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CENTRAL CIRCUITRY IN THE JELLYFISH *AGLANTHA DIGITALE*

III. THE ROOTLET AND PACEMAKER SYSTEMS

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Summary

Tactile stimulation of the subumbrella of *Aglantha digitale* was found to evoke an escape swimming response similar to that evoked by stimulation of the outer surfaces of the margin but that does not involve the ring giant axon. Evidence is presented that conduction around the margin takes place *via* an interconnected system of rootlet interneurones. Confocal microscopy of carboxyfluorescein-filled axons showed that the rootlet neurones run out from the bases of the motor giant axons within the inner nerve ring and come into close contact with those of the neighbouring motor giant axons on either side. Transmission between the rootlet neurones has the properties of chemical synaptic transmission. A distinct type of fast excitatory postsynaptic potential (rootlet PSP) was recorded in motor giant axons following stimulation of nearby axons in 3–5 mmol l^{-1} Mn^{2+}, which lowered the PSP below spike threshold. Immune labelling with anti-syntaxin 1 showed structures tentatively identified as synapses in the inner nerve ring, including some on the rootlet neurones. Neuromuscular junctions were not labelled.

A secondary consequence of stimulating motor giant axons was the triggering of events in the pacemaker system. Triggering was blocked in 105 mmol l^{-1} Mg^{2+}, indicating a synaptic link. Activity in the pacemaker system led indirectly to tentacle contractions (as described in earlier papers in this series), but the contractions were not as sudden or as violent as those seen when escape swimming was mediated by the ring giant axon. Events triggered in the pacemaker system fed back into the motor giants, producing postsynaptic potentials that appeared as humps in the spike after-potential. The conduction velocity of events propagating in the relay system was increased when the rootlet pathway was simultaneously excited (piggyback effect).

With the addition of the rootlet pathway, the number of identified systems concerned with locomotion, feeding and tentacle contractions comes to fourteen, and the list is probably nearly complete.

Key words: Cnidaria, jellyfish, *Aglantha digitale*, hydromedusan behaviour, locomotion, nervous system, central circuitry, syntaxin 1, postsynaptic potential, nerve ring, motor giant axon, rootlet interneurone, intracellular recording, pacemaker system, relay system.

Introduction

Aglantha digitale probably has the most complicated nervous system among known Cnidaria in terms of circuitry, having pathways for both escape and non-escape locomotion (Mackie and Meech, 1995a,b); other medusae that have been studied in detail, such as *Polyorchis penicillatus* (Spencer, 1978, 1979; Spencer and Arkett, 1984), can swim in only one way. The complexity of the wiring in these nervous systems is surprising in a group of animals considered to be primitive, but it is matched in the area of neuroendocrinology where recent studies on the sea pansy *Renilla köllikeri* have also revealed unexpected complexity (Anctil, 1987, 1989; Anctil et al., 1991; Pani et al., 1995). Since the time of Pantin (1952), and as knowledge of these nervous systems has increased, it has become increasingly clear that cnidarian nerves and synapses operate in much the same way as those of higher animals, and the lack of a brain is now seen as an adaptation to radial symmetry rather than an indication of primitiveness (Satterlie and Spencer, 1987). In the case of *A. digitale*, the bundles of nerves running around the margin quite clearly fulfil the role of a central ganglion (Mackie and Meech, 1995a,b). Here, we describe a new and behaviourally important conduction system and its interactions with those described previously.

In *A. digitale*, slow, rhythmic swimming occurs when the animal is fishing for food (Mackie, 1980) and originates in the output of pacemaker neurones in the marginal nerve rings, as in other hydromedusae (Satterlie and Spencer, 1983). Escape swimming follows tactile or vibrational stimulation of the tentacles and outer surfaces of the margin and involves the conduction of action potentials around the margin in the ring giant axon (Roberts and Mackie, 1980). In both types of

swimming, the subumbrellar swimming muscles are excited by impulses propagated in the eight motor giant axons that run up the inside of the bell from their origins in the inner nerve ring. However, during slow swimming, the motor giants conduct Ca^{2+} spikes, while in escape swimming they conduct Na^{+} spikes (Mackie and Meech, 1985). The muscle contractions seen in slow swimming are much weaker than those seen in escape swimming (Donaldson et al., 1980), and the two responses can easily be distinguished by the naked eye. Tentacle contractions accompany swimming in both cases, but are much more sudden and violent in the escape response, during which excitation of the ring giant axon is always accompanied by excitation in the giant axons that run down the tentacles, causing twitch responses. Analysis of the underlying circuitry has revealed two sets of interneurones, the relay and carrier systems, that function in the transfer of information from the pacemaker neurones to the tentacle action systems during slow swimming (Mackie and Meech, 1995a,b), bringing about the slower, graded contractions.

The production of two different sorts of spike in the motor giants has been investigated by intracellular recordings and voltage-clamp and patch-clamp analysis of the membrane channels (Meech and Mackie, 1993a,b, 1995). Na^{+} spikes are generated by fast-rising excitatory postsynaptic potentials (PSPs) representing input from the ring giant (RG) axon, while Ca^{2+} spikes arise from slow PSPs representing input from the pacemaker (P) system. These will be referred to here as RG-PSPs and P-PSPs respectively.

We now find that there is a third type of excitatory synaptic input into the motor giants which, like the RG-PSP, generates Na^{+} spikes but which does not come from the ring giant. The evidence presented in this paper makes it clear that the new type of PSP is due to input from adjacent motor giants transmitted directly between the rootlet interneurones. This rootlet system was first described by Weber et al. (1982), whose summary drawing, based on Lucifer Yellow injections, is reproduced here (Fig. 1). The rootlet neurones, like the lateral neurones and basal plexus that distribute excitation to the muscle sheet (Kerfoot et al., 1985), were found to be dye-coupled to the motor giant and to each other. Each motor giant makes contact with processes of these rootlet neurones in the inner nerve ring. These taper away to a few slender processes in the zone midway between the motor giants, where they appear to overlap with those associated with the neighbouring axon. Unlike Lucifer Yellow, horseradish peroxidase (HRP) injected into motor giants did not penetrate the rootlet system, showing that there was no syncytial continuity between the two, although the motor giants themselves are syncytial structures (Weber et al., 1982). Gap junctions were revealed by electron microscopy at interfaces between motor giants and certain neurones in the inner nerve ring. The latter were thought to be rootlet neurones, so it was concluded that the rootlet system is connected to the motor giants by membrane partitions containing gap junctions. The failure of HRP to enter the rootlet neurones was consistent with this interpretation. In the present paper, we confirm the general picture of the rootlet system presented by these workers.

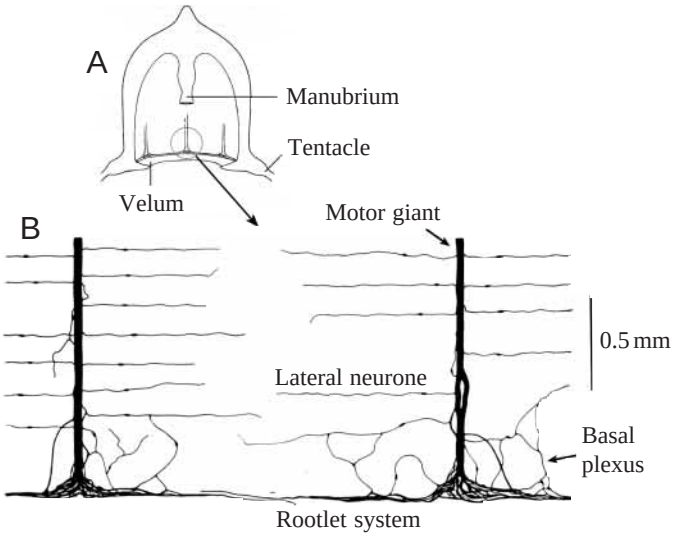

Fig. 1. *Aglantha digitale* cut in half vertically (A) to show (B) the location of motor giant axons and the rootlet system, adapted from Weber et al. (1982) with permission. Bell diameter in A approx. 5 mm.

