The Journal of Experimental Biology 213, 3409-3415

doi:10.1242/jeb.043521

The shadow-induced withdrawal response, dermal photoreceptors, and their input to the higher-order interneuron RPeD11 in the pond snail *Lymnaea stagnalis*

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Accepted 14 July 2010

SUMMARY

The shadow-induced withdrawal response in *Lymnaea stagnalis* is mediated by dermal photoreceptors located on the foot, mantle cavity, and skin around the pneumostome area. Here, we determined whether we could obtain a neural correlate of the withdrawal response elicited by a shadow in a higher-order central neuron that mediates withdrawal behavior. We measured the electrophysiological properties of the higher-order interneuron Right Pedal Dorsal 11 (RPeD11), which has a major role in *Lymnaea* withdrawal behavior. In semi-intact preparations comprising the circumesophageal ganglia, the mantle cavity and the pneumostome, but not the foot and eyes, a light-on stimulus elicited a small short-lasting hyperpolarization and a light-off stimulus elicited a depolarization of RPeD11. We also determined that dermal photoreceptors make a monosynaptic contact with RPeD11. The dermal photoreceptor afferents course to the circumesophageal ganglia *via* the anal and genital nerves to the visceral ganglion, and/or *via* the right internal and external parietal nerves to the parietal ganglion. Finally, in addition to responding to photic stimuli, RPeD11 responds to both mechanical and chemical stimuli delivered to the pneumostome.

Key words: withdrawal behavior, dermal photoreceptor, shadow response, higher-order interneuron, *Lymnaea stagnalis*.

INTRODUCTION

Lymnaea stagnalis is capable of both associative learning and the formation of long-term memory (LTM) (Benjamin et al., 2000; Lukowiak et al., 2008). *Lymnaea stagnalis* is also capable of visuo-vestibular conditioning (VVC) and taste-aversion conditioning, which both involve portions of the whole-animal withdrawal response that is mediated in part by the Right Pedal Dorsal 11 (RPeD11) neuron (Ferguson and Benjamin, 1991a; Ferguson and Benjamin, 1991b; Inoue et al., 1996b; Kawai et al., 2004; Sakakibara, 2006; Sakakibara, 2008; Sakakibara et al., 1998; Syed and Winlow, 1991). RPeD11 also appears to play a role in the stress-enhanced operant conditioning training procedure of aerial respiration that leads to LTM (Martens et al., 2007a; Martens et al., 2007b; Sunada et al., 2010). Although the VVC conditioning paradigm in *Lymnaea* is identical to that in the marine nudibranch *Hermissenda* (Alkon, 1987), the neuronal modifications that underlie conditioned behavior as a result of training appear to be quite different (Sakakibara, 2006; Sakakibara et al., 2005; Tsubata et al., 2003). Ocular photoreceptors are involved in VVC, whereas another type of photoreceptor, the dermal photoreceptor (Dijkgraft, 1935), which is the subject of this report, does not appear to have a role in VVC (Ono et al., 2002).

Dermal photoreceptors in *Lymnaea* play an important role in escape withdrawal behavior (Stoll, 1972). Dermal photoreceptors are necessary for both non-associative and associative learning of the siphon and gill withdrawal responses of *Aplysia* (Lukowiak and Jacklet, 1972; Lukowiak and Sahley, 1981). Because VVC and taste-aversion conditioning both involve withdrawal behavior, which is mediated in part by RPeD11, we evaluated the electrophysiological properties of RPeD11 and its role in associative learning and the subsequent formation of LTM. In the present study, we focused on the shadow-induced withdrawal response of RPeD11, which is mediated by the dermal photoreceptors, as well as the response of RPeD11 to mechanical and/or chemical stimulation of the pneumostome area in semi-intact preparations.

In this study, we show that the shadow-induced withdrawal response, which is mediated by dermal photoreceptors, alters the activity of the higher-order interneuron RPeD11. Moreover, the shadow-induced electrophysiological responses in RPeD11 are mediated in part by mono-synaptic chemical connections originating from the dermal photoreceptors. In addition, RPeD11 responds to mechanical and chemical stimuli applied to the periphery, as well to photic stimuli. [The phototransduction mechanisms of dermal photoreceptors in *Lymnaea* are reported elsewhere (Pankey et al., 2010)].

MATERIALS AND METHODS

Animals

Laboratory-reared freshwater adult pond snails, *L. stagnalis*, with shell lengths of 21.0–26.0 mm were maintained at 20°C in well-aerated water on a 12 h:12 h light:dark cycle (lights on at 08:00 h), and fed cabbage and goldfish/turtle pellets.

