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IDENTIFICATION, CHARACTERISATION AND *IN VITRO* RECONSTRUCTION OF AN INTERNEURONAL NETWORK OF THE SNAIL *HELISOMA TRIVOLVIS*

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Summary

1. We describe three interneurones and their follower cells in the central ganglionic ring of *Helisoma trivolvis.*

2. The largest neurone on the dorsal surface of the left pedal ganglion is shown to be an interneurone that contains dopamine and makes monosynaptic connections with a large number of follower cells in the visceral and left parietal ganglia. This neurone is designated as left pedal dorsal 1 (LPeD1).

3. Another giant neurone is located on the dorsal surface of the right pedal ganglion. Although the position and morphology of this cell, designated right pedal dorsal 1 (RPeD1), are similar to those of LPeD1, it contains serotonin rather than dopamine. This neurone was found to synapse only on LPeD1, no other follower cells have so far been discovered. The connections between LPeD1 and RPeD1 are mutually inhibitory.

4. A small FMRFamide-immunoreactive neurone, identified here as visceral dorsal 4 (VD4), is located on the dorsal surface of the visceral ganglion. This neurone has a large number of follower cells throughout the central ganglionic ring. Among these follower cells are LPeD1 and RPeD1. The transmitter utilized by VD4 at these synapses is probably FMRFamide. In addition, VD4 receives excitatory inputs from LPeD1 that appear to be chemical and monosynaptic.

5. To test further the monosynaptic and specific nature of the connections within the network, the three interneurones were isolated and cultured *in vitro*. In these circumstances, the three neurones extended neurites and formed synapses which, with one exception (occasional electrical coupling between LPeD1 and RPeD1), were of similar type to those observed *invivo*.

6. The identification and characterization of these three interneurones and their follower cells should greatly facilitate future studies of the *Helisoma trivolvis* nervous system. Furthermore, the possibility that this three-cell network can be reconstructed *in vitro* should aid our understanding of the mechanisms underlying synapse formation and neuronal plasticity.

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Key words: mollusc, interneurone, synapse, cell culture, snail, *Helisoma trivolvis.*

Introduction

The identification of interneurones and their connections with follower cells is an important step towards understanding the neuronal basis of behaviour. With the notable exception of the Mauthner neurones of teleost fish (Korn and Faber, 1979), cellular studies of identifiable interneurones in higher animals have usually proved impractical. In the invertebrates, however, individual interneurones that are involved in behaviour patterns such as learning (Kandel, 1976; Byrne *et al.* 1990; Schacher *et al.* 1990) and rhythm generation have been identified (Jacklet, 1989; Harris-Warrick and Marder, 1991). The characterization of such interneurones and their follower cells has enabled analysis of entire neuronal circuits that control specific behaviour patterns and/or their individual components (Getting, 1989). Furthermore, very precise studies of neuronal networks in molluscs have recently been facilitated by the ability to partially reconstruct these circuits *in vitro* (Rayport and Schacher, 1986; Kleinfeld *et al.* 1990*a*,*b*; Syed *et al.* 1990).

Among the molluscs currently in wide use in neurobiological research are the basommatophoran pulmonates, *Helisoma trivolvis*, *Planorbis corneus* and *Lymnaea stagnalis* and the opisthobranchs, *Aplysia californica* and *Tritonia diomedea*. Unlike *Lymnaea*, *Aplysia* and *Tritonia*, however, the central ring ganglia of *Helisoma* and *Planorbis* have only recently begun to be mapped (e.g. Sonetti *et al.* 1982, 1988). In fact, the majority of studies on *Helisoma* have involved the buccal ganglia and its control of feeding behaviour (e.g. Kater, 1974; Granzow and Kater, 1977; Merickel *et al.* 1977; Kaneko *et al.* 1978; Murphy *et al.* 1985; Jones *et al.* 1987; Bulloch *et al.* 1988; Quinlan and Murphy, 1991). Similarly, large identified buccal neurones such as B5 and B19 are being used both *in vivo* and *in vitro* to study various aspects of regeneration, synapse formation and neuronal plasticity (Bulloch, 1989). A number of interneurones have been identified in the central ring ganglia of *Lymnaea*, *Planorbis*, *Aplysia* and *Tritonia*. Knowledge of these interneurones has been fundamental to unravelling the neuronal basis of a number of behaviours (e.g. Kandel, 1976; Getting, 1989; Syed *et al.* 1990, 1991; Syed and Winlow, 1991*a*,*b*). Since the central ganglionic ring of *Helisoma* is similar to that of *Planorbis* and is the mirror image of that of *Lymnaea*, in the present study we searched for interneurones homologous to some of those identified in these species. Using morphological, immunohistochemical, chromatographic, electrophysiological and pharmacological techniques, we have identified a central neuronal network consisting of aminergic and peptidergic interneurones. This interneuronal network acts widely on a variety of follower cells and its identification will greatly facilitate future studies of the nervous system of *Helisoma*.

Materials and methods

Animals

Laboratory-bred stocks of an albino strain of *Helisoma trivolvis* (‘Oregon Red’) of 15–20mm vertical shell height (approximate age 4–6 months) were used unless otherwise specified. These animals were raised as described previously (Jones *et al.* 1987).

Preparation and salines

Central ring ganglia were dissected in normal *Helisoma* saline (in $mmol\,l^{-1}$: NaCl 51.3, KCl 1.7, $CaCl_2$ 4.1, $MgCl_2$ 1.5 and Hepes, 5, pH7.3) and pinned to the silicone rubber (RTV 616 GE) base of 35mm Petri dishes. Zero-Ca^{2+}/high-Mg^{2+} saline contained $CaCl_2$ $0\,mmol\,l^{-1}$, $MgCl_2$ $1.5mmol\,l^{-1}$, $MgSO_4$ $13.5mmol\,l^{-1}$, NaCl and KCl being normal. For high-Ca^{2+}/high-Mg^{2+} saline, the concentrations of both $CaCl_2$ and $MgCl_2$ were raised sixfold, i.e. final concentrations were $CaCl_2$ $24.6mmol\,l^{-1}$, $MgCl_2$ $9\,mmol\,l^{-1}$.

Electrophysiology

Conventional electrophysiological techniques were used for recording intracellular neuronal activity using single-barrelled glass microelectrodes filled with a saturated solution of K_2SO_4 or $1\,mol\,l^{-1}$ KCl. Amplified signals were recorded on chart paper using a Gould four-channel chart recorder (model 2400). Intracellular Lucifer Yellow stains of the neurones were prepared according to the methods of Syed and Winlow (1989).

Whole-mount histochemistry

Central ring ganglia were removed from snails (10–12mm shell height) and pinned out flat on a small piece of silicone rubber by cutting either the cerebral commissure or one of the cerebropedal/cerebropleural connectives.

For glyoxylic acid histofluorescence of monoamines, we modified the whole-mount protocol of Audesirk (1985). Briefly, central ring ganglia were immersed in a solution containing $220mmol\,l^{-1}$ glyoxylic acid (Sigma) plus $40mmol\,l^{-1}$ Hepes prepared freshly in normal *Helisoma* saline and adjusted to pH7.0. After a 30min incubation (at 4 ˚C) the preparations were blotted to remove excess solution, dried under a hair dryer (cool setting), oriented on a glass coverslip and placed in a 100˚C oven for 5min; they were then mounted in a drop of paraffin oil on a slide and viewed under epifluorescence illumination as described by Audesirk (1985).

For whole-mount immunohistochemistry of serotonin- and FMRFamide-containing neurones, pinned preparations were partially desheathed (Syed and Winlow, 1991*a*,*b*) and treated with 0.1% protease (Sigma, type XIV) in normal saline for 5–10min. This treatment was followed by thorough rinsing in cold normal saline. At this point, some preparations were selected for Lucifer Yellow ionophoresis to mark specific neurones (see above). The Lucifer Yellow was allowed to diffuse for 2–3h. All preparations were fixed overnight at 4˚C in Zamboni's fixative (2% paraformaldehyde in $0.1\,mol\,l^{-1}$ phosphate buffer, pH7.2, plus 15% v/v saturated picric acid). Ganglia were rinsed in $0.187\,mol\,l^{-1}$ phosphate-buffered saline (PBS), followed by 4–5 rinses in PBS plus 1% Triton X-100 and then incubated in a blocking solution consisting of 5–10% normal donkey serum, 1% Triton X-100, 0.1% bovine serum albumin and 0.02% sodium azide in PBS.

The preparations were next incubated in primary antisera (diluted 1:200 in blocking solution) for 3–4 days at 4˚C. The primary antibodies used were a polyclonal antiserum generated in goat against a serotonin–BSA conjugate (INCstar Corp.) and a polyclonal antiserum generated in rabbit against a synthetic FMRFamide/succinylated thyroglobulin

conjugate (generously provided by J. Bishop; see O'Donohue *et al.* 1984). After incubation in primary antiserum, the preparations were rinsed thoroughly over a 6–8 h period in PBS plus 1% Triton X-100, then immersed in blocking solution for 1–2h before being incubated overnight (at 4˚C) in secondary antiserum. The secondary antisera used were a donkey anti-goat IgG/fluorescein isothiocyanate conjugate and a donkey anti-rabbit IgG/tetramethylrhodamine isothiocyanate conjugate (both from Jackson Immunoresearch). These labelled antisera were used either singly or together, each at a concentration of 1:50 in blocking solution. The preparations were rinsed thoroughly, first in PBS/1% Triton X-100 and finally in PBS alone. They were then mounted on slides in PBS/glycerol solution (85% glycerol, 15% PBS) with 1% w/v *n*-propyl gallate to reduce fluorophore fading (Giloh and Sedat, 1982). Preparations were viewed under epifluorescence illumination with appropriate filters for fluorescein isothiocyanate, tetramethylrhodamine and Lucifer Yellow. Since the specificity of the FMRFamide antibody for both *Helisoma* and *Lymnaea* has been tested rigorously in our previous studies (Bulloch *et al.* 1988; McKenney *et al.* 1990) we did not repeat preabsorption controls in this study. Furthermore, Richmond *et al.* (1991), utilizing high performance liquid chromatography (HPLC) and radioimmunoassay (RIA) techniques, have demonstrated that VD4 contains true FMRFamide along with FLRFamide and GDPFLRFamide.

High performance liquid chromatography

Identified interneurones were isolated from their respective ganglia as described below. These freshly dissected single neurones were placed into 10 μl of 0.2mol l^{-1} pentafluoropropionic acid (PFPA) and frozen until analysis (within 3h). Immediately before analysis, the samples were individually passed through two freeze–thaw cycles and the contents of the tube were diluted to 100 μl into an autosampler microvial. The chromatographic system consisted of a Waters HPLC pump (model 6000A), an ESA COULOCHEM guard cell at +0.75V, a Waters WISP 710B autosampler, a Beckman Ultrasphere 5μm octadecyl ion-pairing column (4.6mm×45mm) thermostatted at 50˚C, a graphite/cyanoacrylate electrode and a BAS amperometric detector set for +0.65V. The detector signal was digitized and recorded using the Waters/Dynamic Solutions Maxima data acquisition system. The autosampler introduced 90 μl of sample and the separation was performed at 50˚C on a Beckman Ultrasphere 5μm C-18 column (4.6mm×45mm) using a mobile phase consisting of 30mmol l^{-1} trisodium citrate, 10mmol l^{-1} citric acid, 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA), 100mmol l^{-1} sodium perchlorate, 10mmol l^{-1} sodium dodecylsulphate (SDS) at a flow rate of 2ml min^{-1}. Samples were individually processed. Standardization was carried out using freshly prepared external standards in 0.2mol l^{-1} PFPA. The detection limit was 0.1pmol of dopamine or serotonin. In pilot experiments, the level of monamines detected was variable; the data reported here were from snails maintained at 4˚C overnight and dissected in cold saline, a procedure which eliminated this variability.

