Identification and Initial Characterization of a Cluster of Command and Pattern-Generating Neurons Underlying Respiratory Pumping in *Aplysia californica*

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**SUMMARY AND CONCLUSIONS**

1. Respiratory pumping is a relatively stereotyped behavior that occurs both spontaneously and in response to tactile stimuli, anoxia, food presentation, and changes in illumination (10, 20, 37, 39, 42, 43). While considerable progress has been made in elucidating the motor elements underlying this behavior, little is known regarding the cells responsible for its initiation or patterning. This paper describes the identification and initial characterization of a cluster of command and pattern-generating neurons that subserve this behavior.

2. The command and pattern-generating neurons consist of a cluster of at least seven mutually excitatory cells (the L25 cells) located on the left ventral surface of the abdominal ganglion. These cells fire spontaneous bursts of spikes synchronously with the activity of other interneurons and motor neurons activated during spontaneous respiratory pumping.

3. Artificially firing action potentials in the L25 cells to mimic a spontaneous burst produces the same synaptic effects in motor neurons and interneurons activated during respiratory pumping, while hyperpolarizing a single L25 cell can phase shift a subsequently occurring spontaneous burst.

4. The L25 cells are mutually excitatory; spikes in one L25 cell lead to excitatory postsynaptic potentials (EPSPs) and spikes in other L25 cells. At least some of the mutually excitatory effects are mediated via electrical synapses.

5. The L25 cells make mutually inhibitory connections with other interneurons in the abdominal ganglion. They produce slow and long-lasting inhibitory postsynaptic potentials (IPSPs) in the cardiac command neuron L10 and in interneuron L24. L10 and L24 in turn produce fast IPSPs in the L25 cells.

6. Respiratory pumping activity can be elicited by stimulation of siphon skin, and this activity is due in part to activation of the L25 cells. At least part of the activity in L25 cells produced by tactile stimulation of the skin or electrical stimulation of nerves appears to be due to regenerative feedback among the L25 cells.

**INTRODUCTION**

One major focus of studies designed to elucidate the cellular basis of behavior is the analysis of the neural mechanisms underlying the initiation, patterning, and modulation of rhythmic motor activity (For review see Refs. 4, 23, 28, 44, 48, 55.) Despite the fact that a great number of motor behaviors fall into this general category, relatively little progress has been made in understanding the underlying cellular mechanisms, particularly in systems involving longer cycles and in systems subject to larger degrees of modulation. Respiratory pumping in *Aplysia* offers a rel-
Relatively simple test system where these questions can be analyzed.

Respiratory pumping is one of the most conspicuous and frequently occurring behaviors mediated by the abdominal ganglion. Single pumping episodes generally have a duration of about 3–6 s and occur spontaneously at intervals varying between about 30 s and 10 min. This simple fixed action pattern has three nearly synchronous motor components: 1) contraction and withdrawal of gill, siphon, and mantle shelf; 2) closing of the parapodia; and 3) heart inhibition accompanied by a decrease in vasomotor tone (10). While variations in the frequency and amplitude of these motor effects can occur within individual animals, the entire behavior is relatively stereotyped in form and tends to occur in an all-or-none fashion (10, 41). Respiratory pumping occurs spontaneously but can also be triggered by tactile stimulation of the skin and can be modulated by various extrinsic factors such as exposure to food, changes in oxygen levels in the seawater, and escape locomotion (10, 20, 32, 37, 39, 42, 43).

The various motor neuron pools that mediate the components of respiratory pumping have been described in detail. The respiratory (gill, siphon, and mantle shelf) and cardiovascular motor neurons are located in the abdominal ganglion (42, 43, 46, 49, 50, 53, 58) and at least some of the parapodial motor neurons are located in the pedal ganglia (31, 32).

During a spontaneous respiratory pumping cycle, motor neurons innervating the various effector organs receive either excitatory or inhibitory synaptic input from a previously unidentified central command element called interneuron II (35). At the same time, several of the motor cells are released from tonic synaptic input from a spontaneously active neuron, interneuron Xl (cell L24) (10, 35, 41). It has recently been suggested that the synaptic potentials previously attributed to interneuron II are produced by a cluster of at least three interneurons called L25, L26, and L27 (see Ref. 10 for "wiring diagram"). But each of these cells seemed to account for only a limited part of the synaptic input that drives the motor neurons during respiratory pumping. The available data could best be fitted by subdividing these interneurons into two functional groups: a group of mutually excitatory "burst generators" and a second group of interposed "output" interneurons (10).

The identification of interneuron XI and the respiratory interneurons (10) was a first step in elucidating the neural control of respiratory pumping, but the initial analysis of the neural network responsible for generating the pattern of synaptic actions observed in the motor neurons was far from complete. For example, only one burst generator cell (L25) was identified but others appeared to be present, since firing L25 recruited biphasic PSPs back onto itself.