Semi-intact preparation

The circumesophageal ganglia attached to the pneumostome, mantle cavity and internal organ without eyes and buccal ganglia (semi-intact preparation shown in Fig. 1) were dissected from *Lymnaea* with micro-scissors and forceps in *Lymnaea* saline (51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 5.0 mmol l^{-1} $MgCl_2$, 1.5 mmol l^{-1} $CaCl_2$, and 5.0 mmol l^{-1} Hepes, pH 7.9–8.1). Naive semi-intact preparations are

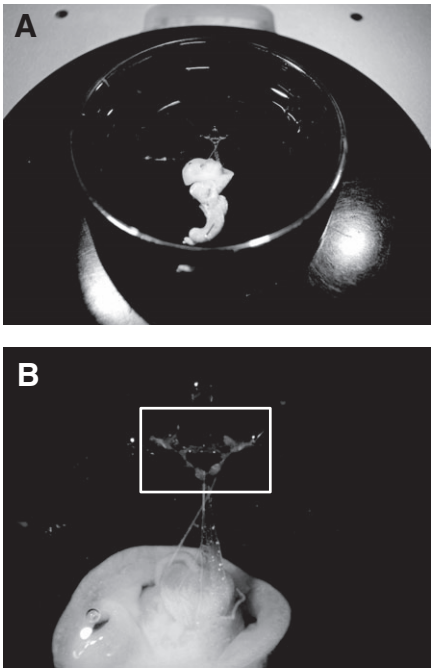

Fig. 1. (A) The semi-intact preparation with the esophageal ganglia and mantle cavity, the skin around the pneumostome and the internal organ in the experimental dish. (B) The neuronal connections of the esophageal ganglia and the other organs are intact and the border is separated using a Vaseline gap. (C) Schematic diagram of the central nervous system, including RPeD11 located in the right pedal ganglion, indicated by a white square in B.

both viable and capable of associative learning (McComb et al., 2005). All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise indicated. The preparation was immobilized on a Silgard-coated culture plate using stainless-steel pins.

Electrophysiology of RPeD11

Intracellular recordings were made from RPeD11 with a 3 mol l^{-1} KCl or, for axonal path finding, a Lucifer yellow CH (LY; 5% solution in 1 mol l^{-1} LiCl: L-453, Molecular Probes, Eugene, OR, USA)-filled glass microelectrode with resistance ranging from 30 to 50 mΩ (measured with a 3 mol l^{-1} KCl-filled electrode). The glass microelectrode, fabricated with a Sutter microelectrode puller (P-2000, Sutter, Novato, CA, USA), was connected by a silver chloride wire to a high-input impedance amplifier (Axoclamp 2B, Molecular Devices, Union City, CA, USA). Voltage responses were recorded on a storage oscilloscope (511A, Tektronix, Beaverton, OR, USA), analyzed using a micro-computer with a Digidata 1322A board (Molecular Devices) with analysis software (pCLAMP, Molecular Devices). The input resistance of RPeD11 was assessed by I-V characteristics obtained using the bridge circuit of an Axoclamp 2B with subtraction of the electrode resistance. RPeD11 is identified by its characteristic inhibitory synaptic input to RPeD1, which is a neuron that mediates aerial respiratory behavior (Inoue et al., 2001; Sangha et al., 2003; Scheibenstock et al., 2002; Syed et al., 1990; Syed et al., 1992). Thus, we simultaneously recorded from RPeD1 and RPeD11. Activation of RPeD11 invariably led to an inhibitory postsynaptic potential (IPSP) in RPeD1. In some cases, the recorded neuron was intracellularly injected with LY by iontophoresis.

To characterize the synaptic input onto RPeD11, either a Ca^{2+}-free saline (51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 6.5 mmol l^{-1} $MgCl_2$, and 5.0 mmol l^{-1} Hepes, pH 7.9–8.1) or a high-Ca^{2+} and high-Mg^{2+} saline (51.3 mmol l^{-1} NaCl, 1.7 mmol l^{-1} KCl, 24 mmol l^{-1} $CaCl_2$, 12 mmol l^{-1} $MgCl_2$, and 5.0 mmol l^{-1} Hepes, pH 7.9–8.1) was perfused at a rate of 6 ml min^{-1}. The high-Ca^{2+}/high-Mg^{2+} saline increased the action potential threshold, thereby blocking or substantially reducing the efficacy of the polysynaptic pathways (Cohen et al., 1978), whereas the Ca^{2+}-free saline inhibited chemical synaptic transmission.

To isolate the circumesophageal ganglia, including the right pedal ganglion where RPeD11 is located, from the pneumostome, mantle cavity and internal organs, a Vaseline gap was placed between the border of the central ring ganglia and the other tissues, keeping the neuronal connection intact.

Photic stimulation

The preparation was maintained in a light-adapted state because illumination was applied for at least 5 min before applying the shadow stimulus. The timing of a 500 ms light-off stimuli from a 50 W Halogen tungsten lamp (HL-10D, Hoya-Schott, Tokyo, Japan) was controlled using a solenoid mechanical shutter (EC-601, Copal, Tokyo, Japan) placed in the light path. The light stimulus illuminated the pneumostome area of the semi-intact preparation using a fiber-optic cable. The light intensity illuminating the preparation was ~700 μW cm^{-2} at 510 nm recorded by a photopower meter (TQ8210, Advantest, Tokyo, Japan).

Tactile stimulation

Tactile stimuli were applied mechanically as gentle small scratches using a stainless-steel pin.

