

DISTRIBUTION OF INTERSEGMENTAL INTERNEURONES THAT CAN RESET THE RESPIRATORY RHYTHM OF THE LOCUST

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Summary

Interneurons in the respiratory rhythm generator of the locust were identified by means of intracellular recording and staining techniques. A description is made of the properties and structures of nine intersegmental neurones which reset the respiratory rhythm when injected with current pulses. All but one of these neurones discharged in phase with expiration. The injection of constant depolarizing current into these interneurons altered the respiratory rate (increase for six, decrease for three). The respiratory rhythm generator extends more posteriorly within the ventral nerve cord than the metathoracic ganglion. In the first fused abdominal ganglion, four individual interneurons were identified descending into the unfused abdominal ganglia. In the first unfused abdominal ganglion an interneurone which reset the respiratory rhythm was found ascending into the metathoracic ganglion. The respiratory rhythm generator also extends more anteriorly within the ventral nerve cord than the metathoracic ganglion. Two interneurons influencing the respiratory rhythm send their axons from the first fused abdominal ganglion into the meta- and mesothoracic ganglia. One of these directly excited a mesothoracic interneurone which also influenced the respiratory rhythm when injected with current. In the suboesophageal ganglion another interneurone was found which, although capable of resetting the respiratory rhythm, was not always active during respiration. We conclude that the respiratory rhythm generator is distributed over abdominal, thoracic and suboesophageal ganglia. At least one part of the respiratory rhythm generator (in the suboesophageal ganglion) is not always active and can be recruited during vigorous respiration. Thus the number of active components in the respiratory rhythm generator is variable and additional elements can be recruited depending on the behavioural situation.

Introduction

Considerable information has been obtained about the neuronal mechanisms underlying certain rhythmic behaviour in insects, such as flight and walking. Surprisingly, a far simpler rhythmic behaviour, respiration, has received relatively

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little attention, although respiratory movements are exhibited in highly dissected preparations so that the neuronal basis of the behaviour can be studied by means of intracellular recordings. The rhythm has been found to be generated at the interneuronal level, and two sets of interneurons have been proposed to drive closer and opener spiracle motoneurons (Burrows, 1974, 1975*a,b*). The first interneurone in the respiratory rhythm-generating system, a neurone capable of resetting the respiratory rhythm, has been identified in the first fused abdominal ganglion (Pearson, 1980). This interneurone descends in the abdominal nerve cord, is active in phase with expiration and probably has a role in coordinating the respiratory rhythm in abdominal ganglia. Another interneurone which coordinates the activity in the thoracic spiracles has also been described (Burrows, 1982). This interneurone ascends from the metathoracic ganglion and inhibits spiracle motoneurons in the mesothoracic ganglion. However, current injection into this neurone does not influence the respiratory rhythm. In the past six years, no further information has been published on the role of interneurons in the respiratory system. This can in part be attributed to the technical difficulty of recording from respiratory interneurons for a sufficient time to investigate their properties. The processes of these interneurons appear to be smaller than those involved in flight (Robertson & Pearson, 1985) and jumping (Gynther & Pearson, 1986).

Some obvious gaps in our knowledge are the exact distribution of the respiratory rhythm generator within the nerve cord, the pattern of interconnections between sensory organs, interneurons and motoneurons, the mechanisms for generating the rhythm and the function of peripheral feedback. In this study we will concentrate only on one problem, the extent to which the respiratory oscillator is distributed within the ventral nerve cord. It is generally assumed that in locusts a primary oscillator is located in the metathoracic ganglion and that this oscillator controls secondary oscillators in the abdominal ganglia (Miller, 1966; Lewis *et al.* 1973). Only one neuronal element of the primary oscillator has been identified (Pearson, 1980) and no neurones in the secondary abdominal oscillators have been identified. There is uncertainty about whether the rhythm generator contains elements rostral to the metathoracic ganglion. Some muscles in the thorax and the neck are involved in respiration but respiratory movements in these muscles cease after cutting the anterior connectives of the metathoracic ganglion. From these results it has been concluded that there is no respiratory rhythmic centre anterior to the metathoracic ganglion (Miller, 1960*b*). However, it is conceivable that neurones in the rhythm generator are located rostral to the metathoracic ganglion even though these ganglia might not be autorhythmic. Changes in abdominal pumping following transection of the meso- and meta-thoracic connectives (Lewis *et al.* 1973) may be due to removal of rostrally located elements of the oscillator.

Our strategy for investigating the distribution of the respiratory rhythm generator within the ventral nerve cord has been to search a variety of ganglia for interneurons which are rhythmically active during respiration and which reset the respiratory rhythm when injected with current.

Materials and methods

Animals

Adult male and female *Locusta migratoria* were obtained from a colony at the University of Alberta. All experiments were performed at room temperature (22–25°C).

Preparation

Following the removal of the wings and legs the animals were mounted dorsal side up on a cork board. To expose the mesothoracic ganglion, a dorsal incision was made in the thorax, and the gut and small muscles overlying the ganglion were removed. Abdominal ganglia were exposed in a similar manner, but the dorsal incision was extended more posteriorly. The suboesophageal ganglion was exposed by opening the head capsule and removing the mandibular and dorsal neck muscles as well as the tentorium. The ganglion from which recordings were obtained was supported on a stainless-steel platform and kept covered with locust saline. Previous studies have shown that expiratory activity occurs almost synchronously in all abdominal segments (Lewis *et al.* 1973), so it was usually monitored by placing a hook electrode on one of nerves 8, 9 or 10 of the metathoracic ganglion. These nerves contain the axons of motoneurons which discharge in phase with expiration (Lewis *et al.* 1973). In some cases electromyogram recordings were obtained from muscle 177 (Lewis *et al.* 1973) which is active in phase with inspiration.

Recording and staining

Intracellular recordings were obtained from neuropile processes of neurones which were rhythmically active in phase with the respiratory rhythm. The recording electrodes were filled with a 5 % solution of Lucifer Yellow in distilled water, which was injected following recording to allow the identification of the recorded neurone. Electrode resistance was in the range 50–200 M Ω . Only the ganglia from which recordings were obtained were processed, as described by Robertson & Pearson (1982). Thus, no information exists on the terminals of stained neurones in other ganglia. The interneurons were identified and numbered according to the three-digit numbering scheme of Robertson & Pearson (1983). The first digit indicates the path of the axon and its position relative to the soma. The two following numerals have been assigned arbitrarily.

Data analysis

Pattern of activity

Phase histograms were calculated by a DEC LSI 11/23 computer to characterize the profile of the neurone's activity during the respiratory cycle. The cycle duration was measured by triggering from the onset of a burst in the expiratory motor nerve to the onset of the following expiratory burst. The cycle duration was

normalized to 1 and divided into 50 bins. The average number of spikes occurring in each bin was then determined.

Effect of constant-current injection

Both positive and negative currents (± 2 –6 nA) were injected into a neurone to examine whether it had an accelerating or decelerating effect on the respiratory rhythm. The effect was assessed by averaging the duration of four respiratory cycles immediately before, during and after the period of increased activity in the neurone. The average of 4–5 of these stimulation experiments and the deviation about the mean were then represented in a histogram.

Resetting of the respiratory rhythm

To examine whether the neurone had a resetting effect on the rhythm, short current pulses were injected (duration 100–300 ms, in one case 1000 ms; current magnitude 2–5 nA). The effect on the respiratory rhythm was calculated by subtracting the duration of the cycle in which the current pulse was applied (t_2) from the duration of the undisturbed cycle immediately preceding this cycle (t_1) and dividing this difference by t_1 ; i.e. $(t_2 - t_1)/t_1$. The effect of stimulation on the first (t_3) and the second cycle (t_4) following the cycle in which the stimulus was applied (t_2) was calculated in a similar way, i.e. $(t_3 - t_1)/t_1$ or $(t_4 - t_1)/t_1$. The phase of application of the stimulus within a respiratory cycle was calculated by dividing the interval between the onset of the cycle in which the stimulus was applied and the onset of the stimulus pulse by the duration of the preceding undisturbed cycle t_1 . For expiratory interneurons a respiratory cycle was taken as the time between the onset of two consecutive bursts of activity in the expiratory motor nerve. For the only inspiratory neurone examined a respiratory cycle was measured as the time between the onset of two consecutive bursts of activity in the inspiratory neurone.

It should be noted that in this study phase 0 is the onset of activity in the expiratory motor nerve, whereas in the study of Pearson (1980) phase 0 is the end of the burst in the stimulated interneurone. This difference causes a shift in phase values but does not affect the result.

Comments on analysis

All stimulation experiments were reproduced 10–30 times for the same neurone in the same animal and, except for neurone 725, each neurone was examined in different animals (326 three times, 327 five times, 328 four times, 329 five times, 377 six times, 516 twice, 720 five times). This was sufficient to assess in a qualitative manner whether there was an effect on the respiratory rhythm. However, because of variability in the respiratory rhythm among animals and in the number of action potentials evoked by current injection it did not enable detailed quantification of the data. Reset curves obtained for the same neurones in different animals had a similar shape, but these phase–response curves could be shifted by as much as 0.2. The variability in the number of spikes evoked by the current injection could have

been due to our recording in different sites in the neurones or to the different levels of spontaneous activity in the neurones. The variable excitation levels of a neurone due to its rhythmic modulation may also cause distortions of reset curves even in the same animal. A pulse given during the phase in which the neurone was depolarized resulted in a much higher spike frequency than a pulse applied during the neurone's inhibitory phase. We did not attempt to control spike numbers at different phases. Therefore, all diagrams in this study can give only qualitative information on the effect of current injection on the respiratory rhythm.

