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NEUROETHOLOGICAL STUDIES OF FREELY SWIMMING *APLYSIA BRASILIANA*

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SUMMARY

Recently developed neurophysiological and behavioural techniques were used to study swimming in the marine gastropod *Aplysia brasiliana*. *Aplysia* swim by bilateral parapodial flapping with an anterior to posterior metachronal wave. Parapodial oscillations are measured from video records. Population recordings from nerves innervating the parapodia during normal swimming in intact *Aplysia* reveal synchronous phasic activity in large efferent units associated with parapodial opening. Isolated brain studies and stimulation of central pathways in intact animals suggest a central pattern generator. We conclude that the output of the neuronal oscillator that controls parapodial flapping radiates synchronously from each pedal ganglion. The putative command to swim originates within the cerebral ganglia.

INTRODUCTION

Invertebrates have been used as model systems to investigate neural control of behaviour (Kandel, 1976; Hoyle, 1977). Their distributed nervous systems have made it possible to study isolated ganglia *in vitro* and to work out detailed connexions among identified neurones. To examine the behavioural role of these neurones, a variety of semi-intact *in vivo* preparations have been developed that are amenable to intracellular analysis. However, most behavioural activities do not occur normally when an animal is surgically opened or simply restrained; it is therefore necessary to record neuronal activity in a freely behaving animal. Furthermore, to correlate ongoing patterns of neuronal activity and behaviour it is necessary to record simultaneously from many neurones. Recording of neural activity during normal behaviour is routine in vertebrates (Phillips, 1973), but has rarely been carried out in invertebrates (Aréchiga & Wiersma, 1969; Wine & Krasne, 1972; Larimer & Eggleston, 1971; Weiss, Cohen & Kupfermann, 1978; Drewes, Landa & McFall, 1978; Magni & Pellegrino, 1978). We have implanted cuff electrodes on various nerves and connectives to record activity in neurosecretory cells (Pinsker & Dudek, 1977), giant sensory neurones (Cobbs &

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Pinsker, 1978), and neurones mediating siphon withdrawal (Kanz, Eberly, Cobbs & Pinsker, 1979) in freely behaving *Aplysia*, a marine gastropod mollusc.

We now use this neuroethological approach to study neuronal populations that become active during a more complex rhythmic behavioural sequence – swimming in *Aplysia brasiliana*. Previous intracellular studies of dissected 'whole animal' preparations of *A. fasciata* (Weevers, 1971) yielded only a small percentage of preparations with recognizable swimming movements for long enough periods to be useful experimentally. Our studies suggest that there is a synchronous neuronal oscillator within each pedal ganglion that underlies parapodial flapping and may receive the command to swim from neurones that originate within the cerebral ganglia.

MATERIALS AND METHODS

Animal maintenance

Aplysia brasiliana (180–280 g) was collected off South Padre Island, Texas. Animals were housed in 18 × 13 × 13 cm perforated plastic cages within large holding tanks containing artificial sea water (Instant Ocean) at 20–23 °C, and maintained on a diet of dried seaweed (Consul Dried Laver). All experiments were performed with animals in a 14 × 17 × 24·5 cm compartment within a 95 l aquarium, with aerated and cooled sea water.

Surgical procedures

To implant cuff electrodes or to perform nerve lesions, the animal was anaesthetized with isotonic $MgCl_2$ solution (*ca.* 25% of body weight) injected into the foot sinuses and placed in a solution of $\frac{1}{2}$ sea water and $\frac{1}{2}$ isotonic $MgCl_2$ for the duration of surgery (0·5–2 h depending on type of procedure). A longitudinal incision (2–2·5 cm) was made dorsally from about the level of the rhinophores caudally, which provided direct access to the circumesophageal ganglia. Post-operatively the animal was injected with sterilized sea water to restore pre-operative body weight, and additional injections were administered if necessary. Approximately 24 h elapsed between surgery and testing. Only animals that were healthy and swam normally were used for electrophysiological analyses.

Electrophysiological procedures

Whole-nerve cuff electrodes (Morris & Maynard, 1970; Hoffer & Marks, 1976; Stein *et al.* 1977) provide a non-invasive monitor of large populations of neurones in freely behaving animals. Multiple-electrode cuff assemblies consisting of stainless steel wire electrodes embedded in silastic tubing (0·51 mm or 0·30 mm i.d.) and separated by a distance of 0·5 cm were implanted surgically on peripheral pedal nerves or central connectives (see Fig. 3, top). A detailed description of the double cuff assembly is given by Cobbs & Pinsker (1978). Each monopolar electrode was referenced to an indifferent lead in the chamber. The headstages of the amplifiers (WP Instruments, Inc., Model DAM-5A) were located close to the water surface and 60 Hz notch filters were used to reduce noise at the time of recording. The electrophysiological data were recorded at $1\frac{7}{8}$ i.p.s. on an FM tape recorder (Hewlett-Packard 3968A). For analogue hard copies the taped data were played back at $\frac{15}{32}$ i.p.s. onto a Gould Brush 440 pen-

recorder, and an adjustable Krohn–Hite filter (set at 15 Hz high pass) was used to attenuate low-frequency noise. For digitized hard copies the taped data were played at real speed into an A/D converter, digitized at 5 KHz and displayed on a printer/plotter (Gould 5000).