This paper describes the identification and initial characterization of a cluster of seven or more L25-like cells that appear to be the command and pattern-generating cells for respiratory pumping. Spontaneous bursts of activity in these cells can precede the synaptic input in the motor neuron pools, while artificially firing spikes in these cells mimics the synaptic input to the motor neurons. Hyperpolarization of a single burst generator (L25) can increase the interburst interval in L25. In addition, these cells are mutually excitatory and make mutually inhibitory connections with interneurons L10 and L24. As a result of their identification it is now possible to begin to examine the synaptic and biophysical mechanisms that underlie the generation and patterning of the bursting behavior and to examine how the bursting pattern can be modulated by extrinsic factors.

METHODS

The experimental preparation and techniques have been previously described in detail (5, 9). All experiments were performed at 15°C with bath temperature regulated with a feedback-controlled thermoelectric cooling unit. In all cases an isolated reflex preparation consisting of the gill, siphon, and a portion of the ink gland with the desheathed abdominal ganglion and its intact peripheral nerves (branchial, genital, and siphon) was utilized (9). The gill and siphon were unrestrained. Neurons activated during spontaneous respiratory pumping were identified based on their characteristic locations, spontaneous input, nerve-evoked input, motor effects, and sensory input (5, 8–10, 12, 22, 40, 42, 50).

The typical procedure used to find cells mediating spontaneous respiratory pumping was first
to impale identified motor neurons (such as L7 or the LD and LB gill and siphon motor neurons) and interneurons (such as L10 and L24) known to receive synaptic input during spontaneous respiratory pumping cycles. The ganglion was then searched for neurons whose activity was synchronous with the synaptic input in various identified follower neurons. Additional tests were then performed as described in RESULTS to determine further if the cell impaled was an L25 cell.

The results represent recordings from 56 L25 cells in 23 different experiments. Many of the illustrations (Figs. 2–5, 8B, 9, and 10) were taken from a single experiment where most of the properties of the L25 cells could be examined.

RESULTS

Location and general properties of command and pattern-generating neurons mediating spontaneous respiratory pumping

The command and pattern-generating neurons mediating spontaneous respiratory pumping consist of a cluster of at least seven relatively small (30–50 μm) cells located on the lateral edge of the left ventral surface of the abdominal ganglion. In some experiments these cells were found among cells on the surface layer of the ganglion but were most typically located several layers deep, usually medial to and below (dorsal to) the LBS siphon motor neurons. In cases where the cells could not be found on the surface, they were exposed by removing some of the larger surface cells in the region where the command and pattern-generating neurons are typically located. In some experiments the cells were penetrated by passing the recording electrode through the outer layer of cells. The command and pattern-generating cells have the general size and appearance of the LE-type sensory neurons but generally lack the distinctive dark rim of the LE cells (8). Because of the similarity of their electrophysiological properties to a single command and pattern-generating cell previously identified as L25 (10), this cell cluster is referred to as L25 cells (Fig. 1).

Command and pattern-generating role of L25 cells

Figure 2 illustrates the typical activity in an L25 cell, two gill motor neurons, and interneuron L24 associated with spontaneous respiratory pumping. Spontaneous bursts of spikes in L25 were associated with EPSPs and spikes in LDG2 and inhibition of motor neuron L7 and interneuron L24. While gill

Fig. 1. Map of the ventral surface of the abdominal ganglion. Circles are the approximate location within the abdominal ganglion of the cell bodies of neurons described in this paper. The L25 cells are the command and pattern-generating neurons, LBS is a siphon motor neuron, L7 and LDG2 are gill motor neurons, while L10 and L24 are interneurons. LDG2 is normally located on the dorsal surface (42) but in some cases is also found on the medial edge of the ventral surface (unpublished observations). The sensory neurons illustrated are the cell bodies of the LE cluster mechanoreceptor neurons that innervate the siphon skin (8). Two L14 cells (ink motor neurons) and L5 are included for orientation.
FIG. 2. Activity in identified neurons during three spontaneous respiratory pumping cycles. Simultaneous intracellular recordings were made from motor neurons (MN) L7 and LDG2 and interneurons (INT) L24 and L25. During each cycle, activity in L25 precedes synaptic input to L7, LDG2, and L24. The large spontaneous IPSPs in LDG2 are produced by L24 (10). Thus at least part of the excitation of LDG2 during a spontaneous burst in L25 is due to disinhibition.

movements were not recorded, high-frequency bursts of spikes in LDG2, like those illustrated in Fig. 2, are sufficient to produce large gill contractions (see Ref. 42). The large spontaneously occurring inhibitory postsynaptic potential (IPSPs) in LDG2 are from L24 (10). Thus, at least part of the excitation in LDG2 during the L25 bursts is due to disinhibition.