Materials and methods

Immunohistology

Specimens of *Aglantha digitale* Müller caught off the dock at the Friday Harbor Laboratories, University of Washington, USA, were kept in glass containers at 7 °C until used. They were dissected in sea water containing 115 mmol l^{-1} Mg^{2+} to immobilize them. The top of the bell was cut off, leaving a cylinder of body wall that was cut vertically and pinned out flat, subumbrella up, in a Sylgard-lined Petri dish using cactus spines as pins. Preparations were fixed for 3–4 h in 4 % paraformaldehyde in 0.1 mol l^{-1} phosphate-buffered saline (PBS) at pH 7.3 followed by several rinses in 0.1 mol l^{-1} PBS containing 0.3 % Triton X-100 and 0.03 % sodium azide (PTA). To visualize general nerve structure and layout, a mouse anti-tubulin antibody (Amersham, N356) diluted 1:50 in PTA was used; to demonstrate possible synaptic areas, a rabbit anti-syntaxin 1 antibody (Calbiochem, 574784) diluted 1:25 or 1:50 was used. Goat serum was added to the incubating solutions at 3–5 %. After 8–12 h, preparations were rinsed in PTA and treated with the appropriate secondary antibodies for a further 8–12 h before washing and mounting in 50 % glycerol containing 0.3 % *n*-propyl gallate. FITC-coupled goat anti-mouse secondary antibody was used to display tubulin and CY5-coupled goat anti-rabbit secondary to display syntaxin 1. In all these experiments, controls were run in which the primary antibody was omitted. Mounted preparations were studied by laser scanning confocal microscopy using a BioRad MRC-600 microscope at Friday Harbor and a Zeiss LSM 410 microscope at the University of Victoria.

Carboxyfluorescein fills

Samples were prepared as above but were pinned out on thin, flexible strips of Sylgard in sea water containing 115 mmol l^{-1} Mg^{2+}. The tips of thin-walled glass electrodes

were filled with 5% carboxyfluorescein filtered through 0.2 μm cellulose acetate membrane filters. The shaft was filled with 2 mol l^{-1} potassium acetate. Dye was injected into giant motor giant axons by ionophoresis, using 1 nA, 2 ms hyperpolarizing current pulses, at 2 pulses s^{-1}. Preparations were left for 5 min to allow the dye to penetrate the rootlet system. The strip of Sylgard bearing the preparation was then inverted and pressed down lightly against a coverglass sealed into a 1 cm diameter hole in the bottom of a small Petri dish with the preparation centred over the hole. This arrangement allowed filled axons to be brought into focus with substage objectives. Observations were made using the BioRad MRC-600 confocal microscope.

Physiology

Observations on the behaviour of whole animals were made in finger bowls under a dissecting microscope. For recordings, preparations were dissected and pinned out as described above for carboxyfluorescein fills. Extracellular recordings were made with polyethylene suction electrodes pulled out to a 30–60 μm internal tip diameter. Intracellular recordings were made with glass microelectrodes filled with filtered 3 mol l^{-1} KCl and having a resistance of 50–60 MΩ. Amplified signals were displayed on an oscilloscope and stored on tape with an instrumentation tape recorder for later study. Stimuli were applied externally through small coaxial bipolar metal electrodes. Temperature was maintained at 10–12 °C using a thermoelectric cooling stage. Further details of these procedures are given in Mackie and Meech (1995a).

Results

Structure

A fairly complete picture of the major components of the nervous system was obtained with anti-tubulin fluorescence microscopy. The motor giant axons, lateral neurones, basal plexus and nerve ring elements were all well shown (Fig. 2A), confirming the general layout of the subumbrellar motor components described from Lucifer Yellow injections by Weber et al. (1982) (see also Fig. 1). However, because the great mass of neurones comprising the inner nerve ring was also stained, it was impossible to follow the rootlet neurones for any distance in these preparations. There was little background staining of the striated muscle sheet in which these elements lie. Nerves running out across the velum were clearly shown. In preparations labelled with anti-syntaxin 1, a low level of immunoreactivity was visible thoughout the cytoplasm of the motor giant axons and nerve ring elements, but there were also numerous, strongly immunoreactive spots within the nerve rings (Fig. 2B). These were restricted to the rootlet neurones and other elements within the nerve rings and were not seen in outlying parts of the nervous system, such as the lateral neurones, basal plexus elements and velar neurites, nor along the main extent of the motor giant axons. In controls omitting the primary antibody, no fluorescence was observed. While these findings are not conclusive, they suggest that the anti-syntaxin 1 immunoreactive spots may be synaptic sites.

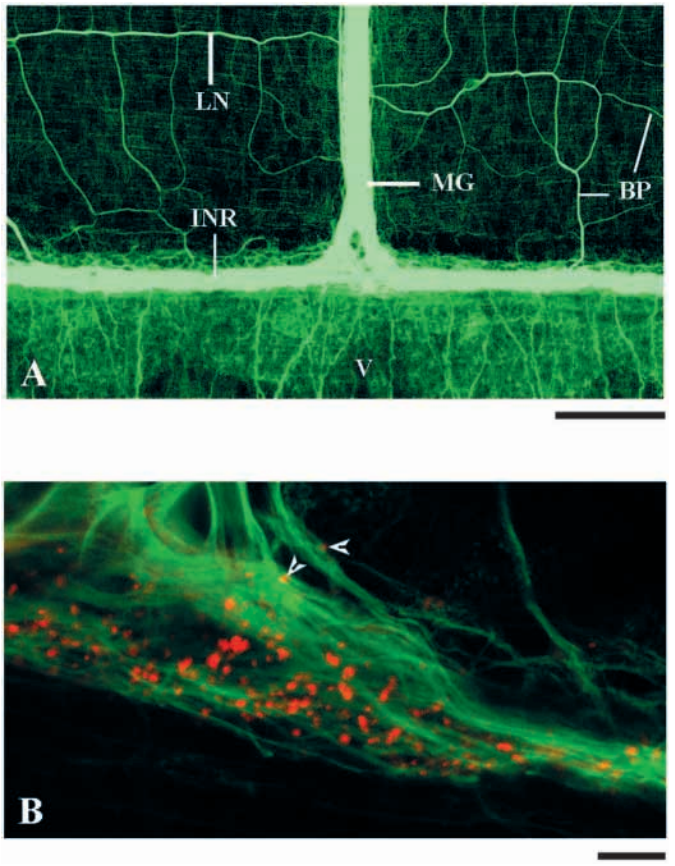

Fig. 2. Confocal images of immune-labelled components. (A) Junction of a motor giant axon (MG) with the inner nerve ring (INR), showing lateral neurones (LN) and the basal plexus (PB). Nerves also run across the velum (V) (anti-tubulin fluorescence microscopy). Scale bar, 100 μm. (B) Rootlet neurones at the base of a motor giant axon and the axons of the inner nerve ring are both shown in green (anti-tubulin staining). An overlay in red (anti-syntaxin 1 staining) showing putative synaptic sites; arrowheads show these sites on the rootlet neurones. Scale bar, 10 μm.

Carboxyfluorescein fills enabled us to follow the rootlet neurones for considerable distances within the inner nerve ring from the point where they connect with the motor giant. In Fig. 3, the two motor giants were 2.18 mm apart at their bases, and the rootlet neurones in Fig. 3A extended past the midpoint between the two axons by at least 230 μm, suggesting a 460 μm zone of overlap, similar to the distance shown in Fig. 1B. The dye became hard to see as it entered the narrow terminal neurites of the rootlet neurones, and the actual overlap zone may therefore be considerably larger. The overlapping rootlet neurones do not end separately, as shown by Weber et al. (1982), but associate very closely so that they can no longer be separately distinguished. This finding is important because it provides a structural basis for the direct interaction between adjacent motor giants *via* the rootlet system suggested by the physiological evidence. Neither the mass of neurites forming the bulk of the inner nerve ring nor the velar nerves were filled.