Chemical stimulation

The chemical stimulus was applied by placing a drop of 0.1 mol l^{-1} KCl or 3% quinidine sulfate around the mantle cavity or pneumostome. The chemicals were quickly washed out using *Lymnaea* saline.

RESULTS

RPeD11 was identified according to the criteria of Inoue et al. (Inoue et al., 1996a), and by its morphologic location and inhibitory effect on RPeD1. The mean (±s.e.m.) resting membrane potential and the input resistance of RPeD11 were –69.4±2.8 mV (N=5) and 21.1±4.1 MΩ (N=6), respectively.

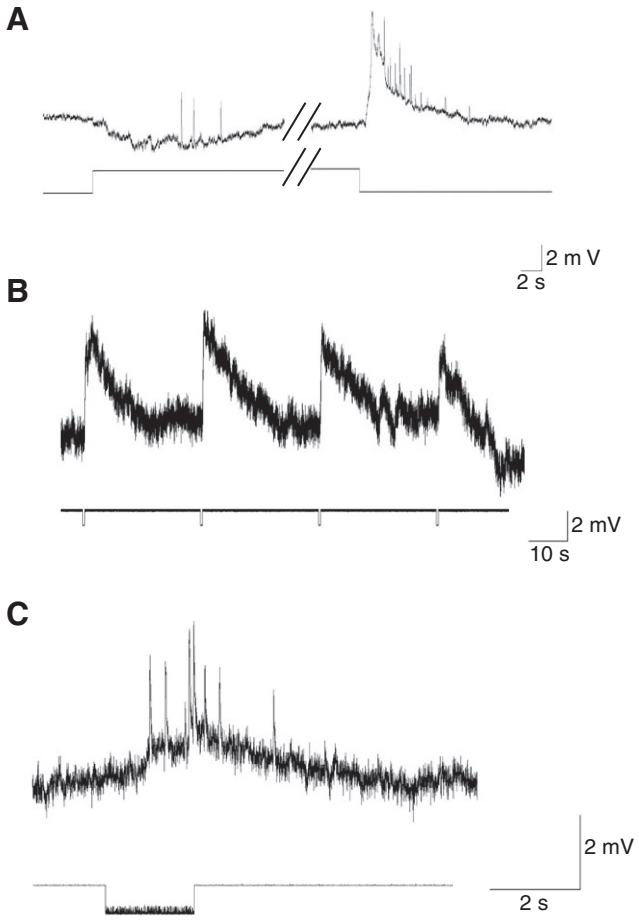

Fig. 2. (A) The light response recorded in RPeD11. The semi-intact preparation was fully in a dark-adapted state, then the light stimulus was applied for 30 s. Note that the light-on stimulus evoked a transient hyperpolarizing response of ~3 mV in amplitude; the membrane potential became depolarized and then recovered to the resting membrane potential level. Soon after the light stimulus was turned off, RPeD11 responded with a depolarizing response. (B) Four successive light-off responses are displayed; (C) the depolarizing response is shown with an expanded time scale. The timing of the light stimulus is shown below each response waveform.

Photic stimulation and the shadow or 'light-off' response

Following 20 min of dark adaptation (i.e. the pneumostome area was not specifically illuminated), RPeD11 responded to the onset of illumination with an approximately 3 mV hyperpolarization (i.e. 'light-on' response). At the cession of the photic stimulus, a much larger depolarization was produced in RPeD11 (i.e. 'light-off' or shadow response). Thus, the 'light-off' or shadow response was much larger than the 'light-on' response and in the opposite direction (i.e. depolarization with the 'light-off' *vs* hyperpolarization with the 'light-on'). That is, RPeD11 responds to the shadow stimulus with a depolarization (Fig. 2B,C). Because both the hyperpolarizing 'light-on' and the depolarizing 'light-off' shadow response in RPeD11 occurred in a semi-intact preparation that did not contain eyes, the source of the photoresponse in RPeD11 must have originated from extraocular photoreceptors (i.e. from dermal photoreceptors) located in and around the pneumostome area.

A typical RPeD11 response to a shadow passed over the pneumostome area is shown in Fig. 2B shows. A 500 ms shadow stimulus induced a 7.8±0.5 mV (N=5) depolarization in RPeD11 that lasted for a mean time of 17 s. This depolarizing response in RPeD11 to the shadow stimulus on the pneumostome area outlasted the shadow stimulus by a factor of 30-fold. Presentation of the shadow stimulus to the pneumostome area once every 30 s continued to evoke an observable depolarizing response in RPeD11 (Fig. 2B). The depolarizing response sometimes decreased in amplitude with successive presentations of the shadow stimulus. Such a decrease in the depolarizing response might represent a neural correlate of decrease in the full pneumostome closing response reported elsewhere in response to repeated exposure of the pneumostome area to a shadow stimulus (Orr et al., 2007). The light-off response shown in Fig. 2C had a long latency (~0.75 s) and initially was composed of only a small depolarization. However, following the initial small depolarization there appeared a series of sharp, larger amplitude depolarizations that were superimposed on the initial depolarization wave.