Results

The aim of this study was to identify, in different ganglia, interneurons which are elements of the respiratory rhythm-generating system. In accordance with previous studies in other motor systems (Friesen *et al.* 1978; Weeks, 1981), a neurone was considered to be an element of the rhythm generator if (1) its cell membrane was rhythmically polarized in phase with the activity in a respiratory motor nerve and (2) current injection into this neurone shifted the phase of the respiratory rhythm (i.e. reset the rhythm). A cell's ability to shift the phase of the respiratory rhythm was always accompanied by its ability either to speed or to slow the respiratory rhythm and this was therefore consistently taken as a third criterion. Only nine interneurons which met these criteria will be described in this study. However, 21 interneurons were found which met the first but failed the second and third criteria, and which may also be elements of the respiratory rhythm generator.

Interneurons in the first fused abdominal ganglion

The metathoracic ganglion consists of four neuromeres, the 'true' metathoracic ganglion and the first three abdominal ganglia which are fused posteriorly to the metathoracic ganglion. In the remainder of this study these abdominal neuromeres will be referred to as the 'fused abdominal ganglia'. The unfused abdominal ganglia are single neuromere ganglia located posterior to the metathoracic ganglion. It is generally assumed that the primary respiratory rhythm generator is located in the metathoracic ganglion (Miller, 1966). However, this assumption is based mostly on lesion experiments which cannot attribute an effect to a specific neuromere of this multineuromere ganglion. Therefore, it remains uncertain whether the primary rhythm generator is located in the true metathoracic ganglion or in one or more of the fused abdominal ganglia. Thus, as a first attempt to identify some of the neuronal elements involved in the generation of the respiratory rhythm, we recorded intracellularly from neurones in the neuropile regions of all four neuromeres of the metathoracic ganglion. In the true metathoracic ganglion we found only one respiratory interneurone and this had no effect on the respiratory rhythm. In the second fused abdominal ganglion we found five interneurons rhythmically active in phase with respiration but none of them affected the respiratory rhythm. No respiratory rhythmic interneurons were

found in the third fused abdominal ganglion. The only interneurons we found that were able to affect the respiratory rhythm had their somata located in the first fused abdominal ganglion. This finding suggests that a major part of the primary respiratory rhythm generator is located within this ganglion. However, it cannot be excluded that there are also undiscovered neurons in other ganglia.

Interneurons 326, 327, 328, 329

Interneurons have been described in the first fused abdominal ganglion which cause an increase in the respiratory rate when injected with constant depolarizing currents (Pearson, 1980). Injection of short pulses of depolarizing currents resets the rhythm. Extracellular recordings from the connectives indicate the presence of more than one pair of these neurons (Pearson, 1980). In this section we will describe four such neurons which are anatomically and physiologically distinct. Each of these neurons was found in at least two different animals and could be re-identified by its shape and physiology. The structure of these interneurons (326, 327, 328, 329) has some common features. The somata of all the neurons are located near the dorsal surface in the lateral region of the first fused abdominal ganglion and their axons leave the ganglion in the abdominal connective contralateral to their somata. The primary neurites run from the somata in an anterior medial direction. Some features, however, allow distinctions to be made between these neurons. 326 has a prominent secondary process arising ipsilateral to the midline and running towards the midline before projecting anteriorly (Fig. 1A). 327 has two prominent secondary processes running anteriorly, ipsi- and contralateral to the midline (Fig. 1B). 328 has two prominent secondary branches arising near the soma and running parallel to the main neurite (Fig. 1C). One of these branches nearly overlaps the main neurite, the other runs more anteriorly to the main neurite. 329 has a prominent secondary branch arising contralateral to the midline and running towards the midline before projecting anteriorly. It also has a characteristic secondary neurite running posteriorly from the midline (Fig. 1D).

All neurons discharged in phase with expiration (see, for example, Fig. 1E,F). The rhythmic depolarizations of these interneurons had several common features. In all neurons these depolarizations commenced prior to the onset of activity in the expiratory motor nerve and reached a maximum coincident with or just after the onset of activity in the motor nerve. In the absence of current injection, spikes occurred on the rising phase of the depolarization and therefore began prior to the onset of expiratory activity (Fig. 1E). If the neuron was only weakly active (activity reduced by injecting negative current) spikes occurred later, almost at the same time as the onset of expiratory activity (Fig. 1F). During expiratory activity the depolarizing input to these neurons was not uniform. The depolarizations of 327, 328 and 329 declined during almost the entire expiratory phase and, in weakly active neurons, spikes were produced only at the onset of expiratory activity, as is shown for 328 (Fig. 1F). The depolarization of 326 was more plateau-like and in the hyperpolarized neuron spikes were sometimes

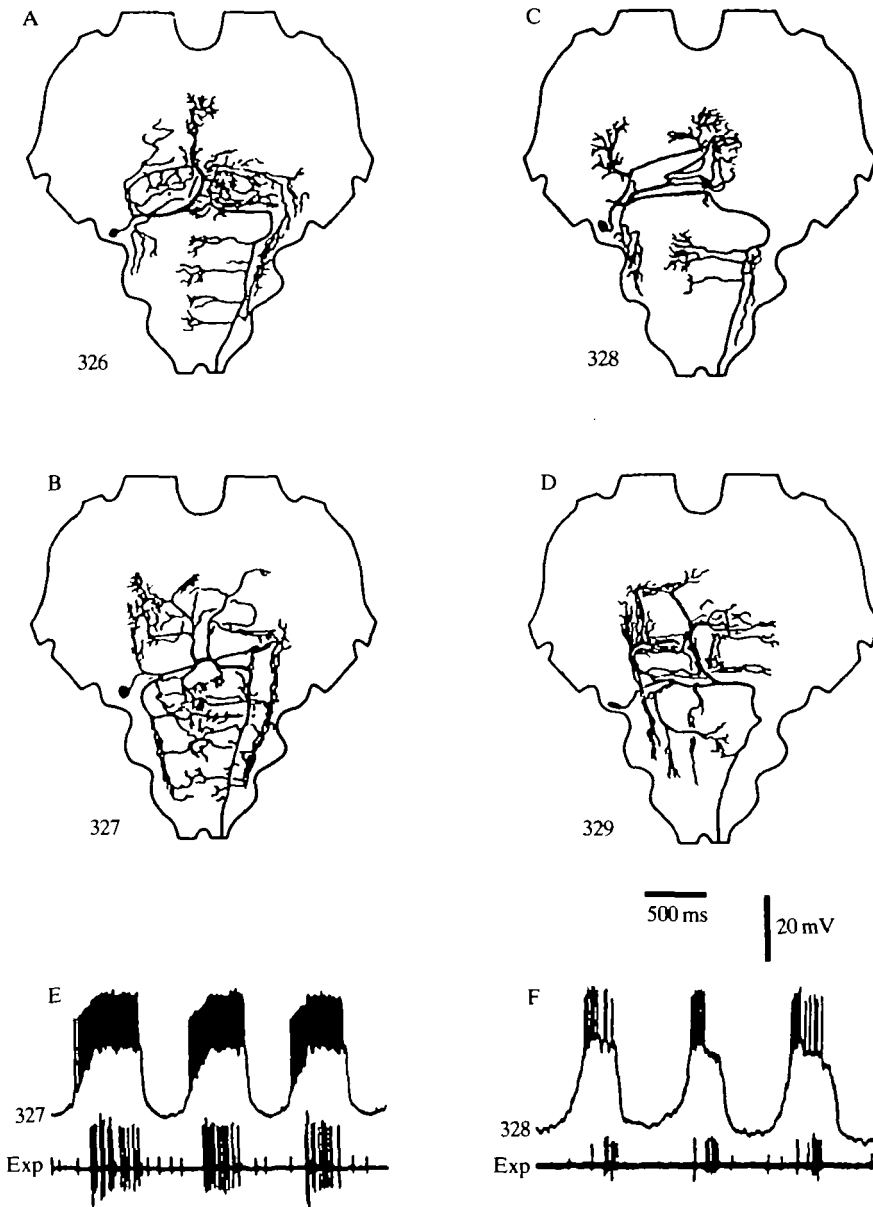


Fig. 1. Characterization of four descending expiratory interneurons in the first fused abdominal ganglion. (A–D) Structure of the descending interneurons 326–329. (E) Intracellular recording from 327 (upper trace), EMG recording from nerve 10 of the metathoracic ganglion (lower trace). Spike activity occurs before the onset and ceases after the offset of expiratory activity (Exp). (F) Intracellular recording from 328 (upper trace), EMG recording from nerve 8 of the metathoracic ganglion (lower trace). 328 was hyperpolarized by negative current to show the shape of the compound depolarization. A phasic depolarization is visible at the onset of expiratory motor activity.

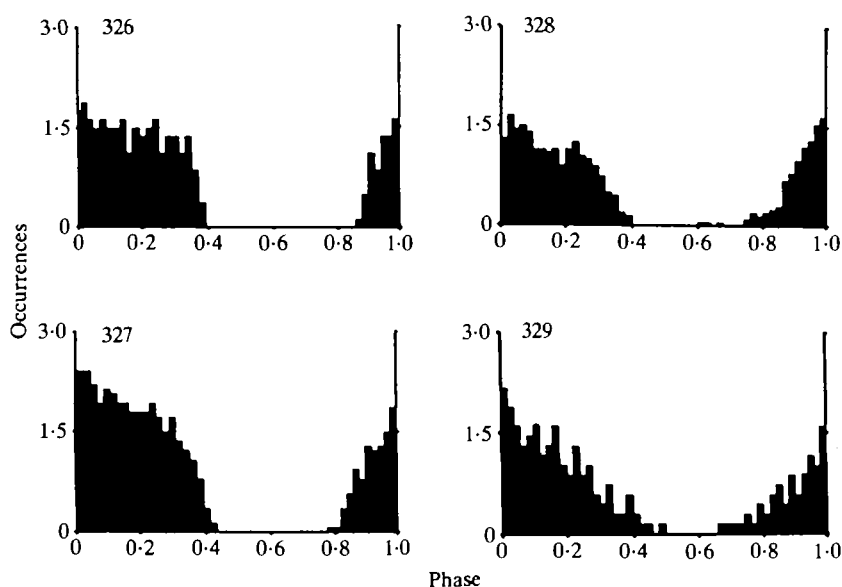


Fig. 2. Phase histograms of the four descending expiratory interneurons. 326: number of cycles (N) = 8, average period (AP) = 528 ms, average bin width (AB) = 10.6 ms. 327: N = 14, AP = 804 ms, AB = 16 ms. 328: N = 20, AP = 655 ms, AB = 13 ms. 329: N = 7, AP = 839 ms, AB = 17 ms.

produced even at the end of the expiratory phase (see, for example, Fig. 4D). Just after the termination of activity in the expiratory motor nerve, all neurones repolarized rapidly and, in the absence of current injection, spikes were usually not produced after the termination of expiratory activity (Fig. 1E). The spike distribution of the interneurons in the absence of current injection is shown in the phase histograms of Fig. 2. Spike activity of all neurones started before the onset of expiratory activity (phase value 0) at a value around 0.8, and activity ceased with the termination of activity in the expiratory nerve at a value around 0.4. These phase histograms also show that the spike activity of these neurones was not uniform during the expiratory phase. For example, it is obvious that spike activity declined in 329 but was steadier in 326.