That activity in L25 is causally related to and not simply correlated with the activity of motor neurons and interneurons activated during spontaneous respiratory pumping is illustrated in Fig. 3. Figure 3A shows a spontaneous burst of spikes in L25 and the associated synaptic input to L7, LDG2, and L24. In Fig. 3B a burst of spikes was produced in L25 by a depolarizing current pulse. This burst of spikes elicited synaptic input to L7, LDG2, and L24 that mimics the spontaneous synaptic input during respiratory pumping. Note that in this example the synaptic input to the follower cells actually began prior to the spike activity in L25 (see also below and DISCUSSION). These results in Fig. 3 indicate that activity in the L25 cells appear to be sufficient to produce at least part of the synaptic input to neurons activated during spontaneous respiratory pumping. These results, however, do not rule out the possibility that the L25 cells are themselves normally driven by another class of burst-generating neurons. Figure 4 illustrates one experiment designed to test this possibility.

Figure 4A illustrates a spontaneous burst of spikes in L25 and the associated synaptic input to L7, LDG2, and L24. Note that in this case the spontaneous burst in L25 is preceded by small fluctuations in potential that increase in frequency. This indicates that the L25 cells may indeed be driven by synaptic input from an unidentified source. Figure 4B
Fig. 3. Comparison of synaptic input and firing patterns in follower cells produced by spontaneous bursts in L25 and by directly firing L25. A: simultaneous intracellular recordings from L7, LDG₂, L24, and L25 during spontaneous burst of activity in L25. B: L25 was hyperpolarized by approximately 30 mV. At arrow hyperpolarization was released, which produced a burst of spikes in L25 and activity in L7, LDG₂, and L24 similar to that produced during a spontaneous burst in L25. At second arrow the cell was again hyperpolarized and spike activity in L25 was abolished, as was the synaptic input to the various follower cells. These results indicate that L25's activity accounts for a large part of the synaptic input associated with the respiratory pumping pattern in these cells. Note that effects of L25 depolarization precede spike activity in L25.

illustrates that this source of synaptic input to an L25 cell can be identified as intrinsic to the group of L25 cells (see also below). In Fig. 4B the L25 cell was transiently hyperpolarized just as the small potential fluctuations that precede spontaneous bursts (see A) began to increase in frequency. Note that the increase in frequency of the potential fluctuations in L25 prior to the hyperpolarization was associated with weak inhibition of L7 and L24 and spikes in LDG₂. With the application of the hyperpolarizing pulse to L25, the frequency of the small potential fluctuations in L25 was reduced and no underlying synaptic input was uncovered. At the same time, hyperpolarization of L25 terminated the initial inhibition of L24 and spike activity in LDG₂. With release of the hyperpolarization, the small potential fluctuations again began to increase in frequency and led to a burst of spikes in L25 and the typical pattern of synaptic input in L7, LDG₂, and L24. These results indicate that hyperpolarization of a single L25 cell can phase shift a spontaneous burst and that the L25 cells are causally involved in the generation of spontaneous bursts. Additional evidence for the causal role of the L25 cells in burst generation is illustrated in Fig. 5.

Mutually excitatory connections among L25 cells

Figure 5 illustrates that the L25 cells appear to produce positive feedback onto them-
selves. In Fig. 5A, a brief burst of spikes was produced in an L25 cell by an intracellular depolarizing current pulse. The initial burst was followed by a long train of spikes. Figure 5B illustrates another example from the same cell but in this case the initial burst of spikes was followed by a long-lasting burst of small potential fluctuation, which presumably underlay the spike train in A. The lower trace of Fig. 5B is a high-gain recording of the small potential fluctuations and illustrates their characteristic biphasic shape.

The small potential fluctuations can be produced not only by firing a brief burst of spikes in an L25 cell but also by releasing an L25 cell from sustained hyperpolarization. Generally the fluctuations increase in frequency until a long-lasting burst of spikes is produced. The interval between the initiation of these small potential fluctuations and subsequent spikes varies. These results indicate that the L25 cells have either intrinsic membrane or network properties that lead to positive feedback as the cells are depolarized. The small potential fluctuations may be due to nonpropagating dendritic spikes or to spikes from other electrically coupled L25 cells. Figures 6 and 7 provide evidence that the L25 cells are mutually excitatory to each other and electrically coupled.

Figure 6 illustrates simultaneous recording from two cells in the L25 cluster. The cells...
FIG. 5. Burst of spikes in L25 results in input onto itself. A: a 1-s intracellular depolarizing current pulse was delivered to an L25 cell, which resulted in 8 spikes. With the termination of the current pulse, L25 continued to fire for 20 s. B: in the same experiment another 1-s depolarizing current pulse was delivered to the L25 cell, which resulted in 9 spikes. In this case the cell did not show continued firing but the initial spike activity produced a long train of biphasic potential fluctuations. Lower trace shows same response at high gain.