In vitro *cell culture preparations*

The current study of *Helisoma* neurones used the procedure for culture of *Lymnaea*

neurones described in detail by Ridgway *et al.* (1991). This procedure was adapted for *Helisoma* by (1) preparation of medium at pH7.4 and (2) use of collagenase/dispase and trypsin at $0.67 mg ml^{-1}$ and $1.33 mg ml^{-1}$, respectively. Plated neurones exhibited neurite outgrowth within 12–24h; intracellular recordings from these sprouted neurones were made after 48h as described earlier. Neurotransmitters such as dopamine and FMRFamide were pressure-ejected through fire-polished glass electrodes using a Picospritzer II (General Valve, Co.).

Results

This paper identifies a network of three *Helisoma* interneurones that are interconnected and that synapse upon a large number of follower cells. The locations of these neurones and their histochemical and morphological properties are described and we then demonstrate the network interactions between these interneurones. Finally, we describe the reconstruction of this network in cell culture and demonstrate that these neurones make specific connections *in vitro* similar to those observed *in vivo*.

Central ring ganglia

With the exception of the paired giant serotonergic cells of the cerebral ganglia (Granzow and Kater, 1979), little is known regarding neurones of the central ring ganglia of *Helisoma* (see Introduction). In order to facilitate studies of *Helisoma*, including the present examination of interneurones, we have prepared a cell map of the cerebral, pedal, pleural, parietal and visceral ganglia (Fig. 1). This map shows (1) individually identifiable giant cells (e.g. LPeD1, RPeD1, previously designated as P1 and P5, respectively, see Haydon *et al.* 1984; McCobb *et al.* 1988) and (2) the major cell clusters (e.g. VA, VB, etc.) and/or ganglionic segments. Details of the morphological, histological and immunohistochemical properties of most of these cells are beyond the scope of the present report and will appear elsewhere (see, for example, Culver *et al.* 1992). However, in the following sections reference is made to a number of groups of follower cells (e.g. Tables 2 and 3).

Left pedal dorsal 1 (LPeD1)

Morphology

The neurone, which we now designate as LPeD1, has the largest soma located on the dorsal surface of the left pedal ganglion, medial to the statocyst (Fig. 1). In adult *Helisoma* (vertical shell diameter 15–20mm), the soma of LPeD1 has a diameter of 100–150 μm. Intracellular Lucifer Yellow staining of this neurone showed it to have a single main axon that passed through the left pleural and parietal ganglia where it branched and projected to the periphery *via* the left internal and external parietal nerves. A fine branch was also observed to enter the visceral ganglion from where it subsequently projected to the periphery *via* the anal nerve (Fig. 2A). Fine axon collaterals of neurone LPeD1 were observed in the left pedal, pleural and parietal ganglia as well as the visceral ganglion (Fig. 2A).

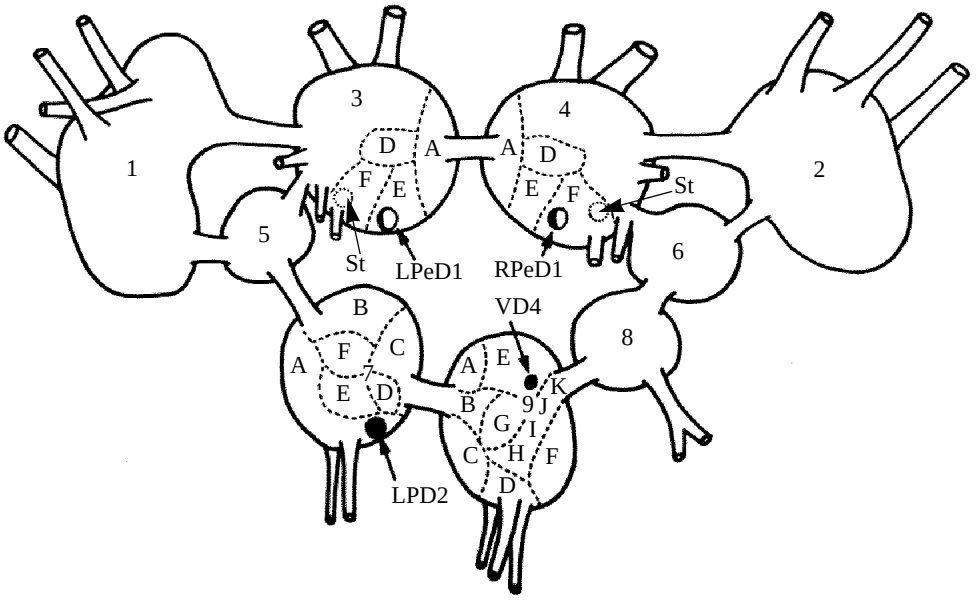


Fig. 1. Diagram of the central ring ganglia of *Helisoma trivolvis* showing the identified neurones and neuronal clusters used in this study. Ganglia are numbered: left and right cerebrals (1, 2); left and right pedals (3, 4); left and right pleurals (5, 6); left and right parietals (7, 8); unpaired visceral (9); the paired buccal ganglia are not shown. The cerebral commissure has been cut and the cerebral ganglia (shown ventral surface up) deflected to reveal the lower ring of ganglia (all shown dorsal surface up). Relevant neuronal clusters are given letter designations: left and right pedal A, D, E and F clusters (L/RPeA, D, E and F); left parietal A to F clusters (LPA, B, C, D, E and F); visceral A to K clusters (VA, B, C, D, E, F, G, H, I, J and K). Individually identifiable neurones: left pedal dorsal 1 (LPeD1); right pedal dorsal 1 (RPeD1); left parietal dorsal 2 (LPD2); visceral dorsal 4 (VD4). The positions of the statocyst organs (St) within the pedal ganglia are also shown. White cells are shown as black, orange cells as partially black. For further details see Culver *et al.* (1992). Not drawn to scale.

Transmitter phenotype

Owing to its apparent homology with the *Lymnaea* giant dopamine cell (RPeD1; Cottrell, 1977; Cottrell *et al.* 1979; McCaman *et al.* 1979), the transmitter of LPeD1 was suspected to be dopamine. Preparations were therefore processed with an induced

Fig. 2. Morphology and transmitter phenotypes of *Helisoma* neurones left pedal dorsal 1 (LPeD1) and right pedal dorsal 1 (RPeD1). Intracellular injection of Lucifer Yellow into LPeD1 (A) and RPeD1 (B) shows their near mirror-image morphologies. Each possesses a single main axon which projects through the ipsilateral pleural and parietal ganglia, bifurcating in the latter to send a branch through the visceral ganglion. Fine arborizations of these axons are found in each of the ganglia (shown in Fig. 1) through which they pass. (C) LPeD1 (identified at arrowhead) emitted the whitish-blue fluorescence associated with the presence of dopamine when the glyoxylic acid technique was applied to whole-mounted ganglia. (D) RPeD1 (at arrowhead) was among the neurones immunoreactive for serotonin in whole-mounted ganglia. Magnification bars: A and B, 200 μm; C and D, 100 μm.

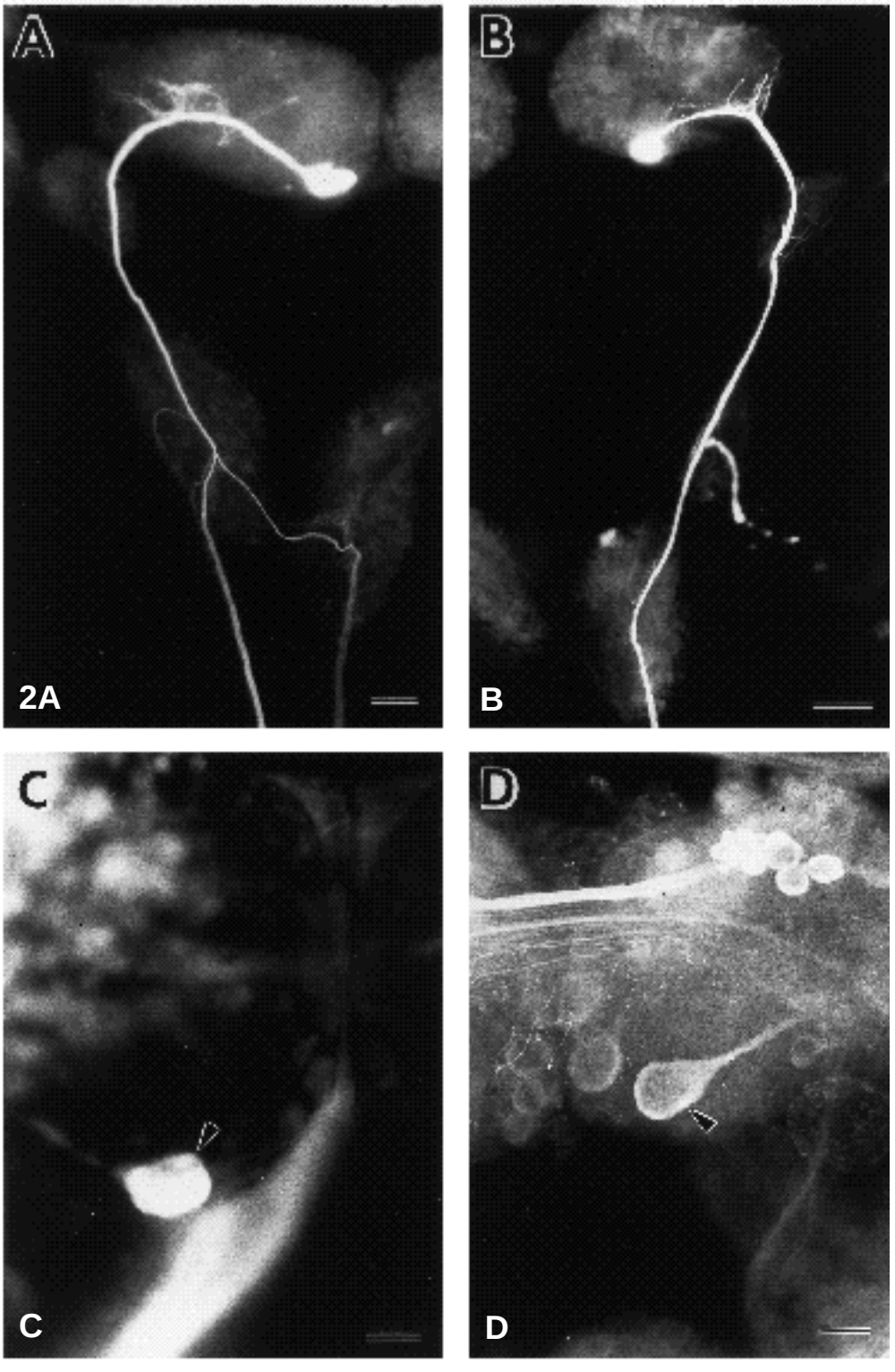

A
2A
B
B
C
C
D
D

Table 1. *Monamine content of neurones LPeD1, RPeD1 and VD4*

	Dopamine		Serotonin	
Neurone	pmol per neurone	mmol l^{-1}* (estimated)	pmol per neurone	mmol l^{-1}* (estimated)
LPeD1	0.74 ± 0.10	0.42	ND	
RPeD1	ND		0.61 ± 0.10	1.16
VD4	ND		ND	

Values are expressed as mean ± standard deviation; ND, not detectable; *N*=10 (LPeD1), 10 (RPeD1), 4 (VD4).

*Neurone diameters of 150 μm (LPeD1) and 100 μm (RPeD1) were assumed for these calculations.