The fluctuation in the response waveform of RPeD11 to the successive shadow stimuli suggested to us that there could be an additional interposed interneuron between the dermal photoreceptors and RPeD11. To better characterize the synaptic input received by RPeD11 following the shadow stimulus, we examined whether the photoresponse was preserved in Ca^{2+}-free saline and/or in high-Ca^{2+}/high-Mg^{2+} saline (Fig. 3).

As can be seen in Fig. 3A the shadow stimulus elicited a large depolarizing input to RPeD11 when the ganglia were in normal saline. However, perfusion of the circumesophageal ganglia with Ca^{2+}-free saline blocked the depolarizing input normally evoked by the shadow stimuli (Fig. 3B). The 'light-off' response to 'light-off' stimuli was completely abolished in Ca^{2+}-free saline. Washing out the Ca^{2+}-free saline with normal *Lymnaea* saline reinstated the depolarizing response elicited by the shadow stimuli (Fig. 3C). These data show that the shadow response was mediated by a chemical synapse onto RPeD11. To indirectly determine whether there was an interposed interneuron between the dermal photoreceptors and RPeD11 in the circumesophageal ganglia, we perfused the circumesophageal ganglia with a high-Ca^{2+}/high-Mg^{2+} saline, which should raise the threshold of any interposed interneurons (Cohen et al., 1978). Perfusion with high-Ca^{2+}/high-Mg^{2+} saline maintained the depolarizing response in RPeD11 elicited by the shadow stimulus, albeit with a smaller amplitude (Fig. 3D). Perfusing with high-Ca^{2+}/high-Mg^{2+} saline tended to abolish/decrease the first component but still preserved the slower one. Washing out the high-Ca^{2+}/high-Mg^{2+} saline with normal *Lymnaea* saline restored the suppressed depolarizing inputs (Fig. 3E). Experiments with perfusion of Ca^{2+}-free and high-Ca^{2+}/high-Mg^{2+} saline were carried out three times and the results were the same in every instance; the light-off response was abolished in Ca^{2+}-free saline, but it was preserved with smaller amplitude in high-Ca^{2+}/high-Mg^{2+} saline, and the response was completely reversible with washing in normal saline. These data suggest that the shadow-induced response inRPeD11 is mediated at least in part by a monosynaptic chemical connection from the dermal photoreceptors. The consistent reduction in response amplitude in high-Ca^{2+}/high-Mg^{2+} saline suggests that polysynaptic connections might also contribute to the response.

Anatomical determination of the dermal photoreceptor input to RPeD11

To begin to determine the anatomical input pathway from the dermal photoreceptors located in the tissue peripheral to RPeD11, LY was first injected into the soma of RPeD11. The fluorescence image of an LY-injected RPeD11 is shown in Fig. 4A, and a Camera-Lucida image is shown in Fig. 4B. RPeD11 has an axon that courses throughout the circumesophageal ring ganglia and then exits from

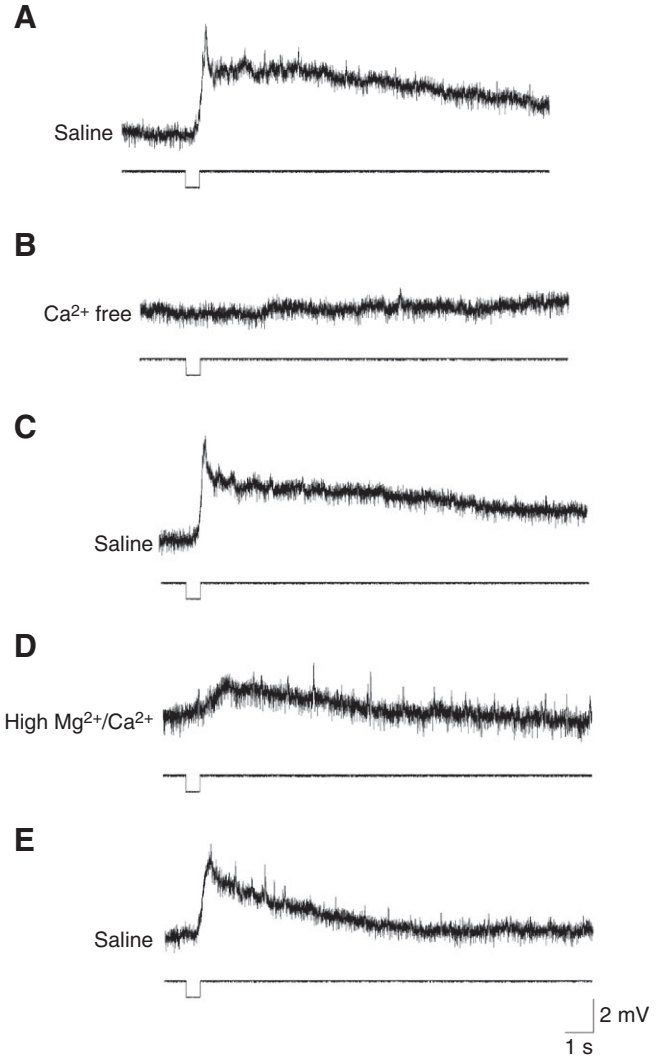

Fig. 3. Light-off responses recorded under different conditions. Panels A through E were successive recordings from the same preparation: (A) normal saline, control recordings; (B) light-induced responses were abolished in Ca^{2+}-free saline; (C) the light-induced depolarization recovered after washing with normal saline; (D) although the light-induced response was decreased in amplitude, RPeD11 still responded to the light-off stimulus in high-Ca^{2+}/high-Mg^{2+} saline; (E) this decrease recovered in normal saline. We performed the same experiment three times, the response to each perfusate was the same in every preparation. The timing of the light stimulus is displayed below each response waveform.

