J. exp. Biol. **134**, 443–450 (1988)
Printed in Great Britain © *The Company of Biologists Limited 1988*

SHORT COMMUNICATION

NON-SPIKING INTERNEURONES IN THE PEDAL GANGLIA OF A SWIMMING MOLLUSC

BY ANDREW N. SPENCER

Department of Zoology, University of Alberta, Edmonton, Alberta, Canada, T6G 2E9

Accepted 13 August 1987

Neurones that produce graded potentials and not action potentials are found in a diverse range of species (Roberts & Bush, 1981), and are usually associated with situations where sensory information is being integrated, for example in the vertebrate retina. Nevertheless, in several arthropod species, non-spiking interneurones can participate in the production of a centrally generated motor pattern (Mendelson, 1971; Pearson & Fourtner, 1975; Heitler & Pearson, 1980; Simmers & Bush, 1980; Takahata, Nagayama & Hisada, 1981; Paul & Mulloney, 1985). Surprisingly, such interneurones with similar functions have not been reported for other phyla. In this study, non-spiking interneurones are described in the pedal ganglia of a mollusc that are probably the source of the motor pattern controlling swimming.

To counteract passive sinking, the small planktonic snail *Cavolinia inflexa* (Fig. 1A) swims vertically almost continuously. Swimming is by symmetrical elevation (towards the dorsal side) and depression of paired wing-like parapodia (Fig. 1C). In strongly swimming individuals the mean frequency of these movements is approximately 6 Hz. The parapodial musculature consists of two layers of obliquely striated muscle fibres on both the ventral and dorsal parapodial surfaces in addition to muscles which span the haemocoelic space.

Snails were collected by horizontal plankton tows at approximately 10 m depth in the Rade de Villefranche and held in running sea water for a maximum of 3 days before experimentation. The shells of snails anaesthetized with a 1:1 mixture of isotonic $MgCl_2$ and sea water were removed and a midline incision was made along the ventral body wall. The gut, buccal mass and salivary glands were removed and the animal was firmly pinned by cactus spines to the Sylgard (Dow Corning) base of a Petri dish to eliminate most movements during swimming. A glass support was positioned under the ganglionic mass for stabilization. For some experiments the central ganglia were isolated and pinned through the cut nerve roots. As the ganglionic sheath is quite thin it was not necessary to remove or digest it with

Key words: non-spiking, interneurone, mollusc, pteropod, swimming, *Cavolinia*.

Pronase. Intracellular recordings were made with micropipettes filled with 2 mol l^{-1} potassium acetate (approx. 35 MΩ) or 5% Lucifer Yellow (approx. 70 MΩ). Neuronal staining with Lucifer Yellow (Stewart, 1978) was achieved by passing a continuous negative current of approx. 5 nA through the recording electrode for 5–30 min. The central ganglionic complex was then dissected out and fixed in 4% paraformaldehyde in sea water for 1 h followed by dehydration through an alcohol series and mounting in methyl salicylate. Conventional d.c. amplification was used with analogue data stored on tape for later analysis. Figs 2 and 3 were made by playback onto a pen-recorder (3 dB down at 125 Hz). Electromyographic recordings, using differential a.c. amplification, were made from a plastic suction electrode filled with sea water attached to the ventral parapodial surface.

The neuronal machinery necessary and sufficient for producing the complete motor pattern was found to be contained in the pair of pedal ganglia (Fig. 1B) which are the largest ganglia of the central ganglionic complex. Ablation of the other ganglia in the complex did not prevent the swimming pattern from being produced.

