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INHIBITION OF THE RESPIRATORY PATTERN-GENERATING NEURONS BY AN IDENTIFIED WHOLE-BODY WITHDRAWAL INTERNEURON OF *LYMNAEA STAGNALIS*

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Summary

Respiration and the whole-body withdrawal are two incompatible behaviors in the freshwater snail *Lymnaea stagnalis*. Whole-body withdrawal behavior is believed to be higher on the behavioral hierarchy than respiratory behavior. A central pattern generator (CPG) underlies respiratory behavior; whole-body withdrawal is mediated by a network of electrically coupled neurons. In this study, we provide evidence that the behavioral hierarchy between the whole-body withdrawal and the respiratory behaviors is established at the interneuronal level. We demonstrate that an identified whole-body withdrawal interneuron inhibits both muscular and neuronal components of the respiratory behavior in *Lymnaea stagnalis*.

A pair of identified, electrically coupled interneurons, termed left and right pedal dorsal 11 (L/RPeD11), coordinates the whole-body withdrawal behavior in *Lymnaea stagnalis*. In the present study, RPeD11 inhibited spontaneously occurring respiratory CPG activity in isolated brain preparations. In addition, electrical stimulation of RPeD11 in a semi-intact preparation also inhibited respiratory CPG interneuron RPeD1.

The synaptic connections between RPeD11 and the respiratory CPG neurons RPeD1 and visceral dorsal 4 (VD4) persisted in the presence of high-Ca^{2+}/high-Mg^{2+} saline, suggesting the possibility that they may be monosynaptic.

In a semi-intact preparation (lung–mantle, pneumostome and central nervous system), electrical stimulation of RPeD11 induced pneumostome and columellar muscle contractions while inhibiting the activity of RPeD1. Moreover, mechanical stimulation of the respiratory orifice, the pneumostome, excited RPeD11, while its effects on the respiratory CPG neuron (RPeD1) were inhibitory.

To determine the monosynaptic nature of connections between RPeD11 and the respiratory CPG neurons in the intact nervous system, we constructed these synapses in culture. RPeD11 and individual respiratory interneurons were isolated from their respective ganglia and co-cultured under conditions that support neurite outgrowth. Following neuritic overlap, RPeD11 was found to establish inhibitory synapses with the respiratory interneurons, supporting the hypothesis that these synaptic connections are likely to be monosynaptic in the intact central nervous system.

Key words: central pattern generator, rhythmic behavior, withdrawal behavior, *in vitro*, synapse formation, mollusc, identified neuron, *Lymnaea stagnalis*.

Introduction

Rhythmic behaviors such as locomotion, respiration and feeding are generally thought to be mediated by discrete groups of synaptically interconnected neurons termed central pattern generators (CPGs) (Delcomyn, 1980; Kristan, 1980; Selverston, 1980; Pearson, 1985, 1993; Getting, 1988, 1989; Calabrese *et al.* 1989; Harris-Warrick and Johnson, 1989; Jacklet, 1989). It therefore seems logical to anticipate that the neuronal basis of behavioral choices may also hinge upon interactions between various CPG neurons. For instance, the neural network underlying a higher-priority behavior, such as an escape response, may also play an active role in terminating other ongoing motor programs.

The neuronal networks mediating higher-priority behaviors have been shown to suppress incompatible motor functions either at the behavioral or at the motor neuronal level (Davis *et al.* 1974*a*,*b*; Kyriakides and McCrohan, 1988; Teyke *et al.* 1990; Arshavsky *et al.* 1994*a*,*b*; Norekian and Satterlie, 1996). Whether the rhythm-generating neurons (CPG neurons) of the

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incompatible behaviors are also directly affected by the neurons of higher-priority behaviors has not yet been determined. Since communications between higher-order CPG neurons mediating different behavioral repertoires are necessary for behaviorally meaningful movements to occur, a detailed knowledge of their interactive capabilities is, therefore, critical for understanding the cellular basis of seemingly distinct motor functions.

The freshwater mollusc *Lymnaea stagnalis* is a useful model system for studying the cellular and molecular mechanisms underlying regeneration and synapse formation (Bulloch and Syed, 1992), the cellular basis of feeding (Benjamin, 1983), locomotion (Haydon and Winlow, 1986; Winlow and Haydon, 1986; Syed *et al.* 1988), respiration (Janse *et al.* 1985; Syed and Winlow, 1991*b*; Syed *et al.* 1991; Moroz and Winlow, 1992), whole-body withdrawal (Sakhorov and S.-Rózsa, 1989; Ferguson and Benjamin, 1991*a*,*b*; Syed and Winlow, 1991*a*; Winlow *et al.* 1992), egg-laying behavior (Ter Maat *et al.* 1989, 1992) and peptidergic signaling mechanisms (Boer *et al.* 1987). Of particular interest to us are two antagonistic motor functions: respiration and whole-body withdrawal behavior. In *Lymnaea stagnalis*, the behavioral and neuronal correlates of these behaviors are well characterized, although the exact cellular mechanisms that coordinate the interactions between these incompatible motor programs are not well understood.

Upon encountering a noxious stimulus, *L. stagnalis* terminates ongoing behaviors, such as feeding, respiration and locomotion, and withdraws its head–foot complex into the shell, an action pattern often referred to as whole-body withdrawal (Ferguson, 1984; Ferguson and Benjamin, 1991*a*,*b*; Syed and Winlow, 1991*a*). This is the only escape response available to the animal and, as it is a factor determining survival, it takes priority over other behaviors such as locomotion and respiration (Winlow *et al.* 1992). A network of identified, electrically coupled neurons, the LPeD11 and RPeD11 neurons, mediates whole-body withdrawal behavior in *L. stagnalis* (Syed and Winlow, 1991*a*).

To accomplish respiratory movements, i.e. opening and closing of its respiratory orifice, the pneumostome, *L. stagnalis* must extend its head–foot complex some distance out of the shell, a behavior that is incompatible with the whole-body withdrawal described above. Both behavioral and neuronal correlates of the respiratory behavior in *L. stagnalis* are well characterized (Syed *et al.* 1990, 1991; Syed and Winlow, 1991*b*). Various environmental stimuli, such as a shadow reflex, a light on–off response or a mechanical touch to the head–foot complex during spontaneously occurring respiratory movements, induce pneumostome closure and an immediate termination of the respiratory behavior (Syed, 1988; Lukowiak *et al.* 1994).

In the present study, we sought to determine the neuronal loci at which the behavioral selection between the withdrawal and respiration programs takes place. We provide direct evidence that an identified, higher-order, whole-body withdrawal interneuron, RPeD11, terminates the respiratory motor programs at the rhythm generator (CPG) level.

Materials and methods

Animals

Laboratory-reared stocks of the freshwater snail *Lymnaea stagnalis* (L.), with an approximate shell length of 25–30 mm, were maintained at 18 °C in well-aerated artificial pond water and fed on lettuce. In order to enhance fictive aerial respiratory activity in the isolated central nervous system (CNS) preparations, animals were kept overnight in non-aerated pond water.

Isolated CNS and semi-intact preparations

Prior to dissection, the animals were anesthetized in a 10 % solution of Listerine (ethanol 21.9 %, menthol 0.042 %) in normal saline. Animals failed to respond to a gentle touch to their head–foot complex within 10 min of Listerine treatment and were, therefore, considered to be anesthetized. These animals recovered completely within 20 min when placed back in normal pond water. The anesthetized animals were deshelled and the central ring ganglia were removed as described elsewhere (Syed and Winlow, 1991*a*,*b*). The isolated CNS was pinned to the bottom of the dissection dish and was maintained in a normal Hepes ($50\,mmol\,l^{-1}$) -buffered *Lymnaea* saline ($51.3\,mmol\,l^{-1}$ NaCl, $1.7\,mmol\,l^{-1}$ KCl, $4.1\,mmol\,l^{-1}$ $CaCl_2$, $1.5\,mmol\,l^{-1}$ $MgCl_2$, see Ridgway *et al.* 1991). The outer connective tissue sheath was removed using fine forceps, whereas the inner connective sheath was softened by applying a crystal of protease (Sigma type XIV) directly to the central ganglia for 15 s, followed by immediate wash-out with cold (4 °C) normal saline. Semi-intact animals were prepared according to previously published methods (Syed *et al.* 1991). Briefly, the pneumostome, lung and mantle cavity with intact innervation from the CNS were isolated and pinned to the bottom of the dish as described above.

