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INTERNEURONAL NETWORK ACTING ON SNAIL NEUROSECRETORY NEURONES (YELLOW CELLS AND YELLOW-GREEN CELLS OF *LYMNAEA*)

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SUMMARY

Neurosecretory cells in *Lymnaea* are the follower cells of a network of interneurones consisting of four different cell types, three of which are inhibitory and one excitatory. One identified interneurone, the visceral white interneurone (VWI), inhibits the Yellow Cells and Yellow-green Cells, or converts Yellow Cells from single spiking to doubletting or bursting. A second interneurone, RPeD1, has weak inhibitory effects on both types of neurosecretory cells but its main effect is to inhibit the VWI and allow the Yellow Cells to be strongly influenced by spontaneous EPSP inputs (input 3).

INTRODUCTION

Networks of interneurones that drive or modulate the activity of follower cells are known for several invertebrate systems. The follower cells are often motoneurones involved in simple behavioural acts such as swimming (e.g. Getting, 1981) or feeding (e.g. Benjamin & Rose, 1980). There are only a few examples where neurosecretory neurones are the follower cells and the best known is probably R15 of *Aplysia* which is excited by interneurone L10 (Kandel, Frazier, Waziri & Coggeshall, 1967). Here a network of interneurones will be described which influence the electrical activity of two types of neurosecretory cells in the brain of the pond snail, *Lymnaea stagnalis*. These are the Yellow Cells and Yellow-green Cells whose name derives from their staining reactions to Alcian Blue-Alcian Yellow in histological studies (Wendelaar Bonga, 1970). They have been identified in electrophysiological experiments and their basic electrical (Benjamin & Swindale, 1975; Benjamin, 1978) and morphological (Swindale & Benjamin, 1976; Benjamin, Slade & Soffe, 1980) features established. There is good evidence that the Yellow Cells play a role in ion and water regulation (Wendelaar Bonga, 1972; Soffe, Benjamin & Slade, 1978).

The opportunity to investigate the interneuronal control of Yellow Cell and Yellow-green Cell activity arose because of the discovery of two different identified interneurones which have postsynaptic effects on these two cell types. One of these is the previously identified giant dopamine-containing interneurone, RPeD1 (Benjamin &

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Winlow, 1981; Winlow, Haydon & Benjamin, 1981) and the other, a newly found cell, the visceral white interneurone (VWI). The discovery of the VWI is particularly significant because its strong inhibitory effects are widespread and influence the spike activity of the neurosecretory cells in all five ganglia where they occur. RPeD1 has weaker inhibitory connections with follower cells and synaptic effects can only be recorded in part of the Yellow Cell and Yellow-green Cell population. The interneuronal control is made more complicated by the presence of mutually inhibitory connections between the interneurones themselves and by the fact that the Yellow Cells receive two further synaptic inputs from unidentified interneurones, one of which can be evoked by activation of RPeD1.

MATERIALS AND METHODS

Standard intracellular recording techniques used in previous studies (Benjamin & Rose, 1979; Benjamin & Winlow, 1981) were used to record from up to four neurones at the same time in isolated *Lymnaea* brains, maintained in HEPES buffered saline (McCrohan & Benjamin, 1980). Current could be passed through the recording electrodes but often a second electrode was inserted for current injection which allowed more accurate measurement of soma membrane potential. Membrane potentials of neurosecretory cells were displayed on a storage oscilloscope or digital voltage meter and current injection monitored on a current to voltage convertor placed between the preparation and ground.

Identification of cells

The location of the Yellow Cells and Yellow-green Cells has been previously described (Swindale & Benjamin, 1976; Benjamin *et al.* 1980). About 30 cells of each type occur in the central ganglia of *Lymnaea* (Soffe *et al.* 1978) usually in mixed clusters close to nerve roots and connectives of the central ganglia but in one location the Yellow Cells occur on their own (Swindale & Benjamin, 1976). This is the 'visceral Yellow Cell cluster' (Benjamin *et al.* 1980) which consists of up to 10 cells located mainly on the dorsal surface of the visceral ganglion close to the connective between the visceral and right parietal ganglion.

Yellow Cells and Yellow-green Cells outside the visceral cluster were identified by dye marking and subsequent staining with neurosecretory stains as described previously (Benjamin & Swindale, 1975). Forty-two cells were identified either as Yellow Cells or Yellow-green Cells in this way and subsequently the cells were shown to have distinctive electrophysiological features often enabling the separation between Yellow Cells and Yellow-green Cells in later experiments to be made on electrophysiological criteria alone. Some Yellow Cells show doublet, triplet or bursting patterns of activity but many show only single spikes even after manipulation of membrane potential (Benjamin, 1978). Mostly the latter type of cell was recorded in the present experiments but examples of doubletting and bursting Yellow Cells are shown in Figs 2C,D and 9C. Yellow-green Cells were smaller in size and never showed doublets or triplets (e.g. Fig. 2A) although up to a quarter fired in long duration bursts lasting from 30–40 s. Certain identification of Yellow Cells could be made in the cas

of the visceral Yellow Cell cluster and this part of the Yellow Cell population was used most commonly in the present experiments. Overall more than 300 Yellow Cells and Yellow-green Cells were recorded.