Each sheet of swimming muscle was found to be innervated by motoneurones whose axons run in two, bilaterally paired parapodial nerves (Fig. 1B). It was possible to record intracellularly from these motoneurones during spontaneous, fictive swimming when the parapodia were restrained, as described above. If the central ganglionic complex was isolated from the animal the full motor pattern could still be recorded from both the motoneurones and interneurones involved in pattern generation, and thus this system meets the major criterion for central pattern generation (Delcomyn, 1980). All motoneurones that were phasically active during these parapodial movements exhibited large amplitude (5–20 mV), plateau-like, depolarizations with each cycle (Figs 2A, 3B). Each depolarization produced a burst of spikes that was propagated in the parapodial nerves and could be recorded from the parapodial musculature. The bursts of spikes were terminated by strong hyperpolarizations that could consist of more than one component. Ionophoresis with Lucifer Yellow showed that all neurones which have axons that leave the pedal ganglia produced spikes when depolarized by current injection. Motoneurones could be identified by stimulating through the recording electrode and monitoring the ipsilateral parapodium for contractions or spikes in the EMG recording. They could

Fig. 1. Diagrammatic view of the shelled pteropod *Cavolinia inflexa*. (A) A snail with parapodia fully expanded outside the shell. (B) Ventral view of the anterior portion of the snail to show the relationship between the parapodia, parapodial nerves and the central ganglionic complex. (C) Series of drawings taken from a ciné-film of swimming in a tethered snail showing a ventral, oblique view during the depression phase with approximately 32 ms between each frame. (D) Illustrations of the four morphological types of non-spiking interneurones in the pedal ganglia that are involved in generating the swimming motor pattern. Drawings made from fixed preparations. At least one elevator and one depressor interneurone of each type has been identified in both hemiganglia, except the type shown in iv, for which an elevator has not been found. The bilateral homologues of this neurone are dye-coupled and therefore both neurones have been illustrated.

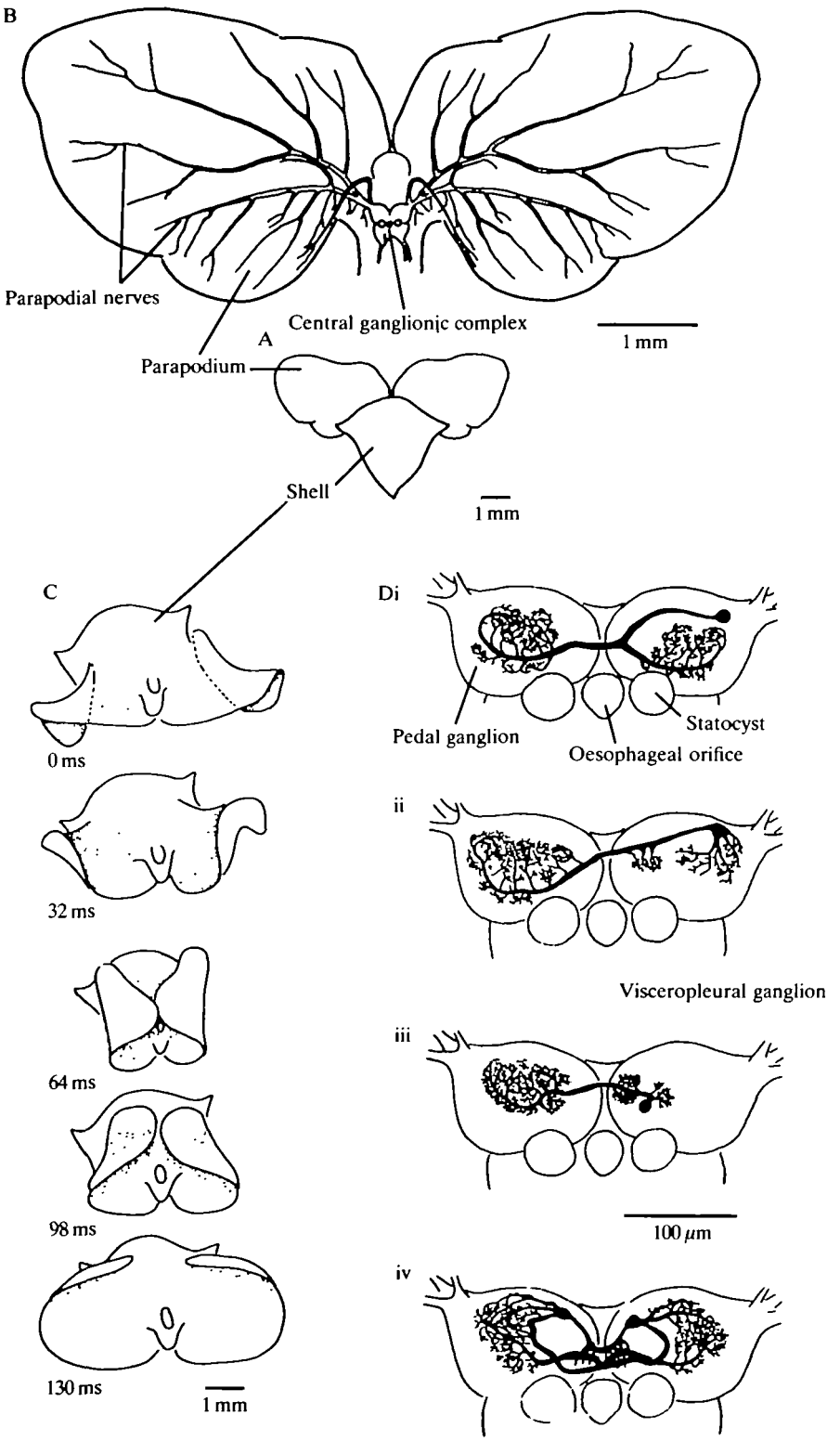
B
Parapodial nerves
Central ganglionic complex
1 mm
A
Parapodium
Shell
1 mm
C
0 ms
32 ms
64 ms
98 ms
130 ms
1 mm
Di
Pedal ganglion
Statocyst
Oesophageal orifice
ii
Visceropleural ganglion
iii
100 μm
iv