are designated L25A and L25B for purposes of the illustration, but as yet it has not been possible to distinguish individual neurons of the L25 cluster from each other. Figure 6A illustrates recordings during a spontaneous burst. Note that there was a relatively short burst of spikes in L25A compared to the longer duration burst in L25B. While the spontaneous spike burst in L25A was short, it was followed by a burst of EPSPs, many of which were one for one with spikes in L25B. In Fig. 6A, a burst of spikes was artificially produced in L25A, which led to a long train of PSPs back onto itself. At least one source of these PSPs is L25B. The spikes in L25A led to a long-lasting slow hyperpolarization of interneuron L10. Firing a burst of spikes in L10 in turn produced fast IPSPs in L25. Mutually inhibitory connections between L10 and L25 (previously called interneuron II) had been inferred (34) but never directly demonstrated. Figure 8B illustrates mutually inhibitory connections between L25 and L24. Just as L25 produced a slow IPSP in L10, it also produced prolonged inhibition of L24 (B1). L24, like L10, produced

Figure 6B illustrates mutually excitatory connections between L25 cells more directly. In Fig. 6B1, spikes produced in L25A with an intracellular depolarizing current pulse led to PSPs in L25B that followed spikes in L25A one for one with a short and constant latency. The EPSPs appeared to facilitate without a concomitant spike broadening (as revealed by high-speed playback), suggesting the possibility of a chemical synapse. It is possible, however, that this facilitation could be due to anomalous rectification or the recruitment of other electrically coupled cells that summate with the PSPs from L25A. Figure 6B2 illustrates that L25B also produced one-for-one EPSPs and spikes in L25A. In addition, these two cells were electrically coupled to each other (see also below). Direct electrical coupling between another pair of cells in the L25 cluster is illustrated in Fig. 7. The coupling appears to be asymmetric but because of the size of the potentials needed to observe these effects (and consequent problems with bridge balancing), the possibility of rectification and a precise estimate of the coupling ratio require further analysis. In conclusion, the mutual excitatory effects among L25 neurons involve electrical and possibly chemical synaptic interconnections as well.

Mutually inhibitory connections between L25 and other interneurons in abdominal ganglion

One of the characteristic features of spontaneous respiratory pumping activity is inhibition of both interneuron L24 and the cardiac command neuron L10 (10, 41). Figure 8 illustrates that the L25 cells make mutually inhibitory connections with these cells. In Fig. 8A1, a burst of spikes in L25 produced by an intracellular depolarizing current pulse caused a long-lasting slow hyperpolarization of interneuron L10. Firing a burst of spikes in L10 in turn produced fast IPSPs in L25 (A2). Mutually inhibitory connections between L10 and L25 (previously called interneuron II) had been inferred (34) but never directly demonstrated. Figure 8B illustrates mutually inhibitory connections between L25 and L24. Just as L25 produced a slow IPSP in L10, it also produced prolonged inhibition of L24 (B1). L24, like L10, produced
FIG. 6. Mutually excitatory connections between L25 cells. A1: simultaneous recordings from two L25 cells during a spontaneous burst. A2: L25A was fired with an intracellular depolarizing current pulse, which produced PSPs and sustained spike activity in L25B and PSPs back onto itself. The PSPs in L25A are one for one with spikes in L25B. L25A was then hyperpolarized (full extent hyperpolarization is clipped by pen recorder) and spike activity in L25B was blocked, as were the PSPs in L25A. B1: directly firing L25A produces EPSPs and spikes in L25B. Note that EPSPs appeared to facilitate. In this case firing L25A did not produce input to itself and no sustained activity was produced in L25B. B2: directly firing L25B produced EPSPs and spikes in L25A.

FIG. 7. Electrical coupling between L25 cells. A: hyperpolarizing L25A led to a small hyperpolarization of L25B. B: hyperpolarization of L25 led to a small hyperpolarization of L25A. Simultaneous high-gain recordings from other neurons in the ganglion revealed that these responses were specific to the L25 cells and therefore not due to polarization of the ground electrodes. Results illustrated here indicate that the coupling may not be symmetrical but further experiments are necessary to test this possibility.
fast IPSPs in L25 ($B_2$). While not illustrated, L10 and L24 also make mutually inhibitory connections with each other (unpublished observations).

Responses of L25 cells to stimulation of siphon skin, individual sensory neurons, peripheral nerves, and connectives

Respiratory pumping is called spontaneous because cyclic activity in the involved motor neurons occurs in the absence of stimulation and when the abdominal ganglion is isolated from the periphery (35, 61). Respiratory pumping and patterns of synaptic input similar to those occurring during spontaneous respiratory pumping can, however, also be triggered by tactile stimulation of the siphon skin and by electrical stimulation of the peripheral nerves and connectives of the abdominal ganglion (22, 37, 42, 43, 52). If the L25 cells are the command and pattern-generating neurons of respiratory pumping, they should be activated by such stimuli.

