Statocyst Hair Cell Activation of Identified Interneurons and Foot Contraction
Motor Neurons in *Hermisenda*

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Pavlovian conditioning of *Hermisenda* produces both light-elicited inhibition of normal positive phototactic behavior and conditioned stimulus (CS)-elicited foot-shortening. Rotation, the unconditioned stimulus (US) elicits foot-shortening and reduced forward ciliary locomotion. The neural circuit supporting ciliary locomotion and its modulation by light is known in some detail. However the neural circuits responsible for rotation-elicited foot-shortening and reduced forward ciliary locomotion are not known. Here we describe components of the neural circuit in *Hermisenda* that produce anterior foot contraction and ciliary activation mediated by statocyst hair cells. We have characterized in semi-intact preparations newly identified pedal ventral contraction motor neurons (VCMNs) and interneurons (I_b). Type I_b interneurons receive polysynaptic input from statocyst hair cells and project directly to VCMNs and cilia activating motor neurons. Depolarization of VCMNs with extrinsic current in normal artificial seawater (ASW) and high-divalent cation ASW, and under conditions where central synaptic transmission was suppressed with 5 mM Ni^{2+} ASW, elicited a contraction of the ipsilateral anterior foot measured from videotape recordings. Mechanical displacement of the statocyst or depolarization of identified statocyst hair cells with extrinsic current elicited spikes and complex EPSPs in type I_b interneurons and complex EPSPs and spikes recorded in VCMNs. Type I_b interneurons are electrically coupled and project to VCMNs and VP1 cilia activating motor neurons located in the contralateral pedal ganglia. The results indicate that statocyst hair cell mediated anterior foot contraction and graviceptive ciliary locomotion involve different interneuronal circuit components from the circuit previously identified as supporting light modulated ciliary locomotion.
Information concerning the direction of gravity relative to body orientation is mediated by the statocysts in invertebrates. In *Hermissenda*, like other gastropod mollusks (Gallin and Wiedehold 1977; Janse 1982, 1983) the statocysts are paired spherical structures containing mechanoreceptor hair cells located around the perimeter of the sensory organ (Alkon 1975; Alkon and Bak 1973; Detwiler and Alkon 1978; Detwiler and Fuortes 1973). Locomotion, influenced by graviceptive input from statocyst hair cells that control reflexes and complex geotactic behaviors has been studied in several invertebrate preparations (Alkon 1974; Arshavsky et al. 1985; Deliagina 1998; Levi et al. 2004; Panchin et al. 1995; Wolff 1975).

Pavlovian conditioning of *Hermissenda* produces both light-elicited inhibition of normal positive phototaxis (Crow and Alkon 1978, 1980; Crow and Offenbach 1983) and conditioned stimulus (CS)-elicited foot contraction (Lederhendler et al. 1986). In the conditioning paradigm, the unconditioned stimulus (US), rotation, produces a clinging reflex consisting of foot-shortening (Lederhendler et al. 1986) and contraction of the body musculature (Alkon 1974) that is mediated by the statocysts (Alkon 1975). In addition, rotation reduces forward ciliary locomotion (Matzel et al. 1990).

The neural circuitry supporting light-elicited ciliary locomotion is now known is some detail (Crow and Tian 2000, 2002a, 2003a), although little is understood about the neural circuitry mediating statocyst elicited foot contraction or graviceptive modulation of ciliary locomotion. In this study we show that the neural circuitry supporting statocyst mediated muscular foot contraction and graviceptive ciliary locomotion contain different interneuronal components from the circuitry responsible for light modulated ciliary locomotion. We have characterized, in semi-intact preparations, newly identified pedal ventral contractile motor neurons (VCMNs) and interneurons (I_b). Type I_b interneurons receive polysynaptic input from statocyst hair cells and
project directly to VCMNs and cilia activating motor neurons (VP1). Labeling of VCMNs revealed that the neurons contained a single axonal process that projected to the foot/body wall region through pedal nerve P2. Extrinsic current depolarization of VCMNs, type Ib interneurons, or stimulation of statocyst hair cells with mechanical taps or direct mechanical displacement of the statocyst elicited contraction of the anterior foot in semi-intact preparations. Fluorescent labeling of type Ib interneurons revealed that axonal processes project to the contralateral pedal ganglion and that type Ib interneurons in each cerebropleural ganglia are electrically coupled. Type Ib interneurons were depolarized by mechanical stimulation of statocyst hair cells or by extrinsic current depolarization of hair cells in different regions of the statocyst. Extrinsic current stimulation of rostral or lateral hair cells depolarized type Ib interneurons; however, caudal hair cells did not exhibit detectable synaptic connections with type Ib interneurons. Depolarization of type Ib interneurons with extrinsic current or statocyst hair cell stimulation elicited complex EPSPs and spikes in VCMNs. In addition, we observed that depolarization of a single type Ib interneuron with extrinsic current was sufficient to elicit contralateral anterior foot contraction. The analysis of the network mediating anterior foot contraction and graviceptive ciliary locomotion suggests that CS-elicited foot-shortening and statocyst-mediated reduced forward locomotion involve different interneurons than the neural circuit controlling light modulated ciliary locomotion. In addition, CS-elicited foot-shortening may involve different sites of interneuronal plasticity from those contributing to light-elicited inhibition of locomotion produced by Pavlovian conditioning.
METHODS

Animals

Adult *Hermissenda crassicornis* were used in the experiments. The animals were obtained from Sea Life Supply, Sand City, CA and maintained in closed artificial seawater aquaria at 14° 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 statocyst hair cells and newly identified type Ib interneurons and type Ib interneurons and VCMNs were collected from partially split-foot semi-intact preparations. Anatomical and electrophysiological criteria were used to identify type Ib interneurons as described previously for type I and II interneurons (Crow and Tian 2000). Surgical desheathing of a small area of the cerebropleural and ventral pedal ganglion was conducted to expose the cell bodies of type Ib interneurons and VCMNs. In semi-intact preparations pedal VCMNs were identified by verifying anterior foot contractions produced by depolarization with extrinsic current. Type Ib interneurons were physiologically identified by eliciting spikes with extrinsic current pulses and recording complex PSPs and spikes in VCMNs.

