J. exp. Biol. (1981), 94, 149–157
With 4 figures
Printed in Great Britain

MORPHOLOGY OF THE GIANT DOPAMINE-CONTAINING NEURONE, R.Pe.D.1, in *LYMNAEA STAGNALIS* REVEALED BY LUCIFER YELLOW CH.

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(*Received* 12 *January* 1981)

SUMMARY

The morphology of the giant dopamine-containing neurone of *Lymnaea stagnalis*, R.Pe.D.1, was studied by injection of Lucifer Yellow CH and by electrophysiological techniques.

R.Pe.D.1 was found to project to the periphery via the nerve trunks of the right parietal and visceral ganglia. Neurites bearing varicosities were found in each ganglion through which the axon passed. The neurites consistently arose from the same regions of the axon and the neuritic fields were similar in each preparation.

INTRODUCTION

The giant neurone, R.Pe.D.1 (right pedal dorsal neurone 1 of Benjamin & Winlow 1981) of *Lymnaea stagnalis* (L.) is known to contain dopamine (Cottrell, Abernethy & Barrand, 1979; McCaman, Ono & McCaman, 1979) and has been shown to have multiple postsynaptic actions on its follower cells which lie in the visceral and right parietal ganglia (Winlow & Benjamin, 1977; Winlow, Haydon & Benjamin, 1981). Little is known of the structure of R.Pe.D.1, but an homologous cell in *Planorbis corneus* has been studied by Pentreath & Berry (1975). This homologous cell does not project to the periphery via the nerve trunks of the pedal ganglia, but via the nerve trunks of the parietal and visceral ganglia. We have determined the structure of R.Pe.D.1 using the fluorescent dye Lucifer Yellow CH (Stewart, 1978). Preliminary results have been presented by Haydon (1981).

MATERIALS AND METHODS

Specimens of *Lymnaea stagnalis* weighing 1·0–3·7 g, were obtained from animal suppliers, kept in Leeds tap water at room temperature and fed on lettuce. Brains were isolated from the animals and prepared for experiments according to the methods of Benjamin & Winlow (1981).

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Morphology

(*a*) *Iontophoretic injection of Lucifer Yellow CH*

Three per cent Lucifer Yellow CH (kindly supplied by Dr W. W. Stewart) was injected iontophoretically into the soma of R.Pe.D.1 from microelectrodes prepared from triangular cross-section electrode glass (Winlow & Benjamin, 1977) or filamented circular cross-section electrode glass. Since only small quantities of Lucifer Yellow were available we filled the tips of the electrodes with this dye and the barrels with Procion Yellow MX4R (Dylon International Ltd). Electrode resistance was 20–80 MΩ and dye was ejected into R.Pe.D.1 with hyperpolarizing current pulses (5–40 nA) of 1 s duration at a rate of 0·5 Hz for 10–40 min. In one preparation dye was ejected for 1 h 30 min. Injection of dye into the cell was terminated either when the soma took on a yellow coloration under blue light or when spontaneous potentials fell to one-third of their original amplitude. In our first 5 preparations the dye was allowed to travel for 1–6 h at 10 °C. The preparation was then fixed overnight in Stieve's fixative. In the remainder of cases (n = 18) the preparation was left overnight (15–23 h) at 10 °C before fixation for 3–3·5 h in Stieve's fixative at 10 °C. The preparations were then dehydrated in alcohols and either cleared in chloroform and whole mounts prepared in DePeX, or cleared and prepared in methyl salicylate. The whole mounts were observed under incident ultraviolet illumination with a Leitz Dialux 20EB compound microscope equipped with a drawing tube and Leica MDa microscope camera. Drawings of individual cells filled by either method were made directly from the specimen and with the aid of monochrome photographs and 35 mm colour transparencies.

Lucifer Yellow CH loses its fluorescence after exposure to u.v. light but we found it possible to store the preparations for many weeks at 10 °C in the darkness. Although some of the finer detail tended to fade, the cell body and main axon branches and larger side branches remained visible.