Electrophysiology

Conventional intracellular recording techniques were used (Syed and Winlow, 1991*a*,*b*). Specifically, glass microelectrodes (1.5 mm internal diameter, WPI) were pulled on a vertical electrode puller (Kopf, 700C) and filled with a saturated solution of K_2SO_4. The tip resistance of these electrodes typically ranged from 25 to 35 MΩ. The intracellular signals were amplified *via* preamplifiers (Getting, model 5; Dagan, 8700), displayed on a storage oscilloscope (Tektronix, R5103N) and recorded on a Gould four-channel chart recorder (Brush 2400). To prepare high-Ca^{2+}/high-Mg^{2+} salines, the concentrations of these divalent cations in the normal saline were raised ([Ca^{2+}] to $24\,mmol\,l^{-1}$ and [Mg^{2+}] to $12\,mmol\,l^{-1}$).

In vitro *cell culture*

Established cell culture techniques for *L. stagnalis* neurons were used as described previously (Syed *et al.* 1990; Ridgway *et al.* 1991). Briefly, the animals were dissected under sterile

conditions and, following a series of antibiotic washes and subsequent enzymatic treatments, the central ring ganglia were pinned to the bottom of the dissection dish. The connective tissue sheath was carefully removed with fine forceps and individual somata were removed using a fire-polished glass pipette coated with Sigma-Cote (Sigma). Neurons were isolated by applying gentle pressure through a Gilmont syringe and were plated onto culture dishes (Falcon, 3001) coated with poly-L-lysine containing brain-conditioned media (see Ridgway *et al.* 1991, for details). Following 24 h of sprouting and subsequent physical overlap between the neurites, both the pre- and postsynaptic neurons were viewed under an inverted microscope (Zeiss Axiovert 135, with Nomarski attachments). The sprouted neurons were impaled using the electrophysiological techniques described above and photographed with a 35 mm camera mounted on the inverted microscope.

Results

In the first series of experiments, we demonstrate that the whole-body withdrawal interneuron RPeD11 terminates the spontaneously occurring respiratory rhythm in the isolated CNS preparation. Evidence is also presented to suggest that this inhibition may be direct as it persists in high-Ca^{2+}/high-Mg^{2+} saline and also in the *in vitro* cell culture. A diagrammatic representation of the central ring ganglia of *L. stagnalis* depicting the locations of the neurons used in this study is presented in Fig. 1A. The identified interneuron RPeD11, involved in whole-body withdrawal, is located on the dorsal surface of the right pedal ganglion. A homolog of RPeD11 is located in the left pedal ganglion and is termed LPeD11 (Syed and Winlow, 1991*a*). Detailed morphological profiles of these cells have been published previously (Syed and Winlow, 1991*a*). These two neurons are electrically coupled to each other and also to a large number of body wall (cerebral A, CeA) and foot musculature (pedal G cluster, PeG) motor neurons (Fig. 1B). Together, these cells constitute the neural network that mediates whole-body withdrawal behavior in *L. stagnalis* (Syed and Winlow, 1991*a*).

Isolated CNS preparations exhibit spontaneous respiratory activity

Fig. 1C shows a summary diagram depicting the synaptic organization of the respiratory CPG neurons (modified from Syed and Winlow, 1991*b*). Brain preparations, isolated from animals that had been maintained overnight under anoxic conditions, exhibited periodic, spontaneous respiratory episodes (Fig. 2A). In this preparation, simultaneous intracellular recordings were made from right pedal dorsal 1 (RPeD1) and visceral dorsal 4 (VD4). As the input 3 interneuron (IP3I) is situated on the opposite side to the VD4

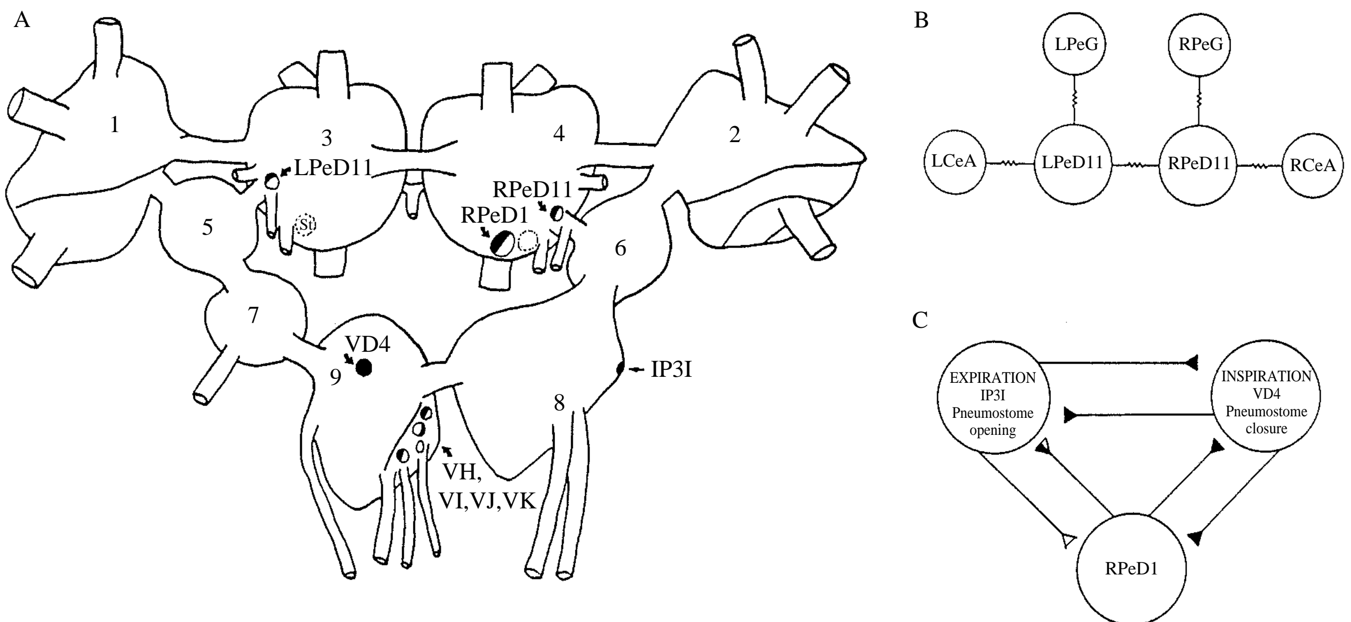


Fig. 1. Schematic diagrams of the central ring ganglia and neuronal networks used in the present study. (A) Diagrammatic representation of the central ring ganglia of *Lymnaea stagnalis* showing the locations of the neurons used in the present study. 1, 2, left and right cerebral ganglia; 3, 4, left and right pedal ganglia; 5, 6, left and right pleural ganglia; 7, 8, left and right parietal ganglia; 9, visceral ganglion; St, statocyst; RPeD11, right pedal dorsal 11; LPeD11, left pedal dorsal 11; RPeD1, right pedal dorsal 1; VD4, visceral dorsal 4; VH, VI, VJ and VK, visceral H, I, J and K cells; IP3I, input 3 interneuron. Shaded areas represent the degree of coloration (darker shades express white pigmentation). (B) Summary diagram of the electrically connected whole-animal withdrawal circuit of *L. stagnalis.* L/RCeA, left and right cerebral A cluster neurons; L/RPeG, left and right pedal G cluster neurons; L/RPeD11, left and right pedal dorsal 11. Modified from Syed and Winlow (1991*a*). (C) A summary of the synaptic connections between *L. stagnalis* respiratory central pattern generator (CPG) neurons. Respiratory interneurons (circles) are interconnected with each other *via* chemical synapses (open and filled symbols represent excitatory and inhibitory synapses, respectively).