The partially desheathed circumesophageal nervous systems were pinned to a SYLGARD (Dow Chemical) stage in a recording chamber filled with 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 preparation was provided by a tungsten-halogen incandescent lamp attached to a fiber optic bundle mounted underneath the recording chamber.
Interneurons, hair cells and pedal VCMNs were impaled 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. Extracellular recordings from pedal nerve P2 and stimulation of pedal nerve P1 were conducted with suction electrodes as previously described (Crow and Tian 2003a). Digitized data were analyzed and prepared for figures using Spike 2 software (Cambridge Electronic Design). Single spikes elicited by brief extrinsic current pulses and trains of action potentials elicited by current steps were applied in the dark through a bridge circuit. Evidence for monosynaptic connections between type I_b interneurons, VCMNs, and VP1 ciliary motor neurons was provided by EPSPs with short and relatively constant latencies and a one-for-one relationship between I_b action potentials and EPSPs recorded in normal ASW and in ASW containing high-divalent cations (3 x Ca^{2+} and 3 x Mg^{2+}).

**Semi-intact preparations**

Semi-intact anterior split-foot *Hermissenda* were prepared by cooling the animals in ASW to between 0° and 1°C followed by isolation of the circumesophageal nervous system from the buccal crest and body, leaving one intact pedal nerve P2. Pedal nerve P2 contains the axons of motor neurons innervating the anterior region of the foot (Richards and Farley 1987). The partially split foot was positioned ventral side up adjacent to the isolated circumesophageal nervous system pinned to the elevated central stage in the recording chamber. The left pedal ganglion was rotated approximately 150° in order to provide for visualization of neuronal cell bodies on the ventral surface. The exposed nervous system and anterior foot were imaged in visible light by a 45-W tungsten-halogen light source projected by a light guide to the central
stage of the recording chamber. For measurements of foot contraction, the foot was visualized in infrared illumination provided by the insertion of an infrared filter (Schott model RG-850) in the light path. A dissecting microscope formed an image of the foot and nervous system in infrared light upon a Dage MTi videocamera connected to a videomonitor and videorecorder. Foot contraction was assessed by video imaging of the anterior region of the foot during depolarization of pedal VCMNs or type Ib interneurons with extrinsic current (5 sec, .5-1.5 nA). Anterior foot contraction was quantified by measuring foot displacement from pre-stimulus baseline positions on a transparency covering the video monitor screen. To rule out the possible contribution of indirect activation of central neurons to foot contraction, pedal VCMNs were depolarized and anterior foot contraction assessed with nervous systems exposed to ASW solutions containing 5 mM Ni^{2+} or high-divalent cations (3 x Ca^{2+} and 3 x Mg^{2+}). For experiments involving suppression of central synaptic transmission, the nervous systems pinned to the elevated central stage were exposed to Ni^{2+} ASW by application inside a vaseline ring that isolated the central ganglion from the normal ASW that contacted the foot and body chambers. The effectiveness of suppression of central synaptic transmission was shown by inhibition of the pedal VCMN complex EPSP elicited by stimulation of pedal nerve 1 in N^{2+} ASW.

Depolarization of statocyst hair cells was produced by a mechanical tap applied to the chamber, or direct mechanical displacement of the statocyst produced by a glass probe. In some experiments trains of action potentials and single spikes were evoked by extrinsic current pulses that depolarized hair cells in identified regions (rostral, lateral, caudal) of the statocyst. Spikes, complex EPSPs and monosynaptic EPSPs were recorded simultaneously from type Ib interneurons and pedal VCMNs or hair cells and type Ib interneurons.
Cell labeling

Type I_b interneurons and pedal VCMNs were impaled with microelectrodes filled with filtered 4% Lucifer yellow in 0.2 M LiCl or 5% 5(6)-carboxyfluorescein in .1M KAc (molecular probes). The electrode tips were filled with the Lucifer yellow in LiCl and backfilled from the shank with 0.2 M LiCl. The connectivity of the I_b interneuron with the pedal VCMN was first established followed by iontophoresis of Lucifer yellow using a constant negative current (1.0 nA) for 20 min or .5nA for 30 min with 5(6)-carboxyfluorescein labeling. Foot contraction elicited by extrinsic current stimulation of pedal VCMNs was verified before dye labeling of pedal VCMNs. After an additional 2 h to allow for diffusion of the Lucifer yellow the ganglion was fixed with 4% paraformaldehyde in 0.2 M cacodylate buffer (pH 7.4) for 3-12 h followed by dehydration in an ascending ETOH series and cleared with methyl salicylate. 5(6)-carboxyfluorescein labeled neurons were visualized in normal ASW. The stained neurons were observed and photographed through a fluorescence microscope (Zeiss, Thornwood, NY).