Electrophysiology

Intracellular recordings were made from R.Pe.D.1 using either triangular cross-section microelectrodes filled with saturated K_2So_4 or, preceding dye injection, via electrodes filled with Lucifer Yellow CH as described above. Lucifer/Procion filled electrodes gave stable recordings comparable with those from K_2SO_4 electrodes. Electrophysiological signals were conventionally displayed and recorded as described by Benjamin & Winlow (1981). Extracellular recordings were made from nerve trunks of the right parietal and visceral ganglia by means of suction electrodes made from glass microelectrodes with their tips broken to such a diameter that the nerve trunks fitted snugly into them. Signals were amplified using a Neurolog NL103 pre-amplifier and an associated NL125 filter system. The criterion we used for evidence of axon projections of R.Pe.D.1 to the extracellularly recorded nerve trunks was that there should be 1:1 following of extracellular to intracellular spikes at a constant latency. In some preparations we stimulated the nerve trunks of the right parietal and visceral ganglia with en passant Ag/AgCl electrodes. The presence of an antidrom

ction potential recorded from the soma of R.Pe.D.1 provided further evidence for an axon projection to the stimulated nerve trunk.

RESULTS

(*a*) *Morphology of R.Pe.D.*1. R.Pe.D.1 has the largest cell body on the right pedal ganglion and lies on the posterior dorsal surface of the ganglion medial to the statocyst. Details of its appearance and size are given elsewhere (Benjamin & Winlow, 1981). The structure of the central nervous system of *Lymnaea stagnalis* is described by Slade, Mills & Winlow (1981) and the suboesophageal ring of ganglia is shown in Fig. 2*a*.

(i) *Axon projections*. Our initial injections of Lucifer Yellow CH revealed branches of R.Pe.D.1 in the right internal and external parietal nerves. A further branch crossed the parieto-visceral connective and projected to the periphery via the anal nerve.

In our later Lucifer injections the dye was allowed to distribute within the neurone for 15–23 h. The same basic pattern of major axon projections emerged (Fig. 1), but a variant axon also appeared. This was absent in some preparations (Fig. 1*a*) and its route to the periphery was variable. In Fig. 1*b* it is shown projecting from the anal nerve whilst in Fig. 1*c* it is shown to project via the intestinal nerve and in Fig. 1*d* via the genital nerve. In one preparation a variant axon was seen in the right internal parietal nerve (Fig. 2*b*) in addition to the invariant axon of this nerve trunk. In two preparations, in addition to the invariant axon of the anal nerve, variant axon branches were present in both the intestinal and genital nerves. These results are summarized in Table 1.

(ii) *Neurites*. Numerous neurites may be observed on R.Pe.D.1 in the right pedal ganglion after Lucifer staining with either the short or long intracellular dye distribution times. To analyse the neuritic fields we have determined where the primary neurites arise from the axon. We have not been able to analyse the secondary and tertiary neurites *per se* but have determined the areas of the CNS in which they are situated. In the right pedal ganglion a characteristic cluster of neurites arises from the axon within 100 μm of the soma and courses towards but does not enter the dorsal pedal commissure (Figs. 1, 2*b*, 3*b*). More distally but still in the right pedal ganglion neurites are clustered along the anterior edge of the axon (Figs. 1, 2*b*). These neurites have never been found to project more anteriorly than the level of the cerebro-pedal connective. Very few neurites arise from the posterior edge of the axon in this ganglion.

Neurites in other ganglia only show up in detail with the long intracellular distribution of the dye. The neurites within the right pleural ganglion are consistently few in number and remain close to the axon; within approximately 20 μm of R.Pe.D.1 (Figs. 1, 2*b*).

The position and pattern of neurites in the right parietal and visceral ganglia are interesting in that they underlie the position of the follower cells of R.Pe.D.1 (Fig. 2) and neurites originating in either ganglion may pass through the parieto-visceral connective into the other ganglion. After the axon of R.Pe.D.1 leaves the right pleural ganglion and enters the right parietal ganglion there are no neurites until *ca.* 50 μm before the first axon branch. These neurites arise from the anterior edge of the axon and run parallel with it towards the visceral ganglion. Very few neurites arise from the