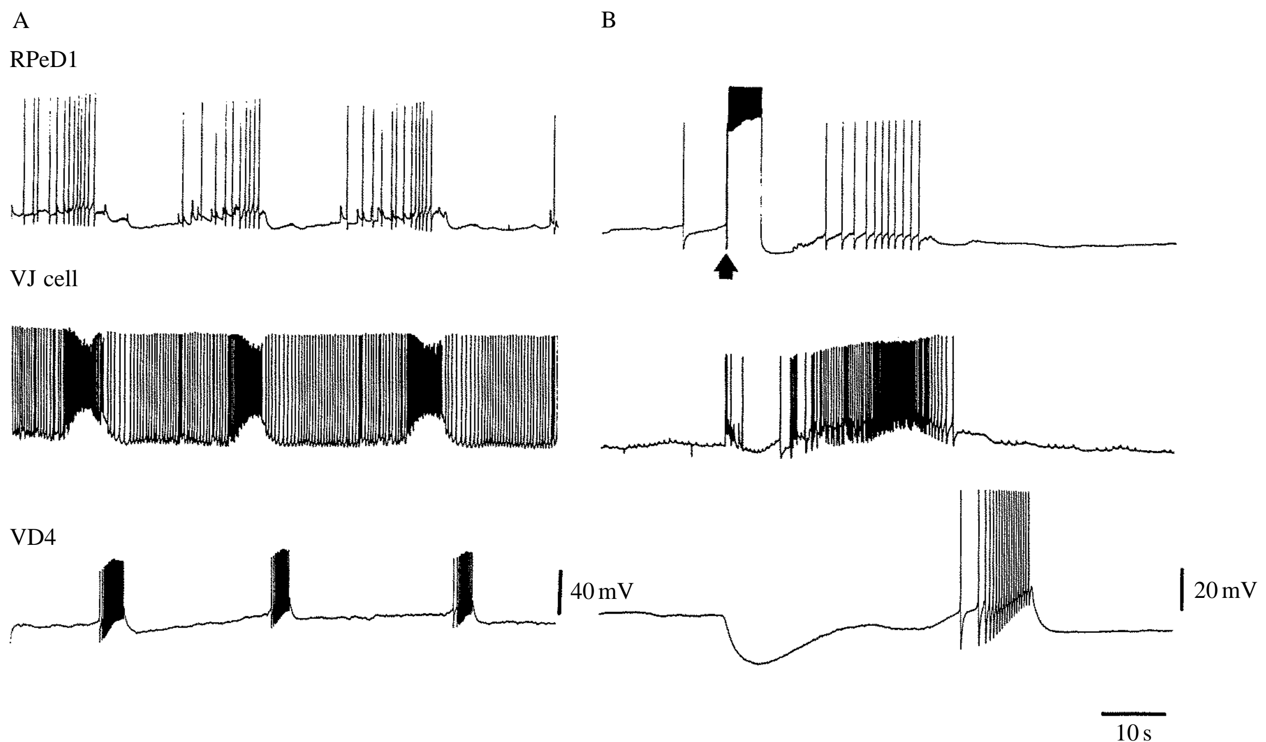


Fig. 2. Respiration-associated rhythmic activity recorded from the isolated central nervous system (CNS) of *Lymnaea stagnalis.* (A) Spontaneously occurring respiratory rhythm recorded simultaneously from interneurons (RPeD1 and VD4) and a motor neuron (VJ cell). Indirect evidence for interneuron IP3 (IP3I) activity was obtained from RPeD1, VJ and VD4 recordings. IP3I excites RPeD1 and VJ, while inhibiting VD4 (producing expiration). Following recovery from its inhibition by IP3I, VD4 fired a burst of spikes (producing inspiration), thereby completing one episode of the respiratory cycle. (B) In a previously quiescent preparation (no respiratory activity), strong electrical stimulation of RPeD1 (at arrow) initiated a single respiratory episode. RPeD1 is believed to excite IP3I by a dual inhibitory and excitatory synapse, whereas its effects on VD4 were inhibitory. Once activated, IP3I excited RPeD1 and VJ while inhibiting VD4. VD4 fired a burst of spikes following its recovery. Vertical scale bars in B are the same as in A, with the exception of VD4.

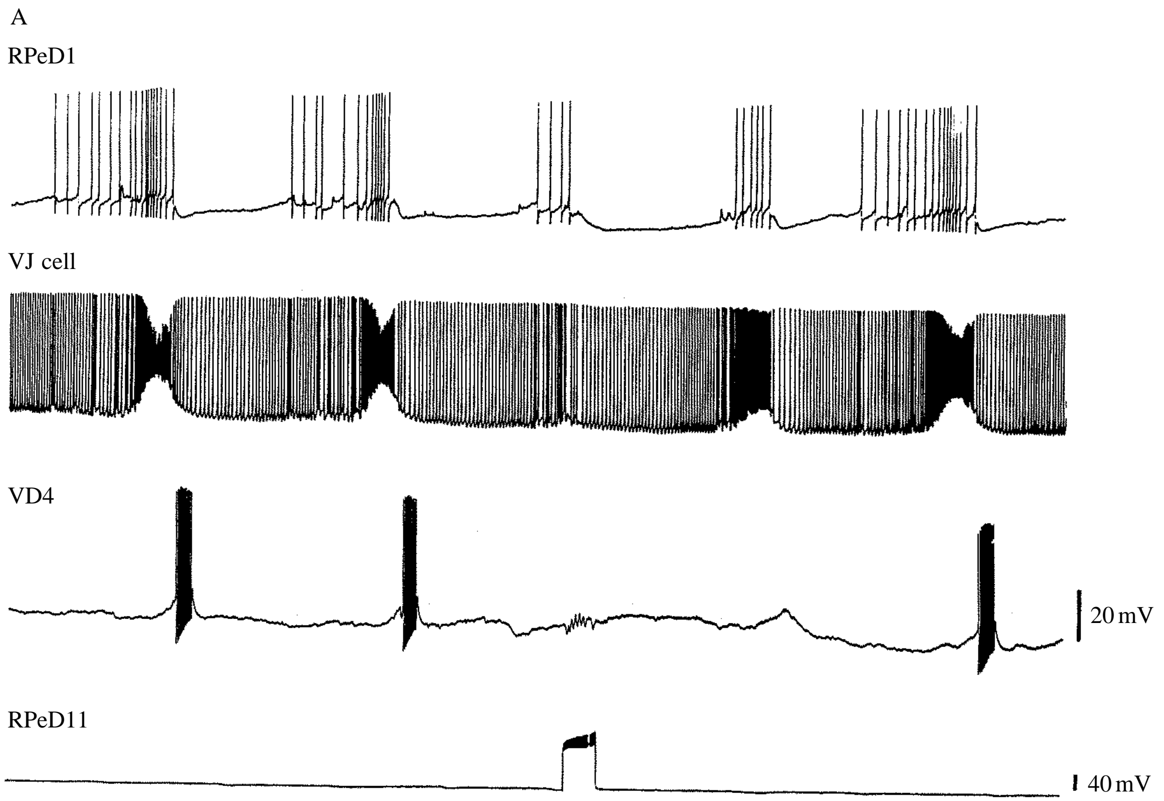
and RPeD1 neurons (Syed *et al.* 1990), indirect evidence for IP3I activity was obtained by recording from a pneumostome opener muscle motor neuron (visceral J, VJ). IP3I activity, which underlies expiration (pneumostome opening), was recorded as a series of characteristic bursts in a VJ cell and RPeD1 (Benjamin and Winlow, 1981). Bursting activity in VD4 represents inspiration (pneumostome closing) (Syed and Winlow, 1991*b*). In previously quiescent preparations, RPeD1 stimulation induced respiratory activity in the CPG network (Syed *et al.* 1990). One such respiratory cycle is shown in Fig. 2B. RPeD1 inhibits VD4 and initiates IP3I activity by a dual inhibitory–excitatory synapse (Syed *et al.* 1990). IP3I in turn excited a VJ cell as well as RPeD1 itself. A combined inhibitory effect of both RPeD1 and IP3I activity caused excitation of VD4 (Fig. 2B; see also Fig. 1C and Syed and Winlow, 1991*b*) *via* mechanisms other than post-inhibitory rebound excitation (Syed *et al.* 1992).

In semi-intact preparations, we have previously demonstrated that a gentle mechanical touch to the pneumostome area terminated respiratory activity (Syed and Winlow, 1991*a*). In the present study, we considered the possibility that this inhibition of the respiratory CPG neurons was mediated by RPeD11.