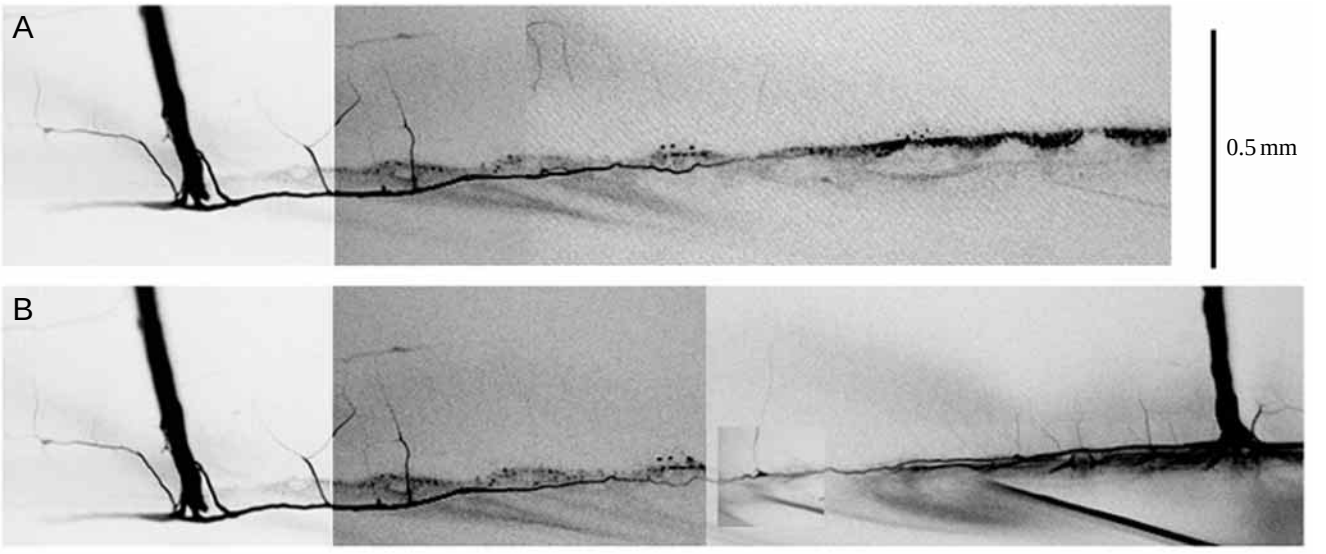

Fig. 3. Confocal images of carboxyfluorescein-injected motor giant axons and their associated rootlet neurones. In A, one axon was injected and the dye spread along the rootlet neurones to the right and could be followed for 1.32 mm. In B, a second axon was injected. Dye spread along the rootlet neurones, showing that they associate closely with the rootlet neurones of the first axon. Scale bar, 0.5 mm.

Behaviour

Stimulation of the inside of the bell in the vicinity of the motor giants with a fine probe, taking care not to touch or cause vibrations in the margin or velum, was found to evoke an escape swimming response of the type associated with Na^+ spikes in the motor giant axons (Mackie and Meech, 1985). As with escape swimming evoked by pinching the tentacles or prodding or vibrating the margin and velum (Roberts and Mackie, 1980), the response consisted of one or two very strong contractions each of which propelled the animal a distance equivalent to several body lengths. The two responses differed, however, in that the response to stimulation of the outer parts invariably included an immediate, strong twitch contraction of the all the tentacles, while subumbrellar stimulation led to graded, incremental tentacular contractions. As it was known from previous work that the twitch contractions of the tentacles seen in the former case are conducted round the margin by the ring giant axon, it seemed likely that the ring giant was not involved in the response that follows stimulation of the subumbrella.

Physiology

If, as the behavioural observations implied, the ring giant was not involved in conducting this escape response around the margin, we had to find another pathway, and none of those so far investigated had the necessary properties. Suspicion therefore fell upon the rootlet interneurones, known from the work of Weber et al. (1982).

The motor giant axons run up the inside of the bell in the strips of ectodermal tissue (radial strands) overlying the endodermal radial canals. Direct stimulation of a motor giant axon by means of a small, coaxial, bipolar electrode placed directly over a radial strand elicited a Na^+ spike that spread to all the other motor giant axons on both sides of the one stimulated. Fig. 4A shows spikes recorded intracellularly (top trace) and extracellularly (bottom trace) from one motor giant axon following stimulation of the adjacent motor giant axon (S1) and a motor giant axon three strands away (S2). The absence from the external trace, in these and many other recordings, of ring giant axon spikes confirms that the ring giant plays no part in conduction of the response. In sea water containing 97–105 mmol l^{-1} Mg^{2+}, transmission between motor giant axons was usually blocked, although the stimulated axon still fired normally, suggesting that chemical synapses are involved in the pathway linking the axons.

When the axon was punctured with the tip of a microelectrode between the stimulating point and the margin, it withered locally and transmission to other axons was blocked, although stimulating the distal, intact part of the axon still evoked action potentials and local muscle responses. Cuts made through the subumbrellar muscle sheet on either side of the motor giant and adjacent to the inner nerve ring confirmed that the transmission route to the other motor giant axons was along the margin. Conduction velocity around the margin in the experiment shown in Fig. 4A was 50 cm s^{-1}. Transection of the inner nerve ring (which includes the rootlet neurones) blocked the spread of the response, while transection of the outer nerve ring did not. These findings, taken in conjunction with the histological evidence presented above, strongly suggest that the rootlet interneurones interconnect to form a conduction pathway involved in the transmission of Na^+ spikes between the motor giant axons.

A second consequence of stimulating over a radial strand was the triggering of impulses in the pacemaker (P) system, which generates rhythmic slow swimming. A single shock was found to trigger one or a series of pacemaker potentials, readily recorded by a suction electrode placed over the inner nerve ring (see Fig. 4B). Depending on the size of the animal, P potentials