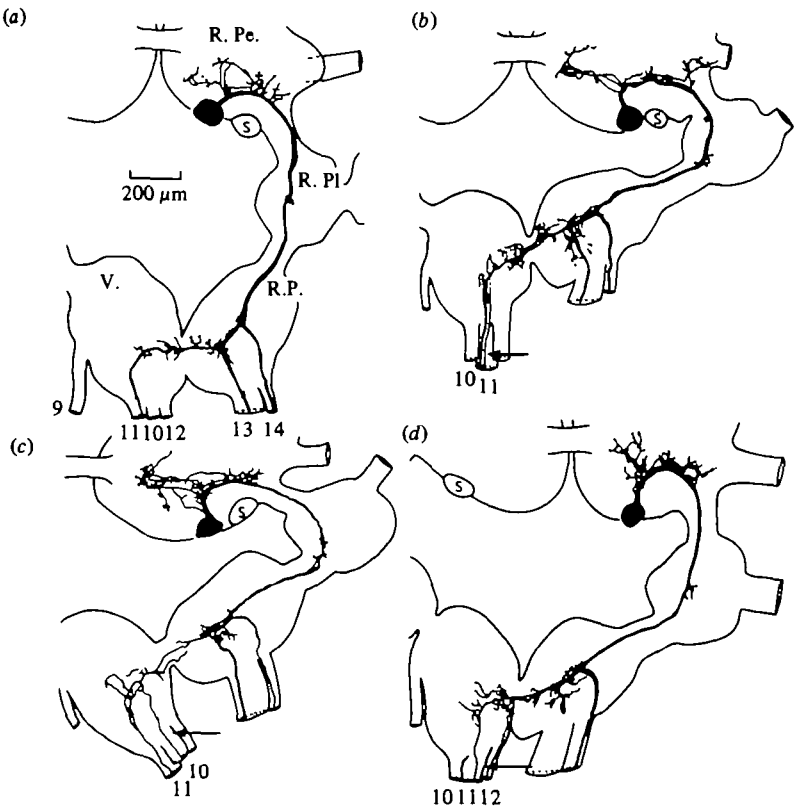

Fig. 1. Camera lucida drawings of R.Pe.D.1 injected with Lucifer Yellow CH which was allowed to travel for 15–23 h before fixation. In (*a*) only the invariant branches were stained. The variant branch (arrows) is seen in the anal nerve (*b*), the intestinal nerve (*c*), and the genital nerve (*d*). Note that the position of the anal nerve appears to change with respect to the intestinal nerve. However, the anal nerve is readily identifiable in all preparations as its site of origin is ventral to that of the intestinal nerve. R.Pe., right pedal ganglion; R.Pl., right pleural ganglion; R.P., right parietal ganglion; V, visceral ganglion. 9, cutaneous pallial nerve; 10, intestinal nerve; 11, anal nerve; 12, genital nerve; 13, internal parietal nerve; 14, external parietal nerve.

posterior edge of the axon until approximately midway between the two invariant axon branches of R.Pe.D.1 in the right parietal ganglion. From these points of origin, neurites are found along the axon up to the parieto-visceral connective. Some of these neurites cross the connective and enter the visceral ganglion. Few neurites arise from the invariant axon which enters the external parietal nerve. However, many neurites arise from the other invariant axon in this ganglion (Figs. 1, 2*b*, 3*c*). As can be seen from Fig. 2*a*, *b*, the neurites in this ganglion approximately underlie the follower cells of R.Pe.D.1, the A gp cells. In the visceral ganglion neurites arise from both the anterior and posterior edges of the axon, some passing through the parieto-visceral connective into the right parietal ganglion. Neurites originating from close to this connective underlie the H, I, J and K cells (Figs. 2*a*, *b*). As the axon bends to project to the periphery in the anal nerve, neurites course towards and underlie the G gp cells (Figs. 2*a*, *b*). Very few neurites arise from the variant axon. Because