In some experiments the nerves or connectives were stimulated through the cuff electrodes with trains of monophasic 1–2 msec pulses (3–20 V) delivered by a Grass S-88 stimulator through an SIU-5 stimulus isolation unit set for direct coupling and reverse polarity.

Following chronic recording experiments the incision was reopened and the brain (cerebral, pleural and pedal ganglia) was isolated by cutting the nerves and connective tissue sheaths. Implanted nerves were cut distal to the electrodes and the isolated brain was pinned in a 60 ml dish perfused with artificial sea water. The electrode wires were reconnected to the same amplifier headstages and signals recorded as before.

The motor fields of peripheral pedal nerves were determined with suction electrodes in a semi-intact, eviscerated preparation. Stimulating the cut distal ends of the nerves (5 s trains, 50 Hz, 2 ms pulses, 15 V through a Grass SD-5 stimulator) produced tetanic contractions in a circumscribed region of the parapodia.

Behavioural procedures

Pre- and post-operative swimming was monitored on videotape (Panasonic model WV-1350 TV camera, Panasonic time-lapse VTR model NV-8030) with the camera positioned directly above the animal. The data were played back one frame at a time (150 ms between successive frames) to measure parapodial oscillations. The linear distance was obtained from the midline of the animal to the edge of the parapodia at three locations (anterior 57%, middle 41%, posterior 25% of the total distance from the tail) along the midline (see Fig. 1*a*). These data were plotted by hand or by computer (PDP 11/45) on a Gould 5000 printer/plotter. In some experiments swimming was measured at different aquarium temperatures with a minimum acclimation time of at least 45 min.

RESULTS AND DISCUSSION

Aplysia swim by flapping (i.e. alternately opening and closing) their bilaterally symmetrical parapodia (Farmer, 1970; Bebbington & Hughes, 1973; Hamilton & Ambrose, 1975). In each cycle a metachronal wave starts in the anterior parapodia and moves posteriorly. A non-invasive behavioural monitor was provided by the video-tape records of swimming animals. Two temporal properties can define the parapodial oscillations (Fig. 1*a*): the period of the swimming oscillator is the time between successive peaks (i.e. maximal openings) in the anterior parapodia, and the metachronal offset is the time between the peaks in the anterior and posterior parapodia (i.e. the anterior to posterior offset). At a given temperature, *Aplysia* swim with a fixed period of parapodial oscillation. Behavioural measures were obtained at normal (*ca.* 20 °C) and at reduced (12 °C) temperature (Fig. 1*b*). At the reduced temperature the period of the swimming oscillator increased from $1{\cdot}86 \pm 0{\cdot}12$ *vs* $3{\cdot}84 \pm 0{\cdot}80$ s (mean $\pm$ S.E.M., $N = 3$); however the anterior to posterior offset remained the same ($0{\cdot}54 \pm 0{\cdot}01$ *vs* $0{\cdot}53 \pm 0{\cdot}03$ s). This resulted in a change in the phase relationship between the anterior and posterior oscillations at lower temperatures. The independence of the period of the swimming