The central nervous system of *Lymnaea* was pinned onto Sylgard with the dorsal side uppermost, allowing simultaneous recording from neurosecretory neurones and the two interneurones. One of these, the giant interneurone, RPeD1, was located on the dorsal surface of the right pedal ganglion and its unique size (100–180 μm diameter) and location allowed it to be identified with absolute certainty in every preparation (see Benjamin & Winlow, 1981). The other was the much smaller VWI (40–60 μm diameter) whose cell body occurred on the dorsal surface of the visceral ganglion usually to the right of the connective joining the visceral ganglion to the left parietal ganglion. Its brilliant white colour usually allowed immediate identification but in preparations where the colour of the cell was less distinct, the identity of the cell could be confirmed quickly by recording it with certain easily identified cells with which it always connects such as the B gp and F gp cells (see Benjamin & Winlow, 1981, for identification of these cell groups) and testing for the excitatory response evoked by a burst of spikes in the VWI (Benjamin, Elliott & Ferguson, 1984). Only rarely was it difficult to find the VWI and certain identification occurred in more than 90% of preparations.

RESULTS

The proposed interneuronal circuit

The network of interneurones controlling the Yellow Cells and Yellow-green Cells is complex and as an introduction its main features will be described here (see also Fig. 11A) as a preliminary to the subsequent presentation of detailed evidence.

The network consists of two identified interneurones RPeD1 and the VWI and two other types of interneurones, so far unidentified, whose effects can be recorded as postsynaptic potentials on other cells in the circuit. These unidentified interneurones produce the so-called input 3 (previously shown to occur on many other cells in the *Lymnaea* brain, Benjamin & Winlow, 1981) and the less widely distributed 'spontaneous inhibitory input'.

Both RPeD1 and type VWI have biphasic (*e–i*, EPSP followed by IPSP) effects on the Yellow Cells and Yellow-green Cells and on each other. In all cases the biphasic potentials have a predominantly inhibitory effect on spike activity. Both interneurones also appear to inhibit input 3 interneurones although this could only be shown indirectly (dashed lines in Fig. 11A). Input 3 strongly excites the Yellow Cells and RPeD1 and inhibits the VWI. The spontaneous inhibitory input was only found in Yellow Cells but its periodic occurrence strongly modulates spike activity.

An important point is that there are no connections from the neurosecretory neurones back onto the interneurones.

It is clear from this description that there are a number of different possible interactions between the four different types of interneurones and the way they control neurosecretory cell activity. These will be considered in detail, following the presentation of data showing the basic connectivity of the system. A functional interpretation

of the role of the various interneuronal types will finally be presented in the Discussion (Fig. 11B).

The follower cells of the VWI

The VWI was always silent, but injection of depolarizing currents for a few seconds evoked a single burst of spikes (Fig. 3A,B), and continuous injection produced further bursts of spikes (Fig. 1E) or less commonly single spiking (Fig. 2A). A burst of spikes in the VWI caused biphasic postsynaptic potentials (BPSPs) in the Yellow Cells (Fig. 1B), Yellow-green Cells (Fig. 1C) and RPeD1 (Fig. 1A). It also inhibited input 3, one of the spontaneously occurring inputs (Fig. 1D,E). The biphasic response in all three cell types consisted of an initial depolarizing phase followed by a longer duration hyperpolarization lasting several seconds (e = EPSP and i = IPSP in Fig. 1 and all subsequent figures in this paper). It was not always possible to see the depolarizing part of the BPSP (e.g. Fig. 4A,D) even in hyperpolarized follower cells and this was particularly true if VWI was repeatedly activated (as in Fig. 5B). The initial depolarizing phase was never seen to elicit spikes and the overall effect of spike activity in the VWI was to inhibit any spike activity in its follower cells. This was a straightforward effect in the case of RPeD1 (Fig. 1C) and the Yellow-green Cells (Fig. 2A), when spike activity was simply prevented or reduced to a low level, so that maintained activity in the VWI suppressed most spike activity for as long as it was activated (Fig. 2A), but the responses in the Yellow Cells were more complicated and depended on whether the Yellow Cells were capable of doubletting or bursting, and the type of activity shown by the VWI itself.

Fig. 2B shows two Yellow Cells which could not be made to burst or double spike by membrane potential manipulation. Here a single burst of spikes in the VWI initially completely inhibited both cells and this was followed by a gradual return to prestimulus frequency. Continuous depolarization of the VWI, which in this case caused it to burst, resulted in an initial longer period of complete inhibition and then a further period when the Yellow cells fired at a reduced rate for as long as burst activity in the VWI was maintained. In contrast the Yellow Cell in Fig. 2C showed some tendency to doublet. Again stimulation of the VWI produced an initial period of inhibition which was followed by doubletting and a gradual return to tonic firing. Many Yellow Cells responded to VWI activation in this way. A more striking example

Fig. 1. VWI postsynaptic effects on RPeD1 (A,C), a Yellow Cell (YC) (B), a Yellow-green Cell (YGC) (C) and input 3 (D,E). (A) Biphasic postsynaptic potential (BPSP) in RPeD1 caused by a burst of spikes in the VWI. The initial depolarizing phase of the BPSP (e) is followed by a long duration hyperpolarization (i). (B) Two bursts of spikes evoked in the VWI by steady depolarization. The first burst hyperpolarizes the YC and stops spike activity, the second reveals the biphasic waveform of the postsynaptic potential. (C) The VWI also evokes a BPSP in this Yellow-green Cell and inhibits the spike activity of RPeD1. (D) Effects of evoked VWI bursts of spikes on the duration of spontaneous input 3 (double-headed arrows) occurring as an Agp neurone and RPeD1. Two uninterrupted spontaneous occurrences of input 3 occur in the left-hand side of the traces and these excite both Agp and RPeD1. The two subsequent occurrences of input 3 are interrupted by the simultaneous activation of the VWI which reduced its duration significantly. Solid arrows indicate the end of the input 3 EPSP. See text for further interpretation of this record. (E) Two periods of VWI activity prevented the occurrence of input 3, which only occurred (double-headed arrows) in the intervening periods when the VWI was silent. Note also the hyperpolarizations on RPeD1 caused by bursts of spikes in the VWI.