Table 2. *Follower cells of* Helisoma *LPeD1*

Follower cells	Postsynaptic response
RPeD1	IPSP
VD4	EPSP
LPA, VB, VH, VK	EPSP
VA, VG, VI	Biphasic PSP
LPD, VC, VE, VJ	IPSP

histofluorescence technique for monoamines, i.e. glyoxylic acid. Isolated central ring ganglia processed in this manner revealed many neurones with the characteristic blue and yellow-green fluorescence associated with dopamine and serotonin respectively (e.g. Audesirk, 1985). The neurone LPeD1 was amongst those with prominent blue fluorescence (Fig. 2C), whereas the giant neurone of the right pedal ganglion (RPeD1) exhibited yellow-green (serotonin) fluorescence (not shown). For further analysis of its transmitter phenotype, neurone LPeD1 was isolated from the ganglion and processed for dopamine and serotonin content by HPLC. Individually isolated somata of neurone LPeD1 were found to contain a high level of dopamine (Table 1; Fig. 3), but serotonin was not detectable.

Follower cells

The follower cells of LPeD1 were identified by making simultaneous intracellular recordings from LPeD1 and a large number of identified neurones and neuronal clusters in the central ring ganglia. These recordings revealed the presence of excitatory, inhibitory and biphasic postsynaptic potentials between LPeD1 and follower neurones (Figs 4–6; Table 2). To obtain evidence for the chemical and monosynaptic nature of these connections, preparations were superfused with zero-Ca^{2+}/high-Mg^{2+} and high-Ca^{2+}/high-Mg^{2+} salines respectively. In all cases tested, zero-Ca^{2+}/high-Mg^{2+} saline treatment blocked excitatory (Fig. 4), inhibitory (Fig. 5) and biphasic connections (Fig. 6) from LPeD1 to its follower cells (*N*=18). As a test for monosynaptic connections, preparations were then superfused with high-Ca^{2+}/high-Mg^{2+} saline, which raises the threshold for action potentials and should reduce the likelihood of polysynaptic transmission (Berry and Pentreath, 1976). In no case tested (*N*=21) was the connection

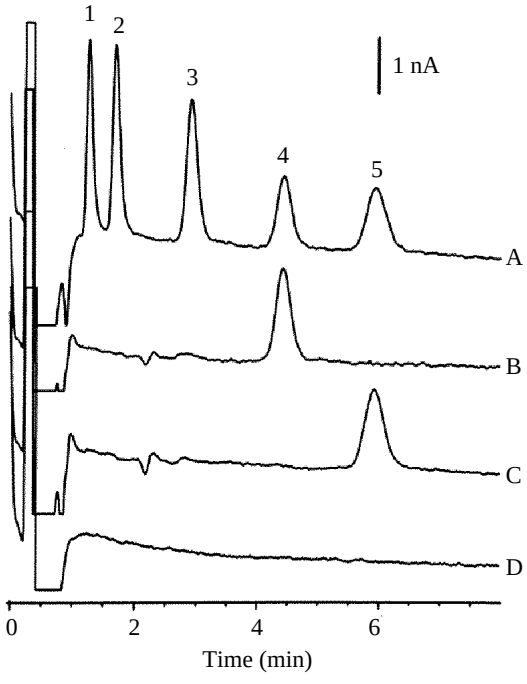


Fig. 3. Identification of dopamine and serotonin in isolated *Helisoma* neurones left pedal dorsal 1 (LPeD1) and right pedal dorsal 1 (RPeD1), respectively, using HPLC. Chromatograms show retention times of (A) 1pmol standards, (B) monoamine content of two LPeD1 neurones, and (C) monoamine content of two RPeD1 neurones, and (D) monoamine content of four VD4 neurones. The standards are as follows: (1) norepinephrine, (2) epinephrine (3) dihydroxybenzyamine (internal standard), (4) dopamine and (5) serotonin. Dopamine was detected only in LPeD1, whereas serotonin was detected only in RPeD1; neither transmitter was detected in visceral dorsal 4 (VD4). Signal intensity (peak height) is expressed in nanoamperes (nA); amount of substance detected is proportional to the area integrated under the peak. The artefact at time zero is due to passage of the solvent front.

from LPeD1 blocked by this treatment, nor was there any obvious increase in latency such as might occur if intervening neurones were involved.

In addition to the follower cells described above, LPeD1 was also found to synapse on both RPeD1 and VD4 interneurones. The postsynaptic effect of LPeD1 on RPeD1 was inhibitory (Fig. 7A) whereas its electrical stimulation excited VD4 (Fig. 7D). These two connections were also blocked in zero-Ca^{2+}/high-Mg^{2+} saline, but were not affected by high-Ca^{2+}/high-Mg^{2+} saline (Fig. 7).

Right pedal dorsal 1 (RPeD1)

Morphology

This is the largest cell present on the dorsal surface of the right pedal ganglion (Figs 1, 2B), with a soma diameter in the range of 80–100 μm in 15–20mm animals. Intracellular

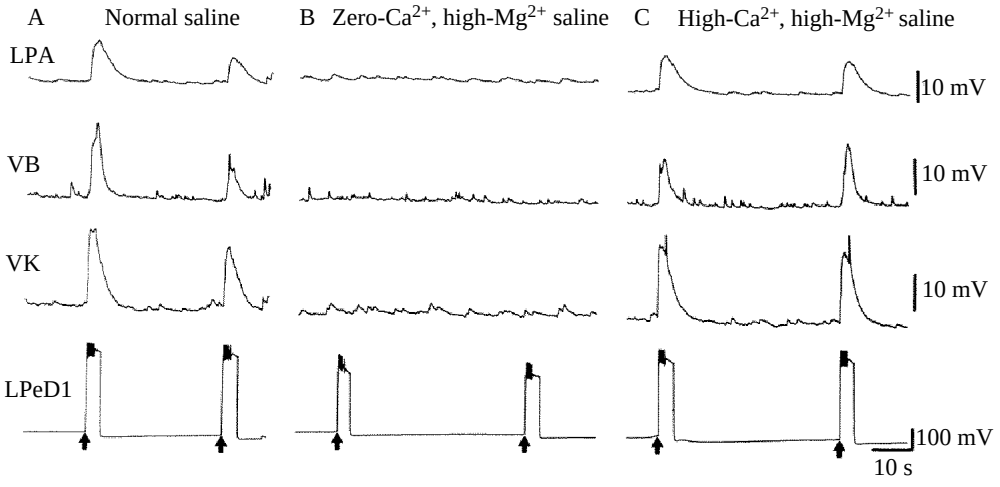


Fig. 4. Electrophysiological evidence for the excitatory chemical and monosynaptic nature of connections between *Helisoma* left pedal dorsal 1 (LPeD1) and selected follower cells. (A) Simultaneous intracellular recordings were made from LPeD1 and follower visceral K (VK), visceral B (VB) and left parietal A (LPA) cluster cells. Action potentials elicited by injection of depolarizing current into LPeD1 (at arrows) excited all three follower cell types. The connections between LPeD1 and these follower cells were blocked in zero-Ca^{2+}/high-Mg^{2+} saline (B), but were unaffected (C) by bathing in high-Ca^{2+}/high-Mg^{2+} saline.

injection of Lucifer Yellow revealed this cell to have a main axon passing through the right pleural, right parietal and visceral ganglia. One branch of this axon projected into the right parietal nerve, whereas the other projected to the periphery *via* the anal nerve. Fine axonal processes were also observed in the right pedal, right pleural, right parietal and visceral ganglia (Fig. 2B).

Transmitter phenotype

Since this cell is also a probable homologue of the *Lymnaea* LPeD1 neurone (Kyriakides *et al.* 1989), whole mounts of central ring ganglia of *Helisoma* were processed for serotonin immunoreactivity using a polyclonal antiserum. This approach revealed many serotonin-immunoreactive somata and fibres throughout the central ring ganglia (data not shown). Amongst many serotonin-immmunoreactive somata in the pedal ganglia, readily identifiable by its size and location was RPeD1 (Fig. 2D). When isolated and processed individually for monoamine content by HPLC, the neurone RPeD1was found to contain a high level of serotonin (Table 1, Fig. 3), but no detectable dopamine.

Follower cells

Despite repeated and rigorous attempts, we failed to locate postsynaptic follower cells of RPeD1 within the pleural, parietal or visceral ganglia. In this context, we made simultaneous recordings from RPeD1 and neurones located in the visceral ganglion,

neurone VD4 and the A–M cluster neurones (20–40 cells tested in each preparation, *N*=7), in the right pleural ganglion, A, B, C cluster neurones (20–25 cells tested in each preparation, *N*=6) and also in the right parietal ganglion, A–F cluster neurones (20–30 cells tested in each preparation, *N*=13). In the left pedal ganglion, however, neurone LPeD1 was found to be inhibited by RPeD1 (Fig. 8), although this connection was not found in all ganglia (observed in 7 out of 10 preparations). In all those seven preparations where this connection was demonstrated, it was found to be chemical and probably monosynaptic in nature based upon the criteria described above (Fig. 8).

Visceral dorsal 4 (VD4)

Morphology

A small, opalescent white cell was identified on the dorsal surface of the visceral ganglion near the right parietal ganglion. On the basis of its probable homology with neurone VD4 in *Lymnaea* (see Discussion), we have designated this cell as VD4 (Fig. 1). Owing to its location and size (50–70 μm in 15–20mm animals), this cell is not as easily discernible as either LPeD1 or RPeD1. When stained with Lucifer Yellow, we found this cell to be a true interneurone, with its axonal projections confined within the central ring ganglia as well as entering the right cerebrobuccal connective (Fig. 9). Neurone VD4 had two main axons which encircled the lower ganglionic ring and axon collaterals in all

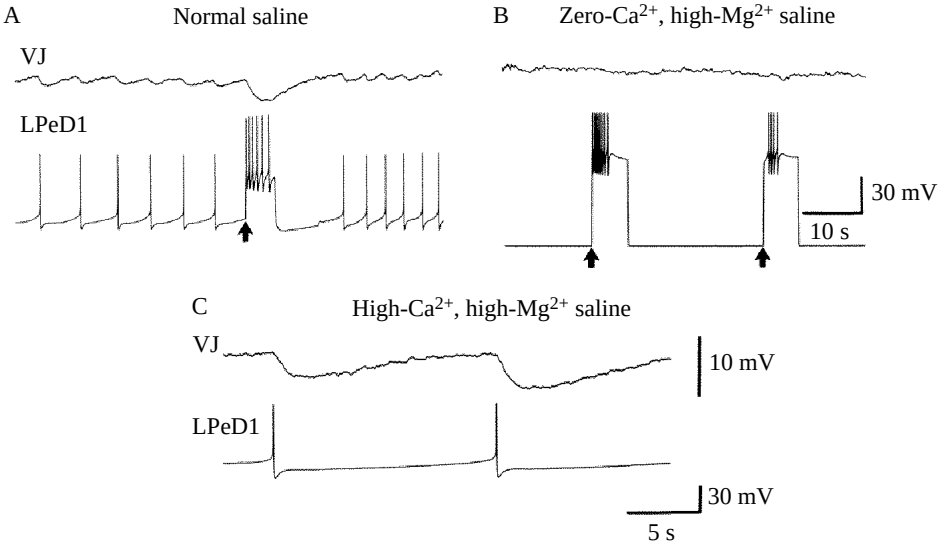


Fig. 5. Example of an inhibitory chemical monosynaptic connection between left pedal dorsal 1 (LPeD1) and a selected follower cell. (A) Either spontaneous or induced (at arrow) action potentials in LPeD1 produced IPSPs in a follower visceral J (VJ) cluster cell recorded in an isolated central ganglionic ring preparation maintained in normal saline. This inhibitory connection was blocked in zero-Ca^{2+}/high-Mg^{2+} saline (B), but not in high-Ca^{2+}/high-Mg^{2+} saline (C).