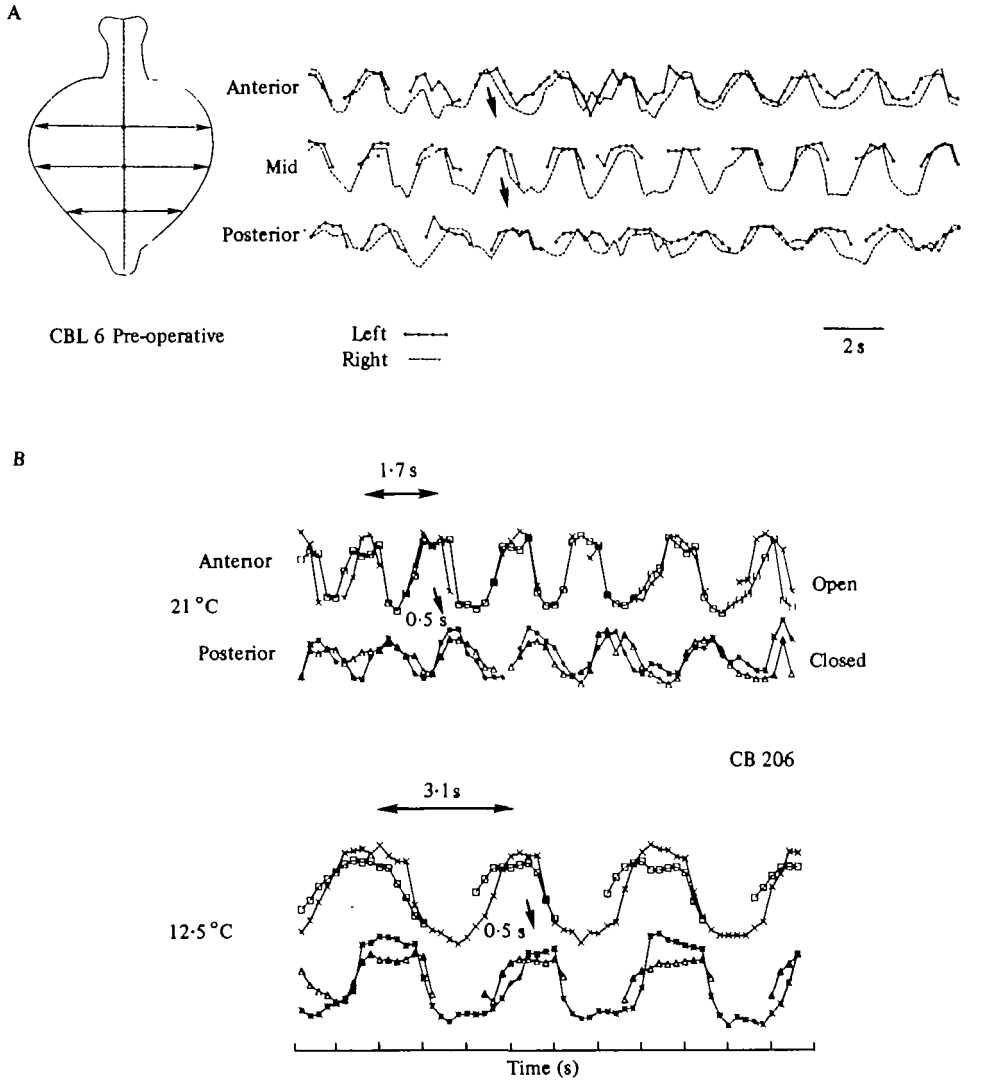

Fig. 1. Parapodial oscillations during swimming. (A) Changes in linear distance from the margin of the parapodia to midline at three locations (left) were measured from videotape records. Bilateral oscillations are plotted for ten swim cycles (right): opening is plotted upwards, and movements of right (– – –) and left (● — ●) parapodia are superimposed. Diagonal arrows indicate the anterior to posterior metachronal offset. Missing data points result from overlap of one parapodium on the other. (B) The period of parapodial flapping (horizontal arrows) increases whereas the temporal offset (diagonal arrows) remains the same at the lower temperature. Note increased amplitude of parapodial movement at the lower temperature.

oscillator and the timing (the absolute offset) of the metachronal wave suggests that they have different underlying mechanisms.

The first step in the neuronal analysis was to determine the critical efferent nerves mediating parapodial movements during swimming by means of lesions and electrical stimulation of peripheral nerves. *Aplysia* do not have a segmentally organized body or