Fig. 1

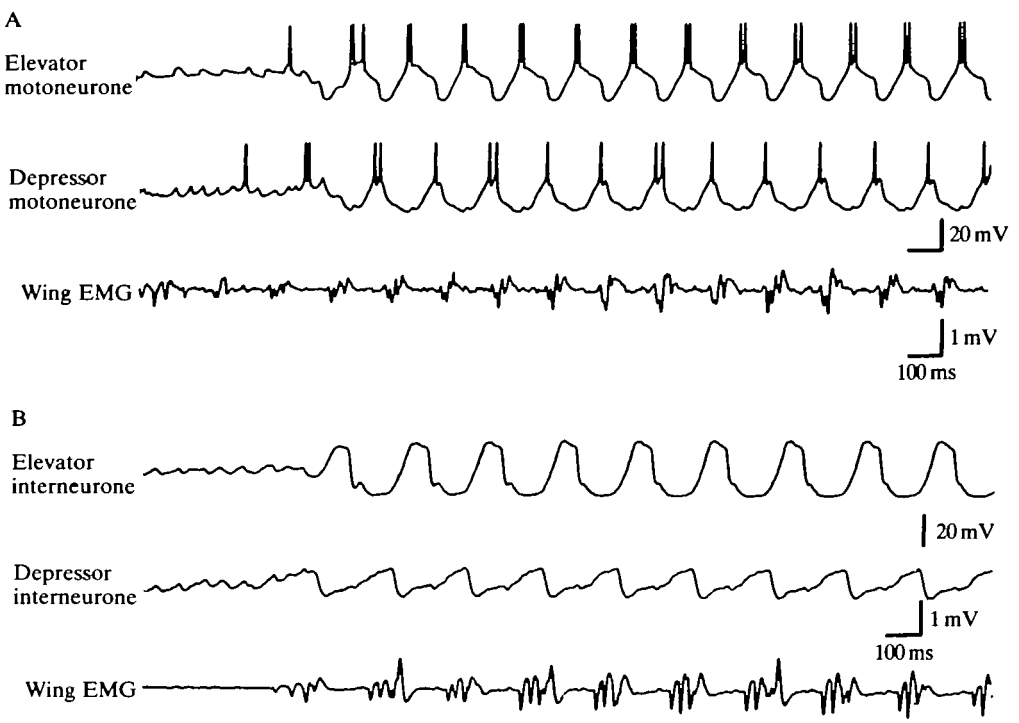

Fig. 2. Intracellular recordings obtained during swimming from motoneurones and non-spiking interneurones in the pedal ganglion of the mollusc *Cavolinia inflexa*. (A) Simultaneous recordings from an elevator (upper trace) and depressor motoneurone (middle trace), before and during a spontaneous bout of swimming showing the characteristic spiking of motoneurones. Note that the spikes are not truncated by the recording method. Parapodial depression was monitored by a suction electrode attached to the ventral parapodial surface which recorded the ipsilateral electromyogram (bottom trace). (B) Simultaneous recordings from an elevator interneurone (upper trace) and a depressor interneurone (middle trace) before and during a spontaneous bout of swimming. Note the lack of spikes. Depolarization in the elevator neurone coincides with hyperpolarization in the depressor neurone; while the animal is not swimming both interneurones receive discrete, simultaneous IPSPs.

be categorized into two broad groups depending on whether the spiking was synchronized with parapodial elevation or depression (Fig. 2A). Such recordings and subsequent staining with Lucifer Yellow showed that there are at least 14 identifiable motoneurones innervating the parapodial musculature in each hemiganglion. The organization and physiology of these motoneurones is very similar to that reported for the gymnosomatous pteropod *Clione limacina* (Satterlie & Spencer, 1985; Arshavsky *et al.* 1985*a*,*b*,*c*,*d*).

In sharp contrast to the motoneurones, all phasically active neurones examined in *Cavolinia*, that did not have any processes projecting outside the pedal ganglia, never produced spikes. Spiking was not seen during penetration of interneurones, or when they were depolarized strongly, or on rebound from strong hyperpolarization.

Recordings made from the somata of interneurones were indistinguishable from those made in the 'dendrites' or major processes and therefore it is unlikely that local generation of spikes occurred but escaped detection. In *Clione*, however, only one of the interneurones may be non-spiking and this neurone (type 12) does not appear necessary for production of the basic rhythm (Arshavsky *et al.* 1985*c*). All the remaining interneurones, including those believed to be essential for pattern generation (types 7 and 8), are capable of producing spikes and normally do so (Arshavsky *et al.* 1985*d*; Satterlie, 1985).