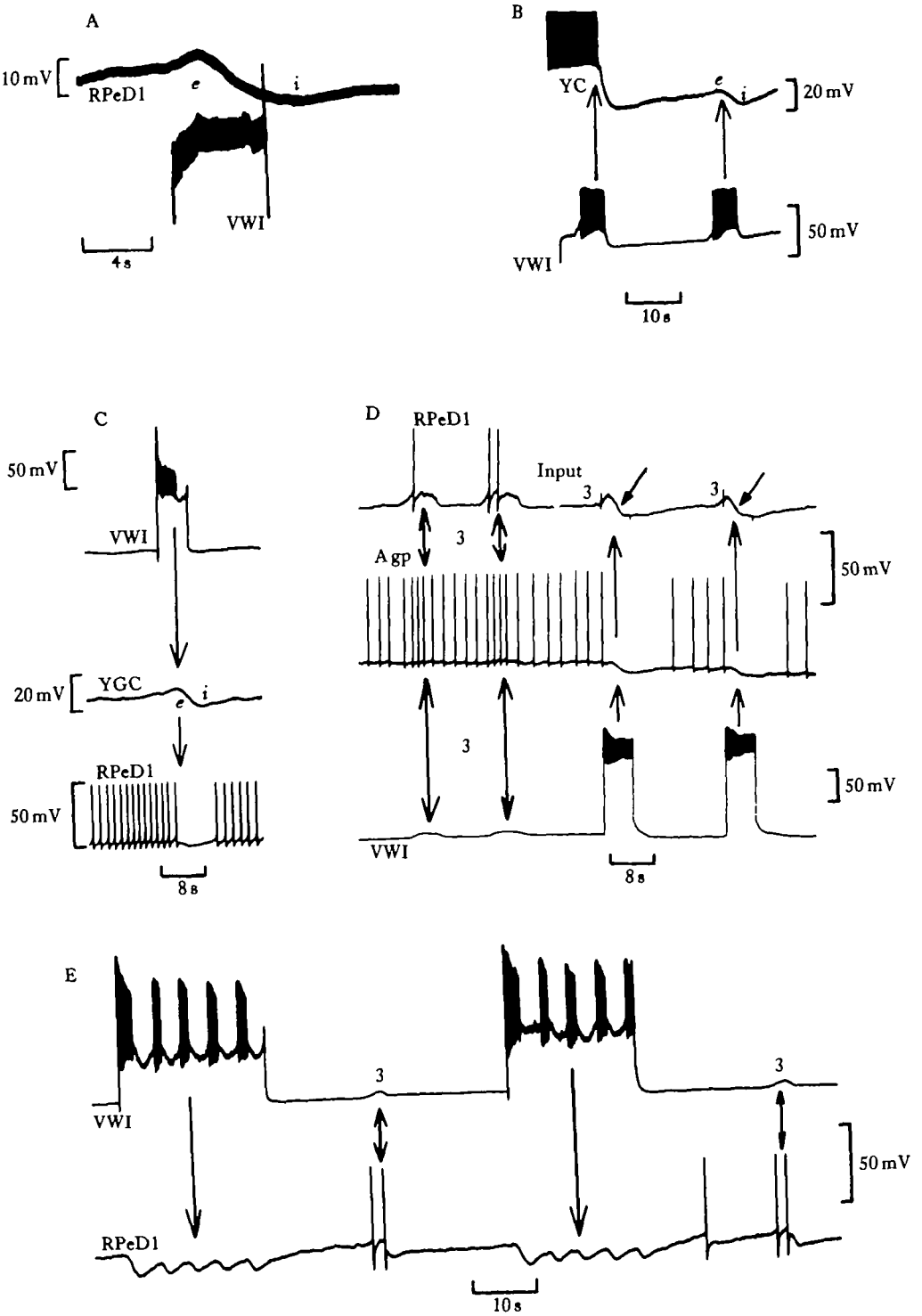
A
10 mV
RPeD1
e
i
VWI
4 s
B
YC
e
i
20 mV
50 mV
VWI
10 s
C
50 mV
VWI
20 mV
YGC
e
i
RPeD1
50 mV
8 s
D
RPeD1
Input
3
3
A gp
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Fig. 1