Figure 9 illustrates the response of L25, L24, LDG2, and L7 to a brief tactile stimulus to the siphon skin. At the arrow, a von Frey hair producing a force of approximately 2 g was delivered to the skin, which led to a prolonged burst of spikes in L25, spikes in LDG2, inhibition of L24, and excitation followed by inhibition in L7. To assess the role of L25 in mediating these responses, L25 was
FIG. 9. Response of L25 and its follower cells to tactile stimulation of the siphon skin. A: at arrow a von Frey hair producing a force of approximately 2 g was presented to the siphon skin for approximately 500 ms. The brief tactile stimulus produced a long-lasting spike discharge in L25 and LDG2, excitation followed by inhibition in L7, and inhibition of L24. B: L25 was hyperpolarized by approximately 30 mV and the same stimulus was again delivered to the siphon skin. EPSPs but no spikes were produced in L25 and the characteristic activity in the follower cells was dramatically reduced.

hypermolarized to remove it functionally from the circuit. When the stimulus was repeated, there was a dramatic reduction in the response of L24 and the motor neurons. Of particular interest is the fact that there was little observable synaptic input to L25 that could account for the pronounced spike discharge produced in Fig. 9A. This experiment, while indicating that the L25 cells make major contributions to the reflex activation of various motor neurons and interneurons in the ganglion, is difficult to interpret due to problems in precisely delivering identical mechanical stimuli with the hand-held von Frey hair.

In other experiments the activity produced in the L25 cells by mechanical stimulation of the skin varied from that illustrated in Fig. 9A. In some experiments an initial burst of several spikes was followed by a brief pause of several seconds and then a delayed high-frequency burst (for example of this type of response see Fig. 10). In other experiments the L25 cells gave the initial burst followed by a slow depolarization, which frequently was composed of small biphasic potential fluctuations that did not lead to a delayed burst. In some experiments only the brief initial spike burst was obtained.

In order to examine more precisely the role of the L25 cells in mediating input from the siphon to motor neurons and interneurons, the tactile stimulus to the skin was mimicked by delivering a brief electric shock to the siphon nerve. Such a shock presumably activates the axons of mechanoreceptor sensory neurons that innervate the siphon skin (8, 19; see Fig. 12-7 of Ref. 33 for examples). Figure 10 illustrates these results. Figure 10A shows the response of L25, L24, LDG2, and L7 to a single shock to the siphon nerve. Note the general similarity of these responses to the responses produced in these same cells to a tactile stimulus to the skin (c.g., Fig. 9A). Just as there was considerable variability between experiments in the response of the L25 cells to mechanical stimulation of the skin, there was also variability...
in their responses to shock of the siphon nerve. In the experiment illustrated in Fig. 10A, the shock produced an initial fast multicomponent EPSP in L25, which was followed by potential fluctuations that increased in frequency and amplitude and eventually culminated in a high-frequency burst of spikes in L25. The shock also produced inhibition of L24, brief short-latency excitation of L7 and LDG2, longer latency inhibition of L7, and longer latency excitation of LDG2. Note that the most intense responses occurred not with the initial shock but later, during spike activity in L25. These results, like the results in Fig. 9, indicate that the L25 cells play an important role in producing the synaptic input to respiratory pumping follower cells following afferent stimulation. To examine the causal role of the L25 cells, an identical shock was delivered but now with L25 hyperpolarized (Fig. 10B). The initial effects in L25, L24, LDG2, and L7 were nearly identical to those of Fig. 10A, (see also C), but the longer latency components were completely abolished. In Fig. 10C the hyperpolarization was removed, the nerve again stimulated, and responses essentially identical to those illustrated in A were obtained. These results indicate that in response to cutaneous or nerve stimulation, L25 plays a major role in mediating the synaptic input to a variety of neurons in the abdominal ganglion. Of particular significance is the fact that hyperpolarization of L25 revealed no underlying synaptic input that could account for the long-latency bursts observed in Fig. 10A and C. Thus, hyperpolarization of L25 not only blocked a major
FIG. 11. Response of L25 to burst of action potentials in a mechanoreceptor sensory neuron. Simultaneous intracellular recordings were made from an L25 cell and the soma of an LE-type mechanoreceptor sensory neuron. A: artificially firing a burst of 14 action potentials in the sensory neuron with a train of depolarizing current pulses triggered a relatively long-lasting burst of spikes in L25. B: spontaneous burst in L25. Note the small potential fluctuations in L25 that precede the spontaneous burst (cf. Fig. 4A). These results indicated that activity in the previously identified sensory neurons innervating the siphon skin accounts for at least part of the ability of skin stimulation and siphon nerve shocks to trigger a burst in L25 (cf. Figs. 9, 10).

FIG. 12. Response of L25 and its follower cells to a brief shock delivered to the left connective. A: simultaneous recordings were made from ink motor neuron L14 (12) and LD motor neuron (probably an LDS siphon motor neuron (50)) and LBS siphon motor neuron, and an L25 cell. For L25, both high- and low-gain recordings are illustrated. At the arrow a single constant-current shock (5 ms duration) was delivered to the left connective producing fast and slow EPSPs in L14, a spike burst in the LD cell and L25, and inhibition of LBS. The spike burst in L25 was preceded by a fast E-IPSP. B: with L25 hyperpolarized the same shock was delivered. The spike burst in L25 was blocked and the input to the LD and LB cell was dramatically reduced, while the response in L14 was unchanged. C: with the hyperpolarization removed and the connective again stimulated, the same responses as in A were obtained.
component of the afferent input to L7, LDG₂, and L24, but it also blocked the input to itself. A major conclusion is that at least part of the response of L25 to afferent input is regenerative.