Two sub-populations of interneurones, comparable with those of motoneurones, could be readily identified from intracellular recordings in *Cavolinia*. When recording from elevator interneurones, depolarizations were seen with each elevation of the parapodia while the depolarizations of depressor interneurones were 180° out of phase with those of elevator interneurones (Fig. 2B). The depolarizations of interneurones had amplitudes as great as 29 mV above a mean resting potential of −52 mV ($N = 16$) and were typically of fairly long duration (80–150 ms), sometimes with several inflections on the rising and falling phases. Simultaneous intracellular recordings showed that depolarizations in one sub-population of interneurones were terminated by strong hyperpolarizations (up to 23 mV below the resting potential) which appeared synchronously in all interneurones that were active at the same phase of the swimming cycle. Frequently there was more than one component to this hyperpolarization since it could be interrupted by a transitory depolarization.

Except for one bilateral pair of interneurones (Fig. 1Div) no other interneurones which were active at the same phase showed dye-coupling, yet in a few cases it was possible to show weak electrical coupling. In *Clione*, however, synchronization of activity in each bilateral population of rhythmically active interneurones (types 7 and 8) does appear to be achieved by electrical coupling (Arshavsky *et al.* 1985*d*). The large alternating hyperpolarizations and depolarizations seen in *Cavolinia* appear to be due mostly to chemical synaptic input since the amplitudes of these compound EPSPs and IPSPs depended on the resting membrane potential (Fig. 3A). Nevertheless, it is also possible that voltage-sensitive currents could be contributing to these rhythmical changes in membrane potential. It is probable that components of the rhythmic depolarizations seen in interneurones are derived from other 'in phase' interneurones which are simultaneously depolarizing. In addition, since the depolarizations of any interneurone were found to coincide with hyperpolarizations of all 'opposite phase' interneurones (Fig. 2B), then a mechanism involving reciprocal inhibition between every elevator and depressor interneurone is conceivable. Such a mechanism has been suggested for swimming in *Clione* (Arshavsky *et al.* 1985*d*; Satterlie, 1985), but in that case antagonism occurs between spiking interneurones (types 7 and 8). If this is the mechanism in *Cavolinia* then graded release of transmitter substances would be necessary to produce the required interactions within and between the two groups of interneurones. Such graded release by non-spiking and spiking interneurones is well-established for arthropods (Burrows & Siegler, 1978; Graubard, 1978; Graubard, Raper & Hartline, 1980).