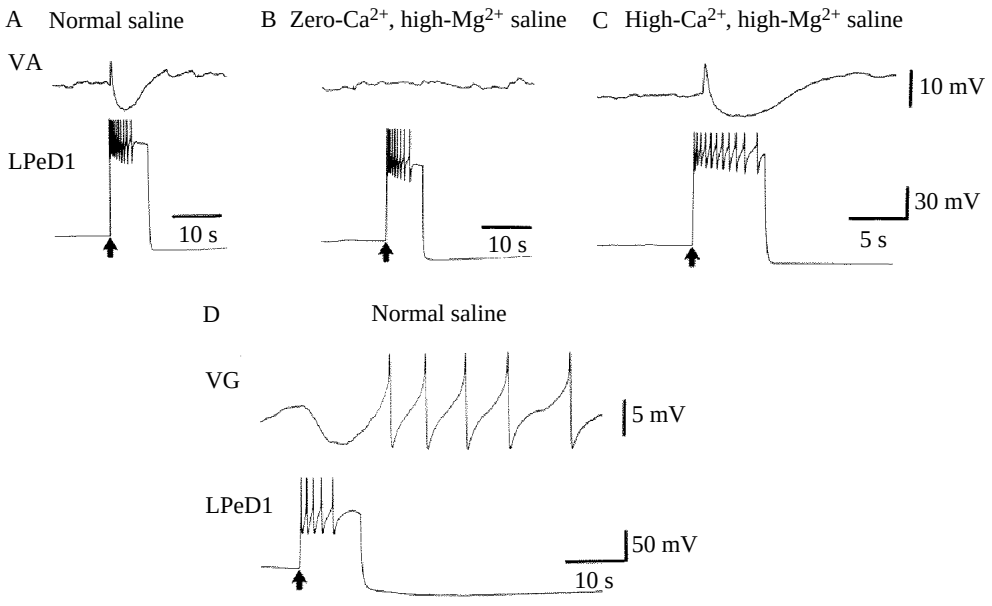


Fig. 6. Examples of biphasic chemical connections between left pedal dorsal 1 (LPeD1) and selected follower cells. (A) Electrical stimulation of LPeD1 (at arrow) caused rapid excitation followed by prolonged inhibition of a follower visceral A (VA) cluster cell. This biphasic response was blocked in zero-Ca^{2+}/high-Mg^{2+} saline (B), but not in high-Ca^{2+}/high-Mg^{2+} saline (C). In another preparation (D), LPeD1 stimulation (at arrow) produced a biphasic response, in this case inhibition followed by excitation, in a follower visceral G (VG) cluster cell.

central ring ganglia. When processed for monoamine content by HPLC, neither dopamine nor serotonin was detected (Table 1, Fig. 3).

Transmitter phenotype

In order to determine the transmitter phenotype of neurone VD4, whole mounts of *Helisoma* central ring ganglia were processed for FMRFamide immunoreactivity using a polyclonal antiserum. A large number of neurones and their fibres, including neurone VD4, were found to be immunoreactive to this antibody. Although FMRFamide immunoreactive somata of VD4 were easily discernible, we confirmed these findings by a double staining procedure. In these preparations, neurone VD4 was first visually identified and stained with Lucifer Yellow dye and then preparations were processed for FMRFamide immunoreactivity (Fig. 9B,C). Neurone VD4, but not LPeD1 and RPeD1, was found to be immunoreactive to the FMRFamide antibody. In a recent study, utilizing a variety of techniques such as HPLC and RIA, Richmond *et al.* (1991) have also shown *Helisoma* VD4 to contain true FMRFamide as well as two other members of the FMRFamide family.

Follower cells

The follower cells of VD4 were found in nearly all ganglia of the central ganglionic

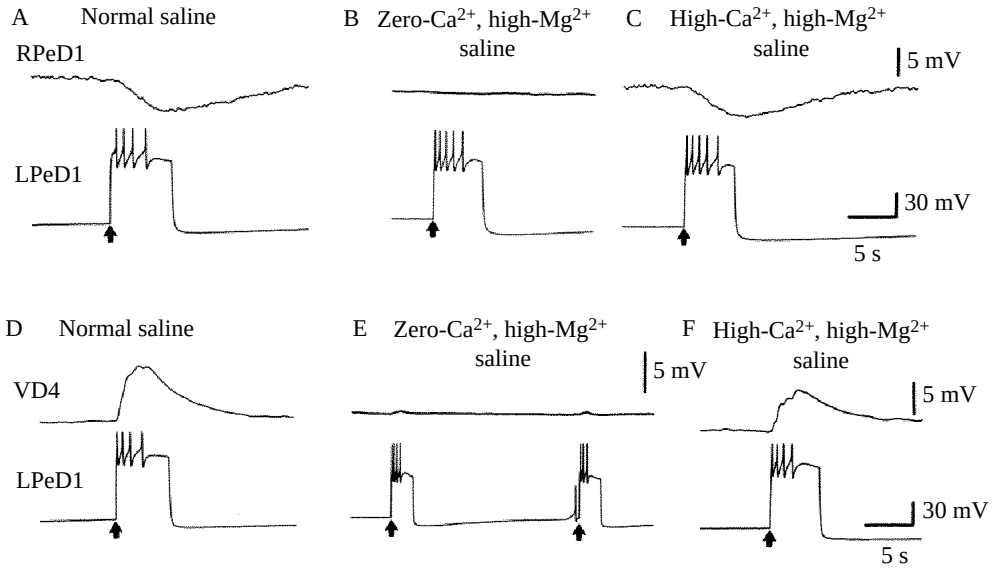


Fig. 7. Chemical monosynaptic connections between *Helisoma* interneurones left pedal dorsal 1 (LPeD1), right pedal dorsal 1 (RPeD1) and visceral dorsal 4 (VD4). (A) In preparations maintained in normal saline, electrical stimulation of LPeD1 (at arrow) inhibited RPeD1. This connection was blocked in zero-Ca^{2+}/high-Mg^{2+} saline (B), but not in high-Ca^{2+}/high-Mg^{2+} saline (C). (D) Whereas the stimulation of LPeD1 (at arrow) excited VD4 in preparations bathed in normal saline, this response was significantly reduced in amplitude when bathed in zero-Ca^{2+}/high-Mg^{2+} saline (E); the response was unaffected by bathing in high-Ca^{2+}/high-Mg^{2+} saline (F).

ring. In most instances, these connections were inhibitory in nature. For example, all of the putative neurosecretory cells examined in the visceral and parietal ganglia (identified on the basis of their white coloration) were found to be inhibited by VD4 (Fig. 10A,B). Furthermore, the effects of VD4 on various pedal ganglia neurones were also inhibitory (e.g. Fig. 10C). In addition to these inhibitory connections, a few excitatory connections were also observed in the pedal, parietal and visceral ganglia (e.g. Fig. 10D) (Table 3). Furthermore, electrical stimulation of VD4 inhibited the activities of both LPeD1 and RPeD1 (Fig. 11A). To test whether the connections between VD4 and LPeD1 and RPeD1 were chemical, we first superfused our preparations with zero-Ca^{2+}/high-Mg^{2+} saline. In this saline the connections between VD4 and its follower cells, e.g. LPeD1 and RPeD1, were significantly reduced in amplitude (Fig. 11B), suggesting that they are chemical in nature. To test the monosynaptic nature of these connections, preparations were superfused with high-Ca^{2+}/high-Mg^{2+} saline. All the connections between VD4 and its follower cells, including LPeD1 and RPeD1, were found to persist under these experimental manipulations (Table 3; Fig. 11C).

Since our immunohistochemical data suggested that neurone VD4 contained an FMRFamide-like peptide, we tested the possibility that FMRFamide and some of its

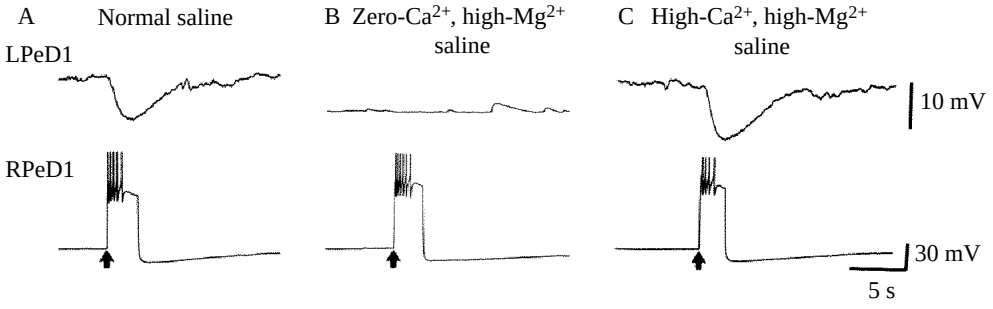


Fig. 8. Electrophysiological evidence for the presence of a chemical monosynaptic connection between *Helisoma* right pedal dorsal 1 (RPeD1) and left pedal dorsal 1 (LPeD1) neurones. (A) Electrical stimulation of RPeD1 (at arrow) inhibited LPeD1 in preparations maintained in normal saline. This inhibitory response was blocked in zero-Ca^{2+}/high-Mg^{2+} saline (B), but not in high-Ca^{2+}/high-Mg^{2+} saline (C).

Table 3. *Follower cells of* Helisoma *VD4*

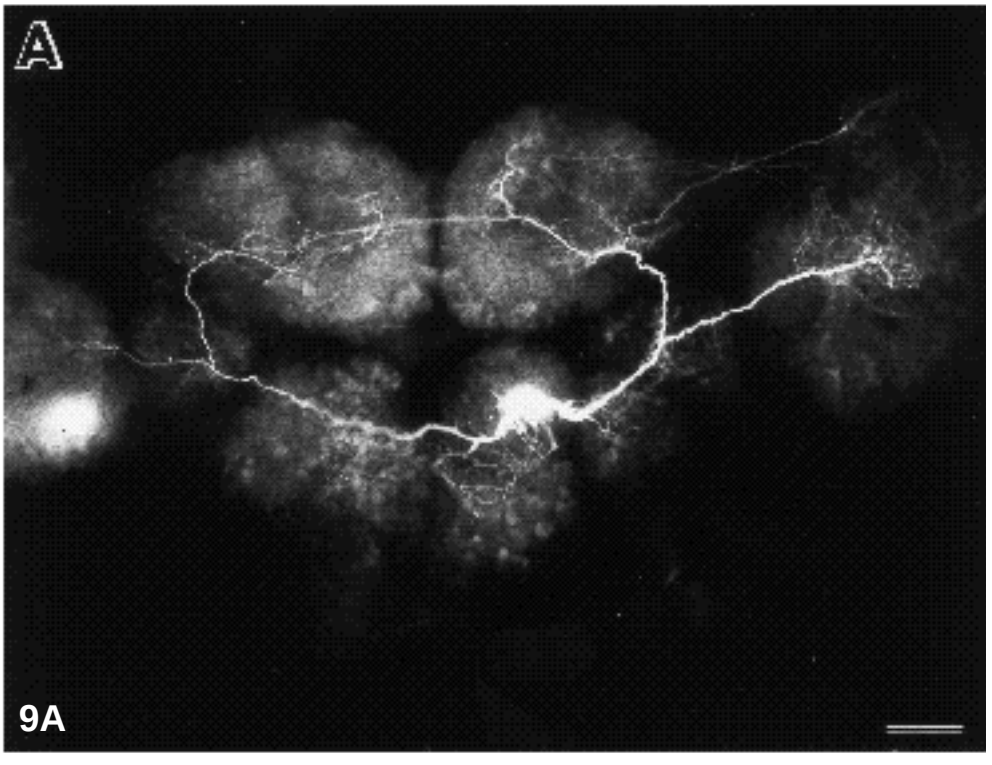
Follower cells	Postsynaptic response
LPeD1	IPSP
RPeD1	IPSP
LPD2, LPB, LPC, LPE L/RPeF, VB, VC, VD, VE, VH, VI, VJ	IPSP
L/RPeA, L/RPeD, VK	EPSP

analogues were active. Simultaneous pressure-ejection of FMRFamide (range 10^{-5}–10^{-7} mol l^{-1}) on follower cells LPeD1 and RPeD1 mimicked the effects of electrical stimulation of VD4 (Fig. 11D). We also tested analogues of FMRFamide such as FLRFamide, SDPFLRFamide and GDPFLRFamide. When bath-applied to LPeD1 or RPeD1, all of these peptides were found to have inhibitory effects similar to either VD4 stimulation or exogenous application of FMRFamide (data not shown).