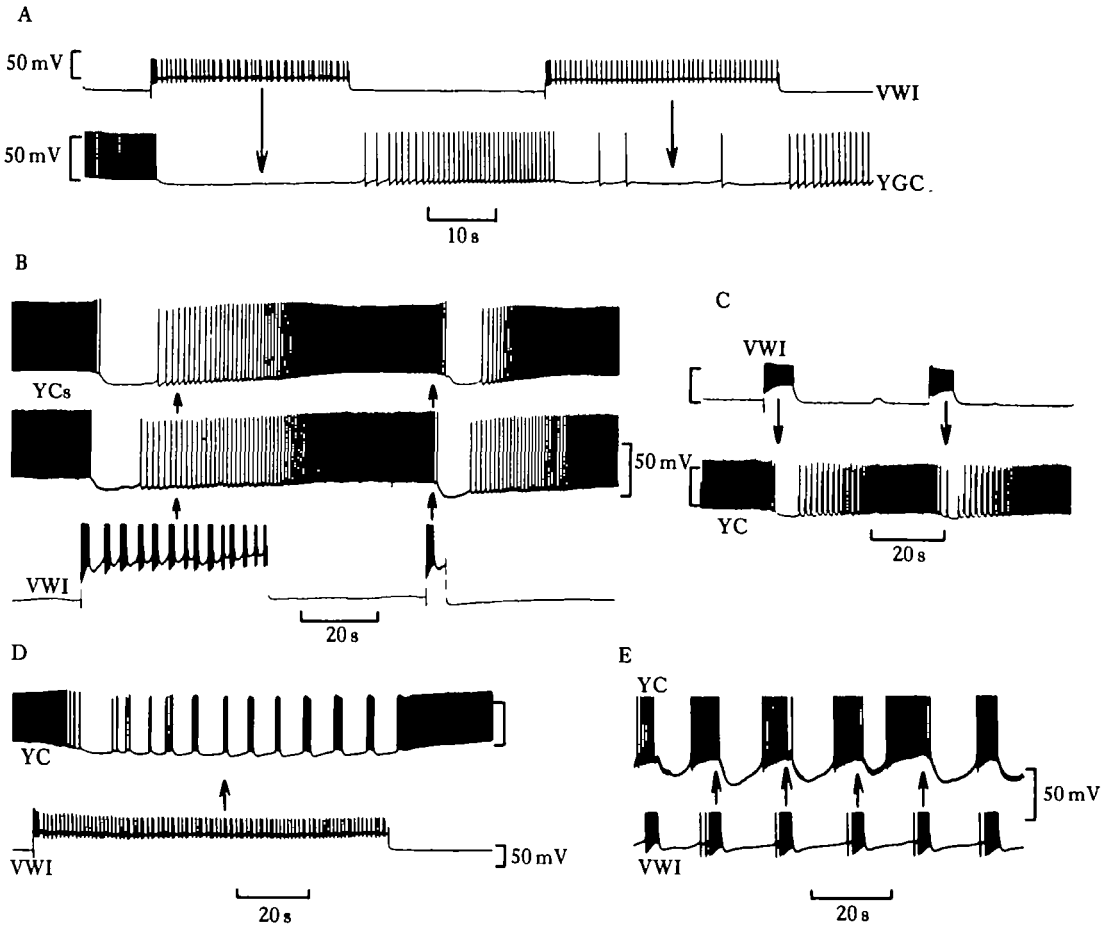


Fig. 2. Effects of the VWI on Yellow Cell and Yellow-green Cell firing activity. (A) Two prolonged periods of VWI spiking inhibit spike activity in the Yellow-green Cell. No Yellow-green Cell (YGC) spikes occurred in the first period of VWI activity but a slightly lower frequency of firing in the VWI during the second period was insufficient completely to suppress YGC spiking. (B) Two single spiking Yellow Cells were completely inhibited initially by VWI bursts but eventually recovered to pre-stimulus firing rates. Prolonged activation of the VWI maintained a lower rate of Yellow Cell firing following initial complete suppression of activity. (C) Doubletting activity in a Yellow Cell occurring in the recovery phase of hyperpolarizing responses caused by bursts of spikes in the VWI. (D) Conversion of a Yellow Cell to bursting by maintained activity in the VWI. (E) Phasic inhibition of a Yellow Cell by the VWI leads to a bursting pattern of activity in the neurosecretory cell.

of spike pattern conversion is shown in Fig. 2D, where burst could be induced in a Yellow Cell by tonic firing of the VWI. This was the same effect produced by application of hyperpolarizing current on high frequency single spiking cells shown previously (Benjamin, 1978, Fig. 2) but here it was produced by interneuronal action. This ability of the VWI to produce bursting in the Yellow Cell was presumably due to the intrinsic tendency of the cell to burst at the appropriate level of underlying membrane potential. However, it is possible to induce burst activity in Yellow Cells showing no spontaneous bursting by phasic inhibition caused by bursts of activity in the VWI (e.g. Fig. 2E). The VWI had to be bursting at a low rate (about once every 20 s in Fig.

2E) to allow the Yellow Cell's membrane potential to recover and reach threshold for firing before the next period of inhibition.