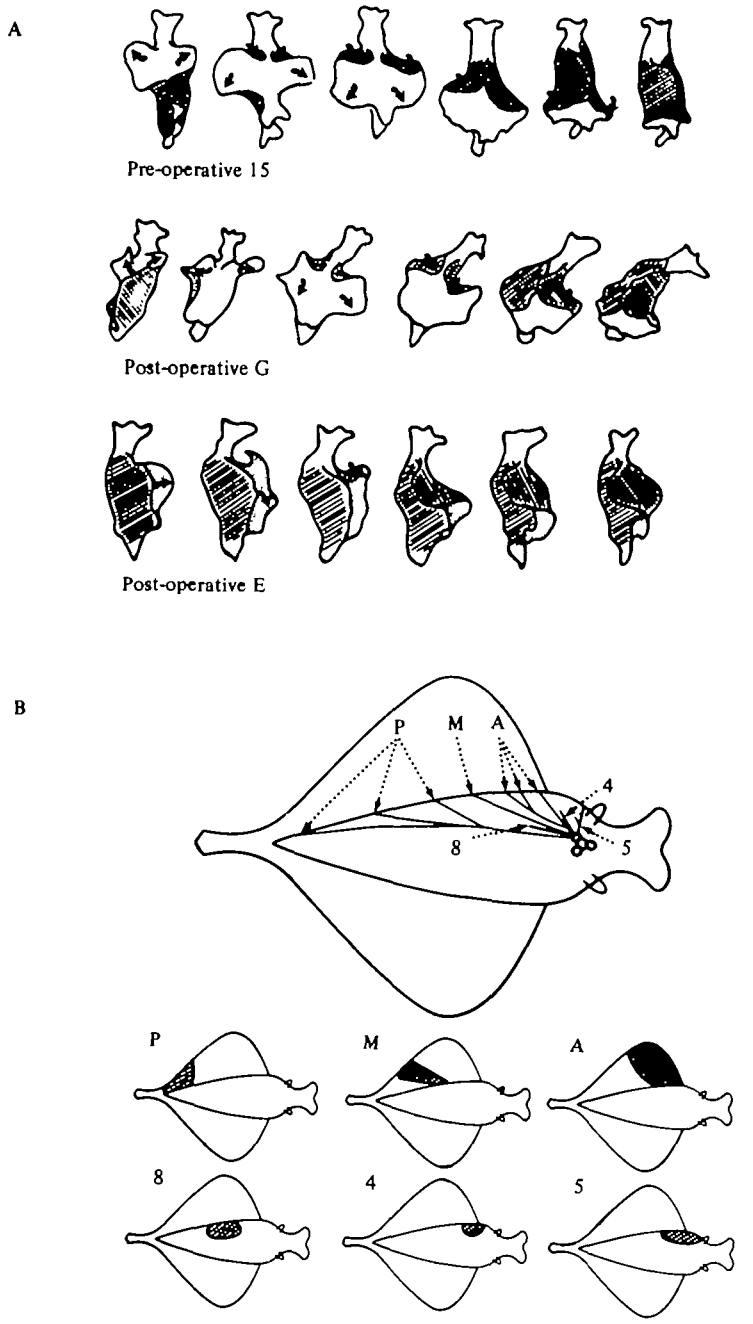

Fig. 2. Peripheral nerve lesions and motor fields. (A) Sequence of parapodial movements during one cycle of swimming in unoperated animal (top), in animal with only anterior, middle and posterior parapodial nerves remaining on right side (middle) and in animal with anterior, middle and posterior nerves cut on left side (bottom). Drawings made from videotape tracings. (B) Peripheral distribution of left pedal nerves innervating foot and parapodia (top). Pedal nerve motor fields (shaded areas) as determined by electrical stimulation (50 Hz; 2 ms pulses; 5 s train at 15 V) of cut distal ends of pedal nerves in parapodia or foot of semi-intact preparation (bottom). Pedal nerve nomenclature adapted from Jahan-Parwar (personal communication).

nervous system; three major nerves radiate from the bilateral pedal ganglia to innervate anterior, middle and posterior parts of the parapodia (Bullock & Horridge, 1965; Jahan-Parwar & Fredman, 1978*a*, *b*). Our lesion studies (Pinsker *et al.* 1978) confirm previous suggestions (Hughes & Tauc, 1962; Dorsett, 1968, Hughes, 1971) that all of the sensory and motor innervation of the parapodia is through these three nerves. Since parapodial flapping is bilaterally symmetrical, unilateral lesions could be performed with the unoperated side as a control. Cutting the anterior, middle and posterior parapodial nerves unilaterally abolished all active parapodial movement on that side during swimming (Fig. 2*a*, bottom), whereas normal flapping was obtained when only these three nerves remained (Fig. 2*a*, middle). Electrical stimulation of cut peripheral nerves in semi-intact preparations indicated an extensive motor field for the anterior nerve and more restricted motor fields for the middle and posterior nerves (Fig. 2*b*). These motor fields are generally similar to the sensory fields described for homologous nerves in *A. fasciata* (Hughes, 1971). In keeping with the more extensive motor field, selective lesions of the anterior nerve produced the most severe impairment of parapodial movement during swimming. These findings suggest that these three peripheral nerves are necessary and sufficient for mediating parapodial flapping during swimming.

Central lesions also produce clear effects on parapodial flapping. Transection of the pedal commissure resulted in lack of co-ordination between right and left parapodia during swimming, however each could independently maintain relatively normal flapping. Bilaterally cutting the cerebro-pedal connectives eliminated all swimming although the efferent nerves were still intact (Jahan-Parwar & Miller, 1978). If the commissure and one cerebro-pedal connective were cut, swimming occurred only with the parapodia contralateral to the cut connective. Neither bilateral transection of the cerebro-pleural connectives nor removal of both pleural ganglia had an effect on parapodial flapping during swimming, in contrast to the findings of Jahan-Parwar & Miller (1978) that transection of the pedal–pleural connective increased the period of parapodial oscillations. These findings suggest that each pedal ganglion contains a neuronal oscillator for rhythmic parapodial movements, bilateral synchrony of parapodial oscillations is mediated by the pathway connecting the pedal ganglia, and the putative command pathway for initiating swimming is the cerebro-pedal connective.

The next step in the neuronal analysis was to record population activity during normal swimming from the critical efferent nerves of the pedal ganglia. Cuff assemblies containing two monopolar electrodes (to determine direction and velocity of impulse conduction) were implanted on various peripheral pedal nerves (Fig. 3). In a quiescent animal, background spontaneous activity in a single parapodial nerve consisted of many medium and small amplitude efferent units (mean amplitude *ca.* 20 μV anterior nerve, 100 μV posterior nerve; conduction velocities from 0·5 to 0·8 m/s) that fired asynchronously (Fig. 3, end of trace). Swimming occurred spontaneously or could be elicited by removing the animal from the substrate. During swimming, a population of larger efferent units (40 μV anterior nerve to 200 μV posterior nerve; 0·5–1·0 m/s conduction velocity) was recruited that fired phasically prior to and during the opening of the parapodia (Fig. 3, beginning of trace). These findings suggest that we can record reliably from a population of efferent neurones whose activity is correlated with parapodial opening. We cannot rule out the possibility that this same population might be active under some other behavioural state, but we did not observe activity in the largest units except during parapodial flapping when an animal was swimming.