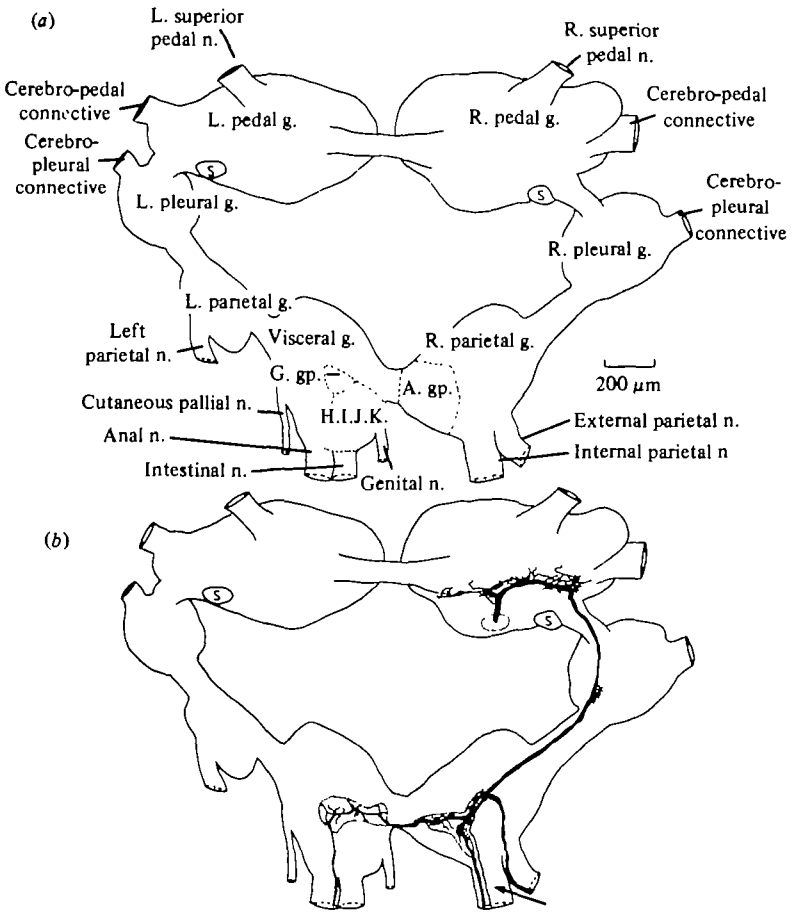

Fig. 2. (*a*) Drawing of the suboesophageal ring of *Lymnaea*. The positions of the follower cells of R.Pe.D.1 are outlined (A gp; G gp; H, I, J, K, cells). (*b*) Same brain as drawn in (*a*). R.Pe.D.1 injected with Lucifer. In the right parietal and visceral ganglia the perimeters of the neuritic fields are outlined. Note the variant axon in the internal parietal nerve trunk (arrow) and that the neuritic fields underlie the somata of many of the follower cells shown in (*a*).

neuritic fields are so dense and complex we have only been able to reconstruct some of the larger neurites. To give an impression of the area of ganglion containing these structures we have outlined their perimeters in Fig. 2*b*. In Fig. 3 we show photomicrographs of the branching patterns and neuritic fields of R.Pe.D.1. However, due to the small diameter of many of the neurites and poor contrast obtained against the autofluorescence of the tissue the total extent of neuritic fields cannot be seen.

In all but two preparations the neurites in each ganglion bear varicosities (Figs. 1, 2*b*, 3*b*). In these two preparations we were unable to electrophysiologically detect connexions between R.Pe.D.1 and its follower cells. In both preparations Lucifer revealed the same neuritic fields as described above, except that in one of these preparations a neurite passed from the right pedal ganglion through the cerebropedal connective and terminated in the right cerebral ganglion. However, there was an absence of varicosities in each neuritic field.

Table 1. *Axon projections of R.Pe.D.1 as revealed by injection of Lucifer Yellow CH*

No. of cells injected	Nerve trunk	No. of times axon branches were observed in nerve trunk: Invariant branch	Variant branch*
23	R. ext. parietal n.	23	—
	R. int. parietal n.	22†	1
	Genital n.	—	5
	Intestinal n.	—	3
	Anal n.	22‡	1

* Variant branches were only observed in preparations where distribution of dye took place for 15–23 h (n = 18). In two preparations two variant branches were observed, one in the genital nerve and one in the intestinal nerve.

† Once we did not observe a branch in the R. int. parietal nerve although the axon coursed towards the nerve and then terminated in the R. parietal ganglion. We believe this to be due to axon damage during dissection (see Fig. 1 *d*).