The evidence that the VWI can inhibit input 3 was indirect because the interneurones responsible for input 3 (see below) were unidentified. The inhibition was indicated by two types of evidence shown in Fig. 1D,E. Activation of the VWI during the spontaneous occurrence of input 3 in RPeD1 and an A gp cell (Fig. 1D) reduced the duration of the compound EPSP indicating that input 3 interneurones were being inhibited, but this evidence is equivocal because VWI also directly inhibits both RPeD1 and the A gp cell and this obscured the depolarizing effect of input 3. However, the occurrence of unitary EPSPs within the complex PSP waveform did cease after about 3 s (arrows) which is about half the duration of the two uninterrupted input 3 EPSPs shown on the left-hand side of the traces in Fig. 1D, so inhibition of input 3 interneurones still seems likely. Further evidence for input 3 inhibition by the VWI is given in Fig. 1E. Here input 3 on both the VWI and RPeD1 only occurred in the intervals when VWI was silent and never during VWI activity (see also Fig. 9 for similar records). The significance of this inhibitory effect of the VWI on input 3 will become clear later. Here it is sufficient to say that input 3 has a powerful excitatory effect on the Yellow Cells and so any activity in the VWI will have the double effect of directly inhibiting the neurosecretory cells but also preventing the strong excitation produced by input 3.

The follower cells of RPeD1

RPeD1 was sometimes silent but usually it fired irregularly at frequencies of below 1 spike s^{-1} except during periodic excitation by input 3 (e.g. Fig. 6B), when it fired for a few seconds at higher frequencies. Even during accelerated firing due to spontaneous excitatory inputs, little effect on the firing activity of neurosecretory neurones was seen. However, injecting several nanoamperes of current *via* a second electrode caused a high frequency burst of spikes in RPeD1 and this inhibited spike activity in both Yellow Cells and Yellow-green Cells (Fig. 4A,B) as well as having a strong inhibitory effect on the VWI (Fig. 9C). This inhibitory response on the neurosecretory cells was caused by the second hyperpolarizing component of a biphasic potential, both components of which were seen when the membrane potential of the neurosecretory cells was set at about −60 mV (Fig. 3A,B). The BPSP consisted of an initial depolarizing wave followed by a longer lasting hyperpolarizing component. A similar BPSP occurred in the VWI following RPeD1 activation (Fig. 3C), but the effect was much stronger so that at firing level the hyperpolarizing phase was large enough (25–30 mV) to prevent spiking even in strongly activated cells (Fig. 9A). Thus a burst of spikes in RPeD1 inevitably inhibited spike activity in VWI but not always in the neurosecretory cells. As well as inhibiting neurosecretory cells, RPeD1, like the VWI, inhibits the interneurones producing input 3 (Winlow *et al.* 1981), but in addition strong activation of RPeD1 also evokes the delayed occurrence of input 3 probably by post-inhibitory rebound (to be considered later, see Fig. 6C).

A comparison of the synaptic effects of VWI and RPeD1 on neurosecretory cells

The effect of an evoked burst of spikes in VWI upon synaptic activity in a Yellow Cell or Yellow-green Cell was compared with the effect of similar activity in RPeD1

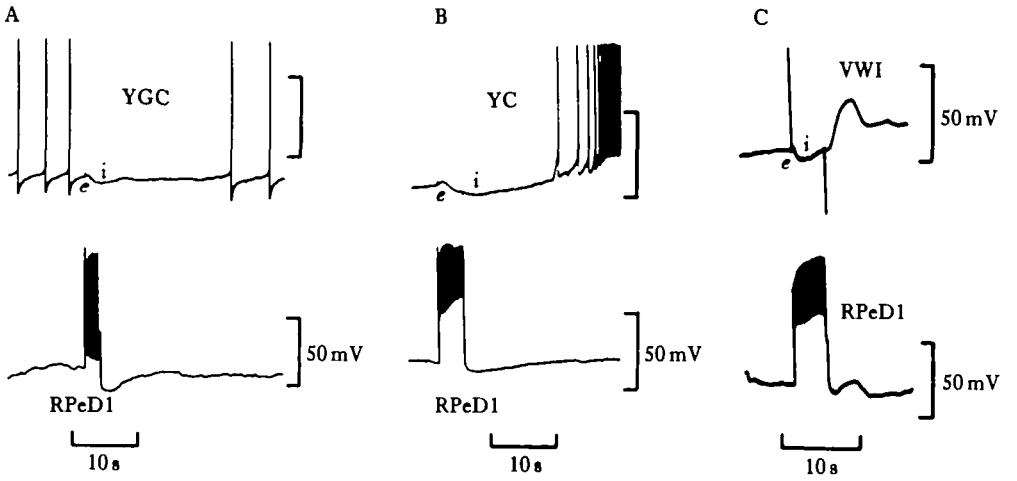


Fig. 3. Postsynaptic responses of (A) a Yellow-green Cell (YGC), (B) a Yellow Cell (YC) (B) and (C) the VWI caused by bursts of spikes in RPeD1. All these responses are biphasic (*e* followed by *i*).