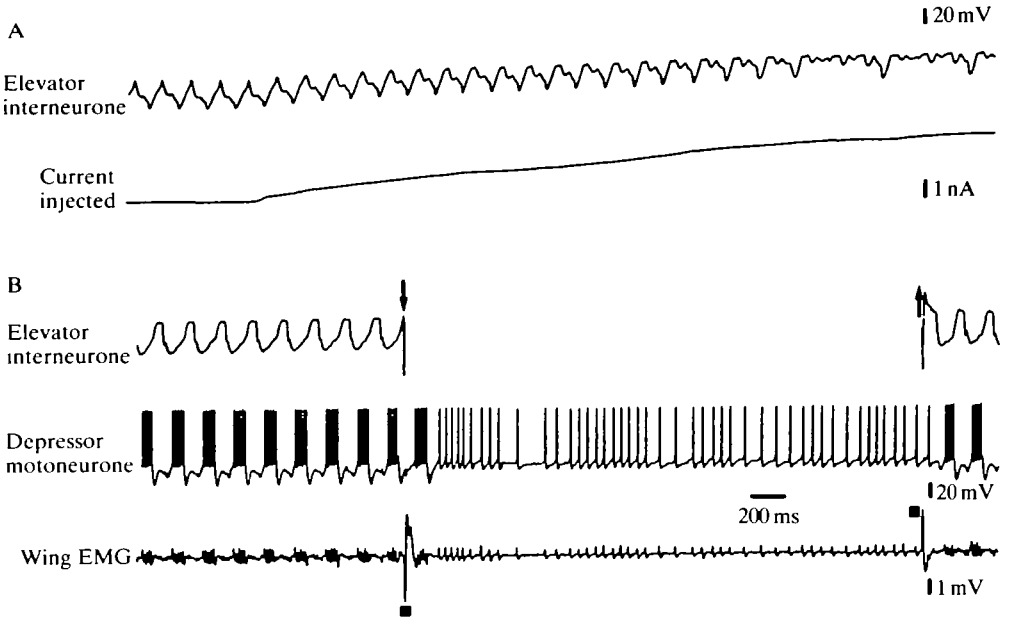

Fig. 3. Intracellular recordings from non-spiking interneurones when current was injected through a bridge circuit to show the nature of the rhythmical membrane potential oscillations and the probable central role of these interneurones in pattern generation. (A) Recording from an elevator interneurone (upper trace) during spontaneous swimming in the isolated ganglionic complex, when depolarizing current (lower trace) was injected through the recording electrode, showing reductions in the amplitude of the rhythmical depolarizations and increases in the amplitude of hyperpolarizations that are proportional to the degree of membrane potential depolarization. At the end of the recording the frequency of swimming had fallen substantially, as can be seen by the reduced frequency of IPSPs. (B) Simultaneous recordings from an elevator interneurone (upper trace) with the same morphology as in Fig. 2B and a depressor motoneurone (middle trace) during a spontaneous bout of swimming. The bottom trace is an EMG monitor of parapodial depression. Between the arrows the interneurone was strongly hyperpolarized by injection of a current of −8 nA (the bridge amplifier could not be balanced during current passing) which suppressed rhythmical output from motoneurones; swimming stopped for the period that the interneurone was hyperpolarized. Note that at this time the motoneurone did not receive the large-amplitude IPSPs that terminate parapodial depression.