RPeD11 inhibits spontaneously occurring rhythmic respiratory activity

To determine the effects of RPeD11 on spontaneously occurring respiratory CPG activity, simultaneous intracellular recordings were made from RPeD11, the respiratory CPG neurons RPeD1 and VD4 and a motor neuron VJ cell. Indirect evidence for the activation of IP3I was again obtained from characteristic excitatory discharges in a VJ cell and RPeD1 (Benjamin and Winlow, 1981). The isolated CNS preparations

Fig. 3. Inhibition of the intracellularly recorded respiratory rhythm by RPeD11. (A) Spontaneously occurring respiratory rhythm as recorded from RPeD1, VJ and VD4. Electrical stimulation of RPeD11 (bottom trace) interrupted the respiratory cycle in the middle of the expiratory phase (IP3I failed to induce its effects on the target RPeD1, VD4 and VJ cells). As a consequence, VD4 failed to fire and the respiratory rhythm was briefly terminated. Note that the subsequent episode of IP3I activity also failed to activate VD4. Normal respiratory activity, however, resumed in the next cycle. (B) Termination of the respiratory rhythmic activity by RPeD11. Repeated stimulation of RPeD11 in an isolated CNS preparation terminated the respiratory rhythmic activity as recorded from the interneurons RPeD1 and VD4 and a motor neuron VJ cell. RPeD11 was stimulated twice with an interval of 15–20 s.



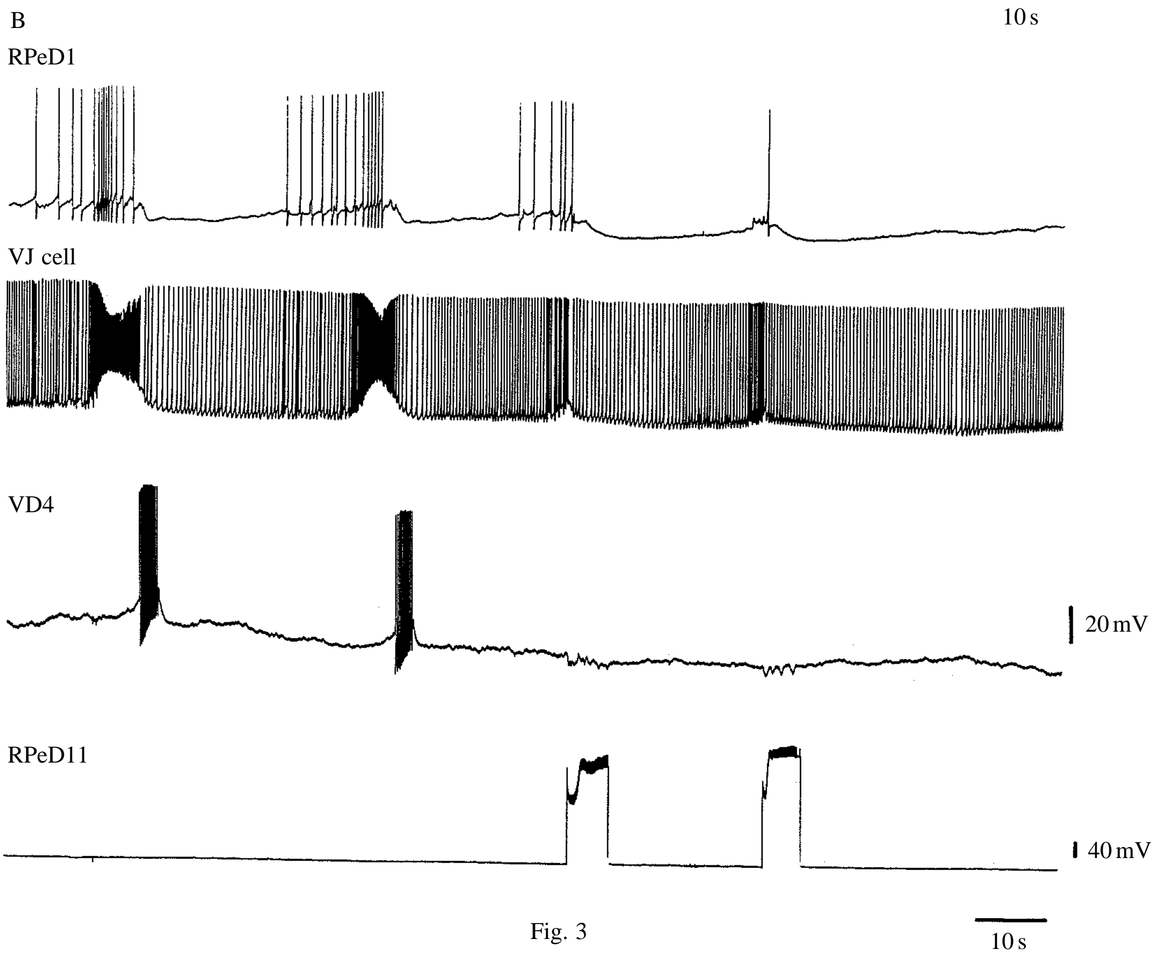


Fig. 3

generated regular, rhythmical respiratory discharges (Fig. 3A), similar to those seen in semi-intact preparations (Syed *et al.* 1991). Brief electrical stimulation of RPeD11 (at a frequency of 3–5 Hz for a duration of 5–10 s) during spontaneously occurring respiratory activity terminated the respiratory rhythm in all of the CPG neurons recorded from various different preparations (N=20) (Fig. 3A). Furthermore, the next episode of respiratory activity in both RPeD1 and the VJ cell was weaker (i.e. fewer spikes in RPeD1, shorter duration of the effect of IP3I bursts on the VJ cell) and uncoordinated, and it failed to activate VD4. Normal rhythmic respiratory activity resumed in the second episode following RPeD11 stimulation (Fig. 3A). A similar but repeated stimulation of RPeD11 (two stimuli at a frequency of 3–5 Hz, with an interval of 15–20 s) during spontaneously occurring respiratory activity terminated the respiratory rhythm for 30–60 min (N=10) (Fig. 3B). These data demonstrate that the electrical stimulation of RPeD11 in an isolated brain preparation is sufficient to inhibit the spontaneously occurring respiratory rhythm.

Synaptic connections between RPeD11 and the respiratory CPG neurons are likely to be monosynaptic

To determine whether RPeD11 inhibited respiratory CPG neurons directly or *via* polysynaptic pathways, simultaneous intracellular recordings were made from RPeD11, RPeD1 and VD4 in isolated brain preparations. In the presence of normal saline, activity in RPeD11 inhibited the activities of both RPeD1 and VD4 (Fig. 4A). Unfortunately, in neither of these preparations was it possible to resolve 1:1 inhibitory postsynaptic potentials (IPSPs) between RPeD11 and RPeD1/VD4. These inhibitory connections, however, persisted in the presence of high-Ca^{2+}/high-Mg^{2+} saline (N=9) (Fig. 4B), suggesting the possibility that they may be monosynaptic in nature. Owing to anatomical constraints (dorsal *versus* ventral), similar data were not obtained for IP3I.

Effects of RPeD11 activity on the respiratory muscles and neuron RPeD1 in a semi-intact preparation

To determine the effects of RPeD11 stimulation on the pneumostome closer muscle (PM), on the columellar muscle (CM) and on the activity of RPeD1, a semi-intact preparation (lung–mantle, pneumostome and CNS, Syed *et al.* 1991) was used. RPeD11 stimulation in these semi-intact preparations induced contractions of both PM and CM, while inhibiting the spontaneous activity in RPeD1 (N=10) (Fig. 5A). These data suggest that activity in RPeD11 is sufficient to inhibit respiratory behavior both at the CPG and at the affector organ level. A noxious stimulus delivered to the pneumostome area (touching with a pair of blunt forceps), however, activated RPeD11 for several seconds, generating either single spikes or bursts of action potentials (Fig. 5B). This excitation of RPeD11, in turn, was correlated with a prolonged inhibition of RPeD1. Taken together, these data demonstrate that, regardless of its stimulation parameters (direct or indirect, short *versus* prolonged bursts of spikes, etc.), RPeD11 activity is sufficient to inhibit the respiratory CPG neuron in the semi-intact animals.