An increase in spike activity evoked either by injecting depolarizing constant current into these neurones or by releasing them from constant hyperpolarizing current resulted in an increase in the respiratory rate (Fig. 3). Injecting short depolarizing pulses into any of these four neurones could reset the respiratory rhythm. Phase-response curves were calculated for all four neurones (Figs 4, 5). For neurone 326, we also evaluated the effect of current injection on the second and third cycle after stimulation, to demonstrate that compensation for the stimulus effect in the first cycle was not made in the subsequent cycles. Short current pulses applied into 326 during the phase in which it was usually inactive (phase range of 0.4–0.6) greatly prolonged the respiratory cycle (Fig. 4A). This effect was lessened if the pulse was applied during expiration, at the time 326 was

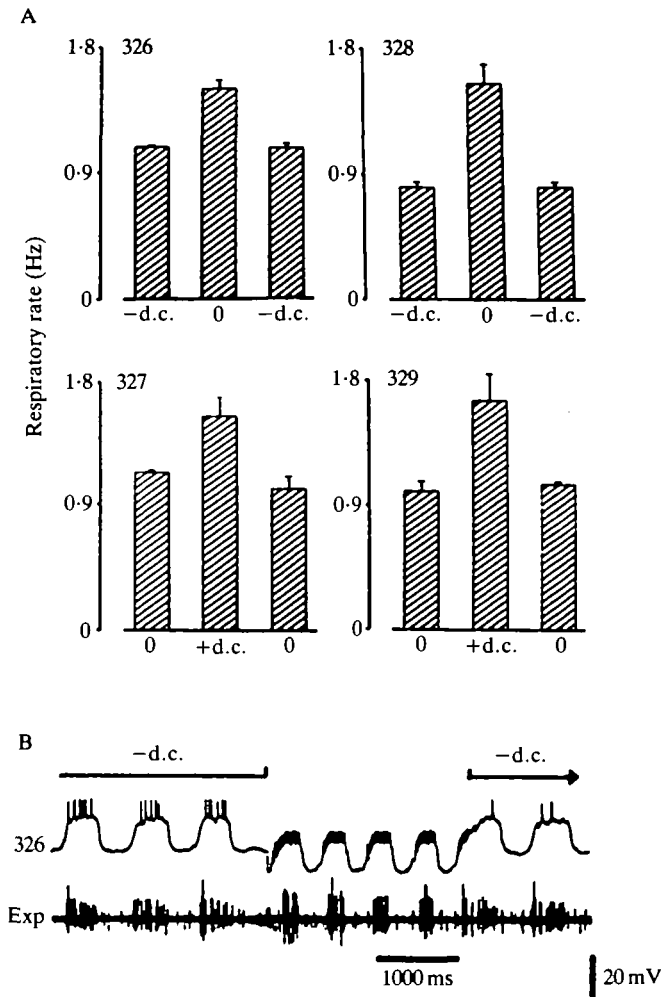


Fig. 3. Activity increase in the descending interneurons causes an increase in the respiratory rate. (A) Each histogram is the average of four stimulation experiments. For each stimulation experiment we calculated the respiratory rate from four subsequent respiratory cycles before, during and after activity increase in the neurone. Activity increase in a neurone was obtained either by release from negative or by injection of positive current (d.c.). The average spike activity of the neurones for each histogram was: 326, -d.c. = 10 Hz, no d.c. = 100 Hz; 327, no d.c. = 72 Hz, +d.c. = 143 Hz; 328, -d.c. = 3 Hz, no d.c. = 100 Hz; 329, no d.c. = 60 Hz, +d.c. = 120 Hz. (B) Stimulation experiment. Release of inhibition in 326 (upper trace) resulted in an increase in the respiratory rate. Lower trace (Exp), EMG recording from nerve 9 of the metathoracic ganglion.

usually active (phase range of 0–0.4). Current pulses applied just before or at the beginning of expiration, in a phase in which 326 was also active (phase range of 0.8–1.0) shortened the first respiratory cycle as did pulses applied at a time when 326 can be active when injected with constant depolarizing current (phase range of

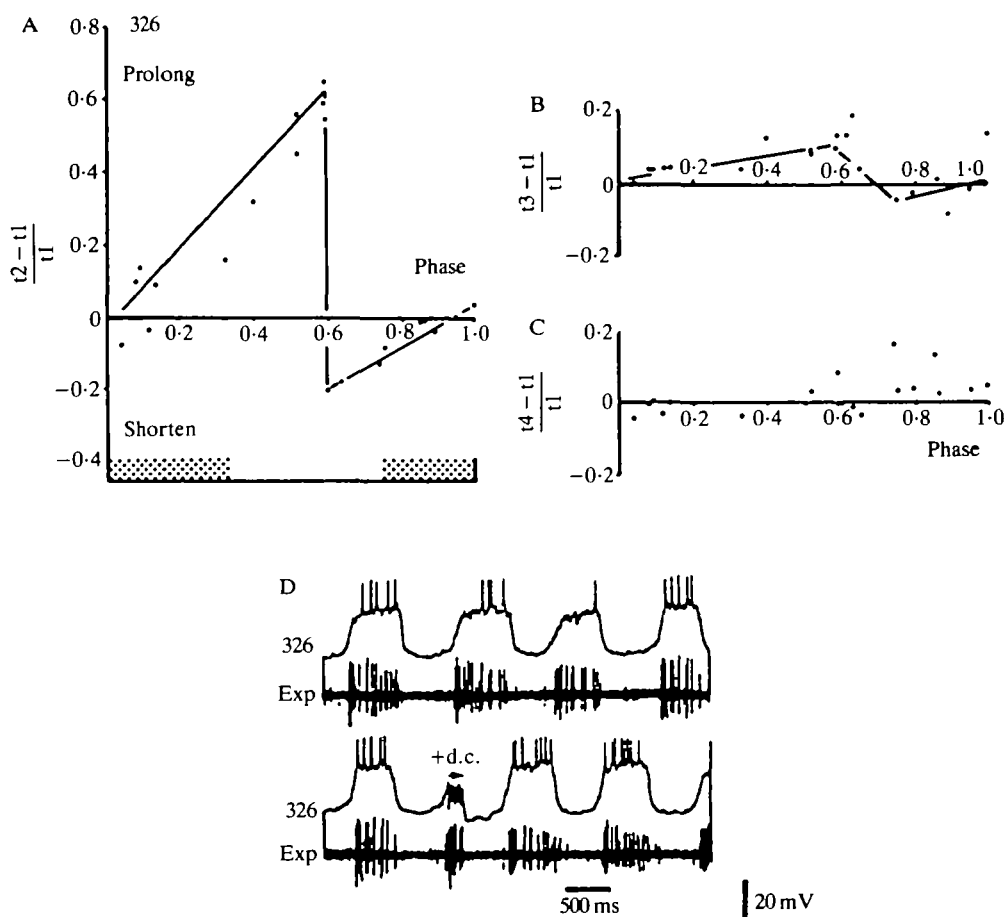


Fig. 4. Resetting of the respiratory rhythm by injecting short current pulses into the descending expiratory interneurone 326. (A) Phase-response curve for the cycle in which the stimulus was applied (t_2). The dotted area indicates the time in the respiratory cycle in which 326 was active. (B) Phase-response curve for the cycle immediately following the cycle in which the stimulus was applied (t_3). (C) Phase-response curve for the second cycle (t_4) following the cycle in which the stimulus was applied. Note that the stimulus effect is not compensated in subsequent cycles. Duration of the stimulus pulses = 300 ms, magnitude of current = 3 nA. (D) Example of a stimulation experiment. The 326 recording in D was obtained in a different animal from that used for A–C. Upper two traces, activity of 326 (upper trace) recorded simultaneously with an EMG of nerve 9 of the metathoracic ganglion (Exp, lower trace). 326 was constantly hyperpolarized (–2 nA). Lower two traces, the current pulse (d.c.) (+3 nA, 150 ms) injected into 326 (upper trace) reset the respiratory rhythm.