RESULTS

Axonal projections of pedal VCMNs

As previously reported, pedal motor neurons that innervate the anterior region of the foot project to postsynaptic targets through pedal nerve P2 (Richards and Farley 1987). A representative example of a 5(6)-carboxyfluorescein filled pedal VCMN is shown in the photograph of the ventral surface of a hemisected circumesophageal nervous system in Fig. 1A. A single labeled axonal process projecting from the pedal VCMN was detected in pedal nerve P2 as indicated by the black arrow in Fig. 1B. Single fluorescent labeled axonal processes detected in P2 could be traced into a region of the anterior foot and body wall (N=5). A drawing of the
anterior split foot semi-intact preparation used to record anterior foot contraction following depolarization of pedal VCMNs and type Ib interneurons is shown in Fig. 1C. Rotation of the pedal ganglion displaced pedal nerve P2 to the bottom quadrant of the ganglion as indicated in the drawing of the pedal ganglia and nerve P2. Depolarization of the pedal VCMN shown in Fig. 1A with a depolarizing current pulse (.5 nA) elicited spikes and a depolarization sufficient to produce contraction of the anterior foot (N=10, Fig. 1D). Newly identified pedal VCMNs and type Ib interneurons were physiologically identified before fluorescent dye labeling. Additional evidence (N=6) for the projection of a pedal VCMN axon in pedal nerve P2 was the detection of a brief and constant latency extracellular spike recorded from nerve P2 (Fig. 2B) elicited by a current evoked action potential in a pedal VCMN (Fig. 2A). In addition, an antidromic spike recorded from a VCMN exhibited a brief and constant latency following suction electrode stimulation of nerve P2 (Fig. 2C). Extracellular spikes recorded from nerve P2 followed one-for-one action potentials in the VCMN elicited by an extrinsic depolarizing current pulse (Fig. 2D-E).

VCMN stimulation elicits anterior foot contraction

Depolarization of pedal VCMNs with 5 sec depolarizing current pulses (.5-.8 nA) elicited contraction of the ipsilateral anterior foot. Fig. 3A1 shows an example of current-elicited spikes recorded from a pedal VCMN in normal ASW. The train of action potentials elicited in the VCMN was sufficient to evoke anterior foot contraction. An example of depolarizing current-elicited spikes recorded in the same pedal VCMN exposed to a high-divalent cation solution (3 x Ca$^{2+}$ and 3 x Mg$^{2+}$) is shown in Fig. 3A2. The high-divalent cation ASW solution did not block foot contraction elicited by depolarization of the pedal VCMN, suggesting that VCMNs do not
indirectly produce contraction through polysynaptic connections with central neurons. The group data (N=5) for average foot contraction measured from video tape recordings is shown in Fig. 3B. For purposes of comparison, pedal VCMN spikes and anterior foot contractions shown in Fig. 3B were elicited by the same level of depolarizing current in normal ASW and high-divalent cation ASW. The smaller contractions detected in high-divalent cation ASW are probably due to the decrease in the number of spikes elicited by the current pulse following the application of the high-divalent ASW. Since multiple spikes are required to elicit foot contraction, summation of PSPs could produce sufficient activity in central polysynaptic pathways to contribute to foot movement. To address this issue, only the central nervous system was exposed to 5 mM Ni\textsuperscript{2+} ASW to suppress synaptic transmission (N=4). As shown in Fig. 4A1 depolarization of a pedal VCMN elicited spikes and a depolarization sufficient to produce verified anterior foot contraction. The effectiveness of suppressing central synaptic transmission was established before examining foot contraction in Ni\textsuperscript{2+} ASW. Pedal nerve P1 contains processes of sensory neurons that project to postsynaptic targets within the circumesophageal nervous system (Crow and Tian unpublished observations). Suction electrode stimulation of pedal nerve P1 evoked a complex EPSP recorded from the VCMN motor neuron that is mediated by activation of central polysynaptic pathways (Fig. 4A2). In the presence of 5 mM Ni\textsuperscript{2+} ASW the same nerve P1 stimulation failed to elicit the complex EPSP recorded from the VCMN (Fig. 4A3). However in 5 mM Ni\textsuperscript{2+} only applied to the circumesophageal nervous system, spikes elicited from the pedal VCMN by an extrinsic current pulse evoked anterior foot contraction (Fig. 4A4).
VCMNs receive synaptic projections from newly identified type I₆ interneurons

Stimulation of statocyst hair cells by a mechanical tap or mechanical displacement of the statocyst resulted in a depolarization of newly identified type I₆ interneurons and VCMN motor neurons (N=10). As shown in Fig. 5A-B, a mechanical tap (arrow Fig. 5A) elicited a single spike recorded in a type I₆ interneuron and a small depolarization and spike recorded from a contralateral pedal VCMN. A stronger mechanical tap (arrow) elicited several spikes recorded from the I₆ interneuron and multiple EPSPs recorded from the contralateral pedal VCMN hyperpolarized to -80 mV to block spike generation (Fig. 5C-D). These results also indicate that other neurons are activated by mechanical stimulation of the statocyst that project to pedal VCMNs since VCMN EPSPs occurred in the absence of spikes recorded from the I₆ interneuron (see Fig. 5D). This observation most likely reflects the convergence of interneuronal projections to pedal VCMNs as-well-as multiple statocyst hair cell synaptic input to type I₆ interneurons.