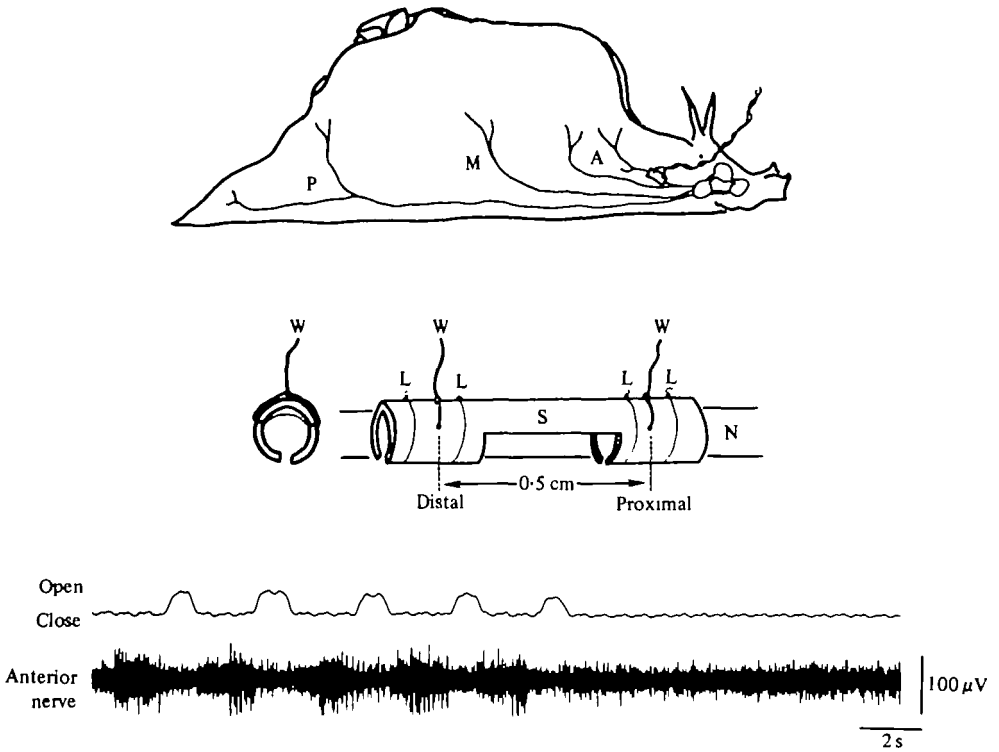

Fig. 3. Population recording with cuff electrode assembly in intact animal. Top: cuff assembly in place on anterior parapodial nerve. Middle: double electrode cuff assembly. S, silastic tubing; W, 25 μm stainless steel wire; L, 6–0 silk thread ligature. Proximal and distal are with reference to ganglia from which the nerve (N) emanates. In the present experiments, electrode separation was typically 0·5 cm. Wire inside tubing has insulation scraped off and is in contact with the nerve. Bottom: upper trace is output of photocell response monitor positioned so that parapodial opening produced an upward deflexion. Exact location of the peak depends on the position of the photocell with respect to the parapodia. Lower trace shows neuronal activity in anterior parapodial nerve recorded from one electrode of cuff assembly. During swimming a single burst of activity occurs in large efferent units on each swim cycle. When animal spontaneously stops swimming, phasic activity in large efferent units ceases.

The efferent motor output underlying locomotion in segmental animals (e.g. cats, cockroaches, leeches) involves a temporal offset between motoneurone activity in adjacent segments that accounts for metachronal offsets between different segmental muscle groups or limbs (Pearson, 1976; Herman *et al.* 1976; Kristan *et al.* 1977; Stein, 1978; Stent *et al.* 1978). If the neuronal mechanisms underlying metachronal parapodial flapping in *Aplysia* are similar to those in segmental animals, then recording during normal swimming should reveal a temporal offset (*ca.* 540 ms) between bursts in ipsilateral anterior and posterior nerves associated with the anterior to posterior wave of parapodial movement. Simultaneous recordings from *contralateral* nerves during swimming revealed synchronous phasic activity in large efferent units in keeping with the bilateral symmetry of the parapodial movements. However, simultaneously recorded phasic activity in *ipsilateral* nerves was also synchronous (Fig. 4): the predicted