0.6–0.8) (Fig. 4A). The stimulating pulses clearly reset the rhythm since no compensation was found in the second and third cycles (Fig. 4B,C). In the second cycle following the stimulus a slowing effect was also visible at phases 0–0.6 and a

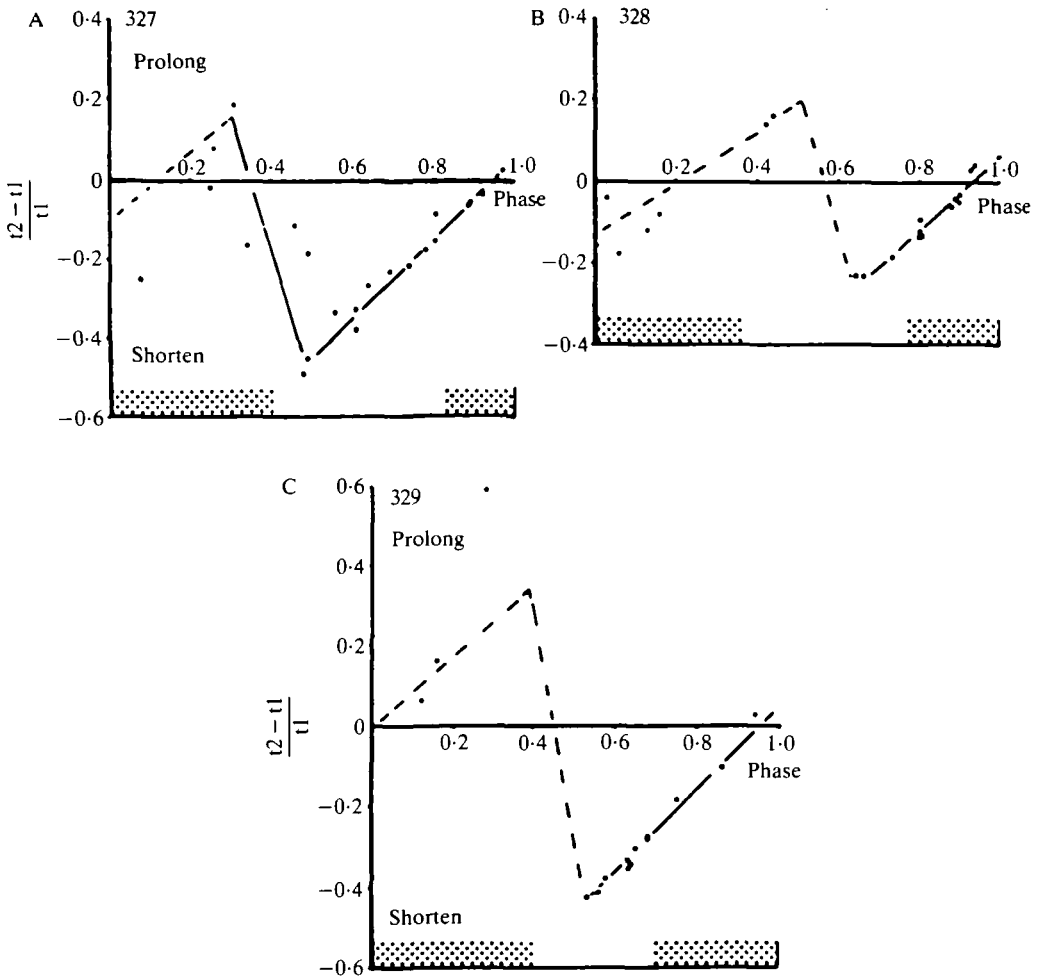


Fig. 5. Phase-response curves of the descending expiratory interneurons 327, 328, 329. (A) 327, duration of pulse = 200 ms, magnitude of current = 5 nA; (B) 328, duration of pulse = 200 ms, magnitude of current = 4 nA; (C) 329, duration of pulse = 300 ms, magnitude of current = 5 nA. In this and all subsequent reset curves dotted lines are drawn if only few values with a high scatter were obtained and solid lines are drawn if many values with a relatively low scatter were obtained. At the bottom of all phase-response curves the dotted areas indicate the average times in the cycle in which the neurones were active.

slight shortening of cycle duration was visible at a phase around 0.8 (Fig. 4B). This weak shortening effect in the second cycle seems to be compensated in the third cycle but, because of the high scatter, this compensation might be not significant (Fig. 4C).

Interneurons 327, 328 and 329 had reset curves similar to the one described for 326 (Fig. 5). A shortening of cycle duration was found with a relatively low scatter if the stimuli were applied with phase values between 0.6 and 1.0. The

interneurone described by Pearson (1980) also has a shortening effect at a similar phase range. Differences between the neurones 327, 328 and 329 were found in the phase range 0–0.6. Since the values in this phase range were relatively highly scattered and for reasons mentioned in Materials and methods, it is not possible to determine whether these differences are significant.

Interneurone 516

Of six inspiratory interneurones found in the metathoracic ganglion only one, interneurone 516, was able to reset the respiratory rhythm. The cell body of interneurone 516 is located in the same dorsolateral region of the first fused abdominal ganglion (Fig. 6A). The primary neurite leaves the soma in an anterior medial direction. Before the main neurite crosses the midline one secondary branch projects anteriorly and another posteriorly. From these branches several processes project towards the lateral neuropile, ipsilateral to the soma. At the midline, a thick secondary branch projects posteriorly from which several smaller processes run into both ipsilateral and contralateral neuropile regions. After crossing the midline, further secondary processes arise anteriorly and posteriorly from the main neurite. These secondary processes and the branches which arise from the main neurite before it crosses the midline are symmetrical. The axon of 516 ascends contralateral to the cell body and projects at least as far as the prothoracic ganglion. Several thin branches project from the axon into the lateral and medial neuropile regions of the metathoracic ganglion.

516 had its maximum activity just after activity had ceased in the expiratory nerve (Fig. 6B). Injection of depolarizing currents into 516 considerably increased the respiratory rate (Fig. 6C,D) and the injection of short depolarizing pulses reset the respiratory rhythm (Fig. 6E,F). A shortening of the respiratory cycle was evoked when the stimulus was applied in the phase range 0–0.8 (Fig. 6E,F). A prolongation of the respiratory cycle was evoked only when the stimulus pulse was applied in a relatively narrow phase range between 0.85 and 1.0 (Fig. 6F).

Interneurone 606

Interneurone 606 has its cell body in a dorsolateral region in the first fused abdominal ganglion (Fig. 7A). The axon ascends ipsilateral to the cell body. In the first and second abdominal segments, as well as in the metathoracic segment, several secondary processes cross the midline and project into the contralateral neuropile. Smaller branches arise from these secondary processes near the midline and project posteriorly. Branches which run posteriorly are also found in the lateral regions ipsilateral to the cell body.

606 was rhythmically active during expiration (Fig. 7B). Although 606 was recorded in four different animals, in only one of them was the quality of the myogram sufficient to obtain a phase histogram. Activity started in this recording at a phase of 0.7 and ceased at a phase of almost 0.5 (Fig. 7D). 606 had two peaks of activity during each cycle. One peak occurred just before the onset of expiratory activity (phase 0.8) and the other after the onset (phase 0.1) (Fig. 7D). Decreasing

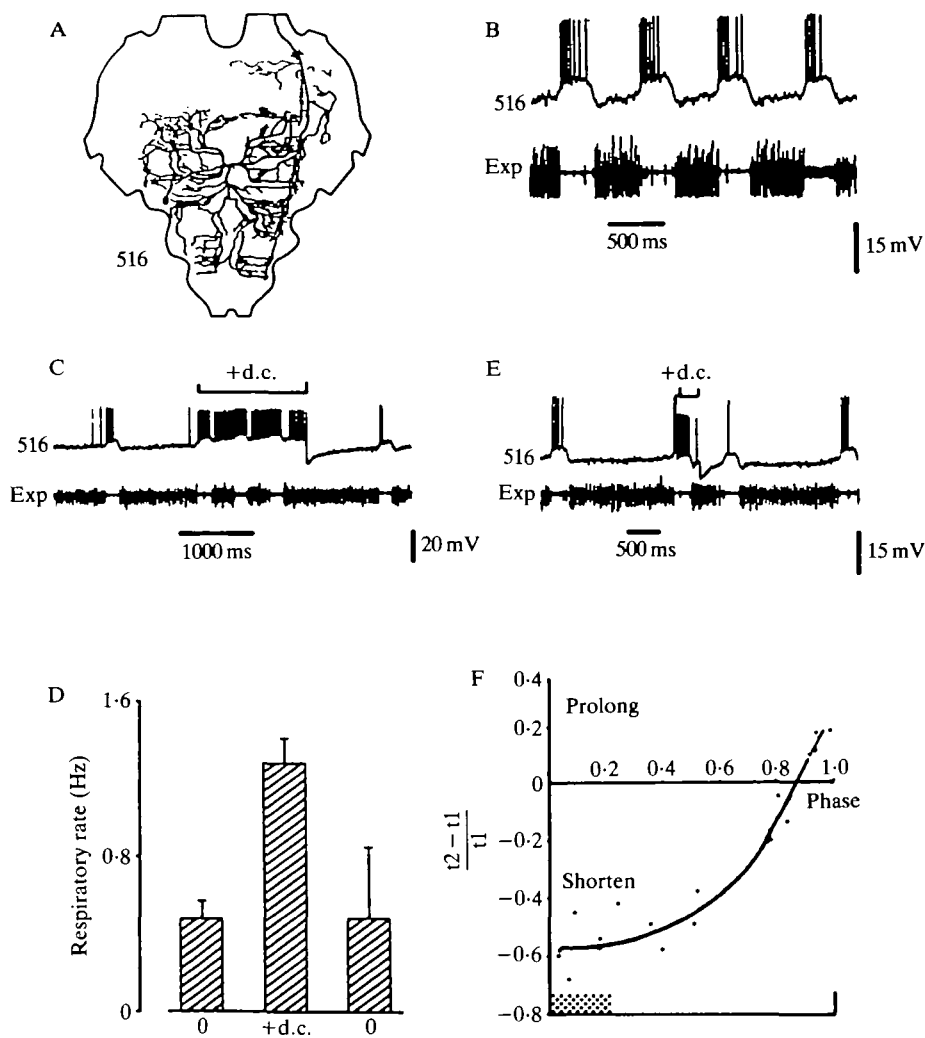


Fig. 6. Characterization of the ascending inspiratory interneurone 516. (A) Structure of the interneurone 516. (B) Activity of 516 (upper trace) recorded with an EMG of nerve 10 of the metathoracic ganglion (Exp) (lower trace). (C) Stimulation experiment. Increase of activity in 516 (upper trace) induced by injecting positive constant current (+2 nA) increased the respiratory rate. EMG recording from nerve 10 of the metathoracic ganglion (lower trace). (D) The activity increase in 516 induced by injecting positive current caused an increase in the respiratory rate. The average activity of 516 was: no d.c. = 12 Hz, +d.c. = 38 Hz. (E) Injection of a short current pulse (300 ms, +2 nA) into 516 (upper trace) caused a resetting of the respiratory rhythm. EMG recording: nerve 10 of the metathoracic ganglion (lower trace). (F) Reset curve calculated from the same recording of interneurone 516 as the recording shown in E. Pulse duration = 300 ms, magnitude of current = +2 nA.