Type I₆ interneurons receive synaptic input from identified hair cells

Extrinsic current stimulation of single hair cells provided evidence that depolarization of type I₆ interneurons is mediated by stimulation of statocyst hair cells. Depolarization of a single statocyst hair cell (Fig. 6A-D) was sufficient to elicit complex EPSPs, and spikes recorded from type I₆ interneurons (N=14). As shown in Fig. 6A, extrinsic current depolarization of a hair cell located in the lateral statocyst evoked spikes recorded in a type I₆ interneuron. Depolarization of a rostral hair cell (Fig. 6C) evoked a complex EPSP recorded from a type I₆ interneuron hyperpolarized to -65 mV to block spike generation (Fig. 6D). The variable latency of type I₆ interneuron EPSPs following hair cell spikes (data not shown) and the absence of a one-for-one
relationship between hair cell spikes and type I_b EPSPs suggested that the synaptic connections between hair cells and type I_b interneurons are polysynaptic. The procedures used in this study for mechanical stimulation of the statocyst that was sufficient to evoke spikes recorded in type I_b interneurons and VCMNs also evoked a depolarizing generator potential with superimposed spikes in identified statocyst hair cells (N=4). An example of a depolarizing generator potential with superimposed spikes recorded from a rostral statocyst hair cell is shown in Fig. 6E. Direct mechanical displacement of the statocyst used in this study produced depolarizing hair cell generator potentials of similar amplitude as previously reported (Alkon and Bak 1973).

Interestingly, the synaptic connections between statocyst hair cells and type I_b interneurons are dependent upon the position of the hair cell on the statocyst. As shown in the simultaneous intracellular recordings from identified hair cells and type I_b interneurons in Fig. 7A1-C2, hair cells located on the rostral and lateral region of the statocyst exhibited connections with type I_b interneurons. Extrinsic current depolarization of rostral and lateral hair cells evoked complex PSPs in type I_b interneurons hyperpolarized to block spike generation. In contrast, hair cells in the caudal region of the statocyst did not exhibit detectable connections with type I_b interneurons (Fig. 7C2). The group summary data for hair cell connections with type I_b interneurons is shown in Table 1. The majority (86%) of rostral and lateral hair cells examined (N=14) projected to type I_b interneurons. None of the caudal hair cells tested (N=6) exhibited a connection with type I_b interneurons. Taken collectively these results indicate that the response to a mechanical tap or direct mechanical displacement of the statocyst recorded in type I_b interneurons is mediated by activation of hair cells located in specific positions on the statocyst.
**Postsynaptic projections of type \( I_b \) interneurons**

Lucifer yellow labeling of type \( I_b \) interneurons revealed that they project axonal processes to the contralateral pedal ganglion (\( N=4 \), Fig. 8A1-8A2). As shown in the Lucifer labeled type \( I_b \) interneuron in Fig. 8A1, the axonal processes from the type \( I_b \) interneuron crosses to the contralateral side in the cerebropleural commissure, courses below the eye and terminates in the contralateral pedal ganglia (black arrowhead, Fig. 8A2). As shown in Fig. 8B-C the labeled contralateral type \( I_b \) interneuron indicated in Fig. 8A1-A2 exhibited a synaptic connection with a pedal VCMN. Depolarization of the type \( I_b \) interneuron with extrinsic current (Fig. 8B) evoked a complex EPSP recorded from the pedal VCMN (Fig. 8C1). Current depolarization of the same pedal VCMN shown in Fig. 8C1 evoked spikes and elicited ipsilateral anterior foot contraction (Fig. 8C2).

Simultaneous intracellular recordings from ipsilateral and contralateral type \( I_b \) interneurons in the cerebropleural ganglia revealed that they are electrically coupled. As shown in the photograph of two type \( I_b \) interneurons labeled with 5(6)-carboxyfluorescein in Fig. 9A, each \( I_b \) interneuron projects to the contralateral pedal ganglia as indicated by the white arrowheads in the pedal ganglia. The axonal processes cross in the cerebropleural commissure (white arrow), a potential site for electrical coupling. Extrinsic current stimulation of one type \( I_b \) interneuron produced a hyperpolarizing electrotonic potential (Fig. 9B) and small hyperpolarizing potential in the contralateral type \( I_b \) interneuron (Fig. 9C). The coupling coefficients between the two type \( I_b \) interneurons ranged from .10 to .19 (\( N=5 \)).
Depolarization of $I_b$ interneurons is sufficient to elicit anterior foot contraction

Depolarization of type $I_b$ interneurons with a 2 sec extrinsic current pulse elicited multiple spikes recorded from both type $I_b$ interneurons and pedal VCMNs (Fig. 10A1-B1). Hyperpolarizing the VCMN below threshold for spike generation (-65 mV) during the period of type $I_b$ depolarization revealed a complex EPSP recorded from the VCMN (Fig. 10A2-B2). As shown in Fig. 10C-D, depolarization of a contralateral type $I_b$ interneuron with a 5 sec 1.5 nA current pulse produced a train of spikes recorded from the VCMN that was sufficient to elicit anterior foot contraction. The mean contraction ($\pm$ SEM) produced by extrinsic current depolarization of type $I_b$ interneurons (N=3) is shown in the summary group data (Fig. 10E).