Reconstruction of the circuit in culture

Since it is often difficult *in vivo* to demonstrate unequivocally that connections

Fig. 9. Morphology and identification of the transmitter phenotype of *Helisoma* visceral dorsal (VD4) neurone. (A) Intracellular ionophoresis of Lucifer Yellow revealed that VD4 is a true interneurone, with all its axonal branches confined within the central ring ganglia. It possesses two main axons which together encircle the lower ganglionic ring and also enter the cerebral ganglia (ganglia are shown in Fig. 1). In double-labelled preparations, VD4 was first marked with Lucifer Yellow (B) and the ganglia were then processed for whole-mount immunohistochemistry (C). Neurone VD4 (at arrowhead in C) was among the visceral ganglion cells that were immunoreactive to an antiserum raised against the tetrapeptide FMRFamide. All magnification bars, 100 μm.


A
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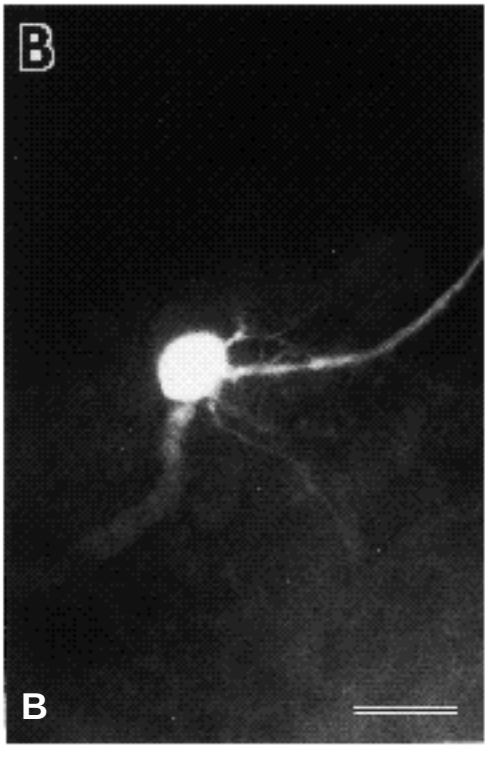
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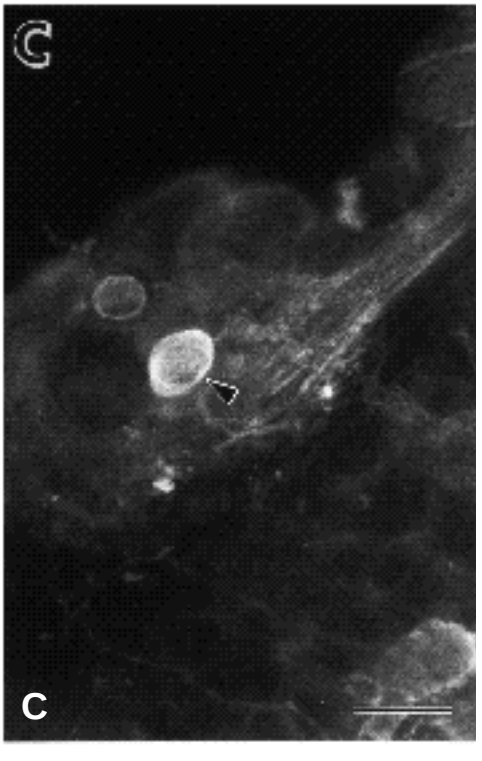
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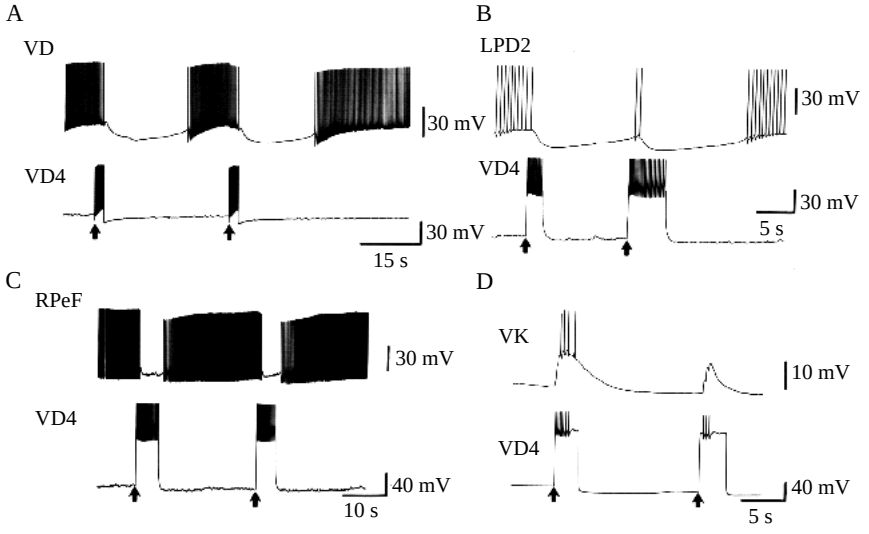

Fig. 10. Follower cells of *Helisoma* visceral dorsal 4 (VD4) neurone identified in the central ring ganglia. The majority of follower cells of VD4, including both neurosecretory (A and B) and non-neurosecretory (C) cell types, were found to be inhibited by VD4. Electrical stimulation of VD4 (at arrows) inhibited visceral D (VD) cluster cells (A), neurone left parietal dorsal 2 (LPD2) (B) and also right pedal F (RPeF) cluster cells (C). (D) Some follower cells were found to be excited by electrical stimulation of VD4, such as visceral K (VK) cluster cells.

between neurones are truly monosynaptic and that the effects of various exogenously applied substances are direct, we turned to *in vitro* cell culture. To test the monosynaptic and specific nature of connections between LPeD1, RPeD1 and VD4, we isolated these neurones from their respective ganglia. When these neurones were plated on poly-L-lysine-coated dishes containing conditioned medium they exhibited neurite outgrowth and also synapse formation (Fig. 12).

We first cultured only one pair of interneurones per dish and tested the ability of the sprouted interneurones to re-establish specific synaptic connections. When simultaneous intracellular recordings were made, these neurones were often found to have established appropriate synaptic connections. For example, a mutually inhibitory chemical connection developed between LPeD1 and RPeD1 in 76% of preparations (N=17) (Fig. 12B,C). In the other 24% of preparations, however, we detected the presence of electrical coupling between these neurones, no chemical connection being observed (Fig. 12D).

When LPeD1 and VD4 were cultured as a pair, LPeD1 developed an appropriate excitatory connection with VD4 (N=7) (Fig. 12E). Similarly, when VD4 was co-cultured with either LPeD1, RPeD1 or both, it re-established its specific inhibitory connections with these cells (N=11) (Fig. 12F). In these same preparations, however, no connections were observed between RPeD1 and VD4, a result which parallels the situation *in vivo*. Although the connections made *in vitro* from VD4 to neurones LPeD1 and RPeD1 were of the same type as those observed *in vivo*, it was difficult to resolve the occurrence of

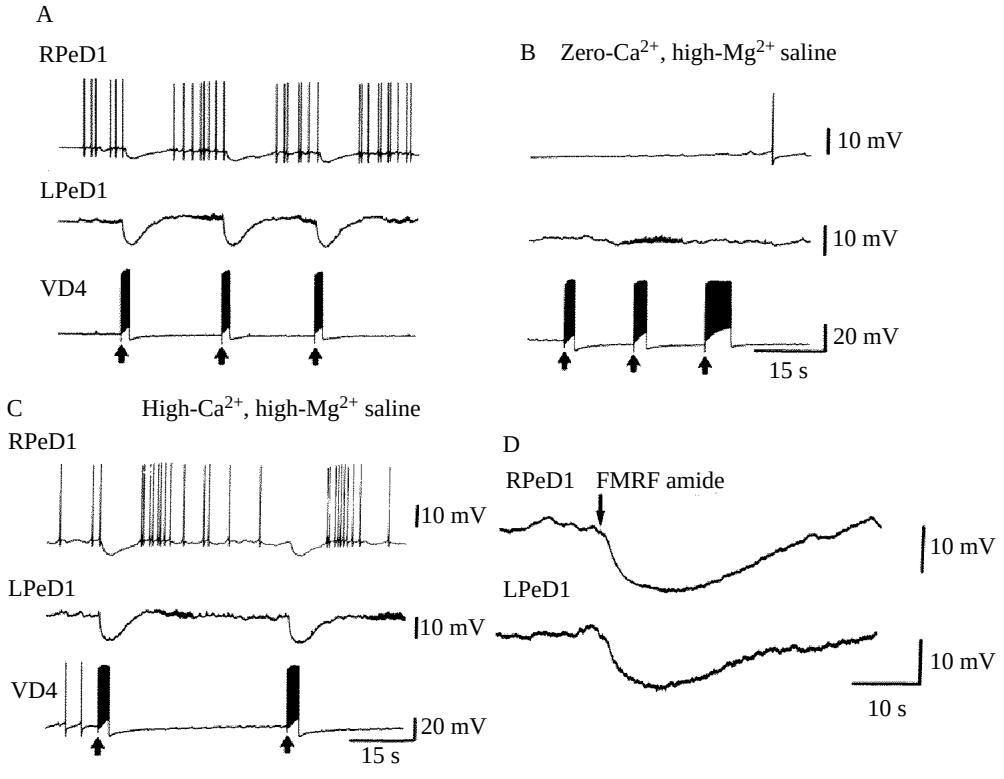

Fig. 11. Chemical monosynaptic connections between *Helisoma* visceral dorsal 4 (VD4) and left pedal dorsal 1 (LPeD1) and right pedal dorsal 1 (RPeD1) neurones. (A) Simultaneous intracellular recordings were made from VD4, LPeD1 and RPeD1 in an isolated ganglionic ring preparation maintained in normal saline. Electrical stimulation of VD4 (at arrows) caused the inhibition of both LPeD1 and RPeD1. The inhibitory effects of VD4 on these two follower cells were blocked in zero-Ca^{2+}/high-Mg^{2+} saline (B), but persisted in high-Ca^{2+}/high-Mg^{2+} saline (C). (D) Direct application of FMRFamide (10^{-6} mol l^{-1} in the pipette) *via* pressure ejection onto the somata of LPeD1 and RPeD1 mimicked the inhibitory effects of VD4 stimulation of these cells.

unitary inhibitory postsynaptic potentials (IPSPs). In contrast, unitary PSPs were often visible in connections made by LPeD1 and RPeD1 onto their follower cells (see Fig. 12B, C and E).

Discussion

Identification of homologous neurones in related molluscan species

The fundamental plan of the molluscan nervous system has remained surprisingly stable, allowing both groups and individual homologous neurones to be identified between ganglia in different classes (Dorsett, 1986; Croll, 1987). This is exemplified by the present study of *Helisoma* where we took advantage of previously identified

interneurones in *Lymnaea* and *Planorbis* and, despite different ganglionic arrangements, were able to identify three homologous interneurones, namely left pedal dorsal 1, right pedal dorsal 1 and visceral dorsal 4.