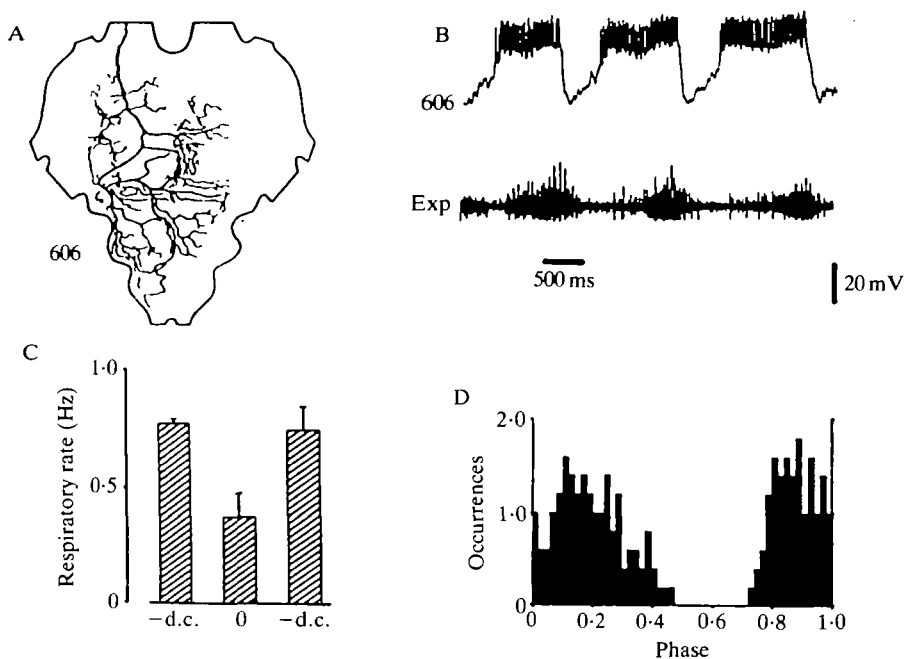


Fig. 7. Characterization of the ascending expiratory interneurone 606. (A) Structure of the interneurone 606. (B) Activity of 606 (upper trace) recorded with an EMG from nerve 8 of the metathoracic ganglion (Exp) (lower trace). (C) Histogram showing that activity increase in 606 causes a decrease in the respiratory rate. The histogram was evaluated as described in Fig. 3. The average activity of 606 was: -d.c. = 2 Hz, no d.c. = 55 Hz. (D) Phase histogram of 606: $N = 5$, average period = 1168 ms, average bin width = 23 ms.

the spike activity in 606 by injecting hyperpolarizing currents increased the respiratory rate (Fig. 7C), and increasing the spike activity in 606 decreased the respiratory rate. Thus current injection into 606 had opposite effects on the respiratory rate from those described for interneurons 326–329. The recordings of 606 were not stable enough to investigate its resetting properties. However, since 606 strongly excited an interneurone which was able to reset the respiratory rhythm we assume that 606 also has the ability to reset this rhythm. This excitatory connection will be described in the following section.

Interneurons in abdominal, mesothoracic and suboesophageal ganglia

To examine whether the respiratory rhythm generator has elements in other ganglia, we recorded from interneurons in the mesothoracic, the suboesophageal and the unfused abdominal ganglia. In this section we describe three interneurons which are located in these ganglia and which affected the respiratory rhythm when injected with constant current.

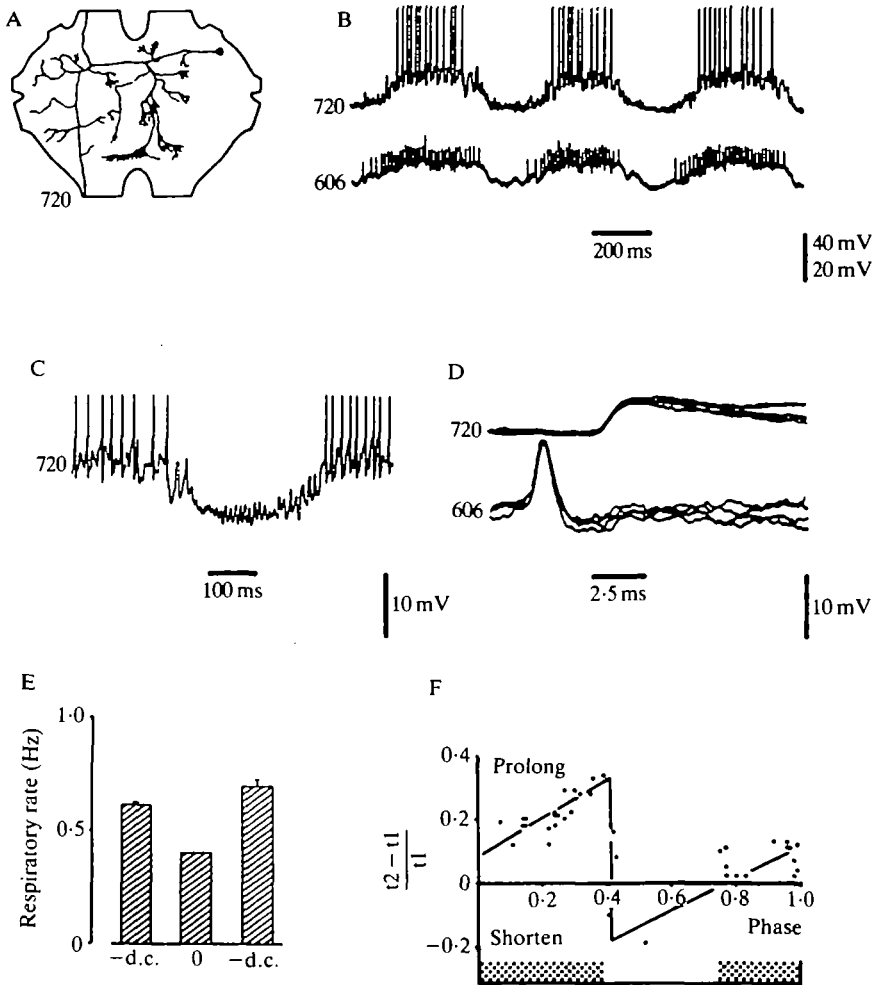


Fig. 8. Characterization of the mesothoracic interneurone 720. (A) Structure of 720. (B) Simultaneous intracellular recording of 720 (upper trace) and 606 (lower trace) during respiration. (C) Intracellular recording of 720. Note the occurrence of many IPSPs during the interburst interval. (D) Each spike in 606 (lower trace) produces an EPSP in 720 (upper trace). The constant latency following of the EPSPs in 720 is displayed by multiple oscilloscope sweeps triggered by the spike in 606. (E) Histogram showing that activity increase in 720 induced by release from negative current (d.c.) (-5 nA) causes a decrease in the respiratory rate. The average activity of 720 was: -d.c. = 6 Hz, no d.c. = 42 Hz. (F) Injection of short current pulses into 720 reset the respiratory rhythm. Duration of current pulse = 200 ms, magnitude of current = 4 nA.

Interneurone 720

Neurone 720 has its cell body posterior to nerve 1 at the lateral margin of the mesothoracic ganglion (Fig. 8A). The main neurite crosses the midline and bifurcates to form an ascending and descending axon in the contralateral connectives. Near the midline of the ipsilateral hemiganglion, a thick secondary

process arises from the main neurite and projects posteriorly, where several smaller branches run into the ipsi- and contralateral neuropile regions. The secondary neurite reaches the posterior edge of the mesothoracic ganglion where it ramifies into a dense bundle of fine branches which spread ipsi- and contralaterally from the branching point. Several branches arise from the descending axon. These relatively fine branches project laterally and medially from the neurone but never reach the midline.

Spike activity in 720 overlapped the activity of interneurone 606 (Fig. 8B) from which it received direct excitatory input. Each spike in 606 was followed 1:1 by an EPSP in 720 (Fig. 8D). In addition to this excitatory input during expiration, 720 also received strong inhibitory input in the interburst interval (Fig. 8C). This inhibitory input was very characteristic of 720 and was not found for the metathoracic expiratory interneurons. Intracellular injection of constant depolarizing current into 720 decreased the respiratory rate, as was also observed for 606 (Fig. 8E). Short depolarizing current pulses (of 200 ms) into 720 reset the rhythm (Fig. 8F). Consistent with the inhibitory effect on the respiratory rhythm, the cycle duration was prolonged if the current pulses were applied in a phase in which 720 was usually active (phase 0–0.4 and phase 0.8–1.0). Current pulses had only a weak shortening effect when applied at a phase in which 720 was usually strongly inhibited (phase 0.5). Thus, for this interneurone the reset curve can explain its inhibitory effect on the respiratory rhythm.