Multiple projections of type $I_b$ interneurons

Previously we showed that the primary pathway for activation of ciliary motor neurons is from type I interneuronal connections with type III$_1$ inhibitory interneurons (Crow and Tian, 2003a). A second pathway that can modulate VP1 ciliary motor neuron spike activity has been identified (Crow and Tian, 2003a). The second pathway involves activation of type II$_b$ interneurons that is sufficient to depolarize VP1 and VP3 ciliary motor neurons. Components of the ciliary motor system modulated by visual input are different from the neural system supporting foot contraction since type $I_c$ and $I_i$ interneurons do not project to pedal VCMNs (see Fig. 13). In this report we provide evidence that statocyst innervation of the ciliary motor system involves different neural pathways from the light-elicited ciliary network. Type $I_b$ interneurons that have been shown to depolarize pedal VCMNs also project to identified VP1 ciliary activating motor neurons. As shown in Fig. 11 the same type $I_b$ interneuron has synaptic connections with both a
physiologically identified VP1 ciliary motor neuron (Fig. 11B) and a physiologically identified pedal VCMN. Motor function was verified by showing that spikes evoked by extrinsic current stimulation of the same type I\textsubscript{b} interneurons were sufficient to elicit anterior foot contraction and cilia movement in semi-intact preparations (N=5). These results indicate that statocyst hair cell depolarization of VP1 ciliary motor neurons and photoreceptor activation of ciliary motor neurons is primarily mediated by two different pathways.

Simultaneous recordings (N=9) from pairs of type I\textsubscript{b} interneurons and VP1 ciliary motor neurons or pedal VCMNs suggested that the synaptic connection is monosynaptic. As shown in Fig. 12A1 a single spike in a type I\textsubscript{b} interneuron evoked a short and relatively constant latency EPSP in a VP1 ciliary motor neuron (Fig. 12B1) that also followed I\textsubscript{b} spikes one-for-one as indicated by the superimposed EPSPs recorded in high-divalent ASW (Fig. 12B2). A single current elicited spike recorded in a type I\textsubscript{b} interneuron (Fig. 12C1) evoked a short and relatively constant latency EPSP recorded in a VCMN (Fig. 12D1) that followed I\textsubscript{b} spikes one-for-one in high-divalent ASW as indicated by the superimposed EPSPs in Fig. 12D2.

DISCUSSION

In this report we have identified anterior foot contraction motor neurons (VCMNs) and characterized synaptic input to VCMNs from statocyst hair cells and type I\textsubscript{b} interneurons. Depolarization of type I\textsubscript{b} interneurons with extrinsic current or mechanical stimulation of statocyst hair cells evoked complex EPSPs and spikes in VCMNs sufficient to elicit contraction of the anterior foot. Direct mechanical stimulation of the statocyst was sufficient to evoke a depolarizing generator potential with superimposed spikes in statocyst hair cells as previously reported (Alkon and Bak 1973). Extrinsic current depolarization of statocyst hair cells evoked
complex EPSPs and spikes recorded in type I\textsubscript{b} interneurons. While depolarization of statocyst hair cells produced strong depolarization of I\textsubscript{b} interneurons, the synaptic connections are presumably polysynaptic. However, the synaptic connection between type I\textsubscript{b} interneurons and VCMNs is direct.

\textit{Neural circuitry contributing to foot-shortening}

Foot-shortening elicited by the CS in conditioned animals and by the US involves a larger contraction of the posterior foot and smaller contraction of the anterior foot near the ventral tentacle (Lederhendler et al. 1986). Previously it was shown that extrinsic current depolarization of a pedal motor neuron (MN1) produced a contraction of the middle region of the ipsilateral foot and a subsequent turning of the posterior foot toward the ipsilateral direction (Goh and Alkon, 1984). In addition, it has been shown that light does not elicit the clinging reflex (Alkon 1974) or foot-shortening before conditioning (Lederhendler et al., 1986). Since light was shown to depolarize MN1 (Goh and Alkon, 1984), it is unlikely that MN1 is a contractile motor neuron innervating the posterior region of the foot. We have observed in semi-intact preparations that stimulation of a neuron on the dorsal surface of the pedal ganglion produces vigorous contraction of the ipsilateral posterior foot (Crow and Tian unpublished observations). Moreover the pedal neuron, like the VCMNs described in this report, is not depolarized by light. We have now identified a number of putative pedal motor neurons with axons projecting in pedal nerve 1 that result in either contraction or elongation of the posterior region of the foot when depolarized with extrinsic current. In addition, depolarization of a single contralateral type I\textsubscript{b} interneuron produces both ciliary movement and posterior foot contraction in semi-intact preparations with only pedal nerve P1 intact (Crow and Tian unpublished observations). These results suggest that
type I_b interneurons project to contractile and ciliary motor neurons innervating all regions of the foot.