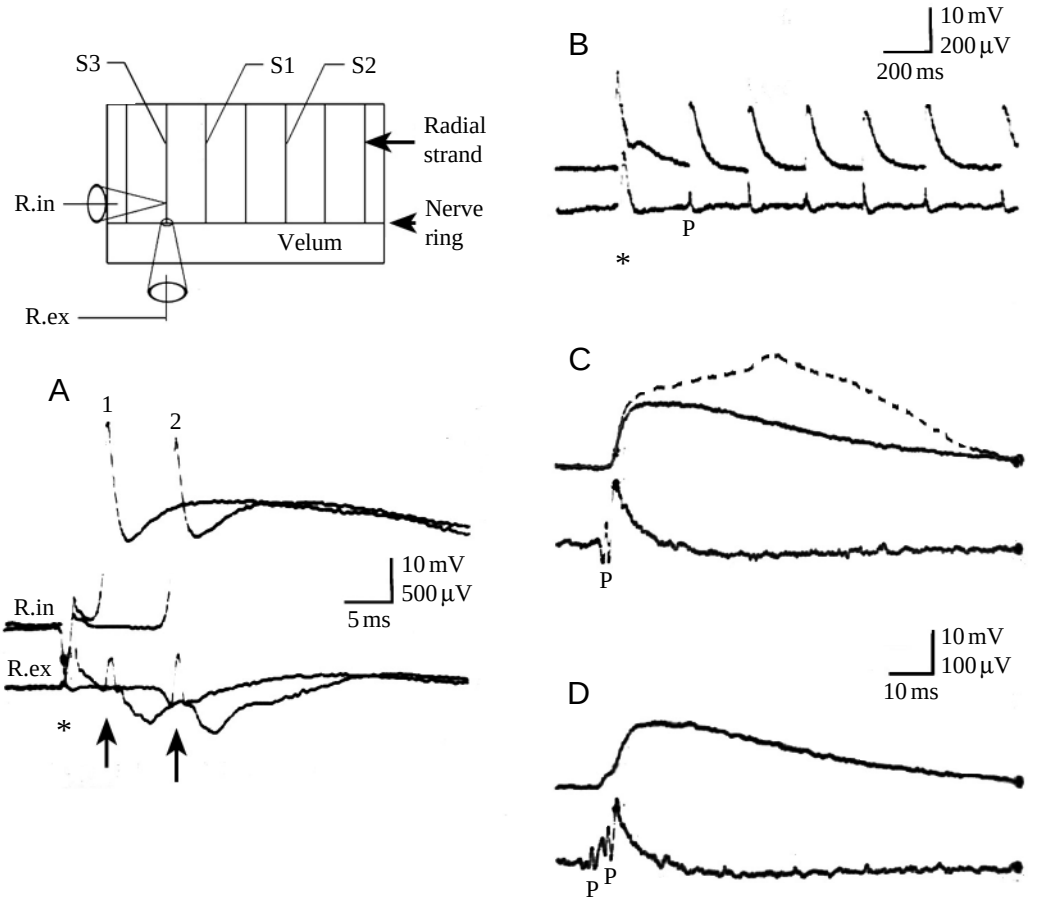

Fig. 4. Stimulation of the motor giant axon excites adjacent motor axons *via* the rootlet pathway and pacemaker (P) system and not *via* the ring giant axon. The inset (top left) shows the experimental arrangement. Intracellular recordings (R.in) were made from a motor giant axon close to its junction with the inner nerve ring, and extracellular recordings (R.ex) were made over the junction. An asterisk marks the shock artefact. The preparation was in 90 mmol l^{-1} Mg^{2+} to reduce muscle responses. (A) Shocks were delivered at S1 and S2, evoking spikes labelled 1 and 2, respectively, on the intracellular recording (upper trace here and elsewhere). In the extracellular recording (lower trace here and elsewhere), the spikes (indicated by arrows) are followed by irregular deflections representing the response of the swimming muscles. (B) A shock at S3 evoked a spike (poorly resolved at this slow sweep speed) followed by a series of pacemaker potentials (one labelled P) in the extracellular recording, each of which evoked a P-PSP (postsynaptic potential) in the motor giant axon. (C) A single P potential and its P-PSP from this series on an expanded time scale. Superimposed on the intracellular recording is a Ca^{2+} spike (dashed line) taken from another preparation in sea water. (D) As for C, but with a double P event and a two-step P-PSP.

are conducted around the margin at 40–60 cm s^{-1} (Meech and Mackie, 1995), probably by the group of large (approximately10 µm in diameter) neurones running in the inner nerve ring described by Weber et al. (1982). These large axons do not extend into or send branches up the radial strands, and P potentials were never recorded from the radial strands, so stimulating a strand could not excite the pacemaker system directly. Creating a lesion in the motor giant between the stimulating point and the margin blocked the production of P potentials in the margin at the same time as it blocked spike propagation in the motor giant. Triggering of P potentials was also blocked in 105 mmol l^{-1} Mg^{2+}. We conclude from these observations that the motor giant axons and/or their rootlet neurones synapse with pacemaker axons in the margin, exciting them whenever they conduct a Na^{+} spike. The P system normally shows a regular pattern of spontaneous bursts (fictive slow swimming) and, if excited by input from a motor giant around the time when it is due to produce a spontaneous burst, the timing may be advanced and a burst may result (Fig. 4B).

Each P event recorded extracellularly was associated with a depolarization in the adjacent motor giant axon (Fig. 4B,C,D). These depolarizations are the ‘slow postsynaptic potentials’ of Meech and Mackie (1995). In sea water, each slow, or P-PSP, generated a Ca^{2+} spike in the motor giant (Fig. 4C), but in water containing more than 83 mmol l^{-1} Mg^{2+} only the PSP was seen. P-PSPs are slowly rising events rarely exceeding 18 mV amplitude, with rounded tops and a long declining phase (τ=60 ms, where τ is the time constant of decay). In some preparations in which [Mg^{2+}] was greater than 87 mmol l^{-1}, the P system fired twice in rapid succession and the correponding PSP was a two-step event (Fig. 4D).

A third consequence of stimulating over a radial strand was the appearance of a small electrical event in the extracellular recording that propagated down to the margin and around it in the outer nerve ring and up the radial strand to the manubrium. We refer to these signals as impulses in the manubrial (M) system, which is involved in feeding behaviour. The response is blocked by cutting the radial strands. M pulses are almost certainly carried by the bundles of small FMRFamide-immunoreactive neurites that run up the radial strands close to the motor giants, connecting sensory elements in the margin with a nerve plexus overlying the muscles in the manubrium (Singla, 1978; Mackie et al., 1985; G. O. Mackie, unpublished

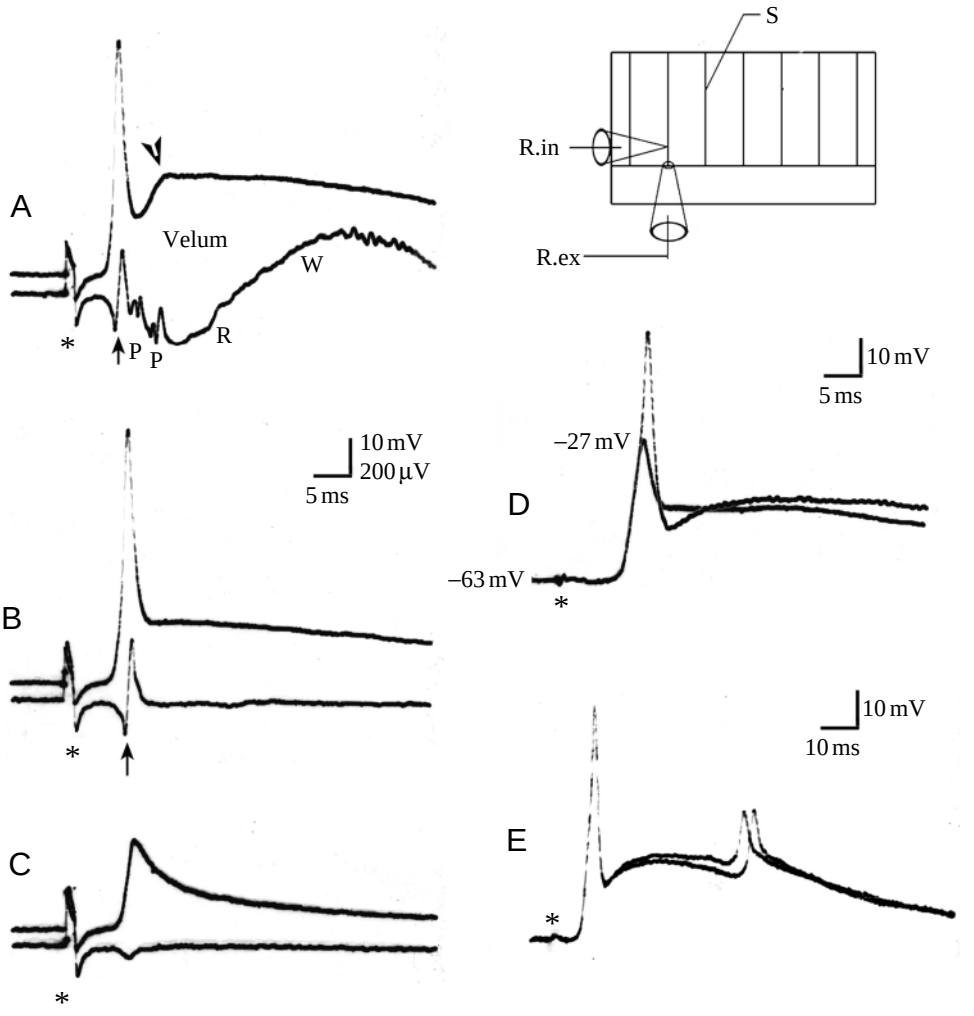

Fig. 5. Communication between the motor giant and the pacemaker (P) system and between rootlet neurones is *via* chemical synapses. Recording from the junctional region (as in Fig. 4) and stimulating on the adjacent giant axon. An asterisk marks the shock artefact. The inset (top right) shows the experimental arrangement (see Fig. 4 for further details). (A) In 3 mmol l^{-1} Mn^{2+}, the spike (arrow in lower trace) was followed by two P potentials which caused postsynaptic potentials that distorted the intracellularly recorded spike after-potential (upper trace; arrowhead). A potential in the relay system (R) and W (the slow wave) depolarization followed the P events in the extracellular recording (lower trace). (B) In 5 mmol l^{-1} Mn^{2+}, the spike no longer triggered the P–R–W sequence and is seen without distortion in both intracellular and extracellular recordings. (C) After a further period in 5 mmol l^{-1} Mn^{2+}, the spike failed, leaving a postsynaptic potential (PSP) representing rootlet input. (D) In 4 mmol l^{-1} Mn^{2+}, a trace in which rootlet input generated a spike is superimposed on a trace in which the spike failed, leaving a large rootlet PSP. (E) In 4 mmol l^{-1} Mn^{2+}, the presynaptic pathway fired twice in response to a single shock but only the first impulse evoked a postsynaptic spike. The second shock produced only a subthreshold rootlet PSP (two similar traces are superimposed).