The siphon skin is innervated by a cluster of primary mechanoreceptor sensory neurons (LE cluster) whose cell bodies are located within the abdominal ganglion (8). Figure 11 illustrates that these cells account for at least some of the excitation produced in the L25 cells by tactile stimulation of the siphon skin. Simultaneous recordings were made from an L25 cell and an LE sensory neuron while a burst of 14 action potentials was initiated in the sensory neuron with a train of depolarizing current pulses (Fig. 11A). The sensory neuron discharge triggered a burst of activity in the L25 cell similar to a spontaneous burst in L25 (Fig. 11B).

Just as the L25 cells are activated by stimulation of the siphon nerve (see above), they are also activated by stimulation of all the other peripheral nerves and connectives of the abdominal ganglion. As with the siphon nerve (Fig. 10), the usual pattern is brief short-latency excitation followed by short-latency inhibition and then slow excitation. In many cases the slow excitation is associated with EPSPs that lead to a long-lasting spike discharge in the L25 cells. A typical example is illustrated in Fig. 12. Figure 12A shows simultaneous recordings from three motor neurons and an L25 cell when a single shock was delivered to the left connective. The response pattern of L25 is as described above. The L14 ink motor neurons received characteristic fast EPSPs followed by slow excitatory input (see also Ref. 13). The LD cell responded with a burst of spikes, while the LB cell showed inhibition. In Fig. 12B, L25 was hyperpolarized and the left connective was again stimulated. There was a dramatic reduction in the input to the LD and LB cell, but the response in L14 was unchanged. The lack of any change in response of the L14 cell is expected, since the L14 cells are not activated during spontaneous respiratory pumping (12; unpublished observations), and directly firing L25 produces no input to L14 (not shown). However, it is clear that just as L25 plays a major role in mediating afferent input from the siphon skin and siphon nerve, it also plays a major role in mediating input to respiratory pumping follower cells produced by electrical stimulation of other pathways. This result is particularly interesting, since connective stimulation is used frequently to mimic sensitizing stimuli to the head of the animal and to synaptically activate neurons in the abdominal ganglion.

DISCUSSION

Respiratory pumping: a possible model system for analyzing mechanisms underlying initiation, patterning, and modulation of cyclic neural activity

While some information is now available on the mechanisms underlying cyclic bursting activity in individual neurons and neural networks with relatively short cycles (10 s), little is known regarding the mechanisms that govern bursting activity with long interburst intervals (1, 3, 24, 27, 29, 36, 38, 47, 54, 56, 57, 59, 62). While the present results do not address specifically the mechanisms underlying the generation of the cyclic activity underlying respiratory pumping, the identification of the command and pattern-generating neurons makes it possible to begin to investigate these questions. Indeed it is interesting that the L25 cells have many of the features which, based on experimental and theoretical studies, have been proposed to be critical for cyclic neural activity (see Ref. 23). These include electrical coupling between bursting neurons and mutually inhibitory connections to other interneurons. The causal role of these features as well as the possible involvement of intrinsic membrane properties and cyclic activity in an electrogenic pump will be the subject of future studies on the L25 cells.

Location of command and pattern-generating neurons

A major obstacle to an analysis of the neural control of respiratory pumping has been the difficulty in identifying major elements having command or pattern-generating functions. In a previous report (10) a single cell identified as L25 was found that seemed to be a command neuron. The present results show that there are multiple L25-like cells with mutually excitatory interconnections. The initial failure to identify more than one L25 cell was due to the fact that the L25 cells are generally located several layers
deep and their typical location is considerably more medial and dorsal than the L25 cell originally identified.

While the L25 cells appear to be a fairly homogeneous population of cells, more detailed analyses may reveal differences in the bursting characteristics (e.g., Ref. 47) as well as differences in their projections to the various follower interneurons and motor neurons activated during respiratory pumping. The present results also do not exclude the possibility that there are other neurons coupled to the L25 cells located in regions of the ganglion outside the area diagrammed in Fig. 1.

Neural circuit for spontaneous and evoked respiratory pumping movements

COMMAND ELEMENT. A simplified wiring diagram of the neural circuit mediating spontaneous and evoked respiratory pumping movements is illustrated in Fig. 13. The major element of this circuit is a cluster of mutually excitatory command neurons identified as the L25 cells. To simplify the diagram only two of these cells are illustrated but recordings have been made from seven such cells in the same experiment, each having similar electrophysiological properties.