Inhibition of the respiratory CPG neurons is mediated by RPeD11 and not via *LPeD11*

Since RPeD11 is electrically coupled to its contralateral homolog LPeD11 (Syed and Winlow, 1991*a*), we tested the possibility that inhibition of the respiratory neurons by RPeD11 may have been mediated indirectly *via* LPeD11. Since saline containing high levels of divalent cations does not perturb electrical connections (Syed and Winlow, 1991*a*), we used intracellular current injection to manipulate the membrane potentials of either RPeD11 or LPeD11 selectively. Simultaneous intracellular activity was recorded from RPeD11, LPeD11 and RPeD1 in an isolated brain preparation. The presence of electrical coupling between RPeD11 and LPeD11 was demonstrated with the passage of both

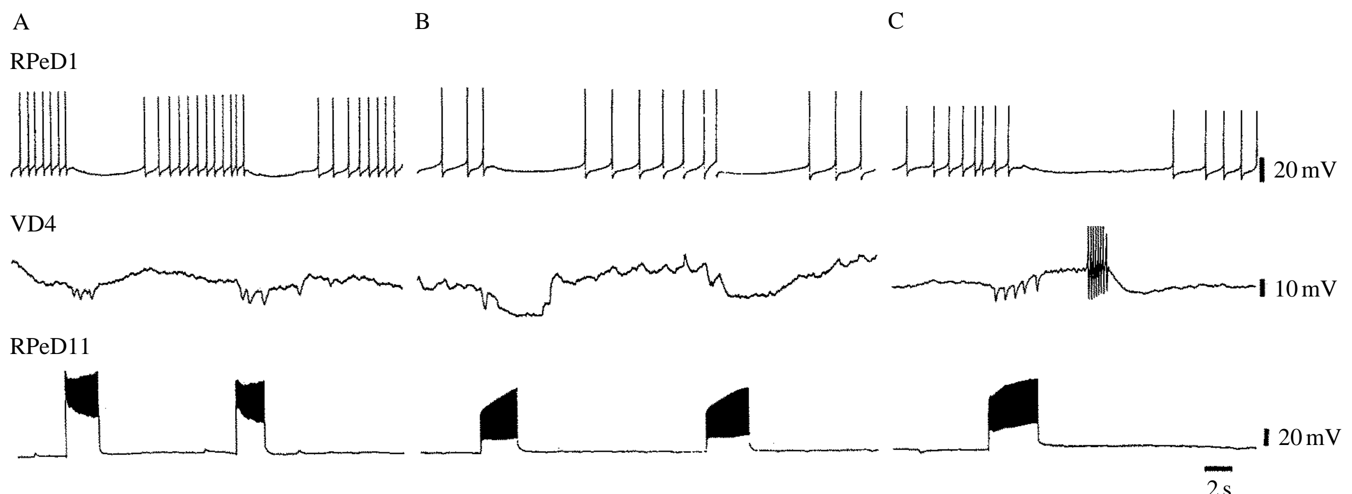


Fig. 4. The monosynaptic nature of connections between RPeD11 and the respiratory neurons RPeD1 and VD4. Simultaneous intracellular recordings were made from RPeD11, VD4 and RPeD1 in an isolated brain preparation. (A) In the presence of normal saline, RPeD11 stimulation inhibited both RPeD1 and VD4. (B) These connections persisted in the high-Ca^{2+}/high-Mg^{2+} saline and also (C) following wash-out.

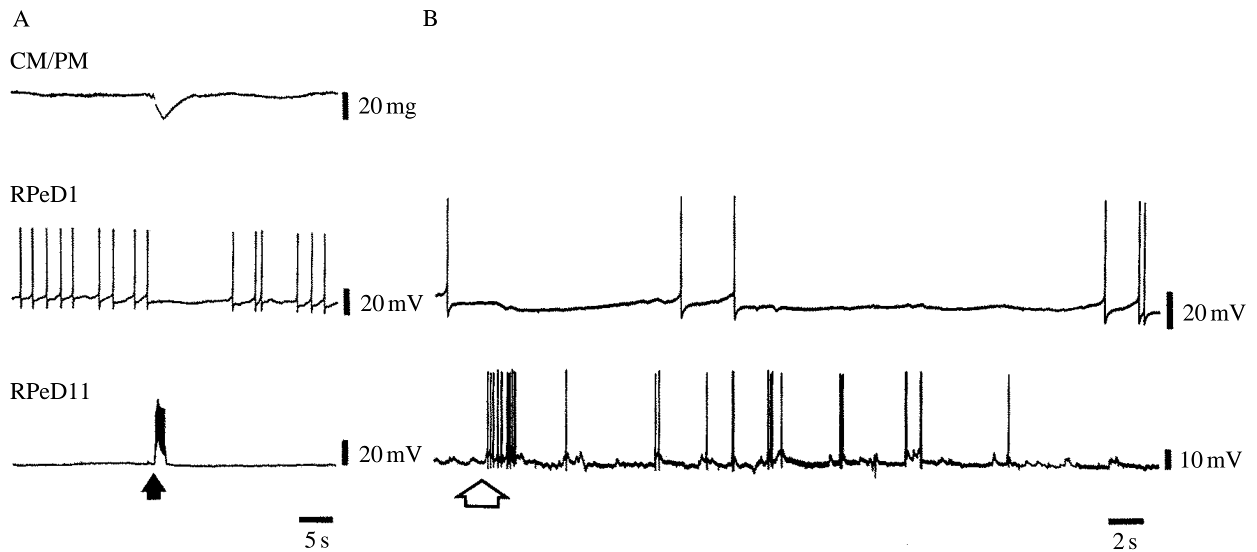


Fig. 5. RPeD11 influences muscular and neuronal components of the respiratory behavior in a semi-intact preparation. (A) In a semi-intact preparation (lung–mantle, pneumostome and CNS), simultaneous intracellular recordings were made from RPeD11 and RPeD1, while muscle contractions were monitored *via* tension transducer recordings, as described previously (Syed and Winlow, 1991*a*). Electrical stimulation of RPeD11 (at the filled arrow) induced contraction of both the columellar muscle (CM) and the pneumostome closer muscle (PM), while inhibiting the activity of RPeD1. (B) Mechanical stimulation of the pneumostome (at open arrow) in the same preparation, but with higher gain, caused a prolonged activation of RPeD11, which in turn inhibited the spiking activity in RPeD1.