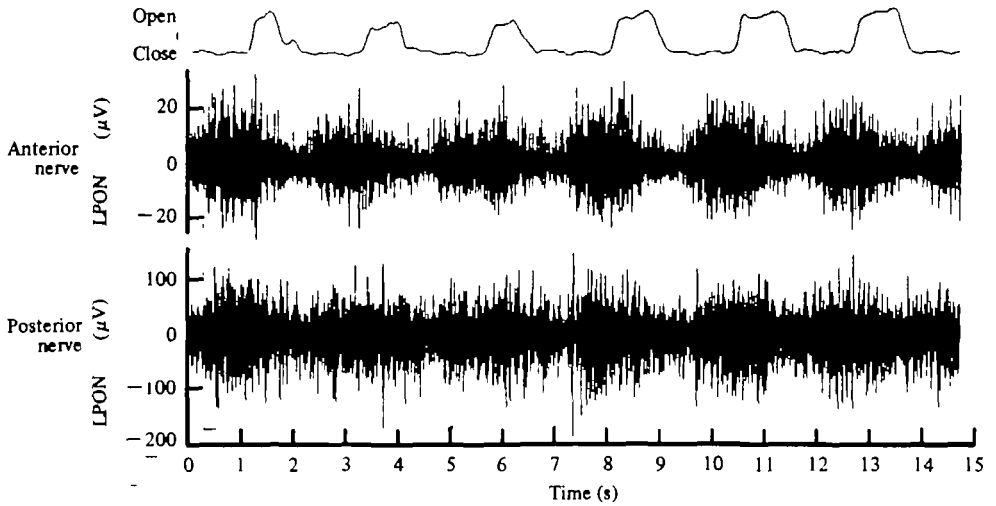

Fig. 4. Synchronous ipsilateral phasic activity in intact animal. Synchronous phasic activity recorded simultaneously from ipsilateral anterior and posterior parapodial nerves. No consistent temporal offset is seen between bursts in anterior and posterior nerves. Neuronal data were digitized at a sampling rate of 200 μs.

540 ms offset between phasic activity in anterior and posterior nerves was absent when recorded near the pedal ganglia. Thus the pattern of efferent neuronal output underlying parapodial flapping during swimming in *Aplysia* consists of phasic bursts of activity that radiate synchronously through all of the parapodial nerves.

Some differences in the time of arrival of efferent activity at the anterior and posterior regions of the parapodia would result from differences in conduction time in nerves of different lengths. However, assuming no decrement in conduction velocity down the axons, this would only account for a relatively small metachronal offset. The average distance from the pedal ganglion to the insertion point of the anterior nerve is about 5 cm, and for the posterior nerve about 17 cm. An efferent impulse with a conduction velocity of 1·0 m/s should reach the motor field for the posterior nerve about 120 ms after it reaches the corresponding point for the anterior nerve, which would be too short a delay to account entirely for the 540 ms behavioural offset in the parapodia. Our present evidence cannot rule out the possibility that conduction velocity is different in the distal regions of the posterior nerve, as has been described in squid stellate nerves (Pumphrey & Young, 1938). However, lowering the temperature from 19 to 13 °C produced a 32% decrease in conduction velocity of the large efferent units associated with parapodial opening (which would increase the calculated neuronal delay from 120 to about 160 ms), yet the anterior to posterior behavioural offset was sometimes shorter at lower temperatures. Thus, the lack of any systematic effect of cooling on the anterior to posterior offset is in keeping with the suggestion that axonal conduction time does not contribute substantially to the timing of the metachronal wave. The independence of the swim period and the speed of the metachronal wave suggests that the former is controlled by the central neuronal oscillator and the latter by a temperature-independent mechanism. Mechanical linkage within the parapodia could provide a simple way of timing the metachronal wave, representing a phyletically more primitive means of

co-ordinating activity in various parts of the parapodia than that described for locomotion in segmental animals. This type of organization may reflect the difference between multiple discrete locomotor appendages or segmentally arranged bands of muscles on the one hand, and a single, mechanically continuous locomotor appendage such as the parapodia on the other. Our inability with present whole-nerve electrodes to record from smaller axons in intact animals does not allow us to rule out the possibility that smaller efferent volleys with the appropriate offset are buried within the phasic bursts in more posterior nerves. It is also possible that a subset of central efferent neurones mediating peripheral inhibition or a peripheral nerve net might contribute to the timing of the metachronal wave.

The fact that the largest units in all three nerves are the ones that are synchronously active only during rhythmic parapodial flapping suggests that they are members of a functionally related population. In intact animals we did not record activity from large units phase-locked with parapodial closing (i.e. between bursts the general amplitude and level of activity was similar to the background activity during quiescence). Since antagonistic populations of motor neurones are so common in locomotor systems, the lack of reliably observable antiphasic activity under these conditions probably results from the small size of the axons that are associated with parapodial closing. In keeping with this suggestion, we have observed smaller amplitude antiphasic activity in peripherally sectioned nerves, presumably due to a decrease in total amount of activity (Fig. 5*b*).

The ultimate goal of this analysis is to reduce the intact animal to a semi-intact preparation for recording intracellularly from the same population of phasically active efferent neurones. It will then be possible to establish whether an individual unit is a motoneurone and to examine synaptic and pacemaker mechanisms within central ganglia. As a step in this direction we established that the cuff electrodes are stable enough to remain in position and provide comparable records when the brain was isolated for *in vitro* examination. Preliminary isolated brain preparations (Fig. 5) showed background spontaneous activity in smaller units, similar to that seen previously when the intact animal was quiescent. A sudden spontaneous onset of activity in large efferent units was sometimes observed synchronously in all nerves innervating the parapodia, and lasted from 30 s to 5 min. In some cases these units fired phasically and, in keeping with *in vivo* activity during swimming, the phasic activity was also synchronous in all parapodial nerves. However, fewer units appeared to contribute to the burst and its length and periodicity were more variable in the isolated brain (Fig. 5). If these findings are not simply due to injury, then sensory feedback in *Aplysia* appears to stabilize the swim oscillator activity cycle and the correlated efferent volley. In other species, the period of efferent neuronal activity underlying rhythmic locomotor movements also undergoes changes when the brain is isolated from sensory feedback (Kristan & Calabrese, 1976; Wilson, 1972; Grillner, 1975).