physiological data). There is no evidence that this system is involved in locomotion and it will be ignored in the present account, but M pulses are labelled in some of the figures.

Recordings made from one motor giant axon following stimulation of another were invariably complicated by the fact that the P system was triggered at the same time. When, as in Fig. 5A, an immediately adjacent axon was stimulated, the first event recorded by the extracellular electrode (lower trace) was the correlate of the Na^+ spike seen in the intracellular recording (upper trace), but this was followed by one or more impulses in the P system, followed in turn by an impulse in the relay (R) system and the accompanying slow wave (W). P–R–W sequences are a normal feature of the preparation. R and W components follow spontaneous P events as well as those evoked by direct stimulation of the inner nerve ring and those, as here, triggered indirectly by excitation of motor giant axons. These events are part of a circuit that functions to cause the tentacles to contract during pacemaker-evoked swimming (Mackie and Meech, 1995a). The P system provides direct input to the motor giants in the form of slow or P-PSPs (Meech and Mackie, 1995). These are represented in Fig. 5A by a hump in the spike after-potential (arrowhead). Addition of 5 mmol l^{-1} Mn^{2+}, which depresses synaptic transmission, rapidly blocked the P events and abolished the hump (Fig. 5B), leaving the spike after-potential in its 'pure' form, a smoothly decaying curve. After a further period in the same solution, during which the preparation was stimulated repeatedly, the spike failed, leaving an underlying excitatory postsynaptic potential attributable to input transmitted *via* the rootlet neurone pathway (rootlet PSP) (Fig. 5C).

Rootlet PSPs are fast-rising events closely resembling the RG-PSPs that follow stimulation of the ring giant axon, which likewise generate Na^+ spikes in the motor giants (Meech and Mackie, 1995). Rootlet PSPs are readily distinguished from P-PSPs because they rise rapidly to a sharp peak, reach larger amplitudes and decay more rapidly (τ=10 ms in Fig. 5C). At their maximum amplitude (Fig. 5D), rootlet PSPs surpass the threshold for Na^+ spike generation, which lies at around –32 mV (Meech and Mackie, 1995). In the preparation shown in Fig. 5E, the stimulated axon occasionally fired twice in response to single shocks, but only the first action potential produced a spike at the recording site. The second produced a

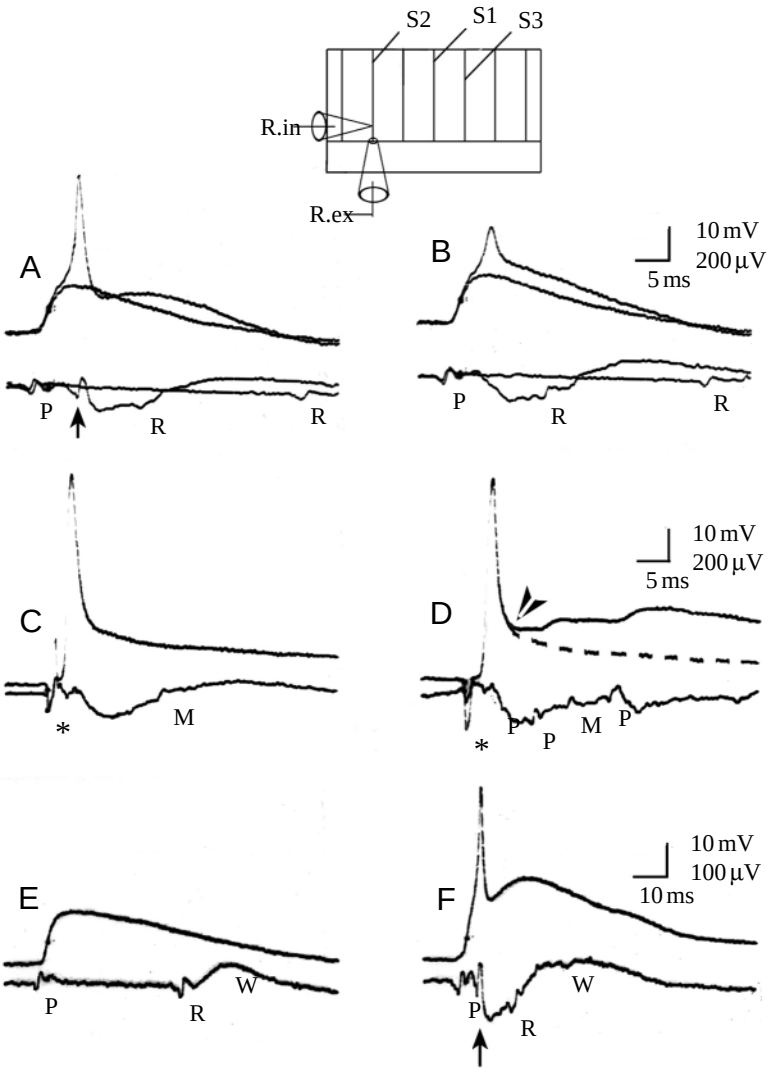

Fig. 6. Properties of the pacemaker and relay systems. (A,B) A preparation in 93 mmol l^{-1} Mg^{2+}. The inset (top right) shows the experimental arrangement (see Fig. 4 for further details). When present, an asterisk marks the shock artefact otherwise the shock artefact is off the recording to the left. Following a stimulus at S1 (see inset), a P-PSP (postsynaptic potential) propagated to the recording site and appeared to produce a Na^+ spike indicated by an arrow in the lower, extracellular recording, but when the spike failed (B) an underlying rootlet PSP (see upper trace) was revealed as the true cause of the spike. These traces are superimposed on recordings of spontaneous pacemaker (P) and P-PSP events. (C,D) In 90 mmol l^{-1} Mg^{2+}, following a stimulus at S2, a spike propagated down to the recording site and is shown in its undistorted form (C), but on another occasion (D) arrival of the spike at the margin triggered the P system to produce a short burst. The P potentials generated P-PSPs, which appear as humps distorting the spike after-potential. The undistorted after-potential is superimposed as a dashed line. The start of the first P-PSP is indicated by an arrowhead. M, activity in the manubrial system. (E) In 90 mmol l^{-1} Mg^{2+}, a spontaneous P–R–W sequence, where W is the slow wave, showing normal P–R latency. (F) The same preparation as in E, stimulating at S3. The P–R latency is reduced compared with E. The arrow marks the spike in the lower, extracellular recording.

rootlet PSP that failed to reach spike threshold. In several other preparations in 3–4 mmol l^{-1} Mn^{2+} or 105 mmol l^{-1} Mg^{2+}, similar abortive rootlet PSPs were obtained by giving two shocks 20–40 s apart.