Many of the rhythmically active, non-spiking interneurones in *Cavolinia* are probably part of the pattern generator. Although injection of short current pulses into interneurones did not reliably produce phase resetting, long-duration hyperpolarization of many interneurones entirely inhibited generation of the rhythm. For example, if an interneurone with the morphology shown in Fig. 1Di was strongly hyperpolarized then rhythmical swimming ceased for the time that this neurone was removed from the network (Fig. 3B). Depolarization of an interneurone could initiate a bout of swimming which continued after the current pulse was removed or it could cause the frequency of the rhythm in other interneurones to decrease

(Fig. 3A). When the snail was not swimming, rhythmically active interneurones were inhibited by a common barrage of IPSPs (Fig. 2B). The parapodia were not retracted when swimming ceased but remained almost fully extended with some maintained muscle tone.

Lucifer Yellow staining revealed at least four morphological types of interneurones in each hemiganglion, and seven functional types; three elevators and four depressors (Fig. 1D). This assumes that there is only one neurone in each morphologically and physiologically distinct class; thus the total number of interneurones that are phasically active during swimming could be far greater. These non-spiking interneurones share some obvious morphological features which are very different from those of motoneurones. Somata were found to be relatively small, 8–30 μm in diameter, while their major processes, particularly where they pass through the commissure to the contralateral ganglion, are surprisingly thick. These processes frequently have as great a diameter as their somata. A similar and relatively simple morphology of the major processes characterizes non-spiking local interneurones involved in the swimmeret pattern generator of the crayfish (Paul & Mulloney, 1985). In *Cavolinia* all interneurones phasically active during swimming have dense fields of finely arborizing neurites in the centres of the neuropile of both the ipsi- and contralateral pedal ganglia with the most extensive arbors usually situated contralaterally to the soma. Because essentially identical electrical activity can be recorded from all parts of these interneurones it is difficult to determine where a cell's inputs and outputs are located. It must be assumed that the morphological characteristics just described are of advantage to a neurone which must transmit signals that are probably not propagated. The comparatively long space-constant that results from such a geometry would ensure that the electrotonic coupling between input and output sites would be considerable (Rall, 1981). However, it should be remembered that spiking neurones in molluscs may also have similar electrical properties (Graubard, 1975; Gorman & Miroli, 1972).

It is not obvious what advantage accrues from using non-spiking interneurones to generate rhythmical motor patterns in *Cavolinia*, especially as *Clione* apparently uses spiking interneurones for the same purpose. Pearson (1986) has suggested that the frequency of a rhythmical pattern can be more easily modified over a greater range by using non-spiking interneurones. It will be necessary to examine control of frequency in these two genera before this idea can be evaluated. This study suggests that we may find that non-spiking interneurones that are essential elements for producing rhythmical and precisely timed motor patterns are present in a wider range of animal groups than had previously been suspected.

I thank the staff at the Statione Zoologique, Villefranche-sur-mer, France for their help and the National Research Council of Canada, CNRS and the Alberta Heritage Foundation for Medical Research for travel and subsistence funds. Research funding was provided by NSERC grant A0419.

REFERENCES

ARSHAVSKY, YU. I., BELOOZEROVA, I. N., ORLOVSKY, G. N., PANCHIN, YU. V. & PAVLOVA, G. A. (1985*a*). Control of locomotion in marine mollusc *Clione limacina*. I. Efferent activity during actual and fictitious swimming. *Expl Brain Res.* **58**, 255–262.

ARSHAVSKY, YU. I., BELOOZEROVA, I. N., ORLOVSKY, G. N., PANCHIN, YU. V. & PAVLOVA, G. A. (1985*b*). Control of locomotion in marine mollusc *Clione limacina*. II. Rhythmic neurons of pedal ganglia. *Expl Brain Res.* **58**, 263–272.

