in III interneurons can efficiently modulate spike activity of CENs.

The graviceptive system modulates spike activity of CENs by excitation of Ib interneurons. In contrast to the monosynaptic inhibitory connection between III interneurons and CENs, Ib interneurons form monosynaptic excitatory connections with both dorsal and ventral CENs. Ib 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, Ib interneurons exhibit low spontaneous spike activity that 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 Ib interneurons are not part of the network controlling visually guided ciliary locomotion. However, graviceptive synaptic input that results in the depolarization of Ib 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 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 upon 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). The dorsal swim interneurons (DSIs) in *Tritonia* and As1-4 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, while in *Hermissenda* the light-dependent activity of CENs is regulated by excitation or inhibition of IIIi interneurons. In addition, the DSIs and As1-4 neurons are 5-HT-IR (Jing and Gillette 1999; Katz et al. 1994; McClellan et al. 1994), in contrast to the Ic, Ii, and Ib interneurons in *Hermissenda* (Tian et al. 2006). Moreover, the 5-HT-IR CPT interneurons in *Hermissenda* do not project to CENs and are inhibited by Ib interneurons.
(Tian et al. 2006). In *Tritonia* the VSI-A and VSI-B interneurons form monosynaptic inhibitory connections with ciliary neurons and have been proposed to mediate touch-evoked inhibition of ciliary crawling. In addition, current-elicited depolarization of C2 interneurons directly excite Pd21 CENs (Audesirk 1978b). However excitatory synaptic input from the DSIs 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 stimulation of all sensory systems. Phototaxis is supported by synaptic excitation and inhibition of IIIi interneurons that inhibit the spike activity of CENs and graviceptive-dependent excitation of Ib 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 I_c synaptic input to III_i interneurons resulting in 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 I_b 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.
FIGURE LEGENDS

Fig1. 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; I_e, I_i, I_b, I_e, II_e and III_i premotor interneurons; CEN, ciliary efferent neurons. Excitatory synapses are indicated by bars and inhibitory synapses by filled circles. Solid lines indicate monosynaptic connections and dashed lines denote polysynaptic connections.

Fig2. Light-evoked changes in the tonic spike activity of II_e and II_i interneurons. Graph of mean spike activity (± SEM) of II_e and II_i interneurons plotted at consecutive 1 min intervals 5 min before light onset (dark-adapted), during 5 min of light (light-adapted), and 5 min after light offset. Light did not evoke rhythmic or phasic firing in either II_e or II_i interneurons. Spontaneous spike frequency of II_i interneurons was significantly higher than the spike frequency of II_e interneurons under dark-adapted conditions. During the 5 min period of illumination II_e interneurons exhibited significant spike frequency accommodation. Light attenuated -1.0 log unit.

Fig3. Electrophysiological identification followed by Lucifer yellow labeling revealed that a subclass of II_e and II_i interneurons, designated as II_e(cp) and II_i(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_i(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.
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 is shown by the inset diagram. Scale bar for both images (A and B) =100 µm.

Fig.4. Type Ie and IIe interneurons exhibit reciprocal excitatory synaptic connections. (A)
Simultaneous recording from a pair of identified type Ie and IIe 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 Ie and IIe 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 Ie and IIe interneurons. A current pulse applied to the Ie
interneuron evoked a depolarization (complex EPSP) recorded from the IIe interneuron. (C)
Simultaneous recording from an identified pair of Ie and IIe interneurons. A current pulse
applied to the IIe interneuron evoked a depolarization (complex EPSP) recorded from the Ie
interneuron. Light attenuated -1.0 log unit.

Fig.5. Identified Ii and IIe(cp) interneurons exhibit reciprocal inhibitory synaptic connections. (A)
Identification of Ii and IIe(cp) interneurons was based upon 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 IPSP recorded from the Ie(cp) interneuron in
normal ASW. (E) Superimposed spikes and Ie(cp) IPSPs recorded in a high-divalent cation
solution (3 X Ca$^{2+}$ and 3 X Mg$^{2+}$). (F) In high-divalent cation ASW a current pulse elicited two spikes from the I$_i$ interneuron and two short-latency IPSPs recorded in the II$_{e(cp)}$ interneuron. Light attenuated -1.0 log unit in part A.

Fig.6. Simultaneous recording from a pair of identified I$_i$ and II$_i$ interneurons and a pair of I$_e$ and II$_i$ interneurons. (A) A current pulse evoked a depolarization of the I$_i$ interneuron and spikes recorded from the II$_i$ interneuron. (B) Current-evoked depolarization of the I$_e$ interneuron elicited an inhibition of spike activity recorded from the II$_i$ interneuron. Identification of type II interneurons. (C) Light evoked a stereotyped depolarization of the II$_e$ interneuron and hyperpolarization of the II$_i$ interneuron. Light initially blocked the IPSPs indicated by the arrow heads shown in the recording from the II$_i$ interneuron. The onset of light is indicated by the arrow above the II$_e$ recording. Simultaneous recordings from pairs of identified II interneurons exhibited both excitation and inhibition. (D) Current-evoked depolarization of a II$_e$ interneuron elicited a depolarization and spike activity recorded from the II$_i$ interneuron. (E) Current-evoked depolarization of a II$_i$ interneuron elicited a hyperpolarization and inhibition of spike activity of the II$_e$ interneuron. Light attenuated -1.0 log unit in the recording of light-elicited activity shown in part C.

Fig.7. I$_e$ interneurons form monosynaptic connections with III$_i$ interneurons. (A) Simultaneous recording from I$_e$ and III$_i$ interneurons revealed that current-evoked depolarization of the I$_e$ interneuron elicited a depolarization and spike activity recorded from the III$_i$ interneuron. (B) Short latency EPSPs recorded in the III$_i$ interneuron followed spikes evoked from the I$_e$ interneuron one-for-one. (C) A current evoked spike in a I$_e$ interneuron in normal ASW elicited a short latency EPSP recorded in the III$_i$ interneuron. (D) Recording in high-divalent cation...
ASW showing superimposed current-evoked spikes in an I_e interneuron and superimposed short-latency EPSPs recorded from the III_i interneuron.

Fig. 8. III_i 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 filled III_i 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 of the bifurcation (white arrows) of the primary III_i axonal process shown in A. (C) High magnification of secondary processes (white arrows) projecting from the III_i interneuron cell body. Eye (E), statocyst (S).

Fig. 9. I_e interneurons regulate CEN spike activity through monosynaptic excitatory connections with III_i interneurons. (A) Simultaneous recording from a I_e interneuron and CEN. Current-evoked depolarization of the I_e interneuron produced an increase in IPSPs recorded in the CEN. III_i interneurons inhibit CENs. (B) Simultaneous recording from a III_i interneuron and an identified CEN in a preparation with a transected pedal commissure. IPSPs recorded in the CEN follow spontaneous spikes in the III_i interneuron one-for-one consistent with a previous report showing a monosynaptic inhibitory connection. Inset: a single current-evoked spike in a III_i interneuron evoked a monosynaptic IPSP in the CEN. (C) In the example from a depolarized III_i interneuron (MP = -40mV), light evoked an increase in spike activity recorded from the III_i interneuron and inhibition of the CEN expressed by an increase in IPSP frequency. (D) Hyperpolarizing the III_i interneuron (MP = -47mV) blocked spike activity elicited by the 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.
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