In the present study we have proposed a standardized system to map the *Helisoma* central ring ganglia. This system is based on that proposed by Benjamin and Winlow

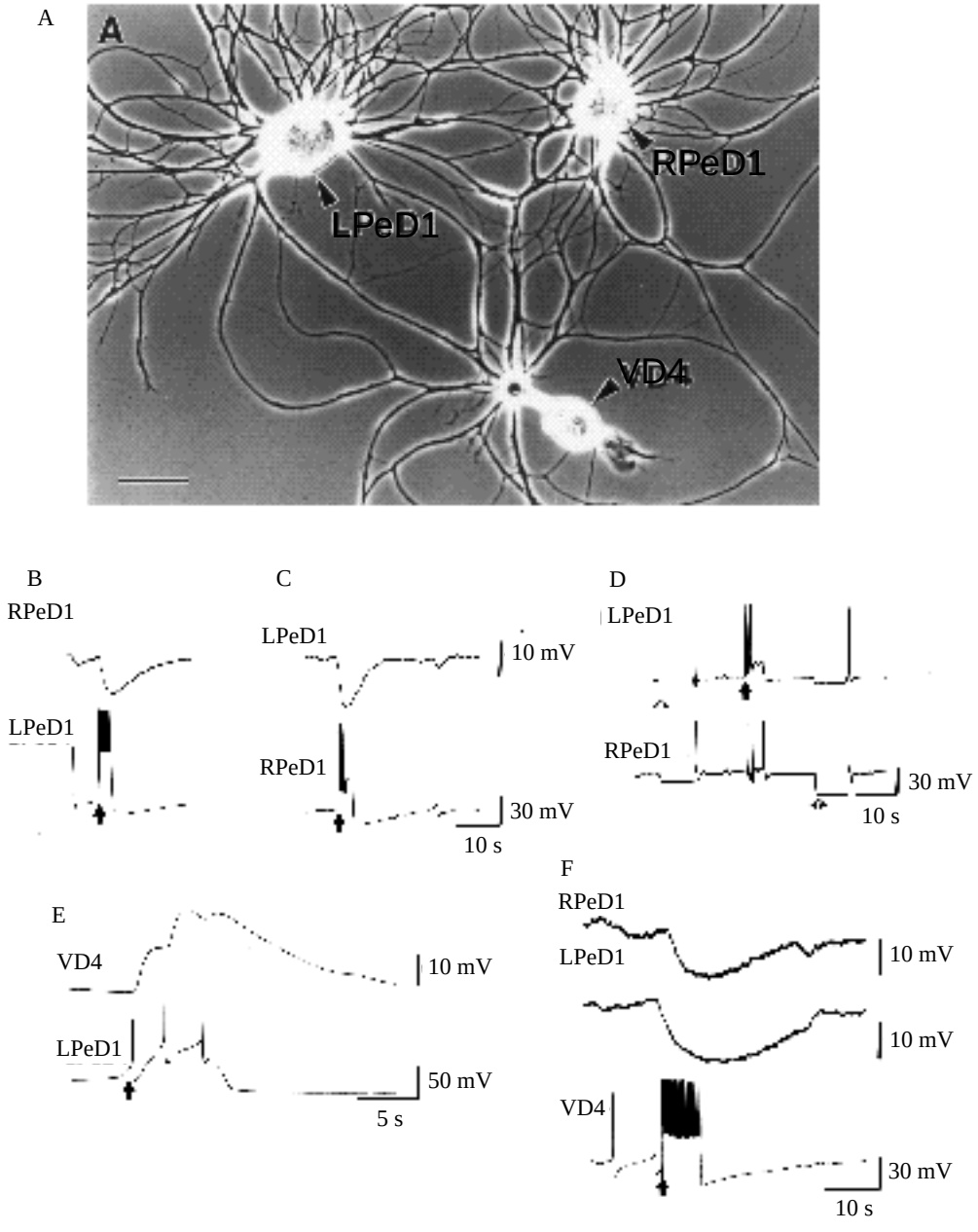

Fig. 12

(1981) for *Lymnaea* which has been successfully used and extended by others. The central ring ganglia of *Helisoma* are the mirror images of those of *Lymnaea*, so the *Lymnaea* nomenclature system can readily be adapted for this species (Culver *et al.* 1992; Richmond *et al.* 1991). Since a large number of laboratories use *Helisoma*, we believe a nomenclature system is required and, in addition, a common system will be a tremendous asset for comparative studies with *Lymnaea*.

Left pedal dorsal 1 (LPeD1)

Berry and Cottrell (1973, 1975) described a multi-action neurone (the giant dopamine cell, GDC) in the sinistral basommatophoran pulmonate *Planorbis corneus*. The cell body of this neurone is located in the left pedal ganglion and was known to contain dopamine (Powell and Cottrell, 1974). In a closely related species, *Lymnaea stagnalis* (L.), a cell similar to the GDC of *Planorbis* was identified in the right pedal ganglion (McCaman *et al.* 1979; Cottrell *et al.* 1979) and was designated as RPeD1 (Benjamin and Winlow, 1981). Since *Lymnaea* is a dextral pulmonate and its central ganglionic ring is a mirror image of that of *Planorbis*, the giant dopamine-containing cells of the two animals are thought to be homologous. These dopaminergic neurones from both *Planorbis* (Berry and Cottrell, 1973, 1975, 1979; Cottrell, 1977) and *Lymnaea* (Winlow *et al.* 1981) are monosynaptically connected to a large number of follower cells in the visceral and right parietal ganglia. Although the exact function of the GDC in *Planorbis* is unclear, in *Lymnaea* the giant dopaminergic neurone RPeD1 is known to be involved in respiratory behaviour (Syed *et al.* 1990; Syed and Winlow, 1991*a*). Earlier, a *Helisoma* neurone was designated as P1 (pedal 1), based primarily on its location and was thought to be the homologue of both the *Planorbis* GDC and *Lymnaea* RPeD1 (McCobb *et al.* 1988). It was also reported that the 'left pedal giant cell' of *Helisoma* contained 0.80pmol per cell of dopamine (McCaman *et al.* 1979), an estimate remarkably close to that of this study (Table 1). The morphology and synaptic contacts of this neurone, however, were previously unknown. We have recently begun to map the nervous system of *Helisoma* and have developed a nomenclature system which is compatible with the system in

Fig. 12. Specificity of synaptic connections formed *in vitro* by *Helisoma* visceral dorsal 4 (VD4), left pedal dorsal 1 (LPeD1) and right pedal dorsal 1 (RPeD1) neurones. (A) Photomicrograph showing the extent of neurite outgrowth exhibited by the three neurones after isolation and plating in conditioned medium for 48h. Magnification bar 100 μm. (B,C) In this plating, neurones LPeD1 and RPeD1 were cultured together. Simultaneous intracellular recordings revealed the presence of appropriate synaptic connections between these two cells. For example, either spontaneous (single) or stimulated (at arrows) action potentials in LPeD1 (B) or RPeD1 (C) inhibited the other cell. These *in vitro* connections were similar to those observed *in vivo*. However, electrotonic coupling was occasionally detected between LPeD1 and RPeD1, as demonstrated by the ability of either hyperpolarizing (open arrows) or depolarizing (filled arrow) current to pass between the two cells shown in D. (E) Re-establishment of the excitatory synaptic connection between LPeD1 and VD4. Electrical stimulation of LPeD1 (at arrow) produced 1:1 EPSPs in VD4. (F) When cultured together with both LPeD1 and RPeD1 (as in A), VD4 re-established its inhibitory synaptic connections. Electrical stimulation of VD4 (at arrow) resulted in inhibition of both LPeD1 and RPeD1, similar to the response found *in vivo* (see Fig. 11).

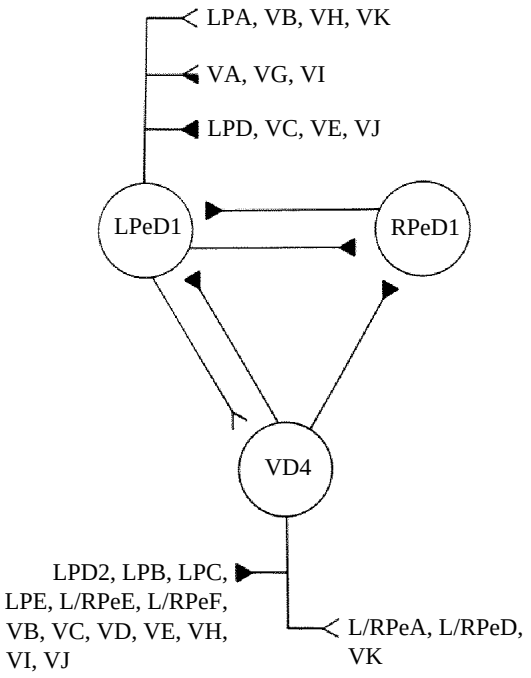

Fig. 13. Summary diagram of the synaptic connections found within the network formed by three *Helisoma* interneurones: left pedal dorsal 1 (LPeD1), right pedal dorsal 1 (RPeD1) and visceral dorsal 4 (VD4). Synaptic connections observed between LPeD1 and identified follower cells, as well as between VD4 and identified follower cells, are also shown. Excitatory connections are represented by the open symbol, inhibitory connections by the closed symbol and biphasic connections by the half-open/half-closed symbol. Abbreviations for follower cells are as given in Fig. 1.

common use for the *Lymnaea* central nervous system. Based upon this naming system for identifiable neurones located on the dorsal surface of the left pedal ganglia, we have designated this cell as *Helisoma* LPeD1. We confirmed using the glyoxylic acid histofluorescence technique and HPLC that this cell contains dopamine and has a similar morphology to that of both the GDC and RPeD1 of *Planorbis* and *Lymnaea*, respectively. Electrophysiologically, LPeD1 of *Helisoma* was also found to make synaptic contacts with a number of cells located in both the visceral and left parietal ganglia which appear to be chemical and monosynaptic. Given the high content of dopamine in the *Helisoma* LPeD1 and the pharmacological characterisation of the synapses made by its homologues in *Planorbis* and *Lymnaea*, dopamine is clearly a strong candidate as the transmitter at LPeD1 synapses in *Helisoma*. The range of pharmacological studies required to establish the identity of the transmitter and receptor subtypes at each of the LPeD1–follower cell connections, however, was beyond the scope of the present study.

It has recently been shown that, in culture, *Helisoma* LPeD1 re-established specific synaptic connections with appropriate follower cells, but not with non-follower cells (Syed *et al.* 1992). Furthermore, *in vitro*, the giant dopamine cells of *Helisoma* and *Lymnaea* make appropriate synapses upon each other's follower cells (Syed *et al.* 1992).

These experiments provide further support for strong homology between the giant dopamine neurones of these different species.

Right pedal dorsal 1 (RPeD1)

A giant neurone present on the dorsal surface of the left pedal ganglion of *Lymnaea* and right pedal ganglion of *Planorbis* is known to contain serotonin (Audesirk, 1985; Cottrell *et al.* 1979; Kyriakides *et al.* 1989). In *Lymnaea*, this serotonergic neurone has been designated as LPeD1, but it has not been found to make synaptic connections with any other neurone (Kyriakides *et al.* 1989). Despite some morphological and electrophysiological information, the function of *Lymnaea* LPeD1 is unknown. A *Helisoma* homologue of *Lymnaea* LPeD1 was apparently used in some cell culture experiments (Haydon *et al.* 1984) where it was referred to as P5. We found this cell to have similar morphology to that of *Lymnaea* LPeD1 (Kyriakides *et al.* 1989) and, like that of *Lymnaea*, this cell also contains serotonin as assayed by glyoxylic acid histofluorescence, immunohistochemistry and HPLC (Table 1). In accordance with our new nomenclature system for *Helisoma*, we now designate this cell as *Helisoma* RPeD1. Although we found this cell to have reciprocal inhibitory connections with LPeD1 in some preparations, the functional significance of RPeD1 is at present unknown. The mutual inhibitory connections between *Lymnaea* LPeD1 and RPeD1 have never been reported in normal preparations. However, following axotomy and regeneration, Benjamin and Allison (1985) found a novel excitatory synapse between RPeD1 and LPeD1.