In many cases spontaneous spike activity in the L25 cells precedes any detectable responses in the follower cells (e.g., Fig. 2). In other cases, however, spike activity in an L25 cell follows the start of synaptic input to the motor neurons and interneurons (cf. Figs. 3A and 4A, which are taken from the same experiment). In addition to the observation that some spontaneous synaptic input to respiratory pumping follower cells precedes spontaneous activity in a given L25 cell, synaptic activity in the follower cells can also precede spike activity in an L25 cell that is artificially depolarized or when an L25 cell is released from tonic hyperpolarization (e.g., Fig. 3B). One interpretation of these observations is that the L25 cells have different spike thresholds and do not fire spikes in perfect synchrony, so that an L25 cell not recorded from can begin to fire and produce some synaptic effects before the entire network is brought to threshold. Figure 6A1 illustrates one spontaneous burst where activity in L25B precedes activity in L25A.

Artificially firing spikes in an L25 cell can mimic the synaptic input in respiratory pumping follower cells that occurs spontaneously, and hyperpolarizing a single L25 cell can phase shift a spontaneous burst. These observations indicate that not only do the L25 cells play a major role in mediating the synaptic input to the follower cells but also that they are causally involved in burst generation. Hyperpolarization of a single L25 cell, however, was never able to prevent completely the spontaneous burst of synaptic input to the follower cells associated with

FIG. 13. Simplified wiring diagram of the neural circuit mediating spontaneous and evoked contractions of the gill. The command and pattern-generating neurons mediating spontaneous respiratory pumping are the L25 cells. While only two are illustrated, there appear to be at least seven of these cells that are mutually excitatory. At least all of the mutually excitatory connections are mediated via electrical synapses. Bursts of spikes in the L25 cells inhibit the cardiac command neuron L10 and interneuron L24 and excite the LD gill and siphon motor neurons. A wide variety of other cells in the ganglion, including the LB siphon motor neurons and motor neuron L7, receive characteristic synaptic input during respiratory pumping cycles, but these cells are not included to simplify the diagram (see Ref. 10). At least some of the excitatory input to the LD cells is mediated by an interneuron (L26, see Refs. 10, 41, 42), which is not illustrated but is interposed between the L25 cells and the LD cells. Activity in gill motor neurons LDG1 and LDG2 causes large contractions of the gill. Tactile stimulation of the siphon causes a reflex withdrawal of the gill, which is mediated in part by monosynaptic connections between mechanoreceptor sensory neurons innervating the siphon skin and the gill motor neurons. Stimulation of the skin also excites the L25 cells, resulting in an enhancement and prolongation of the response produced by the monosynaptic component of the reflex. Solid lines indicate connections believed to be monosynaptic. Synapses labeled FE represent fast excitatory connections while FI and SI represent fast and slow inhibitory connections, respectively.
spontaneous respiratory pumping. This presumably is due to the fact that each L25 cell makes only a limited contribution to the positive feedback among other L25 cells.

Of various command and pattern-generating networks that have been investigated (2, 3, 24–26, 29, 36, 38, 47, 54, 56, 57, 59, 60) this network seems most similar to the cyberchron system in Helisoma (36, 38, 47) and the neurosecretory endogenous oscillators in Lymnaea (60). The cyberchron neurons show many parallels to the L25 neurons. First, they consist of a network of mutually excitatory cells that are electrically coupled to each other. The coupling coefficient of the cyberchron neurons, however, appears larger than that of the L25 cells. Second, a brief intracellular depolarizing current pulse to one cyberchron neuron results in spike activity that greatly outlasts the depolarization. In some cases this procedure leads to the initiation of multiple bursts in the cyberchron cells, but such multiple bursts were never observed in the L25 cells. Third, the cyberchron cells can fire spontaneously but can also be activated by electrical stimulation of peripheral nerves. Finally, hyperpolarization of a cyberchron neuron can phase shift the bursts.

The properties of the L25 cells also bear a number of similarities to the neurosecretory endogenous oscillators in Lymnaea stagnalis (60). These cells, referred to as the “light-yellow cells” (LYC), are mutually excitatory and weakly electrically coupled. They fire spontaneous bursts of spikes at intervals of approximately 10 min. Current injection into one LYC can initiate bursting in a second LYC, while transient hyperpolarization of an LYC cell during a burst can terminate the burst in the one cell as well as the burst in other LYC cells. Like the L25 cells, bursts in the LYC cells do not occur in perfect synchrony.

Aspects of the positive feedback and electrical coupling between the L25 cells are also similar to the trigger-group neurons in Tritonia (25).

OUTPUTS OF L25 CELLS. A wide variety of motor neurons and interneurons in the abdominal ganglion receive characteristic patterns of synaptic input during spontaneous respiratory pumping cycles. Figure 13 illustrates only a small fraction of these cells that include the LD motor neurons and interneurons L10 and L24. L10 is a command neuron for increasing cardiac output and vasomotor tone (41), while the LD cells include gill motor neurons LDG1 and LDG2, siphon motor neurons LDS1–3 (42, 43, 49, 50), and cardiovascular motor neurons LDHE and LDHI (46). While the LD motor neurons are excited during spontaneous respiratory pumping bursts, other gill and siphon motor neurons, such as L7, the L9 group and the LBS cells, are inhibited (42, 43, 49, 50). For a more complete wiring diagram of the cells activated during spontaneous respiratory pumping see Refs. 10, 41, 42.