Differences between visual and graviceptive modulation of ciliary locomotion

Our evidence indicates that anterior foot contraction and statocyst mediated ciliary locomotion involve different interneurons than the neural circuit mediating visually influenced ciliary locomotion (see Fig. 13). Here we report that type I_b interneurons project to both VCMNs and VP1 ciliary motor neurons. However, statocyst mediated ciliary activation is the result of direct type I_b depolarization of VP1 ciliary motor neurons while light results in a disinhibition of VP1 ciliary motor neurons through decreased activity of type III_i interneurons (Crow and Tian 2003a). We have shown previously that type I_c, I_i, II_c and II_i interneurons provide convergent synaptic input to type III_i inhibitory interneurons responsible for inhibition of VP1 and VP3 cilia-activating motor neurons (Crow and Tian, 2003a). In addition, type I_c, I_i, II_c and II_i interneurons that mediate light modulation of VP1 and VP3 neural activity do not project to VCMNs (Crow and Tian, 2003a). However motor neuron VP2 receives synaptic input from the same type III_i interneuron that projects to VP1 and VP3 ciliary motor neurons and thus may be involved in a behavior that is complimentary to ciliary locomotion. Depolarization of motor neuron VP2 elicited a slow lateral movement of the anterior foot and ventral tentacle that may increase the contact area between the ventral foot and the underlying substrate during ciliary locomotion (Crow and Tian, 2003a). Therefore the different motor acts mediated by VP1, VP3 and VP2 motor neurons are not necessarily incompatible, and coactivation may synergistically facilitate ciliary locomotion.
Different behaviors may be controlled by the same neural circuit

It is well-documented that motor systems can support the generation or expression of multiple motor acts (for reviews, see Getting 1989; Kupfermann and Weiss 2001; Marder and Calabrese 1996; Pearson 1993). Representative examples of different behaviors controlled by the same neural network have been identified in *Tritonia* (Popescu and Frost, 2002), *Pleurobranchaea* (Jing and Gillette, 2000), and *Lymnaea* (Syed and Winlow, 1989). Escape locomotion evoked by aversive or noxious stimuli is a closely related locomotor behavior that may be mediated by the same neural network as ciliary locomotion in *Hermisenda*. Ciliary movement is a nonrhythmic, nonmuscular gliding form of locomotion in contrast to escape locomotion which is muscular and rhythmic, involving the generation of foot contractions. However, the two behaviors could be complimentary since increased ciliary activity generated in phase with rhythmic contractions of the pedal musculature during contact of the foot with the substrate would be expected to enhance the speed of escape locomotion. The neural circuit that supports ciliary locomotion in *Hermisenda* could also be reconfigured to generate rhythmic activity in VP1, VP2, VP3 and VCMNs. Light inhibits type I_i interneurons and excites type I_e interneurons and both interneuronal types project to type III_i inhibitory interneurons (Crow and Tian 2003a). Intrinsic rhythmic bursting of type I_e and I_i interneurons could generate periods of excitation and inhibition in type III_i interneurons that would produce phasic excitation of contractile and ciliary motor neurons. Therefore, the neural network supporting ciliary locomotion and its regulation by light could be reconfigured by appropriate sensory input to generate the rhythmic pedal motor program underlying a different behavior, escape locomotion.
Conditioning produces modifications in multiple response systems

Pairings of light and rotation produce conditioning in two different behavioral response systems; ciliary locomotion and foot-shortening. The identification of the neural circuits responsible for ciliary locomotion and foot contraction is consistent with behavioral studies suggesting that CS-elicited foot-shortening and light-elicited inhibition of phototactic behavior may develop independently (Matzel et al., 1990). An alternative hypothesis is that light inhibition of phototaxis is a secondary consequence of CS-elicited foot-shortening. However, it is now clear that conditioning produces light-elicited inhibition of ciliary motor neurons (Crow and Tian 2003b).

In the leech a conditioning procedure consisting of a tactile CS and shock US modifies two different behaviors, stepping and shortening (Sahley and Ready 1988). Classical conditioning in vertebrates exhibits even more complexity of conditioning in multiple response systems (for example see Black and de Toledo, 1972). Moreover, both conditioned foot contraction and conditioned inhibition of phototaxis involve the development or emergence of a new response to the CS, not the potentiation, through US presentations of an already existing response to the CS referred to as reflex potentiation (for review see Sahley and Crow 1998). Consistent with these observations is the finding that light does not depolarize VCMNs or elicit foot-shortening prior to conditioning. In both conditioned responses there is a transfer of functional aspects of the response-evoking properties of the US to the CS (Crow and Alkon 1978; Lederhendler et al. 1986; Matzel et al. 1990). This aspect of conditioning probably accounts for the increased complexity and independence of the circuit supporting the CS and US, and the multiple sites of CS-US interactions within the neural network.
Specificity of hair cell projections to interneurons

Our results showing that caudal hair cells do not project to type Ib interneurons suggest that during conditioning, orientation in the direction of the rotational force vector would elicit foot contraction. Rotation or gravity causes the statoconia to press against motile cilia of hair cells in front of the centrifugal or gravitational force vector, resulting in a depolarizing generator potential and an increase in spike activity (Alkon 1975). Since *Hermissenda* exhibit a negative geotactic response, stimulation of rostral hair cells by a downward orientation would produce foot contraction and initiate turning to reorient in a vertical direction, opposite the gravitational force vector.