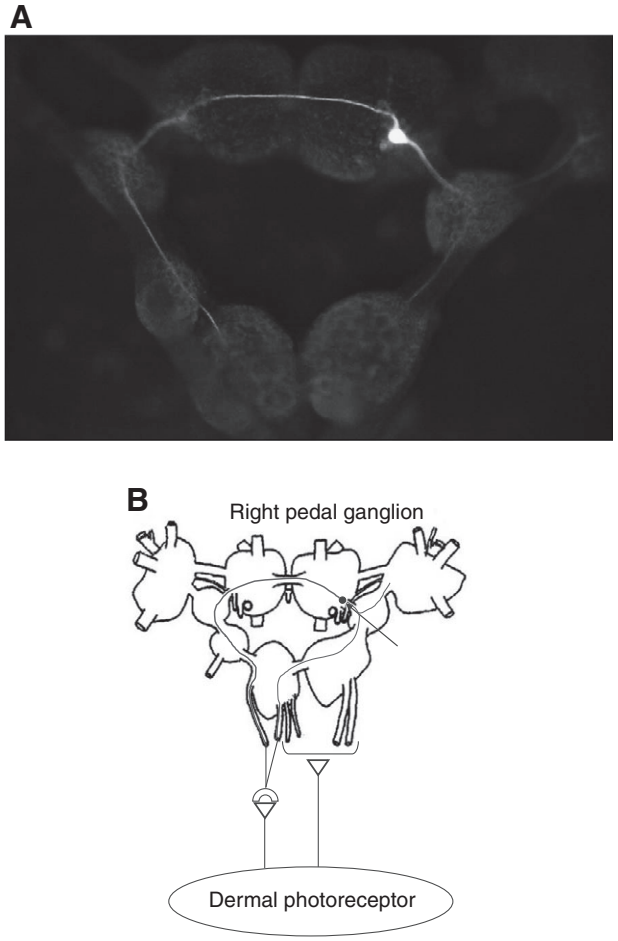

Fig. 4. (A) Fluorescence micrograph of RPeD11 filled with Lucifer yellow. (B) Camera-Lucida drawing obtained from micrograph in A. Axons travel through all of the ganglions and exit from the visceral and parietal ganglion; one axon branches to the cerebral ganglion.

the visceral ganglion. The visceral ganglion has four main nerves that innervate the periphery: (1) the cutaneous pallial nerve; (2) the intestinal nerve; (3) the anal nerve; and (4) the genital nerve (shown from left to right in Fig. 4B) (Slade et al., 1981). Based on these anatomical findings, we performed a series of experiments (selective cutting of the various input nerves) to determine the input pathway by which the dermal photoreceptors communicate with RPeD11.

The simultaneous cutting of the four nerves that exit the visceral ganglion to innervate the periphery (i.e. the cutaneous pallial nerve, the intestinal nerve, the anal nerve and the genital nerve) and the right internal and external parietal nerves originating from the parietal ganglion abolished the shadow-induced response in RPeD11 (Fig. 5A).

When only the cutaneous pallial and intestinal nerve fibers were severed, RPeD11 still responded to the shadow stimulus (Fig. 5B). These findings suggested that the input from dermal photoreceptors entered *via* the anal and the genital nerves that emanate from the visceral ganglion, and/or the right internal and external parietal nerve fibers that emanate from the right parietal ganglion. We then removed all of the peripheral tissue except for the mantle cavity and the pneumostome area. The shadow response in RPeD11 was preserved (Fig. 5C). Conversely, when we cut away all of the circumesophageal ganglia except for the right pedal ganglion, the shadow stimulus did not evoke a response in RPeD11 (Fig. 5D). These findings indicate that dermal photoreceptors are located around the mantle cavity and/or the pneumostome, but not on the pedal ganglion. We performed the selective severance experiment four times and we never observed the light-off response after all four nerves were severed.

Tactile and chemosensory inputs to RPeD11

In addition to photic stimulation of the pneumostome area, synaptic input to RPeD11 can be evoked by other stimulus modalities. For example, application of $0.1\,mol\,l^{-1}$ KCl induced excitatory synaptic responses in RPeD11, but applying a bitter tasting chemical (i.e.