While the L25 cells are causally involved in mediating the synaptic input to the various follower cells, it is not possible to say with certainty to what extent these effects are monosynaptic. Indeed, there is evidence that at least two interneurons, previously identified as L26 and L27, are interposed between the L25 cells and motor neurons activated during spontaneous respiratory pumping (10). With the ability to find the L25 cells reliably, this question will be able to be examined in considerable detail.

INPUTS TO L25 CELLS FROM TACTILE STIMULATION OF SKIN AND ELECTRICAL STIMULATION OF NERVES AND CONNECTIVES. Tactile stimulation of the siphon skin causes reflex withdrawal of the gill and siphon through monosynaptic connections from sensory neurons to motor neurons (Fig. 13 and Refs. 6, 19, 30) and by identified excitatory interneurons interposed between the sensory neurons and the motor neurons (6, 30). The results of the present study indicate that the L25 cells are major interneurons in this reflex pathway (Fig. 13). Tactile stimulation of the siphon or gill or electrical stimulation of the siphon, genital, pericardial, and branchial nerves as well as both pleuroabdominal connectives can cause profound, long-lasting discharges in the L25 cells, which make major contributions to the activity of the motor neurons (Figs. 9, 10, 12). At least part of the activity in the L25 cells produced by such stimuli appears to be due to regenerative feedback among the L25 cells.

There is considerable variability in the response of the L25 cells to stimulation of the siphon skin. In some cases they fire with high-frequency long-lasting discharges (e.g.,
Fig. 9). In other experiments they fire only a small number of spikes for a short period. The reason for this variability is unknown but it may be due to the state of arousal of the animal or uncontrolled variables in the dissection procedure. This variability may account for why the L25 cells make an important contribution to the defensive withdrawal reflex under some circumstances but make only a modest contribution under others (see also Refs. 9, 37, 52).

It has previously been observed that repeated electric shocks to the connective lead to increments in the frequency of IPSPs in L7 and L10. It has been suggested, furthermore, that interneuron II (L25) may be a source of this short-term synaptic modulation, perhaps by an alteration in the endogenous pacemaker rhythm of the interneurons (61). The identification of the L25 cells provides an opportunity to examine this hypothesis directly.

Implications for analysis of gill-withdrawal reflex and its plasticity

The defensive gill-withdrawal reflex has been utilized as a model system to study various forms of simple learning, including short- and long-term habituation, sensitization, and associative learning (14, 15, 45, 51, 52). Given that the L25 cells can make a major contribution to the reflex contraction of the gill, a logical extension of these results is that they may also contribute to modifications of the reflex. Indeed, Pinsker and colleagues (20, 21, 37) have recently suggested, based on extracellular recording from peripheral nerves of intact animals, that activation of the respiratory command network is an important component of both the initiation and the sensitization of reflex-activated siphon and gill withdrawal. Up to this time, all of the plasticity observed in the central nervous system during short- and long-term habituation and sensitization has been attributed to changes in the release of transmitter from the sensory neurons on their various follower motor neurons and interneurons (7, 11, 16–19). At the very least the L25 neurons should reflect such alterations in their input from sensory neurons, being less activated during habituation when sensory neuron transmitter release is reduced and more activated during sensitization when transmitter release is facilitated. In addition though, the properties of the L25 cells could be modulated directly and may thus serve as an additional neural locus contributing to habituation and sensitization.

Recently, Carew et al. (15) have shown that the defensive gill- and siphon-withdrawal reflex undergoes classical conditioning when a weak stimulus to the siphon skin (conditioned stimulus, CS) is paired with a strong stimulus to the tail (unconditioned stimulus, US). After several pairings the gill and siphon withdrawal (conditioned response, CR) is augmented, and this augmentation is pairing specific and persists for at least 2 days. Since both the CS and US can trigger respiratory pumping (32, 42, 43, 52) and the L25 cells can be activated both by input from the siphon skin (Figs. 9 and 10) and by pathways mediating input from the tail (Fig. 12), the L25 cells serve as a point of convergence of the CS and US pathways. It therefore seems possible that changes in the degree of activation of the L25 cells by the CS in response to pairing with the US may contribute to the associative modification.

Both sensitization and classical conditioning produce a large increase in the duration of gill and siphon withdrawal (15, 51). Since monosynaptic connections between sensory neurons and motor neurons are unlikely to show much increase in duration (18, 19), recruitment and enhancement of activity in the L25 cells may be critical in transforming changes in PSP amplitude to changes in the duration of the behavioral responses.

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