In one animal the entrainment of the respiratory oscillator was examined by injecting current pulses into 720 at different constant stimulus frequencies for 25–30 respiratory cycles (Fig. 9). The respiratory oscillator followed in a 1:1 manner with a low scatter ($r = 0.97$ – 0.99 , see also Horsmann *et al.* 1983) in a stimulus frequency range from 0.52 to 0.74 Hz (absolute coordination). Changes in the stimulus frequency caused (1) a change in the frequency of the respiratory oscillator and (2) a shift in the phase at which the oscillator locked to the stimulus (Fig. 9). A decrease in the stimulus frequency caused the respiratory oscillator to advance and an increase in the stimulus frequency was immediately followed by a retardation of the respiratory oscillator relative to the stimulus period (Fig. 9A,B).

Interneurone 377

Interneurone 377 has its cell body in the suboesophageal ganglion at the ventral midline of the anterior labial neuromere (Fig. 10A). Its axon descends in the connectives contralateral to the cell body at least as far as the metathoracic ganglion. The processes of 377 are symmetrically distributed in all the neuromeres of the suboesophageal ganglion. Ipsi- and contralateral to the midline two relatively thick secondary processes project posteriorly into the labial neuromere. Two secondary processes arise from the crossing neurite at the midline and project anteriorly into the mandibular neuromere. Further branches arise from the descending axon.

Interneurone 377 was rhythmically active in phase with expiration but the

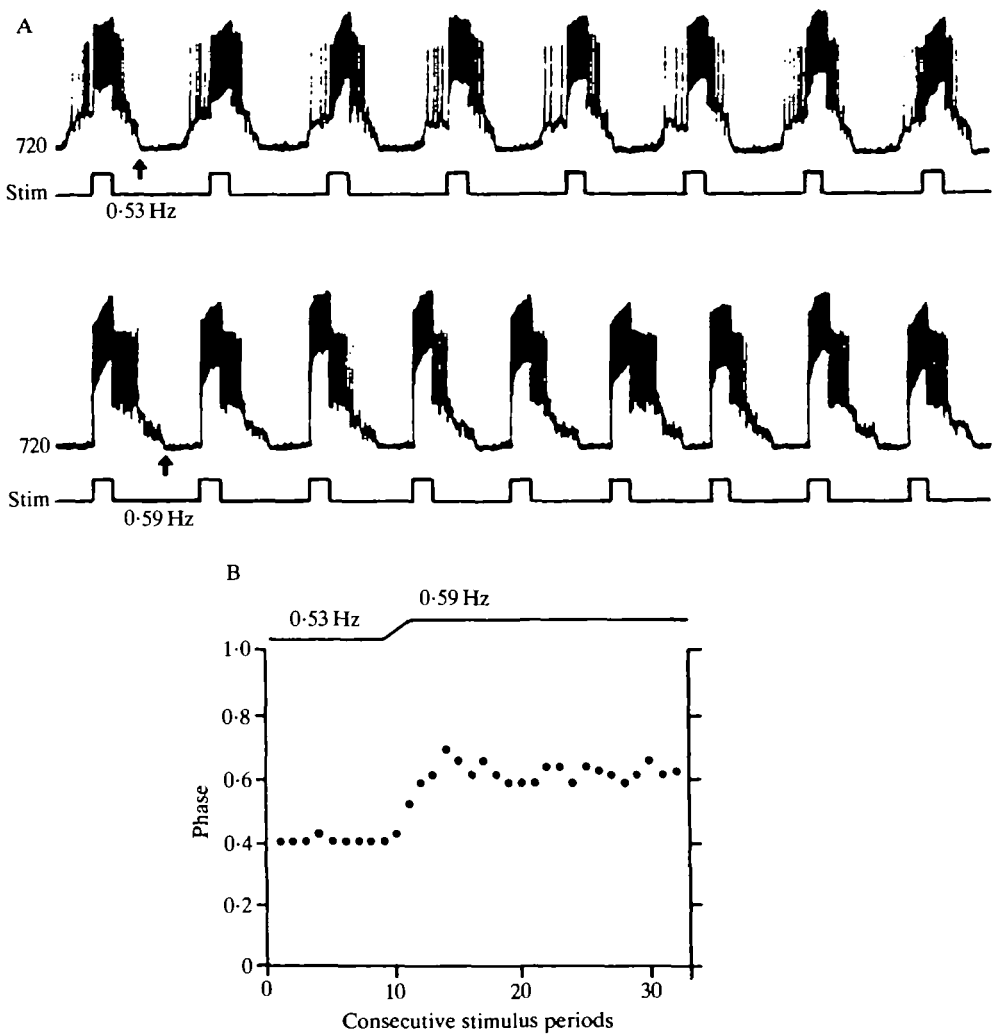


Fig. 9. Entrainment of the respiratory oscillator by injecting current pulses into 720. Duration of the current pulse was 200 ms and the magnitude of current was 4 nA for all experiments. (A) Increase of the stimulus frequency from 0.53 (upper two traces) to 0.59 Hz (lower two traces) increases the respiratory frequency and causes the respiratory oscillator to discharge in a later phase within the stimulus period. The onset of inspiration was used as reference point to monitor the phase shift of the respiratory oscillator (arrows). (B) Sequential phase diagram for one step in the stimulus frequency. An increase of the stimulus frequency from 0.53 to 0.59 Hz is immediately followed by a retardation of the phase. The mean phase distribution for 0.53 Hz stimulation was $p = 0.43$ and for the 0.59 Hz stimulation $p = 0.62$. Values of p were calculated after Horsmann *et al.* 1983. The phases of the inspiratory onset in successive respiratory cycles are plotted with respect to the stimulus period. The stimulus period was the time between two consecutive stimulus pulses, and the phase of the respiratory oscillator within the stimulus cycle was the time from the last stimulus pulse to the onset of inspiratory activity divided by the stimulus period.

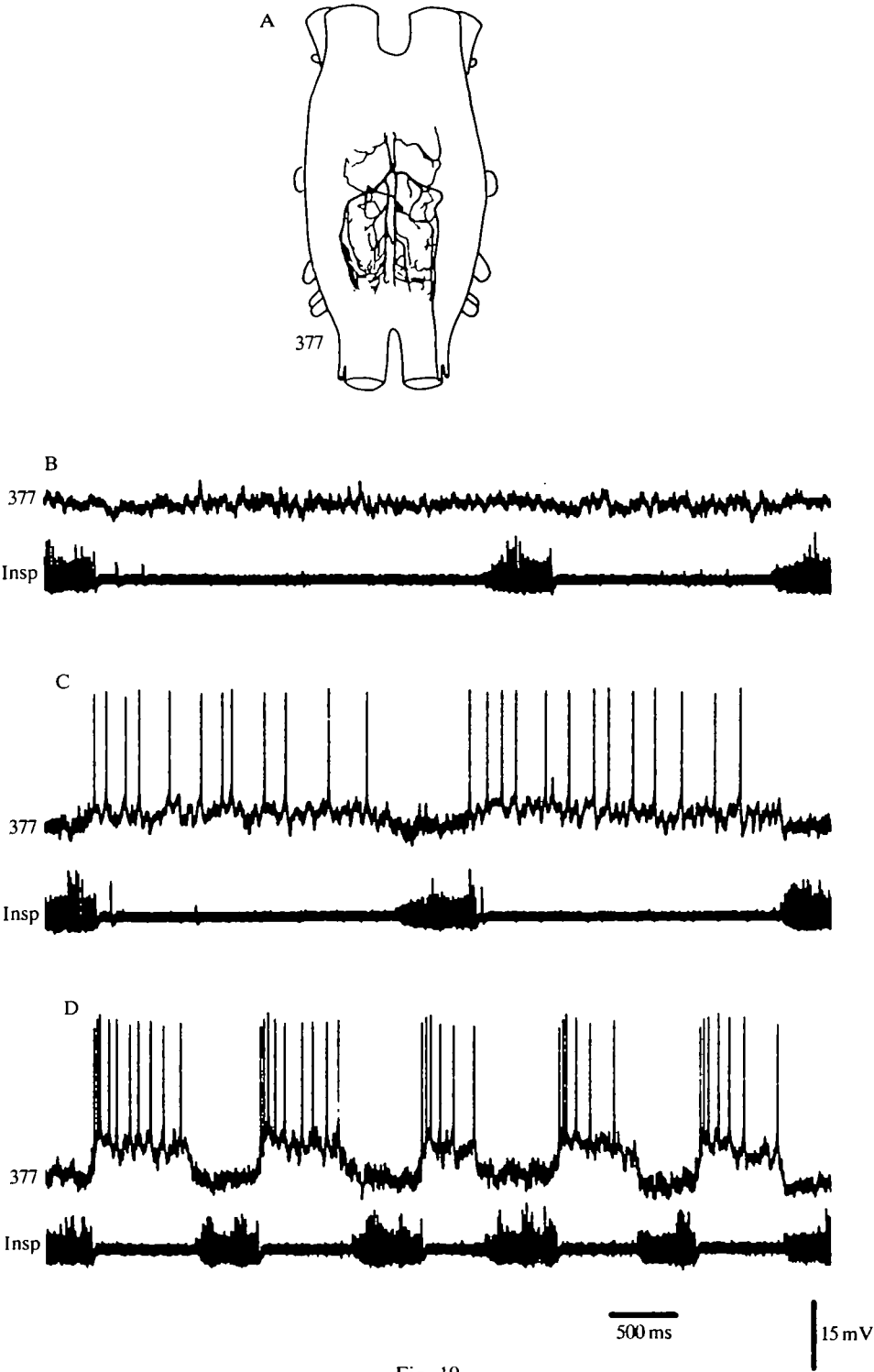


Fig. 10

strength of this activity was variable and it could even fail completely (Fig. 10B). No synaptic modulation was visible during the absence of rhythmic activity in 377, indicating an absence of rhythmic input to it. That this variation was caused by a variable rhythmic input and was not due to individual differences among 377 interneurons in different animals is indicated by the observation of variation during the same 377 recording (Fig. 10B–D). A reproducible alteration of the strength of synaptic input to 377 was obtained in all animals by eliciting rhythmic activity in flight motoneurons by application of a wind stream to the head. The strength of the 377 modulation was considerably increased after this treatment (Fig. 10C,D). During increased activity of 377 a high initial input frequency, which was not present before flight rhythmic activity (Fig. 10C), was visible at the onset of expiration (Fig. 10D). The amplitude of the depolarization was also changed (Fig. 10C,D).