Helisoma RPeD1, in common with the giant dopamine cell of both *Lymnaea* and *Helisoma*, has axons that project to the periphery. Since these neurones have been found to innervate a number of identified neurones, both LPeD1 and RPeD1 are designated as interneurones in accord with common usage.

Visceral dorsal 4 (VD4)

A true interneurone located in the visceral ganglion of *Lymnaea*, with axonal branches confined within the central ring ganglia, was originally identified as the visceral white interneurone (V.W.I.) (Benjamin, 1984). This cell is peptidergic and synapses on many cells in all the central ring ganglia (Benjamin, 1984). A neurone morphologically and electrophysiologically similar to V.W.I. was later described by Janse *et al.* (1985), who named it visceral dorsal 4 (VD4). Extensive electrophysiological and morphological investigations have revealed that V.W.I. and VD4 are synonymous (Syed, 1988). A homologue of the *Lymnaea* VD4 has not yet been identified in *Planorbis* but, in the present study, we appear to have found such a cell in *Helisoma*. In order to be consistent with previously existing maps of *Lymnaea* neurones (Winlow and Benjamin, 1976), we have adopted the nomenclature proposed by Janse *et al.* (1985) (Syed, 1988; Syed and Winlow, 1989, 1991*a*,*b*; Syed *et al.* 1990). Interneurone VD4 of both *Lymnaea* and *Helisoma* are immunoreactive for FMRFamide and have very similar morphologies. Our recent studies have indicated that neurone VD4 of *Lymnaea* does indeed use one of the FMRFamide family of peptides as a transmitter, but also uses a second, as yet unidentified, transmitter (McKenney *et al.* 1990). From the results of the present study of

Helisoma, an FMRFamide peptide (which could be either a tetrapeptide and/or a heptapeptide, see Richmond *et al.* 1991) is a strong candidate as the transmitter used at VD4 to RPeD1 and LPeD1 connections (Fig. 12D); if a second transmitter exists in *Helisoma* VD4, it is unlikely to be dopamine or serotonin (Table 1). However, the apparent absence of unitary EPSPs from VD4 to both LPeD1 and RPeD1 and other follower cells, as well as the long-lasting action of VD4 at some connections, may be due to FMRFamide acting as a local modulator rather than *via* a classical synapse. The interneurones in both species have a number of follower cells in almost all the central ring ganglia. However, the intrinsic properties of these homologous neurones differ slightly. For example, the *Lymnaea* VD4 typically fires bursts of spikes (Syed, 1988; Syed *et al.* 1990) and less commonly single spikes, whereas the *Helisoma* VD4 always fired single spikes (data not shown).

Synaptic specificity of invertebrate neurones in vitro

In addition to being a useful tool for determining the intrinsic properties of identified neurones (e.g. endogenous or conditional bursters) and the monosynaptic nature of their connections, *in vitro* preparations have also proved valuable in the study of synaptic specificity. The mechanisms that determine synaptic specificity, however, are as yet largely obscure. One approach in this area has been to examine synapse formation between identified pre- and postsynaptic neurones cultured *in vitro*. Several invertebrate preparations, such as the leech (see Chiquet and Nicholls, 1987; Nicholls *et al.* 1990) and the molluscs *Aplysia* (Schacher and Proshansky, 1983; Schacher *et al.* 1985; Schacher, 1988; Kleinfeld *et al.* 1990*a*,*b*), *Helisoma* (Haydon, 1988; Cohan *et al.* 1990) and *Lymnaea* (Syed *et al.* 1990), have helped our understanding of specific synapse formation *in vitro*. In almost all of these preparations, neurones have been shown to maintain their intrinsic properties. The literature is divided, however, regarding the specificity of synapses in culture. For instance, although leech identified neurones make specific connections with appropriate targets similar to those seen *in vivo*, some novel connections are also formed (Fuchs *et al.* 1981). Novel connections have also been observed between *Aplysia* neurones (Camardo *et al.* 1983; Schacher *et al.* 1985; Kleinfeld *et al.* 1990*a*,*b*). While the formation of novel connections is interesting and worthy of further study, it does cause difficulties when one is examining the specificity of synapses. In our hands, the synaptic connections formed between specific *Lymnaea* neurones *in vitro* were always appropriate; no inappropriate connections were observed (Syed *et al.* 1990). It is possible that the novel connections observed by others *in vitro* may have existed during early development and were later retracted. Therefore, the presence of novel connections may be transient when examined over a long time course, as has been demonstrated for *in vivo* novel connections of *Helisoma* (Cohan *et al.* 1987). These differences might also be attributed to the differences in tissue culture procedures. For instance, when leech or *Aplysia* neurones are cultured on concanavalin A, the chemical connections usually observed on poly-L-lysine coated dishes are no longer discernible; instead, electrical connections predominate (Lin and Levitan, 1987). Interestingly, substratum conditions are also known to affect the distribution of calcium channels in cultured leech neurones (Ross *et al.* 1988). However, the occurrence of electrical rather than chemical synaptic

connections between LPeD1 and RPeD1 in 24% of *in vitro* preparations is clearly not attributable to different substratum conditions. Although it is possible that the novel electrical synapses observed between LPeD1 and RPeD1 in 24% of *in vitro* preparations might be transient connections that precede chemical synapses, we did not test this possibility rigorously. In these preparations we obtained electrophysiological data 48 h after plating and did not observe inappropriate or novel electrotonic synapses except between LPeD1 and RPeD1. Our data at present, therefore, cannot explain the reasons for the occurrence of electrotonic coupling between LPeD1 and RPeD1. It is, however, possible that these neurones may have different genetic programmes for synapse formation, having a higher degree of plasticity in this regard than the other neurones.

Function of neurones LPeD1, RPeD1 and VD4

The identification of individual neurones and characterization of their synaptic connections is important if we are to understand fully the cellular basis of specific behaviour patterns. In *Lymnaea*, RPeD1, VD4 and the input 3 interneurone (IP3I; as yet unidentified in *Helisoma*) are known to be central pattern-generating interneurones that initiate and control respiratory behaviour (Syed *et al.* 1990; Syed and Winlow, 1991*a*,*b*). In addition to the behavioural significance of these *Lymnaea* neurones, their connectivity patterns are well characterized both *in vivo* (Syed and Winlow, 1991*a*) and *in vitro* (Syed *et al.* 1990). In contrast, the function of the serotonergic cell (LPeD1 in *Lymnaea*, RPeD1 in *Helisoma*) is unknown. Despite apparent locational and morphological similarities between *Lymnaea* neurones RPeD1 and VD4, with *Helisoma* neurones LPeD1 and VD4, respectively, the behavioural function(s) of these *Helisoma* interneurones has yet to be determined.

The interneurones identified in this study and their known follower cells are shown schematically in Fig. 13. The physiological significance of the various neurones and groups of follower neurones in *Helisoma* has yet to be determined. However, in *Lymnaea* the function of many apparently homologous neurones is known. For example, most visceral and parietal neurones are known to be involved in cardiorespiratory control (Buckett *et al.* 1990; Syed and Winlow, 1991*a*,*b*; Syed *et al.* 1991). Most pedal neurones, in contrast, control locomotion (Syed and Winlow, 1989). From these studies it seems reasonable to speculate that LPeD1 and VD4 of *Helisoma* may be part of the circuit that controls cardiorespiratory behaviour. Additionally, neurone VD4 is a multi-action interneurone that may also be involved in controlling the activity of pedal locomotor neurones. The present detailed morphological and electrophysiological characterisation of these interneurones should facilitate future studies of the neural basis of behaviour in *Helisoma*. Furthermore, our new *in vitro* model will provide opportunities to study synapse formation and plasticity in a manner unapproachable in the intact ganglia.

References

AUDESIRK, G. (1985). Amine-containing neurons in the brain of *Lymnaea stagnalis*: distribution and effects of precursors. *Comp. Biochem. Physiol.* **81**A, 359–365.

BENJAMIN, P. R.(1984). Interneuronal network acting on snail neurosecretory neurones (yellow cells and yellow green cells of *Lymnaea*). *J. exp. Biol.* **113**, 165–185.

BENJAMIN, P. R. AND ALLISON, P. (1985). Regeneration of excitatory, inhibitory and biphasic synaptic connections made by a snail giant interneuron. *Proc. R. Soc. Lond. B* **226**, 159–176.

BENJAMIN, P. R. AND WINLOW, W. (1981). The distribution of three wide-acting synaptic inputs to identified neurons in the isolated brain of *Lymnaea stagnalis*. *Comp. Biochem. Physiol.* **70**A, 293–307.

BERRY, M. S. AND COTTRELL, G. A. (1973). Dopamine: Excitatory and inhibitory transmission from a giant dopamine neurone. *Nature New Biology* **242**, 250–253.

BERRY, M. S. AND COTTRELL, G. A. (1975). Excitatory, inhibitory and biphasic synaptic potentials mediated by an identified dopamine containing neurone. *J. Physiol., Lond.* **244**, 589–612.

BERRY, M. S. AND COTTRELL, G. A. (1979). Ionic basis of different synaptic potentials mediated by an identified dopamine-containing neuron in *Planorbis*. *Proc. R. Soc. Lond. B* **203**, 427–444.

BERRY, M. S. AND PENTREATH, V. W. (1976). Criteria for distinguishing between monosynaptic and polysynaptic transmission. *Brain Res.* **105**, 1–20.

BUCKETT, K. J., PETERS, M., DOCKRAY, G. J., VAN MINNEN, J. AND BENJAMIN, P. R.(1990). Regulation of heartbeat in *Lymnaea* by motoneurons containing FMRFamide-like peptides. *J. Neurophysiol.* **63**, 1426–1435.

BULLOCH, A. G. M.(1989). (ed.) Cellular basis of neuronal plasticity. In *Studies in Neuroscience,* no. 7 (series ed. W. Winlow). Manchester: Manchester University Press.

BULLOCH, A. G. M., PRICE, D. A., MURPHY, A. D., LEE, T. D. AND BOWES, H. N. (1988). FMRFamide peptides in *Helisoma*: identification and physiological actions at a peripheral synapse. *J. Neurosci.* **8**, 3459–3469.

BYRNE, J. H., BAXTER, D. V., BUONOMANO, D. V. AND RAYMOND, J. L. (1990). Neuronal and network determinants of simple and higher-order features of associative learning. In *Cold Spring Harbor Symp. quant. Biol.* **LV**, 175–186.

CAMARDO, J., PROSHANSKY, E. AND SCHACHER, S. (1983). Identified *Aplysia* neurones form specific chemical synapses in culture. *J. Neurosci.* **3**, 2614–2620.

CHIQUET, M. AND NICHOLLS, J. G. (1987). Neurite outgrowth and synapse formation by identified leech neurones in culture. *J. exp. Biol.* **132**, 191–206.

COHAN, C. S., ERRICK, J. E. AND XIA, M.-H. (1990). Formation of electrical connections between cultured identified neurons and muscle fibers of the snail *Helisoma*. *Devl Brain. Res.* **56**, 87–97.

COHAN, C. S., HAYDON, P. G., MERCIER, A. J. AND KATER, S. B. (1987). Formation, maintenance and functional uncoupling of connections between identified *Helisoma* neurons *in situ*. *J. Neurobiol.* **18**, 329–341.