The synaptic organization of primary and secondary components of the visual pathway and statocyst pathway of *Hermissenda* has been characterized and described in considerable detail (Akaike and Alkon 1980; Alkon 1973a,b; Alkon and Fuortes 1972; Alkon and Bak 1973; Alkon et al. 1978; Crow and Tian 2000; 2002a; 2003a; Crow et al. 1979; Dennis 1967). The convergence sites providing for synaptic interactions between the CS and US pathways have been identified (Alkon 1973b; Akaike and Alkon 1980; Alkon et al. 1978; Crow and Tian 2000, 2002a, 2002b, 2003a). More recently most of the components of the network supporting ciliary locomotion have now been identified (Crow and Tian 2000; 2002a; 2003a). In addition, modulation of ciliary motor neurons by light before and after conditioning has been described and analyzed (Crow and Tian 2003b). The results presented here provide basic elements for the neural circuit supporting statocyst hair cell mediated foot contraction and statocyst mediated ciliary locomotion and in addition, potential loci for cellular and synaptic plasticity associated with the generation of CS-elicited foot-shortening following Pavlovian conditioning.
REFERENCES


FIGURE LEGENDS

Figure 1. Photograph of components of the circumesophageal nervous system including the location of a dye labeled pedal ventral contractile motor neuron (VCMN) and experimental conditions for measuring anterior foot contraction in semi-intact preparations. A, physiologically identified pedal VCMN injected with 5% 5(6)-carboxyfluorescein. The ventral surface of the hemisected circumesophageal nervous system is facing up in the photograph. Labels: cerebropleural ganglion (CPG), pedal ganglion (PG), and Eye (E). Scale bar in A is 125 µm. B, higher magnification of Fig. 1A showing that the pedal VCMN projects one axonal process located in pedal nerve P2 (black arrow). Scale bar in B is 62.5 µm. C, drawing of the ventral surface of *Hermissenda*. Partial split foot semi-intact preparation depicting one intact pedal nerve P2 connecting the circumesophageal nervous system with the foot and body wall. In the example shown in C the left pedal ganglion was rotated approximately 150° to provide access to neurons on the ventral surface. D, extrinsic current depolarization of the pedal VCMN shown in A elicited contraction of the ipsilateral anterior foot.

Figure 2. Electrophysiological recordings showing that VCMNs project axonal processes in pedal nerve P2. A, a single spike in a VCMN elicited by an extrinsic current pulse is followed by a brief latency extracellular spike recorded in nerve P2 (B). An antidromic spike recorded in the VCMN (C) followed extracellular stimulation of nerve P2. Simultaneous recordings of current elicited spikes from a pedal VCMN (D) and suction electrode recordings from pedal nerve P2 (E). Extracellular spikes recorded from P2 (arrows) followed pedal VCMN action potentials one-for-one.
Figure 3. Anterior foot contraction evoked by current stimulation of VCMNs. A1, intracellular recording from a pedal VCMN in ASW during depolarization with a 5-s 0.8-nA extrinsic current pulse. A2, depolarization of the same VCMN motor neuron in a high-divalent cation solution (3 x Ca$^{2+}$ and 3 x Mg$^{2+}$). B, group data (mean ± SEM) summarizing anterior foot contraction evoked during the 5-s depolarization in ASW and high-divalent cation ASW (N=5).

Figure 4. Pedal VCMN elicited anterior foot contraction is not dependent upon activation of central polysynaptic pathways. A1, an extrinsic current pulse (.5nA 2 sec) elicited spikes from a VCMN in normal ASW and contraction of the ipsilateral anterior foot. A2, a nerve shock (arrow) applied through a suction electrode containing pedal nerve P1 evoked a complex EPSP recorded from the same pedal VCMN as shown in A1. A3, exposure of only the circumesophageal nervous system to 5 mM Ni$^{2+}$ ASW blocked the complex EPSP normally evoked by the nerve shock (arrow). A4, an extrinsic current pulse (.5nA 2 sec) elicited spikes from the same VCMN exposed to 5 mM Ni$^{2+}$ ASW sufficient to elicit contraction of the anterior foot.

Figure 5. Stimulation of statocyst hair cells by mechanical taps evoke depolarization of pedal VCMNs and newly identified type I$_b$ interneurons. A-B, simultaneous intracellular recording from a type I$_b$ interneuron (A) and a VCMN motor neuron (B). A mechanical tap (arrow) evoked a spike in the type I$_b$ interneuron and a depolarization and spike in the VCMN (B). C, a stronger tap (arrow) evoked several spikes recorded from the type I$_b$ interneuron and multiple EPSPs recorded from the VCMN hyperpolarized to -80 mV to block spike generation (D).
Figure 6. A, depolarization of a single lateral statocyst hair cell evoked spikes recorded from a type \( I_b \) interneuron (B). C, depolarization of a rostral statocyst hair cell elicited a complex EPSP recorded in a type \( I_b \) interneuron hyperpolarized to -65 mV to block spike generation (D). E, mechanical stimulation of the statocyst evoked a depolarizing generator potential with superimposed spikes recorded from a rostral statocyst hair cell.

Figure 7. Hair cells in identified locations in the statocyst project to type \( I_b \) interneurons. Simultaneous intracellular recordings from type \( I_b \) interneurons and hair cells located at different positions on the ipsilateral statocyst. A1, extrinsic current stimulation (.5 nA 2 sec) of a rostral hair cell elicited EPSPs recorded in a type \( I_b \) interneuron (A2) hyperpolarized to -80 mV to block spike activity. B1, current stimulation (.5 nA 2 sec) of a lateral hair cell in a different preparation elicited EPSPs in a type \( I_b \) interneuron (B2) hyperpolarized to -72 mV to block spike activity. C1, extrinsic current stimulation of a caudal hair cell did not elicit detectable EPSPs in a type \( I_b \) interneuron (C2) hyperpolarized to -70 mV to block spike activity (see group data, Table 1).