If the phasically active neurones in the isolated brain are from the same population of neurones that fired phasically in the intact animal, then these results also demonstrate that the neuronal oscillator for *Aplysia* swimming is centrally located. Independent evidence for the location of the swimming oscillator comes from transections of central connectives combined with electrical stimulation through implanted cuffs in intact animals. As reported above, bilateral lesions of the cerebro-pedal connectives com-

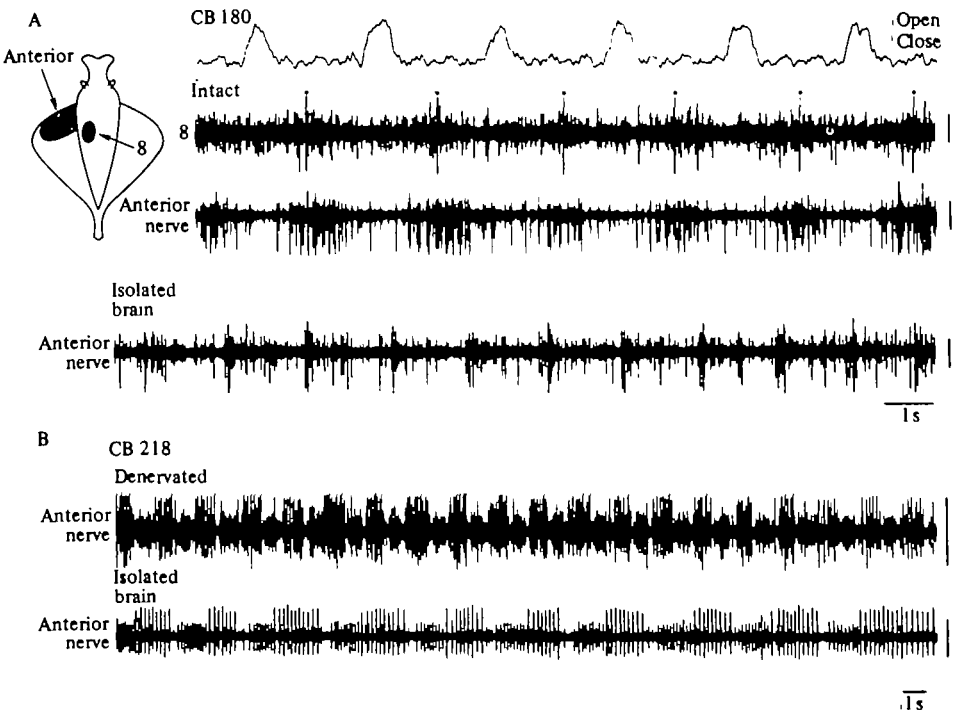

Fig. 5. Phasic pedal nerve activity in reduced preparations. (A) Activity was recorded first *in vivo* during swimming (top three traces) and then in isolated brain (bottom) from same cuff electrode. Note that there is some phasic activity in nerve 8 (middle pedal nerve) which innervates the foot; largest unit in nerve 8 fires once per cycle (dots). Note differences in phasic bursts in anterior parapodial nerve records between *in vivo* and isolated brain preparations. Motor fields (left) of the nerves for this animal were determined in semi-intact preparation. (B) Neuronal activity from anterior parapodial nerve from a different, denervated animal (upper trace). Denervation involved bilaterally cutting the anterior, middle and posterior parapodial nerves at the time the cuff was implanted. The nerve on which the cuff assembly was implanted was cut distal to the cuff. Note alternating large and small amplitude bursts. Lower trace is activity recorded from same electrode in the isolated brain. Note alternating large and small amplitude bursts in what appear to be two individual members of the population, and much longer duration and period of these phasic bursts compared to denervated animal. Vertical bars represent 100 μV.

pletely abolished swimming. However, tonic stimulation (10 Hz) of the cut end of a connective attached to the pedal ganglion could elicit swimming in the intact animal for as long as the stimulus was maintained (Fig. 6). With selective stimulation, elicited parapodial oscillations had an anterior to posterior offset similar to the pre-operative behaviour of the same animal (Fig. 6). This suggests that the command for swimming descends from the cerebral ganglion, but that the phasic activity underlying parapodial flapping is organized within the pedal ganglia. Thus, *Aplysia* swimming resembles locomotion in other species in that the neuronal oscillator is centrally located and its activity is probably modulated by sensory feedback.