While spikes conducted in the P system were easily recognizable in extracellular recordings, those conducted in the rootlet neurones were invisible. The small blip in the extracellular recording of Fig. 5C did not precede the PSP but coincided with its rising phase and cannot, therefore, represent the presynaptic rootlet event. Presumably, the rootlet pathway involves relatively few, small units, insufficient to generate a detectable level of external action current. This is consistent with the histological picture. In practice, however, our inability to record rootlet spikes extracellularly meant that their presence could only be deduced from intracellular recordings and, because the rootlet and P events were propagated at similar conduction velocities and tended to arrive close to one another in time, anomalous recordings in which a motor giant spike appeared to arise from the top of a P-PSP were often seen (see Fig. 6A, upper trace). When such events were observed in 93 mmol l^{-1} Mg^{2+}, the spike sometimes failed, revealing an underlying rootlet PSP surmounting the P-PSP (Fig. 6B, upper trace). On other occasions, the P- and rootlet PSPs were generated simultaneously and fused to form a composite depolarization that could give rise to spikes. Study of numerous recordings suggests that spikes are never generated by P-PSPs but always by rootlet PSPs, even where the two are ‘fused’.

Where the stimulated axon was immediately adjacent to the one from which the recordings were made, the rootlet event nearly always arrived before the P event or events (Fig. 5A). Where the stimulated axon was two or more axons away, the P event arrived at the same time as the rootlet event or slightly ahead of it (Fig. 6A). Reasons for this are suggested in the Discussion.

A number of recordings were made in which an axon was stimulated at its distal end and the recording electrodes were placed proximally, where the axon joined the margin (Fig. 6C,D). The preparation shown in Fig. 6C was in 90 mmol l^{-1} Mg^{2+}, which depresses synaptic transmission, and the giant axon spike is seen in the intracellular recording in its ‘pure’ form with no humps in the after-potential. The spike is represented extracellularly as a smaller deflection than usual

because the external electrode had been placed very slightly to one side of the junction to reduce the size of the spike and so to visualize P events better. There was no indication of triggered P events. In the same preparation, after a period without stimulation, a stimulus produced a giant axon spike that triggered a ripple of three P events, each of which fed back into the motor giant, producing P-PSPs as humps in the decay curve (Fig. 6D). The first of these P-PSPs appeared approximately 2 ms after the peak of the motor giant spike. While accurate measurements were not possible, this delay is approximately right for a two-synapse loop, assuming synaptic delays of 0.8 ms (Kerfoot et al., 1985) and allowing for some conduction time in the local circuit.

A final point clarified by these recordings concerns the 'piggyback' effect, the acceleration of impulses conducted in one system due to depolarizing input from a faster system running in parallel with it (Mackie, 1976). Potentials representing the relay system (R) are typically seen to follow spontaneous and evoked P impulses after 25–40 ms, but the conduction velocity of these events depends on the immediate past history of excitation in the R system and on whether other parallel systems are active at the same time (Mackie and Meech, 1995a). These authors found that R potentials propagate slowly on their own ($<12\ cm\ s^{-1}$), but when triggered and preceded by pacemaker impulses, their conduction velocity can double, and when they follow a burst of ring giant impulses, they can conduct at over $40\ cm\ s^{-1}$. In Fig. 6E,F, stimuli were delivered to a motor giant axon three radial strands away from the recording site. The rootlet pathway sometimes failed to conduct all the way through to the recording point, leaving the P–R–W sequence in which the conduction velocity of the relay system was approximately $15\ cm\ s^{-1}$ (Fig. 6E). On an occasion when the rootlet pathway did not fail and a spike was generated in the motor giant at the recording point (Fig. 6F), the relay system conducted at $33.5\ cm\ s^{-1}$. Acceleration of the conduction velocity of the relay system under similar circumstances is also shown in Fig. 6A,B. We conclude that excitation propagated in the rootlet neurones can exert a direct 'piggybacking' influence on the conduction of events in the relay system. It should be noted that in elevated $[Mg^{2+}]$, the rootlet pathway can fail at any point along the margin, so partial degrees of piggybacking are frequently seen.

Discussion

All the evidence presented here points to the conclusion that the motor giant axons communicate directly with one another *via* rootlet interneurones, which synapse with one another in a zone of overlap. The rootlet neurones are dye-coupled and, presumably, electrically coupled to the motor giant by gap junctions, allowing synaptic currents to flow into the base of the motor giant where we record them. Na^+ spikes arriving at rootlet synapses generate large postsynaptic potentials (the rootlet PSPs), sufficient to evoke postsynaptic Na^+ spikes. Depression of synaptic transmission by the addition of divalent cations allows the rootlet PSP to be seen without the spike. Given the large size and presumably high capacitance of the motor giant, it seems remarkable that such a large PSP can be generated in the motor giant when the synapses are made between a small number of fine neurites several hundred micrometres away. Unfortunately, we were not able to determine the numbers and locations of synapses between rootlet processes because of the difficulty of distinguishing the rootlet neurones from other fine neurites in the immunofluorescence preparations. The results of labelling with anti-syntaxin 1 constitute preliminary evidence that there are numerous synapses in the inner nerve ring, including some involving rootlet neurones. Although there may be many synapses on each neurite in the zone of overlap, and the zone of overlap may be more extensive than revealed by our carboxyfluorescein fills, the rootlet PSPs recorded from the bases of motor giant axons in elevated $[Mg^{2+}]$ and $[Mn^{2+}]$ exhibit a clean waveform without inflections or ripples and do not appear to be spatially summed composite events. A possible answer to this conundrum is that the fast rise of the rootlet PSP may reflect a regenerative contribution from the same kind of T-type Ca^{2+} channels that are present in the motor giant axon membrane (Meech and Mackie, 1993a, 1995). This would explain the unexpected sensitivity of the rootlet PSP to divalent ions compared with the insensitivity of chemical transmission at the neuromuscular junction (see Kerfoot et al., 1985).

The discovery of the rootlet pathway means that we can now distinguish three types of input into the motor giant axons. Slow or P-PSPs, which generate Ca^{2+} spikes, clearly represent input from the pacemaker system because they invariably follow the characteristic P potentials recorded extracellularly. Similarly, input from the ring giant axon can be identified by the fact that it follows ring giant spikes, which are readily recorded both intra- and extracellularly. The rootlet PSP, although resembling the ring giant PSP, can be distinguished from the latter by the fact that it is not preceded by ring giant spikes. In fact, we were not able to record an extracellular correlate of the presynaptic rootlet potentials. This is perhaps to be expected given the histological picture of the rootlet system as a small group of fine neurites. The resemblance between rootlet PSPs and ring giant PSPs is not surprising, given that both serve precisely the same purpose, that of raising the motor giant to the threshold for Na^+ spikes.

In the course of investigating the rootlet pathway, it became clear that excitation of the motor giant axons triggers events in the pacemaker system, so that P potentials propagate around the margin in a close time relationship with those conducted in the rootlet pathway. As the P system in its turn synapses with the motor giants, producing PSPs in them, these events appear postsynaptically and complicate the waveform of spikes produced by the nearly simultaneous rootlet input. Humps in the spike after-potential have been seen previously (see Fig. 1A in Kerfoot et al., 1985) but their significance was puzzling, because recordings of the spike in its 'pure' form showed a smoothly declining after-potential (see Roberts and Mackie, 1980). It is now clear that the humps are P-PSPs.