Despite the occasional complete absence of respiratory rhythmic input to 377, intracellular injection of depolarizing currents into 377 always accelerated the respiratory rhythm (Fig. 11A,B). The effect of injections of current pulses on the respiratory rhythm was phase-dependent. The injection of depolarizing current pulses at the end of the inspiratory phase and during some of the expiratory phase caused a shortening of the respiratory cycle (Fig. 11A,C). The reset curve of 377 was qualitatively similar to that described for expiratory interneurons 326–329 in the metathoracic ganglion (Fig. 11C).

Interneurone 725

Interneurone 725 has its soma in the ventral posterior midline of the first unfused abdominal ganglion (Fig. 12A). The primary neurite projects anteriorly, crosses the midline and bifurcates to send ascending and descending axons into the contralateral connectives. The secondary processes ipsilateral to the soma arise from the primary neurite whereas all contralateral processes arise from the axons.

Interneurone 725 was rhythmically active in phase with expiration (Fig. 12B). The activity of 725 started after the onset of expiratory activity in the metathorax (phase 0.01) and was maximally active in phase 0.2–0.4 (Fig. 12C). Thus, 725 was clearly activated later than the metathoracic expiratory interneurons. Another difference was that fewer spikes were produced in 725 in each respiratory cycle. Intracellular injection of constant depolarizing currents slowed the respiratory rhythm (Fig. 12E). The effects of injections of current pulses were phase-

Fig. 10. Characterization of the interneurone 377, located in the suboesophageal ganglion. (A) Structure of 377. The structure and the activity during flight of 377 have been described elsewhere under the name SD3B (Ramirez, 1986). (B–D) Upper traces: intracellular recording from the same 377, lower traces: EMG recording from muscle 177 (Insp). (B) In the quiescent animal, 377 is sometimes inactive during respiration. (C) Weak rhythmic modulation in 377, two respiratory cycles before flight activity was released by blowing wind on the head. (D) The same recording, but two respiratory cycles after cessation of the wind-induced flight activity. Note (1) the occurrence of a phasic input in 377 which was absent in C and (2) a change in the depolarization amplitude from C to D.

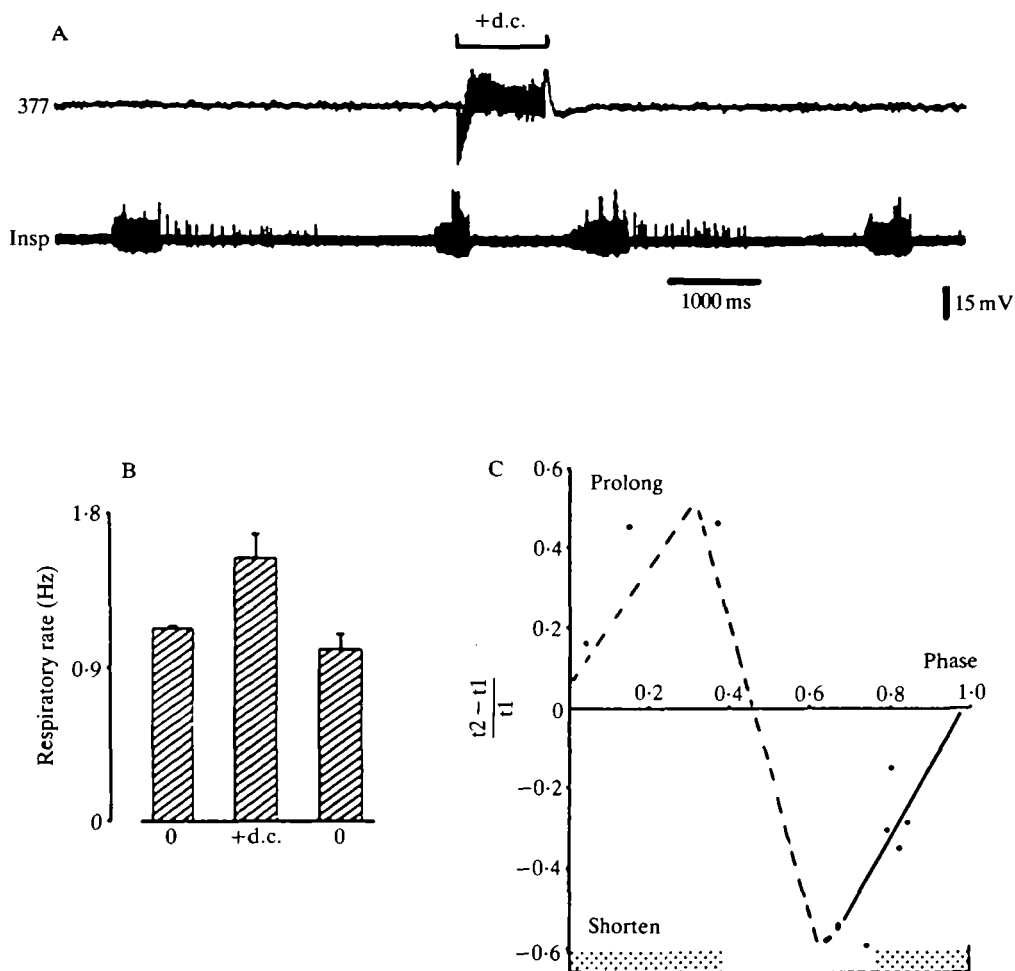


Fig. 11. The influence of 377 on the respiratory rhythm. (A) Injection of a current pulse (3 nA, 1000 ms) causes a shortening of the respiratory cycle. The recording in A is a.c. filtered. (B) Injection of constant depolarizing current (+3 nA) increased the respiratory rate. Average activity of 377: no d.c. = 0, +d.c. = 40 Hz. (C) Reset curve of 377. Pulse duration = 1000 ms, magnitude of current pulse = 3 nA. The dotted area at the bottom of C indicates the activity distribution of 377, were 377 to have been active. In this stimulation experiment 377 was recorded with an inspiratory myogram, but the reset curve was normalized to an expiratory cycle by shifting the ordinate.

dependent and the injections prolonged the cycle period at the phases examined (phase 0.2–0.8) (Fig. 12D,F).

Discussion

Interneurones in the metathoracic ganglion

Studies on various insects, such as dragonfly larvae (Mill, 1970), dobsonfly

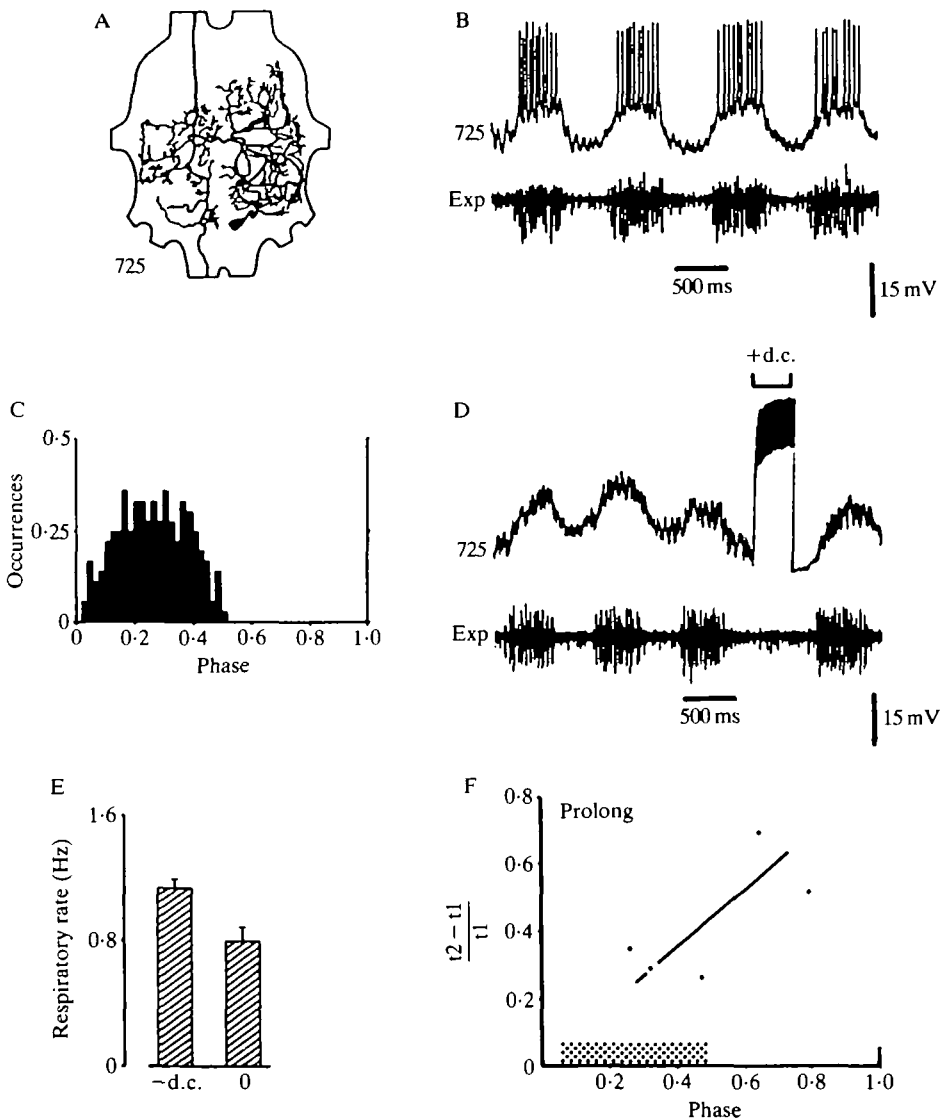


Fig. 12. Characterization of the interneurone 725 located in the first unfused abdominal ganglion. (A) Structure of 725. (B) Activity of 725 (upper trace) recorded with an EMG of nerve 8 in the metathoracic ganglion (Exp) (lower trace). (C) Phase histogram of 725. $N = 36$, average period = 873 ms, average bin width = 17.5 ms. (D) A current pulse injected into 725 (upper trace) causes a prolongation of the respiratory cycle. Lower trace, EMG of nerve 8 in the metathoracic ganglion. Duration of the current pulse = 350 ms, magnitude of current = +2 nA. 725 was hyperpolarized by constant negative current (d.c.) (–3 nA). (E) Histogram showing that release of inhibition in 725 causes a decrease in the respiratory rate. –d.c. = 0 Hz, no d.c. = 40 Hz. (F) Phase-response curve. Duration of the pulses = 350 ms, magnitude of current = +2 nA.