‡ Once the axon did not enter the visceral ganglion and thus the anal nerve. Again, we believe this was due to axon damage during dissection.

(*b*) *Electrophysiology*. R.Pe.D.1 has a characteristic action potential and characteristic synaptic inputs as described by Benjamin & Winlow (1981). The invariant branches of R.Pe.D.1 can be recorded repeatedly and easily using suction electrodes as shown in Fig. 4. Typical delays between the peak of the intracellular action potential and that of the extracellularly recorded spike are as follows: right external parietal nerve, 11 ms (Fig. 4*a*); right internal parietal nerve, 13 ms (Fig. 4*b*); anal nerve 14 ms (Fig. 4*c*). However, considerable variability of delay can occur according to the position of the suction electrode on the nerve trunk. It has not proved possible to demonstrate the presence of the variant branches of R.Pe.D.1 in the genital or intestinal nerves by extracellular recording even in preparations where we subsequently demonstrated their presence by injection of Lucifer Yellow. However, extracellular stimulation of the nerve trunks with pairs of Ag/AgCl electrodes reveals the presence of the variant axon of R.Pe.D.1 (Figs. 4*d–h*).

DISCUSSION

The neurone R.Pe.D.1, of the dextral pulmonate *Lymnaea stagnalis* (L.) appears to be the mirror image of the homologous giant dopamine-containing cell lying in the left pedal ganglion of the sinistral pulmonate, *Planorbis corneus* (Pentreath & Berry, 1975). One difference between these two neurones is that the giant dopamine-containing cell of *Planorbis* has an additional axon branch which passes through the contralateral pedal ganglion and around the nerve ring. The axon of R.Pe.D.1 arises from the soma and passes out of the right pedal ganglion into the right pleural, right parietal and visceral ganglia. Invariant axon branches project to the periphery, via the external and internal parietal nerves and the anal nerve. The variant axon branch of R.Pe.D.1 projects to the periphery via the anal, intestinal, genital or internal parietal nerve (Figs. 1, 2). Variations in the pathway of axons to the periphery are not un-