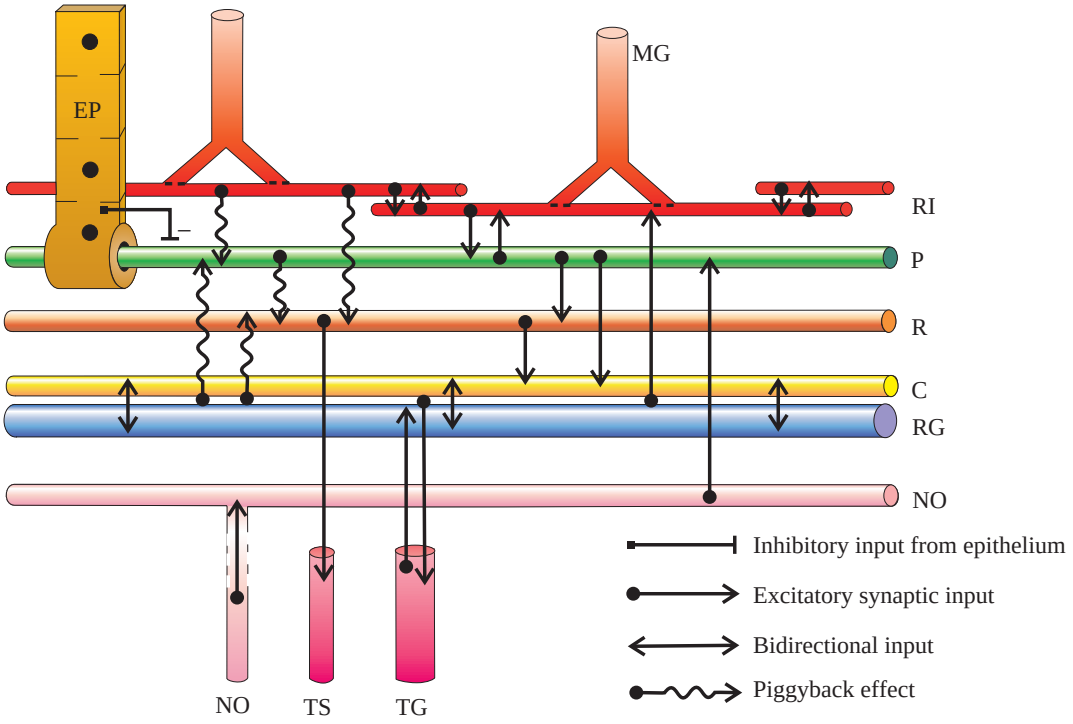

Fig. 7. The principal pathways involved in the control of locomotion and tentacle contractions. The diagram is based on Mackie and Meech (1995a), with the addition of the rootlet interneurone system (this study), the epithelial pathway (Mackie and Singla, 1997) and the nitric oxide pathway (Moroz et al., 1997). Gap junctions are shown as incomplete membrane partitions at the junctions of the rootlet system with the motor giants and between the epithelial cells. A minus sign indicates inhibitory input. All other inputs, including the bidirectional input, are excitatory. The piggyback effect is explained in the text. EP, epithelial pathway; MG, motor giant axon; P, pacemaker system; RI, rootlet interneurone system; R, relay system; C, carrier system; RG, ring giant axon; NO, nitric oxide pathway; TG, tentacle giant system; TS, tentacle slow system.

The mechanism whereby the tentacles contract during escape responses evoked by subumbrellar stimulation has not been worked out in detail, but we know from previous work (Mackie and Meech, 1995a) that triggering of P potentials singly or in bursts will occur when motor giants fire, and this will result in activation of the relay system and the pathways to the tentacles; the tentacular contractions produced will be graded events of the type seen during slow swimming rather than the violent contractions associated with escape behaviour in which the ring giant spreads the response.

Because the rootlet system and P pathways conduct at similar velocities and the two PSPs appear almost synchronously, the spike may appear to rise from a P-PSP, but analysis of such cases always shows that a rootlet PSP was present and actually generated the spike. Under experimental conditions (elevated [Mg^{2+}]), the two events can sum to produce a spike, although neither would come close to the spike threshold on its own. Thus, in a preparation containing elevated [Mg^{2+}] (Fig. 6A,B), a 14 mV rootlet PSP surmounting a 15 mV P-PSP almost reached spike threshold. This might suggest that summing of the two sorts of input contributes to spike production under normal conditions, but this seems unlikely, or at least unnecessary, because the rootlet PSP can easily achieve spike threshold on its own (Fig. 5D). Where a P-PSP surmounted an abortive rootlet PSP, spikes were never generated even though the summed event surpassed spike threshold. It appears that Na^+ spike production calls for a rapidly rising PSP, not just one that exceeds the threshold.

When a motor giant axon immediately adjacent to the one serving for the recordings is stimulated, the rootlet PSP is recorded ahead of the slow PSP, but when the stimulated axon is two or more axons away, the slow PSP is seen coincidently with or leading the rootlet PSP. The rootlet pathway from the immediately adjacent axon involves only one synapse, while that going through the pacemaker system must involve two (Fig. 7). The additional synapse would delay the arrival of the P event and its PSP. How does the P event catch up with and overtake the rootlet event when more distant axons are stimulated? We envisage the P neurones as long units that may extend without interruption through the territories of several motor giant rootlet systems. Once impulses are initiated in the P system, they will travel for long distances along the margin at the high conduction velocity characteristic of this system (40–60 cm s^{-1}), whereas the rootlet pathway will be interrupted by synapses that introduce a delay at each overlap zone. It must be said, however, that we do not know how long the P neurones are, how many there are and how they are kept in synchrony so that they appear to propagate as a single unit. The fact that synchrony sometimes breaks down in elevated [Mg^{2+}] (Fig. 4D) suggests that they are laterally interconnected by synapses and ‘piggyback’ each other when the synapses are operating normally.

An interesting aspect of this nervous system is the feedback loop operating between the motor giants and the P system. Synaptic transmission occurs between the two systems in both directions. This means that whenever a motor giant fires, whether because it is stimulated itself, because other motor giants are stimulated or because the ring giant axon is stimulated, the P system will also be excited after a delay of approximately 1 ms, and at least one P impulse will be seen. If

the P system is about to produce a spontaneous burst, a burst will be triggered, and a series of P-PSPs will be recorded in the motor giant (as in Fig. 4B). While a single P-PSP occurring within a few milliseconds of a spike in the motor giant is unlikely to have any behavioural consequences, the later events in a triggered P burst would certainly evoke Ca^{2+} spikes and slow swimming in an animal in its natural habitat. This may explain the observation that escape swims are sometimes followed by slow swims.

Bidirectional transmission between the rootlet neurones and the P system could be brought about by separate synapses polarized in opposite directions or by symmetrical synapses (i.e. single synapses with vesicles on each side), as described in *Cyanea capillata* (Anderson and Grünert, 1988). Symmetrical synapses have not been reported between rootlet neurones and pacemaker neurones, but occur elsewhere in the nervous system of *A. digitale* (Mackie, 1989) and might be present here. Our recordings showing feedback from the P system to the motor giants are very reminiscent of the recordings of Anderson (1985) obtained by inserting electrodes into neurones on either side of symmetrical synapses in *C. capillata* in which, if synaptic transmission from neurone A to neurone B produced a spike in B, a PSP appeared in the form of a hump on the declining slope of the presynaptic (A) spike. Anderson and Spencer (1989) review the general occurrence and possible significance of symmetrical synapses in cnidarians and other animals.

An inventory of the conduction system

In Fig. 7, we present an updated version of our previous wiring diagram (see Fig. 8 in Mackie and Meech, 1995a) modified to include the rootlet pathway. In addition to the rootlet pathway, there are four physiologically recognizable, through-conducting systems of neurones running around the margin and directly concerned with swimming or with the contractions of the tentacles that accompany swimming. The *pacemaker* system initiates slow, rhythmic swimming; the *ring giant axon* coordinates escape swimming; and the *relay* and *carrier* systems transfer information from the pacemakers to the ring giant and tentacles during slow swimming. Tentacle contractions themselves are of two sorts, twitch responses mediated by the *tentacle giant axons* (which also provide sensory input to the ring giant) and slow contractions mediated by a diffuse, *slowly conducting tentacle system*. Two other pathways concerned with locomotion have been described since the original wiring diagram appeared and are included in Fig. 7, the touch-sensitive *exumbrellar epithelium*, excitation in which inhibits the locomotory pacemakers (Mackie and Singla, 1997), and a plexus of *nitric-oxide-synthase-reactive neurones* (NO system) located in the tentacles and outer nerve ring. Nitric oxide, presumably secreted by the NO neurites in the nerve ring, excites the swimming pacemakers (Moroz et al., 1997). Including these two systems along with the *motor giants* and the *rootlet neurones* brings the total number of known conduction pathways involved in swimming and tentacle contraction to ten but, in the interests of simplicity, we have omitted several components from Fig. 7: the *lateral and basal plexus neurones* that spread excitation across the subumbrellar muscle sheet (Kerfoot et al., 1985), the vibration-sensitive *hair cell mechanoreceptors* that excite the ring giant (Arkett et al., 1988) and the *statocyst nerves*, whose input regulates the directionality of locomotion (Mackie, 1980). Including these components brings the total to thirteen.