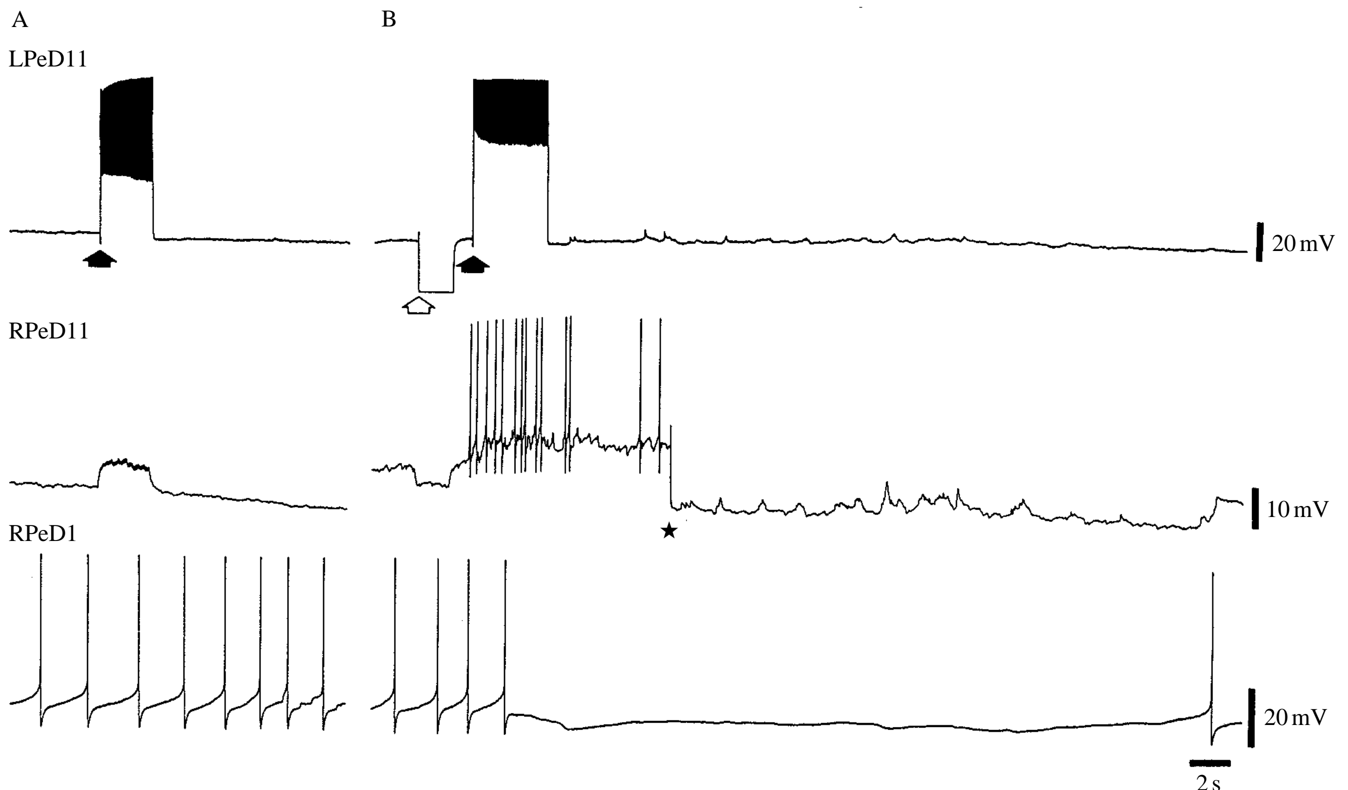


Fig. 6. The inhibition of RPeD1 by RPeD11 is not indirectly mediated by electrical coupling with LPeD11. Simultaneous intracellular recordings of L/RPeD11 and RPeD1 in an isolated brain preparation. (A) With RPeD11 held at its resting membrane potential (–65 mV), depolarizing current injection in LPeD11 (at filled arrow) generated electrotonic potentials in RPeD11 but failed to inhibit RPeD1 activity. (B) RPeD11 was depolarized near its firing threshold (–55 mV) *via* current injection through the recording electrode. Hyperpolarizing (open arrow) and depolarizing (filled arrow) current pulses were injected into LPeD11 and the passage of current into RPeD11 was monitored as an index of electrical coupling between the cells. Stimulation of LPeD11 generated spikes in RPeD11, which in turn inhibited RPeD1. ★ represents the time when RPeD11 was repolarized to its resting level.

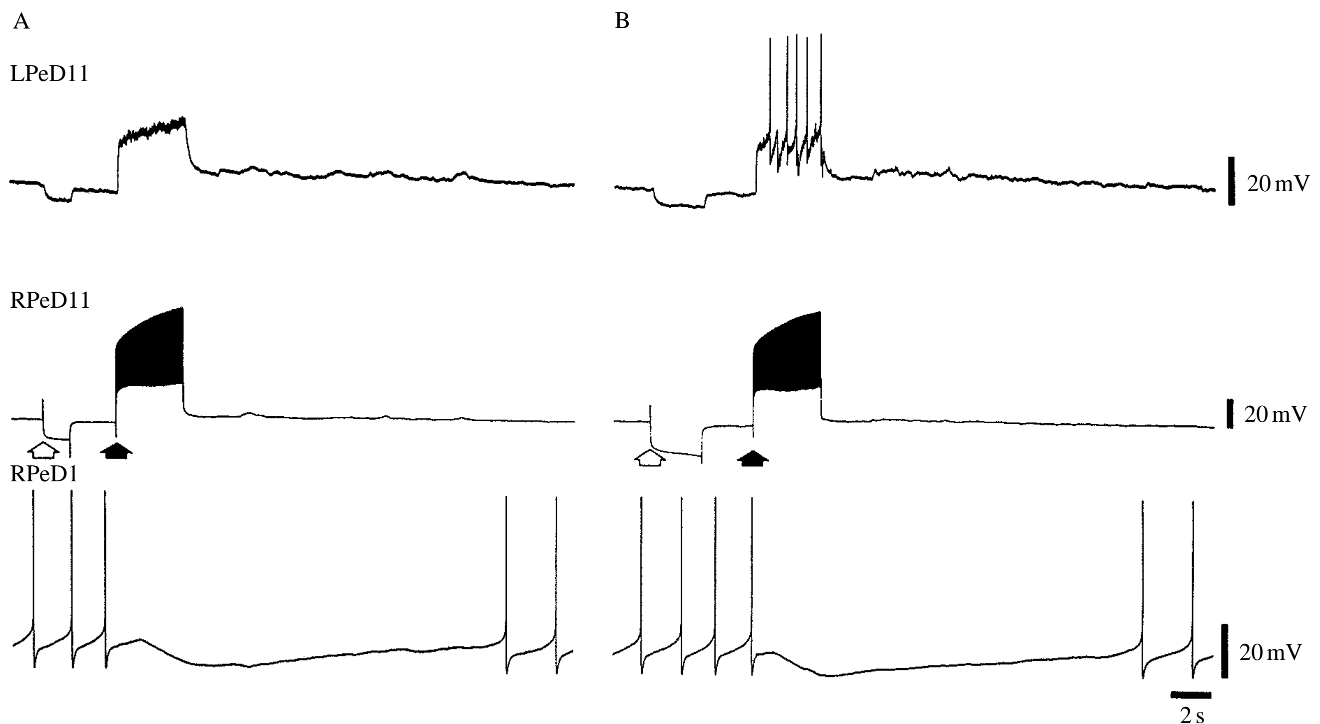


Fig. 7. LPeD11 does not contribute to RPeD11-induced inhibition of RPeD1. Hyperpolarizing (open arrow) and depolarizing (filled arrow) current pulses were injected into RPeD11 to demonstrate the presence of electrical coupling between LPeD11 and RPeD11. (A) RPeD11 stimulation did not generate spikes in LPeD11 maintained at rest (−65 mV), but RPeD1 was inhibited. (B) RPeD11 stimulation generated spikes in a depolarized (−60 mV) LPeD11. Action potentials in LPeD11 did not alter RPeD11-induced inhibition of RPeD1.

hyperpolarizing and depolarizing current pulses between the cells (Fig. 6). Stimulation of LPeD11 with depolarizing current injection produced electrotonic potentials in RPeD11 maintained at rest (−65 mV). It did not, however, inhibit RPeD1 activity (Fig. 6A). RPeD11 was then depolarized near its firing threshold (−55 mV) and LPeD11 was stimulated once again. LPeD11 now successfully generated spikes in RPeD11 which, in turn, inhibited RPeD1 activity (Fig. 6B). These experiments were highly reproducible, and the data (*N*=7) suggest that RPeD1 is inhibited directly by RPeD11 and not *via* LPeD11. To determine whether LPeD11 contributes to RPeD11-induced inhibition of RPeD1, the current was injected into RPeD11 in order to activate a previously depolarized LPeD11 (held near its firing threshold). Under these experimental conditions, regardless of the spiking activity in LPeD11, activity in RPeD11 produced a similar inhibition in RPeD1 (Fig. 7A,B). These data serve to demonstrate further that RPeD1 inhibition is mediated by RPeD11 and that LPeD11 does not contribute significantly to this inhibition.

In vitro *reconstruction of synaptic connections between RPeD11 and the respiratory CPG (RPeD1, VD4 and IP3I)*

Although the synaptic connections between RPeD11 and the respiratory CPG neurons persisted in the presence of high levels of divalent cations, it was impossible to discern 1:1 IPSPs in the target cells. Moreover, since RPeD11 has extensive electrotonic couplings with a large number of other motor neurons (Syed and Winlow, 1991*a*), it was difficult to obtain unequivocal evidence that the connections were indeed direct. Furthermore, owing to anatomical constraints, we had yet to confirm the nature of the synaptic connections between RPeD11 and the IP3I in isolated brain preparations *in vivo*. Therefore, in order to determine the nature of these connections, we utilized *in vitro* cell culture, where specific synapses between *L. stagnalis* neurons can be reconstructed reliably.

Individual cell somata of the respiratory CPG neurons RPeD1, VD4 and IP3I were isolated from their respective ganglia and cultured *in vitro* with RPeD11 under conditions that support neurite outgrowth and synapse formation (Syed *et al.* 1990; Ridgway *et al.* 1991). When cultured with an individual respiratory interneuron, RPeD11 always formed inhibitory synapses. Specifically, when co-cultured with RPeD1 (*N*=13, Fig. 8A), VD4 (*N*=7, Fig. 9A) or IP3I (*N*=3, Fig. 10A), RPeD11 formed inhibitory synapses with these cells (Figs 8B, 9B, 10B) that were similar to those observed *in vivo* (Figs 3, 4). In these *in vitro* preparations, in the absence of other synaptic and non-synaptic influences, unitary 1:1 IPSPs were often discernible, particularly between RPeD11 and VD4 and between RPeD11 and IP3I (Figs 9B, 10B). These data demonstrate that the inhibitory connections between RPeD11 and the respiratory CPG neurons can be reconstructed *in vitro*, providing us with an opportunity to investigate in detail the pharmacological basis of synaptic interactions between these cells.