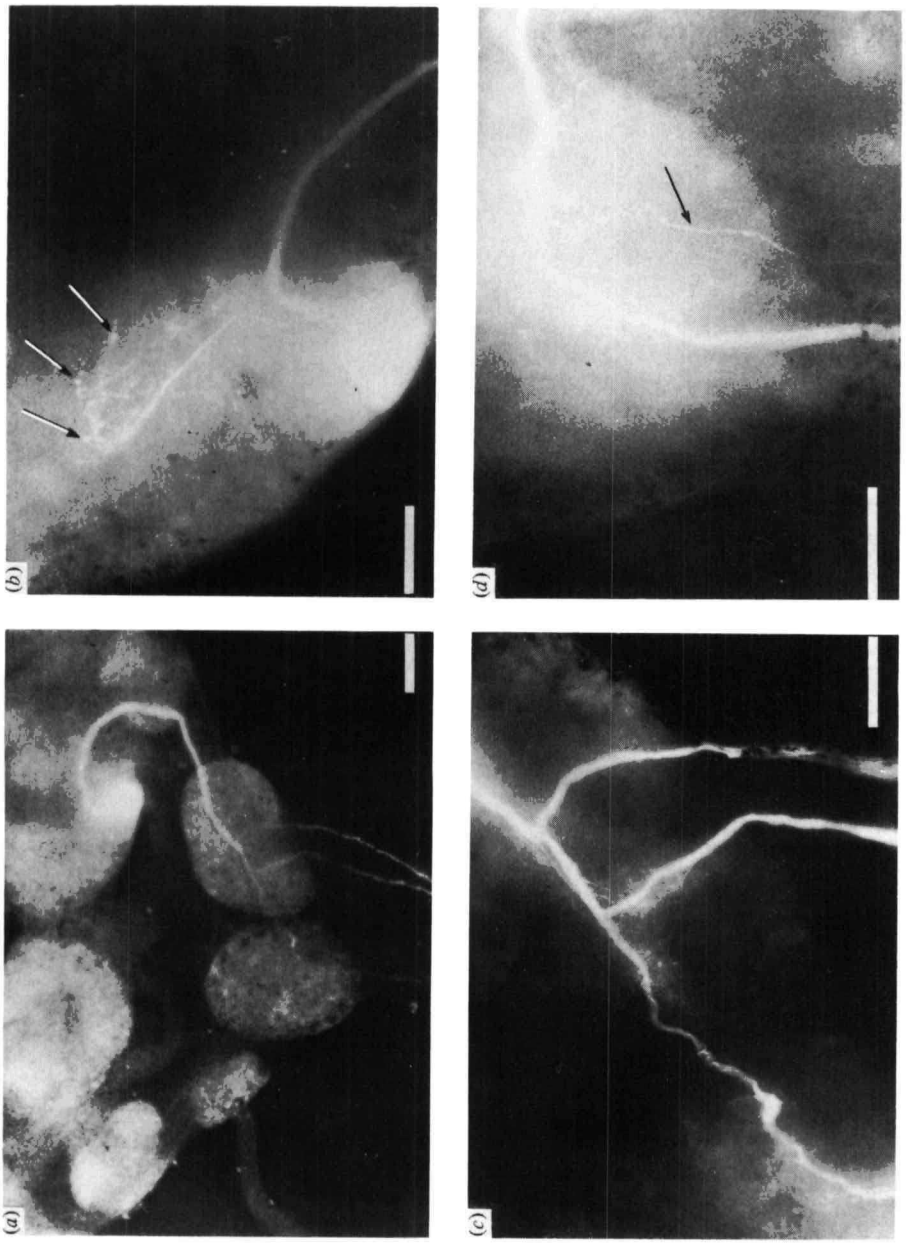

Fig. 3. Photomicrographs of R.Pe.D.1 injected with Lucifer Yellow CH. (*a*) The invariant axon projections of R.Pe.D.1. (*b*) The soma, axon, neurites and varicosities (arrows) of R.Pe.D.1 in the right pedal ganglion. (*c*) Invariant axon branches and neurites of R.Pe.D.1 in the right parietal and visceral ganglia. (*d*) The visceral ganglion showing the variant axon (arrow) and invariant axon. (*a*) and (*c*) are from one preparation (drawn in Fig. 1*b*) and (*b*) and (*d*) from another preparation (drawn in Fig. 1*c*). Calibration (*a*) 200 μm; (*b*)–(*d*) 100 μm.