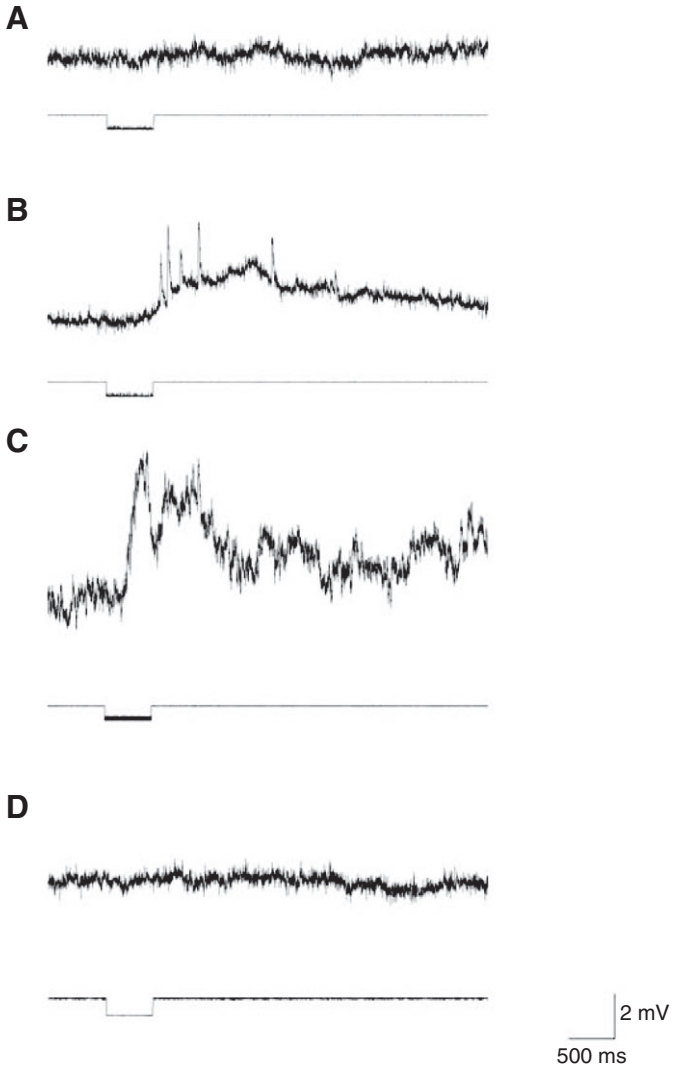

Fig. 5. The light-off response disappeared when the right two nerve fibers of the visceral ganglion and two fibers of the parietal ganglion were cut (A), whereas the response was preserved when the right-most two fibers of the visceral ganglion were cut (B). (C) When we removed the internal organs leaving the mantle cavity and the skin around the pneumostome intact, the light-off response was still observed. (D) Removal of other ganglia, except the right parietal ganglion, abolished the light-induced response. We performed the selective severance experiment four times, and the result was the same in every instance.

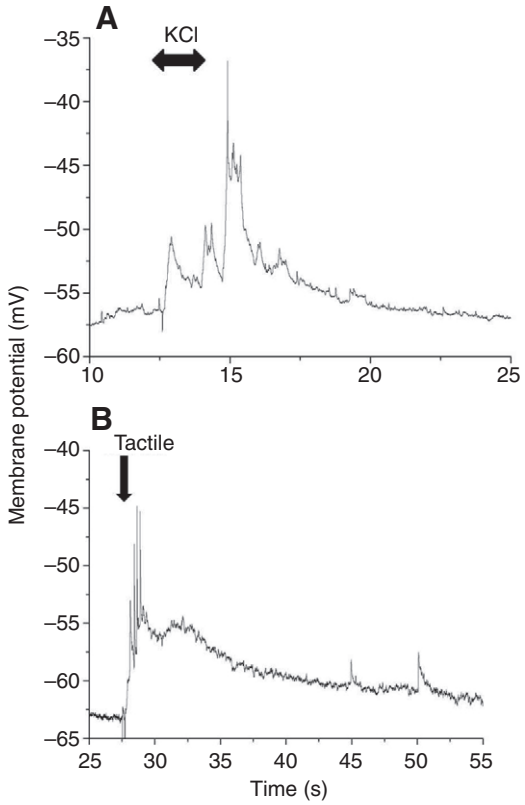

Fig. 6. Multi-modal response in RPeD11 is shown. The light-off stimulus elicited a depolarizing response (not shown), then 100 mmol l^{-1} KCl applied around the mantle cavity induced a 10 mV depolarization (A). A tactile stimulus was manually applied to the mantle cavity using a sharp insect pin. RPeD11 was depolarized by more than 10 mV with impulse generation (B). RPeD11 responded to a tactile stimulus and KCl application in all preparations (N=6).

3% quinidine sulfate; data not shown) did not elicit a response in RPeD11 (Fig. 6A). Tactile stimulation of the pneumostome area resulted in excitatory synaptic input (i.e. depolarization) to RPeD11 (Fig. 6B). The data shown in Fig. 6 were obtained from the same semi-intact preparation that is shown in Fig. 5. Every time we tried to observe the multi-modal response, RPeD11 responded the same way in six out of six preparations.