To this list of physiologically distinct (and in some cases histologically identified) systems involved in locomotion and tentacle control may be added at least one more well-defined through-conduction pathway, the *manubrial system*, composed of FMRFamide-immunoreactive nerves, which mediates feeding responses between the margin and the manubrium (Mackie et al., 1985; G. O. Mackie, unpublished observations), bringing the total of known conduction pathways to fourteen.

While many details of the circuitry remain to be worked out, it seems doubtful that any major conduction system still remains undetected because we can assign all the electrical signals recorded from the nerve rings to one or other of the known systems, and most aspects of the animal's behaviour can be explained on the basis of interactions among them. The work on *A. digitale* fully supports the view expressed by other workers (e.g. Satterlie and Spencer, 1987; McFarlane et al., 1989) that cnidarian nervous systems, far from being 'simple', can be as complex as those of arthropods.

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References

Anctil, M. (1987). Bioactivity of FMRFamide and related peptides on a contractile system of the coelenterate *Renilla köllikeri*. *J. Comp. Physiol.* **157**, 31–38.

Anctil, M. (1989). The antiquity of monoaminergic neurotransmitters: evidence from Cnidaria. In *Evolution of the First Nervous Systems* (ed. P. A. V. Anderson), pp. 141–155. New York: Plenum.

Anctil, M., Pani, A. K. and Ali, M. A. (1991). Modulation of rhythmic contractions by melatonin *via* cyclic GMP in the coelenterate *Renilla köllikeri*. *J. Comp. Physiol.* **161**, 569–575.

Anderson, P. A. V. (1985). Physiology of a bidirectional, excitatory, chemical synapse. *J. Neurophysiol.* **53**, 821–835.

Anderson, P. A. V. and Grünert, U. (1988). Three-dimensional structure of bidirectional, excitatory chemical synapses in the jellyfish *Cyanea capillata*. *Synapse* **2**, 606–613.

Anderson, P. A. V. and Spencer, A. N. (1989). The importance of cnidarian synapses for neurobiology. *J. Neurobiol.* **20**, 435–457.

Arkett, S. A., Mackie, G. O. and Meech, R. W. (1988). Hair cell mechanoreception in the jellyfish *Aglantha digitale*. *J. Exp. Biol.* **135**, 329–342.

Donaldson, S., Mackie, G. O. and Roberts, A. (1980). Preliminary observations on escape swimming and giant neurons in *Aglantha digitale* (Hydromedusae: Trachylina). *Can. J. Zool.* **58**, 549–552.

Kerfoot, P. A. H., Mackie, G. O., Meech, R. W., Roberts, A. and Singla, C. L. (1985). Neuromuscular transmission in the jellyfish *Aglantha digitale*. *J. Exp. Biol.* **116**, 1–25.

Mackie, G. O. (1976). The control of fast and slow muscle contractions in the siphonophore stem. In *Coelenterate Ecology and Behavior* (ed. G. O. Mackie), pp. 647–659. New York: Plenum.

Mackie, G. O. (1980). Slow swimming and cyclical 'fishing' behavior in *Aglantha digitale* (Hydromedusae: Trachylina). *Can. J. Fish. Aquat. Sci.* **37**, 1550–1556.

Mackie, G. O. (1989). Evolution of cnidarian giant axons. In *Evolution of the First Nervous Systems* (ed. P. A. V. Anderson), pp. 395–407. New York: Plenum.

Mackie, G. O. and Meech, R. W. (1985). Separate sodium and calcium spikes in the same axon. *Nature* **313**, 791–793.

Mackie, G. O. and Meech, R. W. (1995a). Central circuitry in the jellyfish *Aglantha digitale*. I. The relay system. *J. Exp. Biol.* **198**, 2261–2270.

Mackie, G. O. and Meech, R. W. (1995b). Central circuitry in the jellyfish *Aglantha digitale*. II. The ring giant and carrier systems. *J. Exp. Biol.* **198**, 2271–2278.

Mackie, G. O. and Singla, C. L. (1997). The role of epithelial conduction in the behaviour of *Aglantha digitale* (O. F. Müller, 1776) (Hydromedusae: Rhopalonematidae). Proceedings of the Sixth International Conference on Coelenterate Biology. Nat. Natuurhist. Museum, Leiden, pp. 307–313.

Mackie, G. O., Singla, C. L. and Stell, W. K. (1985). Distribution of nerve elements showing FMRFamide-like immunoreactivity in hydromedusae. *Acta Zool.* **66**, 199–210.

McFarlane, I. D., Graff, D. and Grimmelikhuijzen, C. J. P. (1989). Evolution of conducting systems and neurotransmitters in the Anthozoa. In *Evolution of the First Nervous Systems* (ed. P. A. V. Anderson), pp. 111–127. New York: Plenum.

Meech, R. W. and Mackie, G. O. (1993a). Ionic currents in giant motor axons of the jellyfish, *Aglantha digitale*. *J. Neurophysiol.* **69**, 884–893.

Meech, R. W. and Mackie, G. O. (1993b). Potassium channel family in giant motor axons of *Aglantha digitale*. *J. Neurophysiol.* **69**, 894–901.

Meech, R. W. and Mackie, G. O. (1995). Synaptic potentials and threshold currents underlying spike production in motor giant axons of *Aglantha digitale*. *J. Neurophysiol.* **74**, 1662–1670.

Moroz, L. L., Mackie, G. O. and Meech, R. W. (1997). The distribution and action of nitric oxide in the jellyfish *Aglantha digitale* (Hydromedusae: Trachylina). *J. Physiol., Lond.* **504**, 13P.

Pani, A. K., Anctil, M. and Umbriaco, D. (1995). Neuronal localization and evoked release of norepinephrine in the cnidarian *Renilla köllikeri*. *J. Exp. Zool.* **272**, 1–12.

Pantin, C. F. A. (1952). The elementary nervous system. *Proc. R. Soc. Lond. B* **140**, 147–168.

Roberts, A. and Mackie, G. O. (1980). The giant axon escape system of a hydrozoan medusa, *Aglantha digitale*. *J. Exp. Biol.* **84**, 303–318.

Satterlie, R. A. and Spencer, A. N. (1983). Neuronal control of locomotion in hydrozoan medusae: a comparative study. *J. Comp. Physiol.* **150**, 195–207.

Satterlie, R. A. and Spencer, A. N. (1987). Organization of conducting systems in 'simple' invertebrates: Porifera, Cnidaria and Ctenophora. In *Nervous Systems in Invertebrates* (ed. M. A. Ali), pp. 213–264. New York: Plenum.

Singla, C. L. (1978). Locomotion and neuromuscular system of *Aglantha digitale*. *Cell Tissue Res.* **188**, 317–327.

Spencer, A. N. (1978). Neurobiology of *Polyorchis*. I. Functions of effector systems. *J. Neurobiol.* **9**, 143–157.

Spencer, A. N. (1979). Neurobiology of *Polyorchis*. II. Structure of effector systems. *J. Neurobiol.* **10**, 95–117.

Spencer, A. N. and Arkett, S. A. (1984). Radial symmetry and the organization of central neurones in a hydrozoan jellyfish. *J. Exp. Biol.* **110**, 69–90.

Weber, C., Singla, C. L. and Kerfoot, P. A. H. (1982). Microanatomy of the subumbrellar motor innervation in *Aglantha digitale* (Hydromedusae: Trachylina). *Cell Tissue Res.* **223**, 305–312.