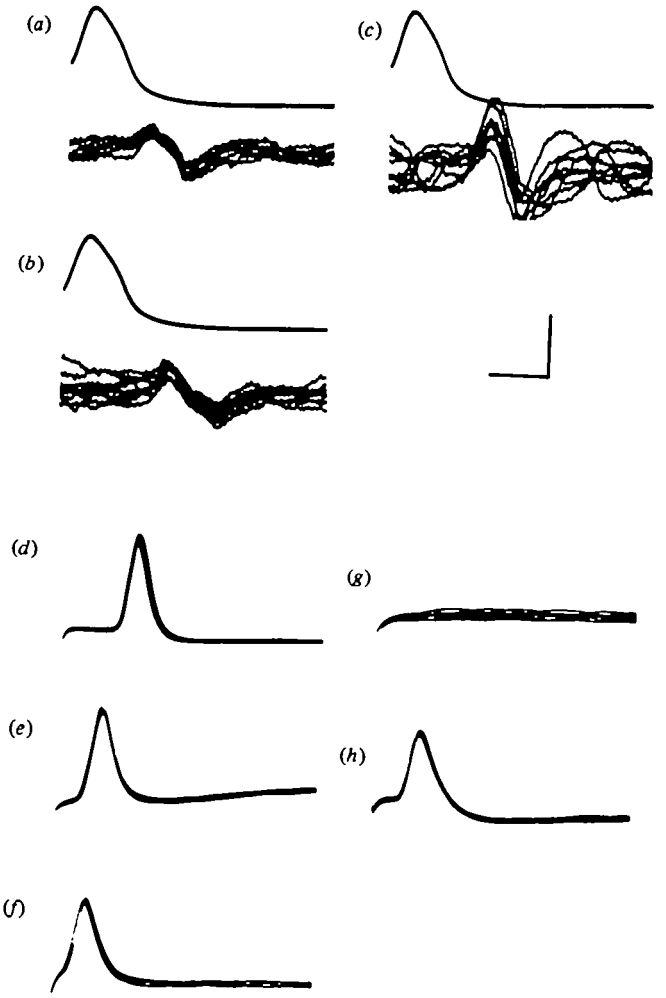

Fig. 4. Electrophysiological evidence for axon projections of R.Pe.D.1. (*a–c*) Ten superimposed sweeps. Oscilloscope sweep triggered by the ascending phase of the intracellularly recorded spike, showing 1 : 1 intra–extracellularly recorded spikes with constant latency. Upper, Intracellular spike. Lower, Extracellular spike. (*a*) External parietal nerve. (*b*) Internal parietal nerve. (*c*) Anal nerve. (*d–h*) Intracellular recordings from the soma of R.Pe.D.1 during extracellular stimulation of the external parietal nerve, (*d*); internal parietal nerve, (*e*); genital nerve, (*f*); intestinal nerve, (*g*); and anal nerve, (*h*). Antidromic action potentials are present in (*d*)–(*f*) and (*h*). In each pair of traces ten superimposed sweeps are shown. The oscilloscope sweep was triggered by the extracellular stimulus pulse. Voltage calibration: (*a–c*) upper traces 50 mV; lower traces 500 μV. (*d–h*) 40 mV. Time calibration: 10 ms.

common in molluscs. Winlow & Kandel (1976) demonstrated that identified neurones of the *Aplysia* abdominal ganglion have both variant and invariant branches. However the neuritic fields of R.Pe.D.1 are constant in position, presumably indicating that R.Pe.D.1 always makes synaptic connexions in the same regions of the brain. For example neuritic fields in the right parietal and visceral ganglia underlie the somata of the follower cells of R.Pe.D.1 (Benjamin & Winlow, 1981). This finding may aid in localization of follower cells of other presynaptic neurones in future studies.

Varicosities

In each neuritic field of R.Pe.D.1 we noticed varicose structures. These are not uncommon in molluscan preparations. Benjamin & Ings (1972), using the Golgi–Cox technique on *Lymnaea stagnalis*, Blackshaw (1976), using Procion on *Archidoris*, and Stewart (1978), using Lucifer Yellow on *Hermissenda*, all observed varicose structures by light microscopy. The ultrastructure of the varicosities was not determined.

Pentreath & Berry (1975) injected tritiated dopamine into the soma of the giant dopamine-containing cell of *Planorbis corneus*, and by the use of light and electron microscope autoradiography they found varicosities which contained vesicles. Varicosities have been found to be presynaptic terminals in *Aplysia* (Bailey *et al.*, 1979) and in stellate cells of the rat visual cortex (Peters & Proskauer, 1980). From these results obtained in *Planorbis*, *Aplysia*, and the rat we tentatively conclude that varicosities in *Lymnaea* are the presynaptic sites for chemical transmission. However, this must be substantiated by electron microscopic studies. Furthermore, Winlow *et al.* (1981) have shown that R.Pe.D.1 monosynaptically connects with follower cells in the visceral and right parietal ganglia, and Winlow & Haydon (unpublished observations) have found R.Pe.D.1 to connect with follower cells in the right pleural and right pedal ganglia.

An interesting feature of the varicosities is that they were absent in the preparations in which there were no recordable connexions between R.Pe.D.1 and its follower cells. We do not know why these synaptic connexions were absent but if the varicosities of R.Pe.D.1 are its presynaptic terminals then we have demonstrated an interesting example of a morphological change associated with a functional change.

From this study we can conclude that whilst R.Pe.D.1 has both variant and invariant axon projections it has consistent neuritic fields which underlie the position of its follower cells. Each neuritic field contains varicosities which may be the presynaptic terminals for chemical transmission.

This work was supported by an SRC grant to Dr W. Winlow.

The authors wish to thank Dr W. W. Stewart for supplying the Lucifer Yellow used in this study. Thanks are also due to Dr A. V. Holden and Mrs C. T. Slade for reading and criticizing an earlier draft of the manuscript, to Miss H. Davies for her expert technical assistance and to Mr D. Johanson for photographic assistance.

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