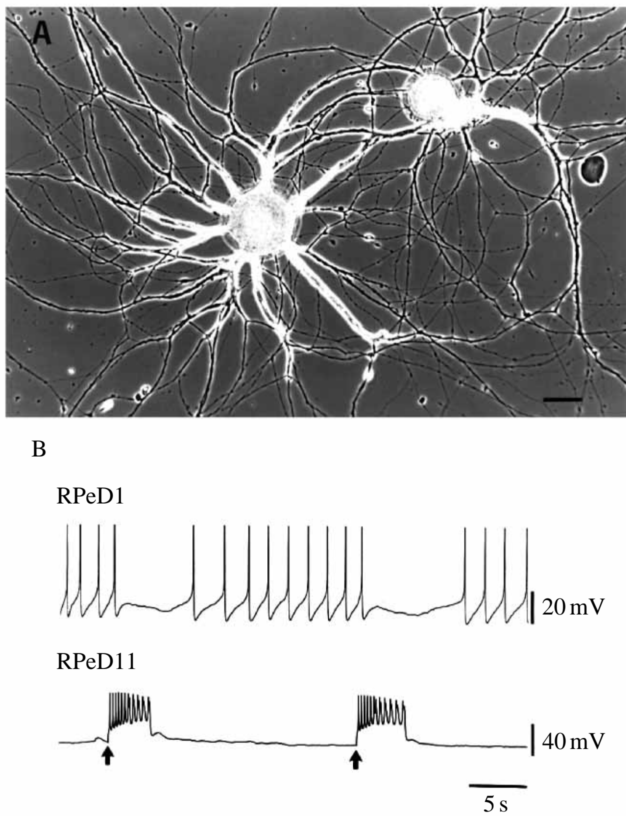


Fig. 8. *In vitro* reconstruction of synapses between RPeD11 and RPeD1. (A) RPeD11 and RPeD1 were isolated *in vitro* under culture conditions that support neurite outgrowth. Both cells exhibited robust sprouting. Scale bar, 50 μm. (B) Following neuritic overlap (24 h post-plating), intracellular recordings were made. Injections of depolarizing current into RPeD11 (at arrows) inhibited spontaneous activity in RPeD1.

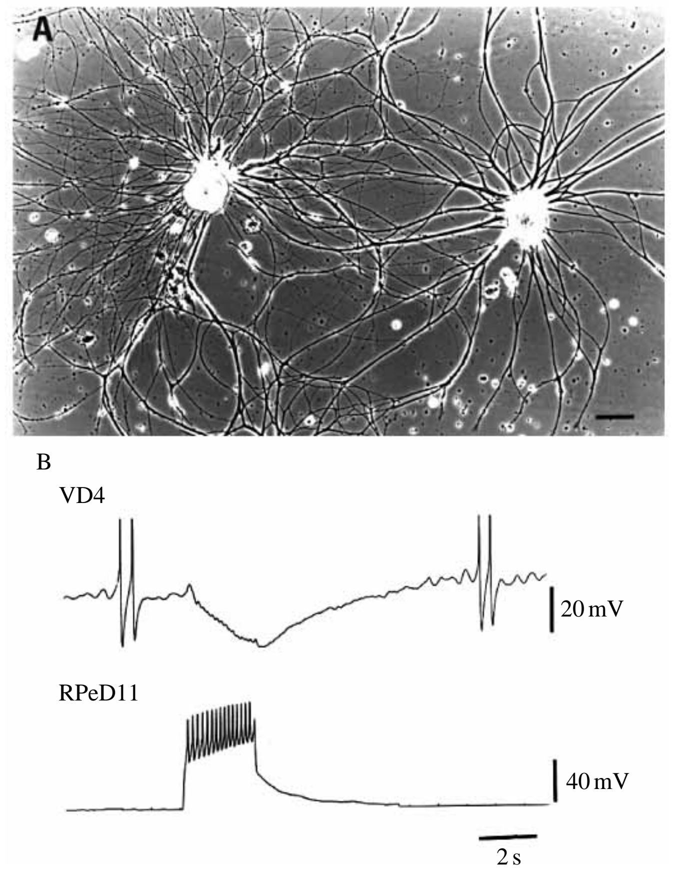


Fig. 9. Establishment of synaptic connections between RPeD11 and VD4 in culture. (A) RPeD11 and VD4 were plated in close proximity to obtain neuritic overlap. Scale bar, 50 μm. (B) Simultaneous intracellular recordings from RPeD11 and VD4 revealed an inhibitory chemical synapse between these cells. VD4 was depolarized to its firing threshold in this preparation.

Discussion

In this study, we have provided evidence that a higher-order, whole-body withdrawal interneuron RPeD11 directly inhibits respiratory CPG neurons. At the neuronal level, our data are consistent with the behavioral observation that stimuli which induce whole-body withdrawal terminate respiratory behavior (Winlow *et al.* 1992).

Whole-body withdrawal and escape responses have been shown to take priority over other behaviors in molluscs, including *L. stagnalis* (Syed and Winlow, 1991*a*; Winlow *et al.* 1992), *Aplysia californica* (Walters and Erickson, 1986), *Planorbis corneus*(Arshavsky *et al.* 1994*a*,*b*), *Helix lucorum* (Balaban, 1983; Maximova and Balaban, 1984), *Tritonia diomedia* (Getting, 1977) and *Clione limacina* (Huang and Satterlie, 1990; Norekian and Satterlie, 1996). The sole exceptions appears to be *Pleurobranchaea* and the holoplanktonic pteropod mollusc *Clione limacina*, where feeding behavior dominates other behaviors, such as head withdrawal, righting and mating (Davis *et al.* 1974*a*; Norekian and Satterlie, 1996). In most animals, the dominance of escape responses over other behaviors is thought to stem from the whole-body withdrawal neurons dominating other neural networks that mediate incompatible behaviors. In most of these species, inhibitory synaptic connections between withdrawal-associated neurons and those involved in the incompatible behaviors have been observed. Since the inhibitory effects of withdrawal neurons were primarily observed either at the behavioral or at the motor neuronal level (see Introduction), the extent to which the withdrawal circuit directly influenced neurons at the level of higher-order neurons remained undetermined.

We have previously demonstrated that the respiratory motor neurons were monosynaptically influenced by RPeD11 (Syed and Winlow, 1991*a*). In the present study, we provide evidence for the inhibition of *L. stagnalis* respiratory CPG interneurons (RPeD1, VD4, IP3I) by a whole-body withdrawal neuron RPeD11. Our data are, therefore, consistent with the hypothesis that neurons mediating higher-priority motor functions inhibit incompatible behaviors not only at the motor neuronal but also at the higher-order CPG neuron level. The behavioral significance of such an extensive suppression of motor command, at all levels of its motor output, would be to ensure an effective and efficient termination of an incompatible behavior.