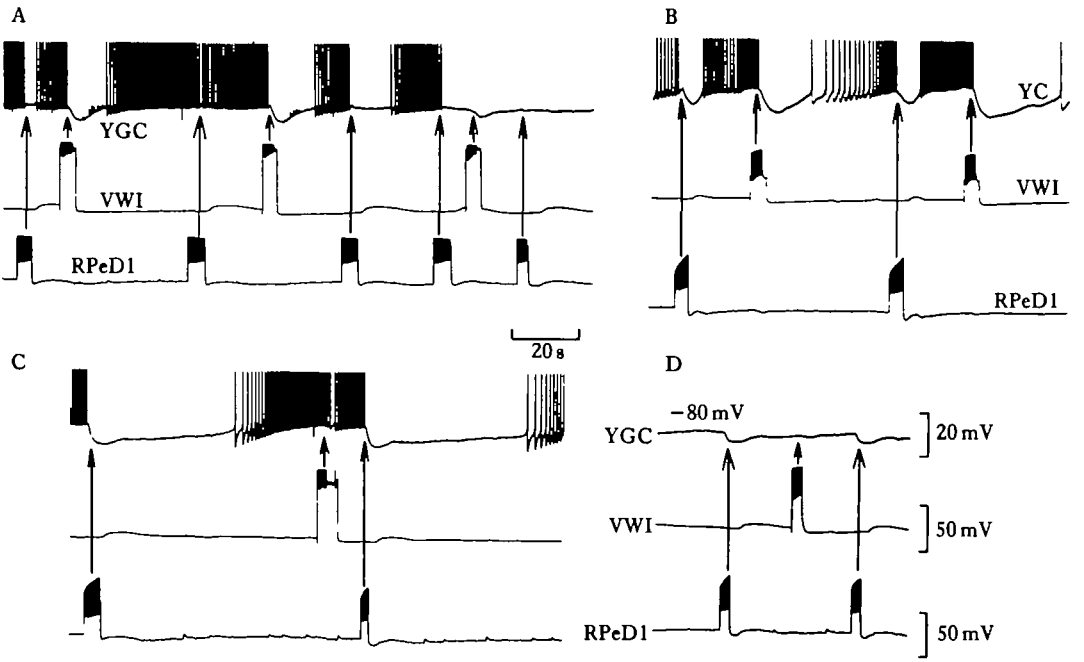


Fig. 4. Comparison of the postsynaptic effects of RPeD1 and the VWI on Yellow Cells (YC) and Yellow-green Cells (YGC). The amplitude and duration of the hyperpolarizing response is usually much greater for the VWI response compared with that for RPeD1 for both Yellow-green Cells (A) and Yellow Cells (B). The single exception to this is shown in (C) and (D) (same cells in both) where the strength of the responses on a Yellow-green Cell was opposite to that in (A).

upon the same neurosecretory cell. In more than 50 neurosecretory cells, the VWI induced a hyperpolarizing response in the neurosecretory cell which was larger in amplitude and longer in duration than that produced by RPeD1 (e.g. Fig. 4A,B). If the cell was first inhibited by RPeD1 and then VWI activation followed rapidly, ther

the cell was further hyperpolarized (Fig. 4A, right side of YGC trace). In one cell (a probable Yellow-green Cell), the strength of the two interneurones' effects was reversed (Fig. 4C,D). Thus in Fig. 4D, RPeD1 still caused a strong hyperpolarizing response in the neurosecretory cell at −80 mV whereas alternate activation of VWI produced almost no change in membrane potential.

The two types of interneurone could produce different amplitudes of hyperpolarization if they were affecting different ionic conductance mechanisms and this