COTTRELL, G. A. (1977). Identified amine-containing neurones and their synaptic connexions. *Neuroscience* **2**, 1–18.

COTTRELL, G. A., ABERNATHY, K. B. AND BARRAND, M. A. (1979). Large amine-containing neurons in the central ganglia of *Lymnaea stagnalis*. *Neuroscience* **4**, 685–689.

CROLL, R. P. (1987). Identified neurons and cellular homologues. In *Nervous Systems in Invertebrates* (ed. M. A. Ali), pp. 41–58. New York: Plenum Press.

CULVER, T., SYED, N. I., RIDGWAY, R. L., BULLOCH, A. G. M. AND LUKOWIAK, K. (1992). Neuronal mapping studies on the central nervous system of the pulmonate snail *Helisoma trivolvis*. *Acta Biol. Hung.* (in press).

DORSETT, D. A. (1986). Brain to cells: The neuroanatomy of selected gastropod species. In *The Mollusca,* vol. 9, *Neurobiology and Behavior,* part 2 (ed. A. O. D. Willows), pp. 107–187. New York, London: Academic Press.

FUCHS, P. A., NICHOLLS, J. G. AND READY, D. F.(1981). Membrane properties and selective connexions of identified leech neurones in culture. *J. Physiol., Lond.* **316**, 203–223.

GETTING, P. (1989). Emerging properties governing the operation of neural networks. *A. Rev. Neurosci.* **12**, 185–204.

GILOH, M. AND SEDAT, J. W. (1982). Fluorescence microscopy: Reduced photobleaching of rhodamine and fluorescein protein conjugates by *n*-propyl gallate. *Science* **217**, 1252–1253.

GRANZOW, B. AND KATER, S. B. (1977). Identified higher order neurons controlling the feeding motor program of *Helisoma*. *Neuroscience* **2**, 1049–1063.

HARRIS-WARRICK, R. M. AND MARDER, E. (1991). Modulation of neural networks for behavior. *A. Rev. Neurosci.* **14**, 39–57.

HAYDON, P. G. (1988). The formation of chemical synapses between cell-cultured neuronal somata. *J. Neurosci.* **8**, 1032–1038.

HAYDON, P. G., MCCOBB, D. P. AND KATER, S. B. (1984). Serotonin selectively inhibits growth cone motility and synaptogenesis of specific identified neurons. *Science* **226**, 561–564.

JACKLET, J. W. (1989). (ed.) *Neuronal and Cellular Oscillators*. New York, Basel: M. Dekker Inc.

JANSE, C., VAN DER WILT, C. J., VAN DER PLAS, J. AND VAN DER ROEST, M. (1985). Central and peripheral neurones involved in oxygen perception in the pulmonate snail *Lymnaea stagnalis* (Mollusca, Gastropoda). *Comp. Biochem. Physiol.* **82**A, 459–467.

JONES, P. G., ROSSER, S. J. AND BULLOCH, A. G. M. (1987). Glutamate suppression of feeding and the underlying output of effector neurons in *Helisoma*. *Brain Res.* **437**, 56–68.

KANDEL, E. R. (1976). *Cellular Basis of Behaviour*. San Francisco: W. H. Freeman and Co.

KANEKO, C. R. S., MERICKEL, M. AND KATER, S. B.(1978). Centrally programmed feeding in *Helisoma*: Identification and characteristics of an electrically coupled pre-motor neuron network. *Brain Res.* **146**, 1–21.

KATER, S. B.(1974). Feeding in *Helisoma trivolvis*: the morphological and physiological bases of a fixed action pattern. *Am. Zool.* **14**, 1017–1036.

KLEINFELD, D., PARSONS, T. D., RACCUIA-BEHLING, F., SALZBERG, B. M. AND OBAID, A. L. (1990*a*). Foreign connections are formed *in vitro* by *Aplysia* interneurone L10 and its *in vivo* followers and non-followers. *J. exp. Biol.* **154**, 237–255.

KLEINFELD, D., RACCUIA-BEHLING, F. AND CHIEL, H. J. (1990*b*). Circuits constructed from identified *Aplysia* neurons exhibit multiple patterns of persistent activity. *Biophys. J.* **57**, 697–715.

KORN, H. AND FABER, D. S. (1979). Electrical interactions between vertebrate neurons: Field effects and electrotonic coupling. In *The Neurosciences, Fourth Study Program*, (ed. F. O. Schmitt and F. G. Worden), pp. 333–358. Cambridge, Massachusetts: MIT Press.

KYRIAKIDES, M., MCCROHAN, C. R., SLADE, C. T., SYED, N. I. AND WINLOW, W.(1989). The morphology and electrophysiology of the neurones of the pedal ganglia of *Lymnaea stagnalis* (L.) *Comp. Biochem. Physiol.* **93**A, 861–876.

LIN, S. S. AND LEVITAN, I. B. (1987). Concanavalin A alters synaptic specificity between cultured *Aplysia* neurons. *Science* **237**, 648–650.

MCCAMAN, M. W., ONO, J. K. AND MCCAMAN, R. E. (1979). Dopamine measurements in molluscan ganglia and neurons using a new, sensitive assay. *J. Neurochem.* **32**, 1111–1113.

MCCOBB, D. P., HAYDON, P. G. AND KATER, S. B. (1988). Dopamine and serotonin regulation of neurite outgrowth of different identified neurons. *J. Neurosci. Res.* **19**, 19–26.

MCKENNEY, K., SYED, N. I. AND BULLOCH, A. G. M. (1990). The search for a classical transmitter in a peptidergic interneuron. *Soc. Neurosci. Abstr.* **16**, 549.

MERICKEL, M. B., EYMAN, E. D. AND KATER, S. B. (1977). Analysis of a network of electrically coupled neurons producing rhythmic activity in the snail *Helisoma trivolvis. IEEE Trans. Biomed. Eng.* **BME-24**, 277–287.

MURPHY, A. D., LUKOWIAK, K. AND STELL, W. K. (1985). Peptidergic modulation of patterned motor activity in identified neurons of *Helisoma*. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7140–7144.

NICHOLLS, J. G., LIU, Y., PAYTON, B. W. AND KUFFLER, D. P. (1990). The specificity of synapse formation by identified leech neurones in culture. *J. exp. Biol.* **153**, 141–154.

O'DONOHUE, T. L., BISHOP, J. F., CHRONWALL, B. M., GROOME, J. AND WATSON III, W. H. (1984). Characterization and distribution of FMRFamide immunoreactivity in the rat central nervous system. *Peptides* **5**, 563–568.

POWELL, B. AND COTTRELL, G. A. (1974). Dopamine in an identified neurone of *Planorbis corneus*. *J. Neurochem.* **22**, 605–606.

QUINLAN, E. M. AND MURPHY, A. D. (1991). Glutamate as a putative neurotransmitter in the buccal central pattern generator of *Helisoma trivolvis. J. Neurophysiol.* **66**, 1264–1271.

RAYPORT, S. G. AND SCHACHER, S. (1986). Synaptic plasticity *in vitro*: Cell culture of identified *Aplysia* neurons mediating short-term habituation and sensitization. *J. Neurosci.* **6**, 759–763.

RICHMOND, J. E., FUNTE, L. R., SMITH, W. L., PRICE, D. A. AND HAYDON, P. G. (1991). Activation of a peptidergic synapse locally modulates postsynaptic calcium influx. *J. exp. Biol.* **161**, 257–271.

RIDGWAY, R. L., SYED, N. I., BULLOCH, A. G. M. AND LUKOWIAK, K. (1991). Nerve Growth Factor (NGF) induces sprouting by specific neurons of the snail, *Lymnaea stagnalis*. *J. Neurobiol.* **22**, 377–390.

ROSS, W. N., ARECHIGA, H. AND NICHOLLS, J. G. (1988). Influence of substrate on the distribution of calcium channels in identified leech neurons in culture. *Proc. natn. Acad. Sci. U.S.A.* **85**, 4075–4078.

SCHACHER, S. (1988). Identified *Aplysia* neurons maintained in dissociated cell culture: Regeneration,

synapse formation and synaptic plasticity. In *Cell Culture Approaches to Invertebrate Neuroscience* (ed. D. J. Beadle, G. Lees and S. B. Kater), pp. 53–84. New York: Academic Press.

SCHACHER, S., GLANZMAN, D., BARZILAI, A., DASH, P., GRANT, S. G. N., KELLER, F., MAYFORD, M. AND KANDEL, E. R. (1990). Long term facilitation in *Aplysia*: persistant phosphorylation and structural changes. *Cold Spring Harbor Symp. quant. Biol.* **LV**, 187–202.

SCHACHER, S. AND PROSHANSKY, E. (1983). Neurite regeneration by *Aplysia* neurons in dissociated cell culture: Modulation by *Aplysia* hemolymph and the presence of the initial axonal segment. *J. Neurosci.* **3**, 2403–2413.

SCHACHER, S., RAYPORT, S. G. AND AMBRON, R. T. (1985). Giant *Aplysia* neuron R2 reliably forms strong chemical connections *in vitro*. *J. Neurosci.* **5**, 2851–2856.

SONETTI, D., BIONDI, C., FERRETTI, M. E., PORTOLAN, A. AND FASOLO, A.(1988). Neuromediators in the CNS of the freshwater snail *Planorbis corneus*: Localization and effects on the adenylate cyclase system of specific ganglia. *Symp. Biol. Hung.* **36**, 309–327.

SONETTI, D., RASSU, M. A. AND LOMBARDO, F.(1982). Mapping of neurons by retrograde cobalt filling of the tentacular nerves of *Planorbis corneus*. *Comp. Biochem. Physiol.* **73**A, 47–56.

SYED, N. I. (1988). Neural control of locomotion in *Lymnaea*. PhD thesis, University of Leeds, Leeds, UK.

SYED, N. I., BULLOCH, A. G. M. AND LUKOWIAK, K. L. (1990). *In vitro* reconstruction of the respiratory central pattern generator (CPG) of the mollusc *Lymnaea*. *Science* **250**, 282–285.

SYED, N. I., HARRISON, D. AND WINLOW, W. (1991). Respiratory behavior in the pond snail *Lymnaea stagnalis*. I. Behavioral analysis and the identification of motor neurons. *J. comp. Physiol.* **169**, 541–555.

SYED, N. I., LUKOWIAK, K. AND BULLOCH, A. G. M. (1992). Specific *in vitro* cynaptogenesis between identified *Lymnaea* and *Helisoma* neurons. *Neuro Report* **3**, 793–796.

SYED, N. I. AND WINLOW, W. (1989). Morphology and electrophysiology of neurons innervating the ciliated locomotor epithelium of *Lymnaea stagnalis* (L.). *Comp. Biochem. Physiol.* **93**A, 633–644.

SYED, N. I. AND WINLOW, W. (1991*a*). Respiratory behavior in the pond snail *Lymnaea stagnalis*. II. Neural elements of the central pattern generator (CPG). *J. comp. Physiol.* **169**, 557–568.

SYED, N. I. AND WINLOW, W. (1991*b*). Coordination of locomotor and cardiorespiratory networks of *Lymnaea stagnalis* by a pair of identified interneurones. *J. exp. Biol.* **158**, 37–62.

WINLOW, W. AND BENJAMIN, P. R. (1976). Neuronal mapping in the brain of the pond-snail, *Lymnaea stagnalis* (L.). In *Neurobiology of Invertebrates, Gastropoda Brain* (ed. J. Salanki), pp. 41–59. Budapest: Akademiai Kiado.

WINLOW, W., HAYDON, P. G. AND BENJAMIN, P. R. (1981). Multiple postsynaptic actions of the giant dopamine-containing neurone R.Pe.D.1 of *Lymnaea stagnalis*. *J. exp. Biol.* **94**, 137–148.