ARSHAVSKY, YU. I., BELOOZEROVA, I. N., ORLOVSKY, G. N., PANCHIN, YU. V. & PAVLOVA, G. A. (1985*c*). Control of locomotion in marine mollusc *Clione limacina*. III. On the origin of locomotory rhythm. *Expl Brain Res.* **58**, 273–284.

ARSHAVSKY, YU. I., BELOOZEROVA, I. N., ORLOVSKY, G. N., PANCHIN, YU. V. & PAVLOVA, G. A. (1985*d*). Control of locomotion in marine mollusc *Clione limacina*. IV. Role of type 12 interneurons. *Expl Brain Res.* **58**, 285–293.

BURROWS, M. & SIEGLER, M. V. S. (1978). Graded synaptic transmission between local interneurones and motor neurones in the metathoracic ganglion of the locust. *J. Physiol., Lond.* **285**, 231–255.

DELCOMYN, F. (1980). Neural basis of rhythmic behaviour in animals. *Science* **210**, 492–498.

GORMAN, A. L. F. & MIROLLI, M. (1972). The passive electrical properties of the membrane of a molluscan neurone. *J. Physiol., Lond.* **227**, 35–49.

GRAUBARD, K. (1975). Voltage attenuation within *Aplysia* neurons: the effect of branching pattern. *Brain Res.* **88**, 325–332.

GRAUBARD, K. (1978). Synaptic transmission without action potentials: input–output properties of a non-spiking presynaptic neuron. *J. Neurophysiol.* **41**, 1014–1025.

GRAUBARD, K., RAPER, J. A. & HARTLINE, D. K. (1980). Graded synaptic transmission between spiking neurons. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3733–3735.

HEITLER, W. J. & PEARSON, K. G. (1980). Non-spiking interactions and local interneurones in the central pattern generator of the crayfish swimmeret system. *Brain Res.* **187**, 206–211.

MENDELSON, M. (1971). Oscillator neurons in crustacean ganglia. *Science* **171**, 1170–1173.

PAUL, D. H. & MULLONEY, B. (1985). Non-spiking local interneuron in the motor pattern generator for the crayfish swimmeret. *J. Neurophysiol.* **54**, 28–39.

PEARSON, K. (1986). Neuronal circuits for patterning motor activity in invertebrates. In *Comparative Neurobiology: Modes of Communication in the Nervous System* (ed. M. J. Cohen & F. Strumwasser), pp. 225–244. New York: John Wiley & Sons, Inc.

PEARSON, K. & FOURTNER, C. R. (1975). Nonspiking interneurons in walking system of the cockroach. *J. Neurophysiol.* **38**, 33–51.

RALL, W. (1981). Functional aspects of neuronal geometry. In *Neurones Without Impulses: Their Significance for Vertebrate and Invertebrate Nervous Systems*, Soc. exp. Biol. Seminar Series 6 (ed. A. Roberts & B. M. H. Bush), pp. 223–254. Cambridge: Cambridge University Press.

ROBERTS, A. & BUSH, B. M. H. (eds) (1981). *Neurones Without Impulses: Their Significance for Vertebrate and Invertebrate Nervous Systems*, Soc. exp. Biol. Seminar Series 6. Cambridge: Cambridge University Press.

SATTERLIE, R. A. (1985). Reciprocal inhibition and postinhibitory rebound produce reverberation in a locomotion pattern generator. *Science* **229**, 402–404.

SATTERLIE, R. A. & SPENCER, A. N. (1985). Swimming in the pteropod mollusc. *Clione limacina*. II. Physiology. *J. exp. Biol.* **116**, 205–222.

SIMMERS, A. J. & BUSH, B. M. H. (1980). Non-spiking neurones controlling ventilation in crabs. *Brain Res.* **197**, 247–252.

STEWART, W. W. (1978). Functional connections between cells as revealed by dye coupling with a highly fluorescent napthalimide tracer. *Cell* **14**, 741–759.

TAKAHATA, M., NAGAYAMA, T. & HISADA, M. (1981). Physiological and morphological characterization of anaxonic non-spiking interneurons in the crayfish motor control system. *Brain Res.* **226**, 309–314.