To specify the detailed pattern of neuronal activity that underlies parapodial flapping it will be necessary to describe the simultaneous firing of many members of the population in swimming animals and to recognize these same units in semi-intact or isolated

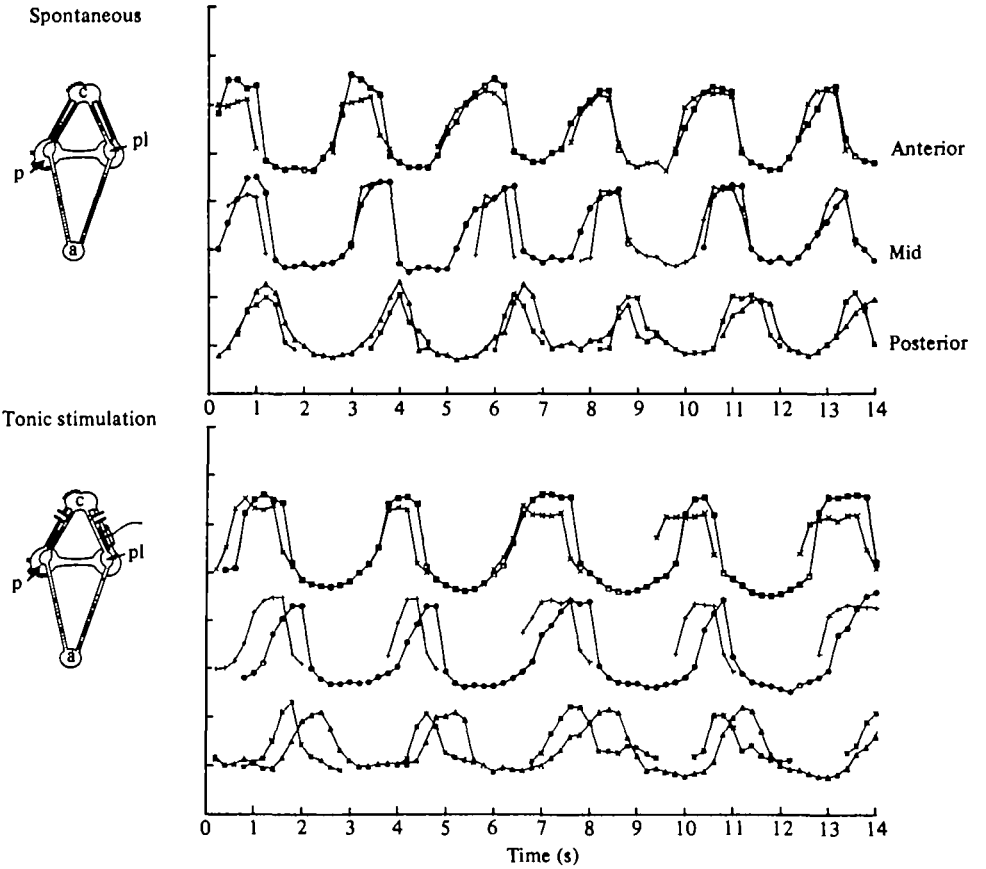

Fig. 6. Stimulation of cut putative command pathway in intact animal. Pre-operative measures (top) from videotape recordings of normal parapodial oscillations during spontaneous swimming (2·4 s period; 0·75 s anterior to posterior offset for left and right sides). The animal then received bilateral cerebro-pedal connective lesions and a cuff was implanted on the right side (bottom left). The animal no longer swims spontaneously, but tonic electrical stimulation (bottom; 10 Hz, 1·5 ms pulses at 5 V) elicits swimming (2·9 s period; 0·75 s offset on right side; 1·0 s offset on left side). Despite the basic similarities there are differences in period and bilateral synchrony that may be due to the artificial nature of the unilateral electrical stimulation. Inset: c, cerebral; p, pedal; pl, pleural; a, abdominal ganglion.

neuronal preparations. A major limitation of the present approach is that there is too much activity in the intact swimming animal in whole peripheral nerves to be analysed by currently available spike separation techniques that depend on unitary waveforms (Camp & Pinsker, 1979). More selective recordings from smaller populations within the nerve would be helpful in this regard.

We have applied a variety of techniques to analyse swimming in *Aplysia*. Our neuroethological approach emphasizes recording of large neuronal populations during normal behaviour in intact animals as an important prerequisite for a cellular analysis. The availability of criteria for normal behaviour in intact animals provides evidence that the recorded pattern of neuronal activity is normal. Once this pattern of activity is characterized it should be possible to determine in what ways the pattern of activity in more

reduced preparations is different. Given the difficulty of obtaining normal swimming in semi-intact preparations, demonstrating that a given unit is a parapodial motoneurone is not sufficient evidence that this unit is involved in swimming, since the parapodia are also involved in a variety of other spontaneous behavioural activities (Hening *et al.* 1976) and reflexes (Jahn-Parwar & Fredman, 1978*a*, *b*). With the present approach it should be possible to gradually reduce the intact animal to a semi-intact preparation and thereby relate the neuronal recordings during natural behaviour to intracellular recordings from individual members of the population (Kanz *et al.* 1979).

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