DISCUSSION

RPeD11, first identified by Syed and Winlow (Syed and Winlow, 1991), is a higher-order interneuron that is the dominant member of an electronically coupled network of neurons that mediate the escape withdrawal response in *Lymnaea* (Inoue et al., 1996a; Inoue et al., 1996b; Syed and Winlow, 1991). This neuron is located in the right pedal ganglion and its activation inhibits certain behaviors (e.g. feeding, aerial respiration, locomotion) that are incompatible with the whole-animal withdrawal response into its shell. The whole animal withdrawal response, however, is not at an all-or-none behavior, in that the response can be graded depending on the specific aversive stimulus and the intensity of the stimulus applied to the snail (H.S., T.H., K.L. and M.S., unpublished). Although a mildly aversive stimulus might activate RPeD11, it may only elicit a local withdrawal response. Thus, for example, a weak tactile stimulus applied to the pneumostome only elicits pneumostome closure, not the whole animal withdrawal response (Lukowiak et al., 1996).

In the present study, we recorded from RPeD11 in a semi-intact preparation comprising the central ring ganglia, the pneumostome and the mantle cavity, and determined the response to photic stimuli applied to the pneumostome and adjacent areas. *Lymnaea* respond to a shadow cast over the pneumostome by closing the pneumostome (Orr et al., 2007; Stoll, 1972). To our knowledge this is the first time a so-called dermal photo response has been shown to elicit synaptic activity in a higher-order interneuron in a molluscan preparation. In addition, these experiments allowed us to examine whether the pneumostome closure behavior elicited by the shadow stimulus was mediated in part by RPeD11. Furthermore, many of the behaviors that undergo associative learning and the subsequent formation of LTM in *Lymnaea* involve withdrawal behaviors (e.g. classical

conditioning of the vestibular-photic system and taste aversion, as well as operant conditioning of aerial respiratory behaviors) that are most likely to also involve RPeD11 activation. Our present study is the first step in determining the possible causal role of this neuron in both associative learning and memory (e.g. LTM) formation.

In *Lymnaea*, as well as in other mollusks, dermal photoreceptors are reported to mediate a shadow-induced withdrawal response that is likely to be part of a constellation of anti-predator responses (Lukowiak and Jacklet, 1972; Lukowiak and Sahley, 1981; Orr et al., 2007). In the present study, we found that the dermal photoreceptors also mediate a 'light-on' response. That is, we unexpectedly found that shinning light onto the pneumostome area resulted in a photic-induced hyperpolarization of RPeD11. Thus, the light-on stimulus did not elicit a withdrawal response (e.g. closure of the pneumostome) because RPeD11 was made less active. In other gastropod mollusks (e.g. *Aplysia* and *Hermissenda*), however, a 'light-on' stimulus elicits a siphon withdrawal response (Lukowiak and Jacklet, 1972) and a foot contraction (Alkon, 1974). In addition, the 'light-on' stimulus can serve as a conditional stimulus in a classical conditioning procedure that, when paired with a strong tactile stimulus to the gill, ultimately leads to the 'light-on' stimulus eliciting the gill withdrawal response (Lukowiak and Sahley, 1981) and, when paired with a vigorous rotation, ultimately leads to the 'light-on' stimulus eliciting the unconditional response (Alkon, 1983). Here, however, we concentrated primarily on the response of RPeD11 to a shadow stimulus. The shadow or 'light-off' stimulus induced a depolarization of ~8 mV in amplitude. Because this semi-intact preparation does not contain eyes, we concluded that this input to RPeD11 was mediated by dermal photoreceptors that make a chemical monosynaptic connection, and our lesion experiment confirmed that the photoreceptors must be located on the mantle or in the pneumostome area.

In the initial studies of RPeD11 (Syed and Winlow, 1991), attention was primarily focused on its synaptic connections within the central nervous system to identified interneurons and motor neurons. Following the findings of Ferguson and Benjamin (Ferguson and Benjamin, 1991b), it came to be accepted that RPeD11, as well as the interneurons to which it was electrically coupled (e.g. LPeD11), mediated the whole-animal withdrawal response. Thus, RPeD11 activation inhibited the circuits that mediate behaviors incompatible with whole-animal withdrawal and excited those neurons and circuits involved in the withdrawal of the snail into its shell (Inoue et al., 1996b). Although Ferguson and Benjamin (Ferguson and Benjamin, 1991b) examined the response of various motor neurons downstream from RPeD11 in relation to sensory stimulation (both photic and tactile), they did not report whether such stimuli affected RPeD11 activity. Inoue et al. (Inoue et al., 1996b) discovered a central mechanosensory neuron, RPD3, that is activated by tactile stimulus of the periphery, including the pneumostome area, which makes an excitatory synaptic connection with RPeD11. To our knowledge, the data reported here are the first to examine the effect of the shadow stimulus on RPeD11 activity. Because the shadow stimulus elicits portions of the whole animal withdrawal response, we hypothesized that the shadow stimulus would elicit RPeD11 activity. Our data are also in agreement with those of Stoll (Stoll, 1972), who demonstrated that the shadow response is mediated solely by dermal photoreceptors.