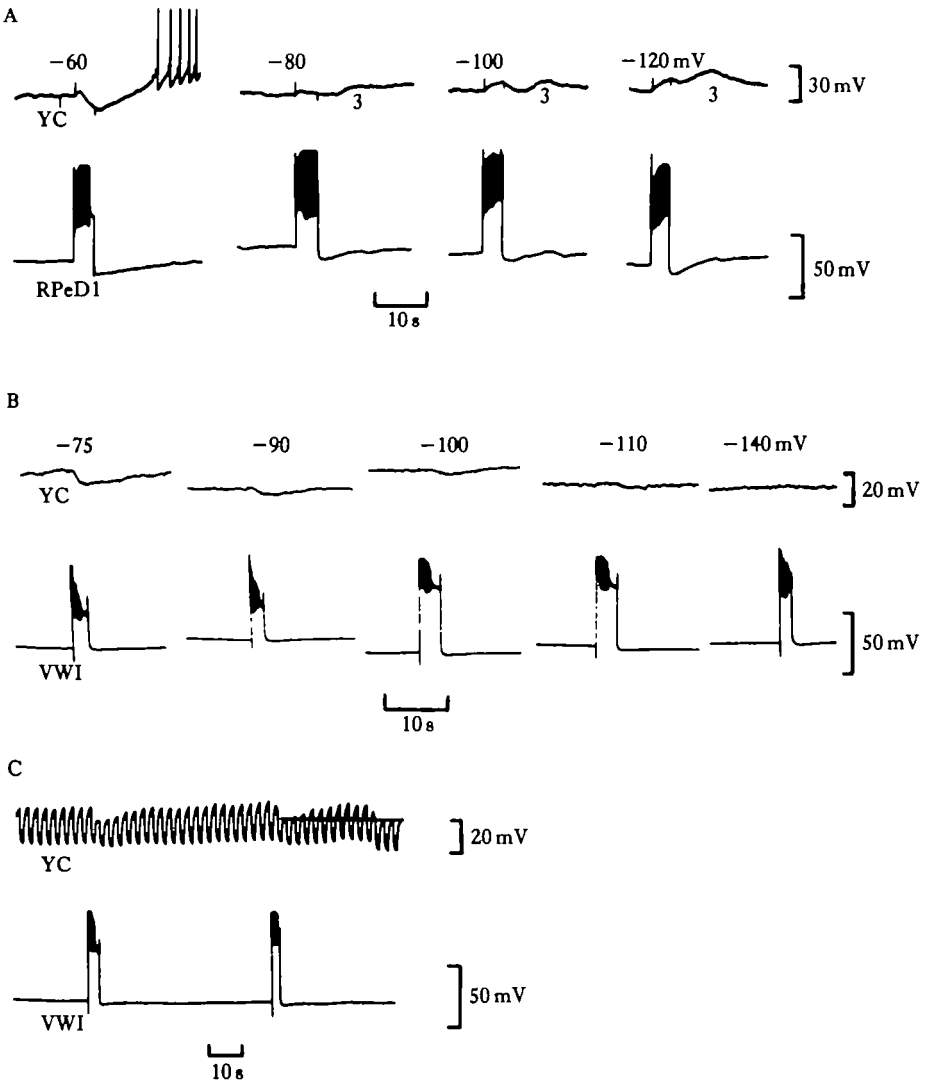


Fig. 5. Effect of membrane potential on the postsynaptic responses of Yellow Cells to activation of (A) RPeD1 and (B) the VWI. (C) Conductance increase during postsynaptic response in a Yellow Cell (YC) caused by the VWI. Constant current pulses applied to the Yellow Cell *via* a second microelectrode. To test that the increase in conductance during the hyperpolarizing phase of BPSP was not due to the rectification properties of the postsynaptic membrane, the membrane potential of the Yellow Cell was artificially changed to the maximum hyperpolarization occurring during the PSP (horizontal line). The amplitude of the hyperpolarizing potential due to square current pulses was still lower during the PSP.

is supported by different reversal potentials for the postsynaptic responses (Fig. 5). Fig. 5A shows that the hyperpolarizing component of RPeD1 induced BPSP reversed to a depolarizing potential at a more negative membrane potential than −80 mV, so that between −120 mV and −100 mV the PSP had an overall depolarizing waveform whereas at −60 mV it had an initial depolarizing phase followed by a second hyperpolarizing one. Strong activation of RPeD1 also resulted in a delayed activation of input 3 (see next section) so that in Fig. 5A all the postsynaptic responses were followed by a second depolarizing wave due to input 3. The results of Fig. 5B, when VWI responses at various membrane potentials were measured, contrasts strikingly with those produced by RPeD1. At a membrane potential of −80 mV the BPSP still showed a marked hyperpolarization and this component of the response only disappeared at −120 mV. Further hyperpolarization did not reverse the potential and this was a consistent finding. These records also showed that the initial depolarizing phase of the BPSP response to the VWI was labile and tended to disappear with repeated activation of the interneurone (for instance as the sequence of repeated activation of the VWI from −75 to −100 mV was made).

These results can readily explain the difference in the ability of the two interneurones to prevent spike activity in the neurosecretory cells. The VWI consistently and more readily inhibited Yellow Cells and Yellow-green Cells by shifting the membrane potential to a more negative value than RPeD1. The results shown in Fig. 5C indicate that the PSP in response to VWI was caused by a conventional conductance increase mechanism and that this could not be accounted for simply by the rectification properties of the membrane.

The follower cells of input 3

Input 3 was a spontaneously occurring compound synaptic input arising from unidentified interneuronal sources within the brain which was found on many cells in the right parietal and visceral ganglion of *Lymnaea* (Benjamin & Winlow, 1981). The similar waveform of this input on different cells suggested that it was due to the activity of at least two different coupled neurones which had common follower cell connections in the *Lymnaea* CNS. Input 3 was the only excitatory synaptic input recorded in RPeD1 (Benjamin & Winlow, 1981) so recording the neurone with other cells allowed input 3 to be identified elsewhere on the basis of simultaneous occurrence and identical waveform (Fig. 6A–D). Alternatively, input 3 could also be evoked by strong activation of RPeD1 (Winlow *et al.* 1981 and Fig. 6C) so that in preparations where it did not occur spontaneously it could be evoked readily.

Experiments of the type shown in Fig. 6 demonstrated that input 3 was present on the Yellow Cells of the visceral ganglia, the VWI and RPeD1. Fig. 6A identifies input 3 on a Yellow Cell by showing its simultaneous occurrence on a hyperpolarized Yellow Cell and RPeD1. At recorded membrane potential it was excitatory and caused an increase of spike activity in RPeD1 (Fig. 6B, bottom trace) or evoked spikes in a silent Yellow Cell (Fig. 6E, top trace). On the VWI, input 3 normally occurred as a depolarizing potential (Fig. 6C). However, depolarizing the VWI to firing level reversed its polarity so that it was actually an IPSP which inhibited firing (Fig. 6D).

Most of the records shown in the present paper were from non-bursty Yellow Cells and input 3 accelerated spike activity or triggered Yellow Cells into activity if they