larvae (Fitch & Kammer, 1982), adult cockroaches (Farley *et al.* 1967) and adult locusts (Miller, 1960*a*) have revealed that the respiratory rhythm is generated in more than one ganglion. Some of these ganglia are able to generate a rhythm in respiratory motoneurons when isolated from the rest of the nervous system. However, lesion experiments have shown that the relative importance of these ganglia in the generation of the respiratory rhythm is not uniform. One ganglion usually leads the respiratory rhythm (primary respiratory oscillator) and coordinates the rhythmic activity in the other ganglia (secondary oscillators). In different insect species, the primary respiratory oscillator is located in different ganglia. In dragonfly larvae it is located in the most posterior abdominal ganglion (the eighth) (Komatsu, 1982) and in dobsonfly larvae in the third abdominal ganglion (Fitch & Kammer, 1982). In adult locusts (Miller, 1966), crickets (Huber, 1960) and cockroaches (Farley, 1967) the primary respiratory oscillator is located somewhere in the metathoracic ganglion, although it is not known whether it is in the 'true' metathoracic ganglion or in one of the three abdominal ganglia which are fused to it.

It is assumed that intersegmental interneurons located in or associated with the primary oscillator coordinate the activity in the secondary oscillators. In the dragonfly a pair of ascending interneurons has been described which might be responsible for such coordination (Komatsu, 1984). In crickets, cockroaches and locusts it has been demonstrated that burst activity related to expiration is present in abdominal connectives (Huber, 1960; Farley *et al.* 1967; Miller, 1966) and it is assumed that this activity is caused by interneurons descending from the metathoracic ganglion and coordinating the secondary oscillators which are located in the abdominal ganglia (Lewis *et al.* 1973). By means of intracellular recordings, descending interneurons have been identified in the locust metathoracic ganglion which discharge rhythmically in phase with expiration and which also affect the respiratory rhythm when stimulated (Pearson, 1980). The properties of these interneurons are consistent with the properties proposed for the coordinating interneurons by Lewis (1973). However, the exact number of these coordinating interneurons has not been established.

In this study we present evidence for the existence of at least four descending interneurons in each half of the first fused abdominal ganglion. These interneurons have a number of properties in common. All have a rising depolarization which starts before the onset of expiratory activity at a phase of approximately 0.7 and which reaches its maximum at the onset of expiratory motor activity. Activity in these interneurons continues throughout the expiratory phase (phase range 0–0.4) and ceases synchronously with the end of expiratory motor activity (phase 0.4). All these neurons accelerate the respiratory rhythm when injected with constant depolarizing current and reset the respiratory rhythm when injected with short depolarizing current pulses. The somata of these neurons are all located in the same region of the first fused abdominal ganglion. Since no other expiratory interneurons were found which descended to the abdominal ganglia and which also reset the respiratory rhythm, it seems likely that the effects observed by Lewis

et al. (1973) are caused by these neurones. This would imply that the primary respiratory rhythm generator of locusts is not located in the 'true' metathoracic ganglion but in the first fused abdominal ganglion. Consistent with this suggestion is the finding that 606 and 516, which both have an effect on the respiratory rhythm, were also located in the first fused abdominal ganglion.

Ascending influence from the abdominal ganglia to the metathoracic ganglion

There is general agreement that the primary respiratory oscillator controls the secondary respiratory oscillators in the abdominal ganglia (Miller, 1960a; Lewis *et al.* 1973; Pearson, 1980). However, it is not known whether the secondary oscillators also have an effect on the primary oscillator. The existence of interactions between the secondary oscillators and the primary oscillator is indicated by the discovery of interneurone 725. This interneurone is located in the first unfused abdominal ganglion and strongly affects the respiratory rhythm in the first fused abdominal ganglion (primary oscillator). Activity in 725 started after the onset of activity in the descending interneurons located in the first fused abdominal ganglion as well as after the onset of activity in the expiratory motor nerve of this ganglion. Therefore, 725 activity was too late to have an immediate effect on the onset of the expiratory phase in the primary oscillator. However, 725 could act through the neuronal elements of the primary oscillator on the next expiratory cycle. This is very likely, since stimulation of 725 clearly prolonged the onset of the next expiratory phase in the primary oscillator.

What might be the functional relevance of feedback from the secondary to the primary oscillator? It is known that the respiratory rhythm depends on the animal's fat supplies and egg loads and that experimental loading of the abdomen can strongly affect the overall respiratory motor pattern (Giszter, 1986). Such an adaptation of the rhythm to loads in the abdomen might be caused by direct feedback from sensory receptors to the primary oscillator but might also be caused by more indirect feedback *via* the secondary oscillator. Neurone 725 might be a good candidate for mediating feedback from the secondary to the primary oscillator. The number of interneurons in the segmental abdominal ganglia which have an effect on the overall respiratory rhythm is unknown. Of six different respiratory interneurons which were identified in the segmental abdominal ganglia only interneurone 725 had an effect on the respiratory rhythm of the first fused abdominal ganglion. Neurone 725 also has a descending axon and therefore may also affect oscillators in more posterior abdominal ganglia.

Anterior distribution of the respiratory rhythm generator into the thoracic and suboesophageal ganglia

The question of whether the respiratory oscillator is distributed in ganglia anterior to the metathoracic ganglion is unresolved. Hoyle (1959) described a rhythm in the motor axons innervating spiracle 2 which is produced in the isolated mesothoracic ganglion but it has been questioned whether this rhythm is related to the respiratory rhythm observed in the intact locust (Miller, 1960b). Thus, Miller

(1960a) has stated that there appears to be no rhythmic centre anterior to the metathoracic ganglion. Nevertheless, it is known that, in the intact animal, muscles in the thorax and the neck contract rhythmically during respiration (see Miller, 1960a). This rhythmic activity could be derived through at least three possible pathways: (1) through interneurons ascending from the primary oscillator which are not elements of the respiratory rhythm-generating system; (2) through interneurons ascending from the primary oscillator which are elements of the respiratory rhythm-generating system; and (3) through interneurons located in the thoracic ganglia and suboesophageal ganglion which are elements of the respiratory rhythm-generating system.

Until now there has been evidence only for the first possibility. One inspiratory interneurone has been found which ascends from the metathoracic ganglion into the meso- and prothoracic ganglia and affects several thoracic spiracles without having any effect on the respiratory rhythm itself (Burrows, 1982). Some clues for the two other possibilities were presented in this study. Consistent with the second possibility, we found two interneurons which have their somata in the first fused abdominal ganglion and which ascend into the thoracic ganglia. These neurones, the inspiratory interneurone 516 and the expiratory interneurone 606, both have an effect on the respiratory rhythm. The third possibility, which was the most unlikely considering the results of the lesion experiments (Miller, 1960a), became more likely as a result of the findings presented in this study. We showed that 606 excited interneurone 720 which is located in the mesothoracic ganglion. Intracellular stimulation of 720 had a strong effect on the respiratory rhythm. Constant depolarizing currents slowed the respiratory rhythm and injection of short depolarizing pulses reset and entrained the rhythm. The coupling of the respiratory oscillator to 720 stimulation was very strong and therefore there is no doubt that the respiratory rhythm generator has elements in the mesothoracic ganglion.

A further extension of the respiratory rhythm generator into the suboesophageal ganglion is also indicated by the identification of the descending interneurone 377 which is located in the suboesophageal ganglion and which descends at least into the metathoracic ganglion. Continuous depolarization of 377 accelerated the respiratory rhythm and injection of short pulses into 377 reset the respiratory rhythm. These properties indicate that 377 is an element of the respiratory rhythm-generating system. However, we demonstrated that 377 was not always active during respiration, indicating that 377 is not necessary for the generation of the respiratory rhythm. This is not surprising since all the interneurons described in this study could be artificially removed from the respiratory rhythm-generating system, by applying hyperpolarizing currents, without stopping respiratory rhythmic activity. Thus, none of the nine neuronal elements of the respiratory rhythm generator described in this study was necessary for the generation of the respiratory rhythm. This is also consistent with the findings in the flight rhythm-generating system of the locust. None of the known rhythm-generating elements for flight is necessary (Robertson & Pearson, 1983; 1985). However, the finding that the level of activity in interneurone 377 can

change, and in some animals cease completely, is unusual since it demonstrates that the number of neuronal elements in the respiratory rhythm-generating system may vary. The additional recruitment of interneurons such as 377 during situations when the oxygen consumption is high seems to be a mechanism to accelerate the respiratory rhythm generator, and might also be a mechanism to activate additional muscles. Indeed, it is known that the muscles of the thorax and the neck are only activated during vigorous respiration, for example after flight, when 377 is strongly active (Miller, 1960a). At this time the respiratory rate is also increased. An additional recruitment of neuronal elements also shows that the number of active components in a rhythm generator may not be fixed but can be adapted to the behavioural situation.

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