Extraocular photosensation in gastropods has been studied in *Aplysia* (Dijkgraft, 1935; Lukowiak and Jacklet, 1972; Lukowiak and Sahley, 1981; Lyons et al., 2006; Stoll, 1979), *Hermissenda* (Hodgson and Crow, 1991; Jerussi and Alkon, 1981) and *Lymnaea* (Chono et al., 2002; Ono et al., 2002; Stoll, 1972; Stoll, 1973). In *Aplysia*, there are two types of photosensitive elements present in the peripheral tissue; one is responsible for the 'light on' response and the other is responsible for the 'light off' response (Stoll, 1979). By contrast, in *Lymnaea* there does not appear to be a dermally mediated 'light-on' behavioral response (Stoll, 1973). There is, however, a 'light on' response in *Lymnaea* originating from the ocular photoreceptors (Sakakibara, 2006; Sakakibara et al., 2005) and a 'light off' behavioral response mediated by dermal photoreceptors (Chono et al., 2002; Orr et al., 2007). Thus, our finding that dermal photoreceptors signal the 'light-on' stimulus to RPeD11 is novel and suggests that it might be more difficult to elicit the whole-body withdrawal response when the snail is illuminated than when it is in the dark. We have not yet tested this hypothesis and to our knowledge, such experiments have not been performed by anyone else. We know that the threshold to elicit a number of withdrawal responses in *Lymnaea* is reduced when the snail detects a predator (Orr et al., 2007), indicating that these behaviors are modified by the snail's environment. It is also possible that dermal photoreceptors play a role in *Lymnaea* to entrain its circadian rhythm, as in other species (Berson et al., 2002; Fernald, 2004; Fernald, 2006; Lyons et al., 2006). This issue, as well as the role played by RPeD11 in mediating and/or modulating the animal's circadian rhythm, requires further study. It is possible that sensitivity to the stimuli needed to elicit the whole-animal withdrawal response depends on the circadian rhythm.

Our experiments also demonstrate that input from the dermal photoreceptors to RPeD11 is achieved *via* both a mono and a polysynaptic chemical synapse. The shadow stimulus-evoked input to RPeD11 was abolished in Ca^{2+}-free saline, whereas it was still apparent in a saline that raises the threshold of any interposed interneuron. Thus, we are reasonably confident that there is direct input from the dermal photoreceptors to RPeD11. We observed that the first component of the light-induced depolarization seemed to be abolished/diminished in high-Ca^{2+}/high-Mg^{2+} saline, but we could still record the slower second synaptic component. It is possible that the first component that is abolished by the high divalent cation saline, originates from the electrically connected LPeD11 through gap junctions. That is, the electronic properties of the RPeD11/LPeD11 circuit might be altered by the high divalent cation saline such that input arising from activation of LPeD11 by the shadow stimulus might not be observed. We know that LPeD11 responds to a shadow stimulus in much the same manner as RPeD11 (data not shown). It is quite unlikely that the initial component would come from a polysynaptic input because the interposition of an 'extra' synaptic connection should increase the response latency. It is worth mentioning that previously we have reported that in high-Ca^{2+}/high-Mg^{2+} saline there is a tendency for the latency of a monosynaptic connection to slow down (Sakakibara et al., 2005). Ocular photoreceptor cells have monosynaptic connections with statocyst hair cells, and we observed that the latency of the photoresponse in hair cells, which is normally 0.3 s with even the brightest light, was at least 50 ms longer in high-Ca^{2+}/high-Mg^{2+} saline (Sakakibara et al., 2005).

Moreover, there are other sensory neurons located in the periphery that signal other noxious stimuli (e.g. touch, aversive chemicals such as KCl). Each of these stimuli can elicit the whole-animal withdrawal response. We also performed selective cutting experiments that delineated the probable pathway by which the information from the periphery travels to RPeD11. Our surgical manipulation data rule out the possibility that central neurons are directly responding to the shadow stimuli. Thus, we conclude that the dermal photoreceptors directly convey information regarding the shadow

stimulus to RPeD11, which is important because it is thought that the shadow-induced withdrawal response is a *Lymnaea* anti-predator behavior (Orr et al., 2007). Furthermore, in contrast to *Hermissenda*, where pedal neurons P7, P8, P9 and P10, which are in part responsible for its defensive escape behaviors (i.e. clinging to a rock rather than withdrawing), receive input solely from ocular photoreceptors (Hodgson and Crow, 1991), RPeD11 in *Lymnaea* receives input from dermal photoreceptors and activates a withdrawal response.

Our initial experiments that are reported here will serve as the basis for a better understanding of the important role that RPeD11 plays in the causal neuronal basis of associative learning and the subsequent formation of LTM in the *Lymnaea* model system. We have preliminary data that the shadow stimulus can be used instead of a tactile stimulus to the pneumostome area to produce long-term memory following an operant conditioning procedure (K.L., unpublished).

LIST OF ABBREVIATIONS

IPSP	inhibitory postsynaptic potential
LTM	long term memory
LY	Lucifer yellow
RPeD11	Right Pedal Dorsal 11 neuron

ACKNOWLEDGEMENTS

This study was financially supported by a grant from Tokai University for the promotion of Scientific Research to M.S. and by CIHR to K.L.

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