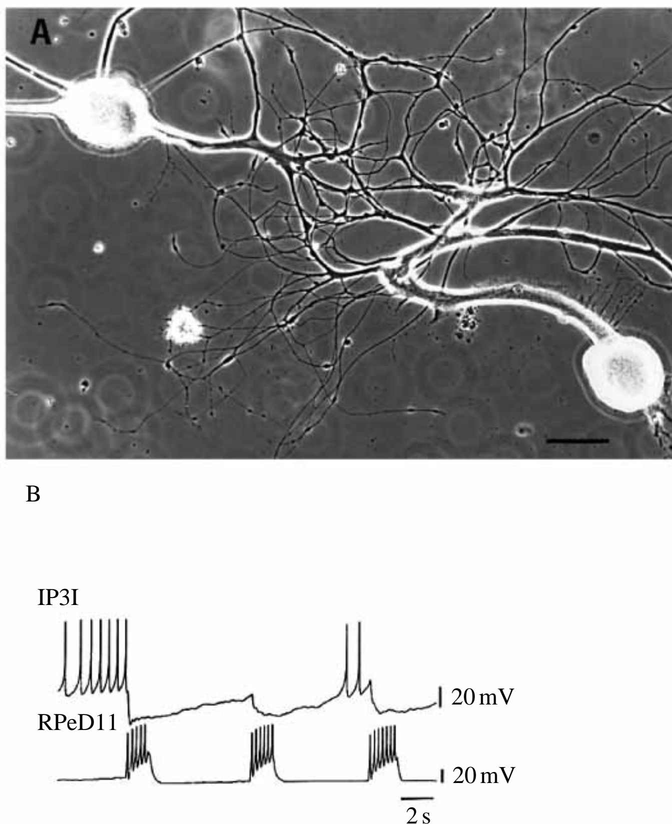


Fig. 10. Specific synapse formation between cultured neurons RPeD11 and IP3I. (A) RPeD11 and IP3I exhibited robust sprouting *in vitro*. Scale bar, 50 μm. (B) Intracellular recordings from these cells revealed the presence of an inhibitory synapse between RPeD11 and IP3I. Since IP3I was quiescent *in vitro*, it was depolarized by current injection to demonstrate the inhibitory effects of RPeD11.

Neuronal basis of coordination between the respiratory and whole-body withdrawal behaviors

As described above, during spontaneously occurring respiratory movements in a freely behaving animal, a gentle mechanical touch to the pneumostome area causes its closure and the termination of the respiratory behavior (Lukowiak *et al.* 1994). When the animal is not ventilating, however, a stronger stimulus applied to the head–foot complex of both *L. stagnalis* (Sakhorov and S.-Rózsa, 1989) and *Planorbis corneus* (Arshavsky *et al.* 1994*b*) causes a forceful opening of the pneumostome. The behavioral significance of these pneumostome openings may be to reduce the body volume, by removing air from the lung and hemolymph from the mantle cavity (Sakhorov and S.-Rózsa, 1989; Arshavsky *et al.* 1994*b*). In *Helix lucorum*, however, noxious stimuli that induce whole-body withdrawal always cause pneumostome closure (Balaban, 1983).

In both *Helix lucorum* and *Planorbis corneus*, the neurons that control withdrawal behavior appear to control the pneumostome as well. For instance, a command neuron, LPa3, underlies whole-body withdrawal behavior in *Helix lucorum*. In addition to activating withdrawal reflexes, electrical stimulation of LPa3 in a semi-intact preparation induces pneumostome closing movements (Balaban, 1983; Vehovszky *et al.* 1989). In *Planorbis corneus*, the electrical stimulation of the withdrawal neuron DRN1 induces pneumostome opening movements (Arshavsky *et al.* 1994*b*). These data, therefore, suggest that, in addition to inducing withdrawal reflexes, the neurons controlling withdrawal behavior may also exert motor control over the pneumostome musculature.

This generic involvement of the pneumostome in both respiratory and withdrawal behaviors led some researchers to suggest that these two motor functions were controlled by the same network (Sakhorov and S.-Rózsa, 1989). For instance, Sakhorov and S.-Rózsa (1989) raised the possibility that the IP3I activity (pneumostome openings as observed during respiration) in *L. stagnalis* may be a component of the whole-animal withdrawal behavior. Our data, however, are not consistent with their hypothesis. We have previously demonstrated, in a semi-intact preparation, that a gentle mechanical touch to the pneumostome (inducing whole-body withdrawal) terminates IP3I activity in the middle of its discharge (Syed and Winlow, 1991*a*), suggesting that it is not a component of the withdrawal reflex. In the present study, we have demonstrated inhibitory synaptic connections between the withdrawal neuron RPeD11 and the respiratory CPG neurons (RPeD1, VD4 and IP3I). Moreover, we have also demonstrated in a semi-intact preparation that a noxious stimulus delivered to the pneumostome area inhibits the respiratory interneuron RPeD1 (Fig. 6). Taken together, both the behavioral analysis and the network configuration reinforce our view that pneumostome opening and the underlying motor discharges (IP3I activity), as observed during active respiratory movements, are not a component of the withdrawal reflex.

If the neurons involved in mediating the respiratory and withdrawal behaviors are different, how are these two behaviors coordinated? Owing to the lack of information regarding the identity of the respiratory neurons in both *Helix lucorum* and *Planorbis corneus*, the synaptic relationships between withdrawal and respiratory neurons have not been worked out. In *L. stagnalis*, however, we have now provided direct evidence that the withdrawal neuron RPeD11 not only terminates the respiratory rhythm at the higher CPG level but also induces pneumostome closure. We believe that RPeD11 may achieve this task not only by inhibiting the respiratory CPG neurons directly but also by inducing the contractions of both withdrawal (columellar) and pneumostome opener muscles.

In vitro *synaptic connections between RPeD11 and respiratory CPG neurons*

To deduce the direct *versus* indirect nature of synaptic connections between RPeD11 and the respiratory CPG neurons, we recorded intracellularly in a solution containing a high concentration of divalent cations. Under these experimental conditions, the synaptic connections between RPeD11 and the respiratory CPG neurons persisted, suggesting that they may be monosynaptic. Moreover, stimulation of other electrically connected neurons, such as LPeD11, did not mimic the effect of RPeD11 stimulation on the target cells, again suggesting that RPeD11 may affect its targets directly. Similar methods have

previously been used to demonstrate monosynaptic connections in *L. stagnalis* (Syed and Winlow, 1991*a*). However, owing to extensive electrotonic coupling between RPeD11 and other connected cells (L/RCeA, L/RPeG), providing unequivocal evidence for direct *versus* indirect polysynaptic pathways was still difficult. The presence of electrically coupled neurons in any given circuit often makes it difficult to determine the monosynaptic nature of connections between the neurons in question. Various biochemical and cell deletion techniques, such as treatment with solutions containing a high level of divalent cations (Berry and Pentreath, 1976), enzyme injections (Marder, 1984) and photoinactivation of the neurons (Miller and Selverston, 1979; Elliott and Kleindienst, 1990; Syed and Winlow, 1991*a*), have elucidated the roles of individual electrically coupled neurons in various circuits. Owing to the extensive electrical coupling between the many motor neurons underlying *L. stagnalis* withdrawal behavior, these approaches are much less feasible in this case.

To demonstrate that the connections between RPeD11 and the respiratory CPG neurons were likely to be monosynaptic, we constructed these synapses *in vitro*. Adult *L. stagnalis* neurons, like those of many other invertebrate species, regrow their processes in culture and reform specific synapses similar to those observed *in vivo* (Bulloch and Syed, 1992). In a number of these invertebrate species, inappropriate synapses may also form in culture (Chiquet and Nicholls, 1987; Kleinfeld *et al.* 1990). In *L. stagnalis*, however, we found RPeD11 to establish inhibitory synapses with its target cells *in vitro*. Although the synapses between RPeD11 and the respiratory neurons described in this study were of the appropriate type (inhibitory), the compound IPSPs were of larger amplitude and longer duration than those recorded in the *in vivo* preparations. The augmentation of synaptic responses *in vitro* may have been due in part to the absence of the synaptic and non-synaptic interactions with a multitude of other cells that often make it difficult to resolve such subtle responses *in vivo*.

In summary, we have provided evidence that the neuronal networks underlying the respiratory and whole-body withdrawal behaviors are organized in a hierarchical manner, with the withdrawal network taking priority over the respiratory circuit. RPeD11, which plays a key role in coordinating the whole-animal withdrawal behavior, also inhibits respiratory CPG neurons directly and, therefore, serves as a main determinant of the behavioral hierarchy between whole-body withdrawal and respiration. These data provide a unique example in which an incompatible behavior, i.e. respiration, is terminated by the whole-body withdrawal neuron RPeD11 at all levels of CPG organization. Specifically, RPeD11 terminated respiratory rhythm generation at the rhythm generator (CPG) level (Fig. 3A,B), motor command was terminated at the motor neuronal level (Syed and Winlow, 1991*b*) and the expression of behavior was further curtailed at the muscular level (Fig. 5A). In conclusion, our data suggest that, in order for it to be effective, a neuronal circuit situated hierarchically higher than the others must terminate incompatible motor commands simultaneously at all levels of motor output organization.

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