Figure 8. Photographs of a physiologically identified contralateral type \( I_b \) interneuron labeled with Lucifer yellow. A1, Lucifer yellow labeling showed that type \( I_b \) interneurons project to the contralateral pedal ganglia. Eye (E), cerebropleural ganglia (CPG), pedal ganglia (PG). Dye labeled type \( I_b \) interneuron soma indicated by black arrowhead. Scale bar in A1 is 62.5 \( \mu \)m. A2, same preparation as in A1 showing different plane of focus revealing an axonal projection to the contralateral pedal ganglia (black arrowhead). Scale bar in A2 is 62.5 \( \mu \)m. B1, extrinsic current stimulation of the type \( I_b \) interneuron shown in A1-A2 evoked a complex EPSP recorded in the
VCMN hyperpolarized to -65 mV (C1). C2, extrinsic current stimulation of the same VCMN produced spikes and ipsilateral anterior foot contraction.

Figure 9. A, photograph of the dorsal surface of the circumesophageal nervous system showing two identified type I_b interneurons (ipsilateral and contralateral) labeled with 5% 5(6)-carboxyfluorescein. Each type I_b interneuron projects to the contralateral pedal ganglia (white arrowheads). White arrow indicates cerebropleural commissure connecting each CPG. Scale bar in A is 150 µm. B-C, simultaneous intracellular recordings from two type I_b interneurons. Extrinsic current stimulation of one type I_b interneuron produced a hyperpolarizing electrotonic potential (B) and a smaller potential in the contralateral type I_b (C) indicating electrical coupling between the two type I_b interneurons. The likely site of the lateral coupling is in the cerebropleural commissure indicated by the white arrow in A.

Figure 10. Depolarization of I_b interneurons with extrinsic current produces a depolarization of VCMNs that is sufficient to elicit foot contraction. A1, depolarization of a type I_b interneuron with a 2-s extrinsic current pulse produced a depolarization and spike train recorded from a VCMN (B1). A2, hyperpolarizing the VCMN to -65 mV during the period of stimulation of the type I_b interneuron revealed a complex EPSP recorded in the VCMN motor neuron (B2). C, depolarization of a type I_b interneuron with a 5-s 1.5 nA current pulse elicited a train of spikes recorded in the VCMN (D) that was sufficient to produce anterior foot contraction. E, summary group data (mean ± SEM) for type I_b interneuron elicited foot contraction (N=3).
Figure 11. Newly identified type \( I_b \) interneurons project to both pedal VCMNs and VP1 ciliary activating motor neurons. Simultaneous intracellular recordings from the same type \( I_b \) interneuron and a VP1 ciliary motor neuron or a pedal VCMN. A1, extrinsic current stimulation of a type \( I_b \) interneuron evoked spikes recorded from a physiologically identified VP1 ciliary motor neuron (B). A2, extrinsic current stimulation of the same type \( I_b \) interneuron evoked spikes in a physiologically identified VCMN (C).

Figure 12. Type \( I_b \) interneurons directly excite pedal VCMNs and VP1 ciliary activating motor neurons. Simultaneous recordings from type \( I_b \) interneurons and VP1 ciliary motor neurons or pedal VCMNs in a high-divalent cation solution (3 x Ca\(^{2+}\) and 3 x Mg\(^{2+}\)). A1, a single spike generated in a type \( I_b \) interneuron by a current pulse evoked a monosynaptic EPSP recorded from a VP1 motor neuron (B1). The latency between the type \( I_b \) spike and VP1 is short, relatively constant, and one-for-one as shown by three consecutive superimposed spike (A2) and evoked superimposed EPSPs (B2). C1, a single spike generated in a type \( I_b \) interneuron by a current pulse evoked a monosynaptic EPSP recorded from a VCMN (D1). EPSPs recorded from the VCMN followed spikes in the type \( I_b \) interneuron one-for-one with a brief and relatively constant latency as indicated by three consecutive superimposed spike (C2) and superimposed EPSPs recorded from the VCMN (D2).

Figure 13. Neural network depicting components of the *Hermissenda* visual and graviceptive pathways. Diagram of synaptic connections between identified photoreceptors and interneurons, statocyst hair cells and interneurons, and interneurons and contractile and ciliary motor neurons. Previously identified interneurons in the visual pathway designated as types \( I_c \), \( I_i \) and \( III_i \), and
newly identified interneurons designated as type I₀. VP1 cells are ventral ciliary motor neurons and VCMN and CMN are contractile motor neurons. Filled triangles, inhibitory synapses, and open triangles, excitatory synapses. Solid lines indicate established monosynaptic connections, dashed lines, polysynaptic connections with potential interneurons not yet identified.
<table>
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</table>
FIGURE 4

A1

VCMN

ASW

50 mV

1 sec

A2

VCMN

ASW

5 mV

250 ms

A3

VCMN

Ni^{2+} ASW

5 mV

250 ms

A4

VCMN

Ni^{2+} ASW

50 mV

1 sec
FIGURE 6

A

Hair Cell

50 mV

B

\( I_b \)

25 mV

1 sec

C

Hair Cell

25 mV

D

\( I_b \)

5 mV

2 sec

E

Hair Cell

20 mV

200 ms
FIGURE 12
HIGH DIVERGENT ASW

A1  A2
\[ I_b \] 25 mV  \[ I_b \] 25 mV

B1  B2
\[ V_{PI} \] 2 mV  \[ V_{PI} \] 2 mV
\[ 200 \text{ ms} \]

C1  C2
\[ I_b \] 20 mV  \[ I_b \] 20 mV

D1  D2
\[ V_{CMN} \] 1 mV  \[ V_{CMN} \] 1 mV
\[ 200 \text{ ms} \]