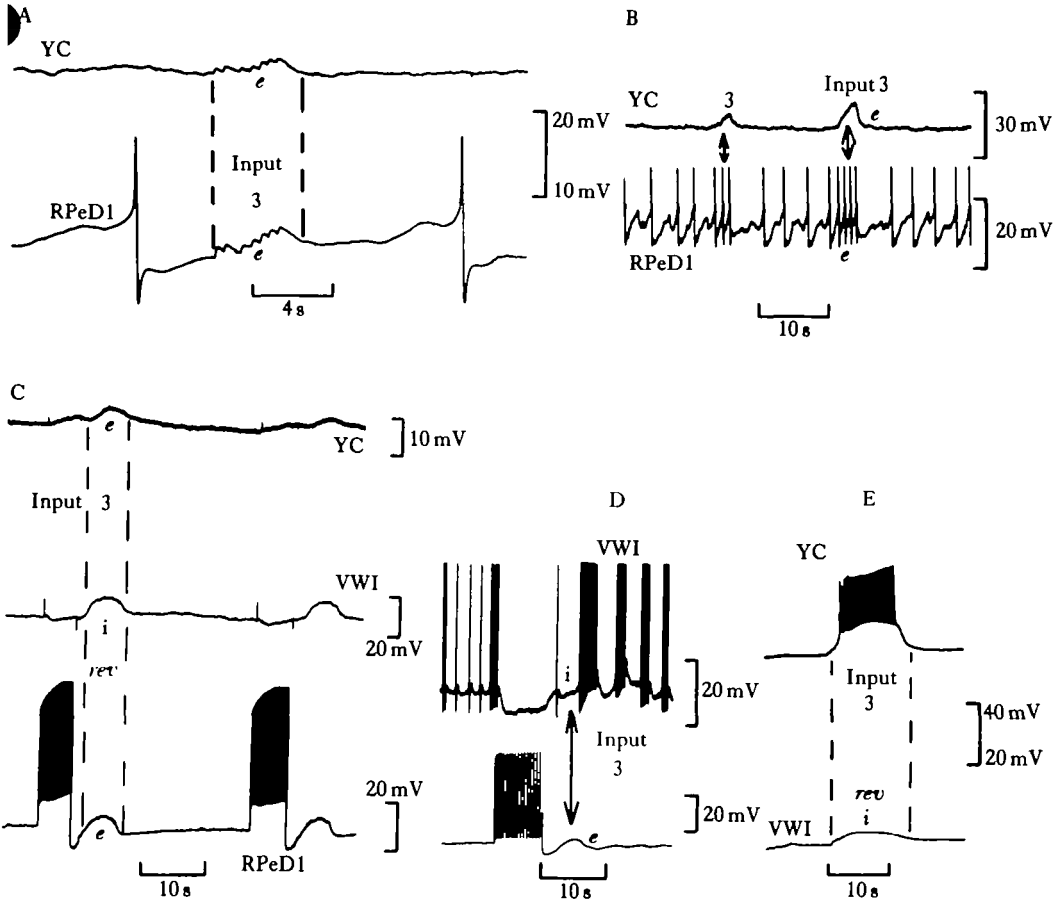


Fig. 6. Occurrence and effects on spike activity of input 3 on Yellow Cells, RPeD1 and the VWI. (A) Input 3 occurs as a compound EPSP (*e*) on a visceral ganglion Yellow Cell (YC) and RPeD1. Both cells are hyperpolarized. (B) At recorded membrane potential input 3 accelerates the firing pattern of RPeD1. The occurrence of input 3 is monitored on the hyperpolarized Yellow Cell (top trace). (C) Delayed occurrences of input 3 on all three cell types following strong bursts of spikes in RPeD1. In the VWI input 3 appears as a depolarizing potential at recorded membrane potential but is in fact a reversed inhibitory postsynaptic potential (*rev*). Note that the Yellow Cell is strongly hyperpolarized so that the direct synaptic effect of RPeD1 is an overall depolarization because the hyperpolarizing phase of the BPSP is reversed (see Fig. 7A). (D) The VWI is depolarized to firing level and input 3 on VWI is clearly seen as a hyperpolarizing potential. (E) In a silent Yellow Cell input 3 evokes a burst of spikes. This spontaneous input 3 also occurs on the VWI.

were in a hyperpolarized state (Figs 6E, 7B, 8). The effects of input 3 on bursty Yellow Cells have been described earlier (Benjamin, 1978) and there it was shown that input 3 could trigger bursts which far outlasted the stimulus and also entrained the burst rhythm under certain circumstances. In silent non-bursty Yellow Cells the increase in spike activity due to input 3 coincided with the duration of the synaptic input (Fig. 6E).

In summary it can be said that input 3 had strong effects on the spike activity on the three cell types where it occurred, excitatory in the case of the Yellow Cells and RPeD1 and inhibitory in the case of the VWI.

Follower cells of the spontaneous inhibitory input

This is the fourth type of synaptic input to be described in the present study. It often occurred periodically at a frequency of every 20–30 s or less (Fig. 7) on Yellow Cells and stopped firing for several seconds (Fig. 7). It occurred on cells of the visceral Yellow Cell cluster (Fig. 7A), on certain other giant neurones of the visceral right parietal ganglia (Soffe, 1979) but was absent on RPeD1 and the VWI (not shown). Neither was it found on the Yellow-green Cells. No obvious linkage has been found between the spontaneous inhibitory input on the Yellow Cells and activity in RPeD1 and the VWI.

Interactions between synaptic effects on neurosecretory neurones

In this section the interactions between the four different types of synaptic effects on neurosecretory cells will be described. The analysis is based mainly on recordings from the Yellow Cells of the 'visceral Yellow Cell cluster', which receive all four of the inputs, but the information of the interactive effects of RPeD1 and the VWI inputs and their own mutually inhibitory connections also applied to those Yellow-green Cells from which recordings were made.

Interactions between RPeD1 inhibition and input 3 excitation

A strong burst of spikes in RPeD1 inhibited the Yellow Cells but this also led to a delayed activation of input 3 which had the opposite (excitatory) effect on the same cell. This resulted in an initial rapid hyperpolarization caused by direct RPeD1 inhibition followed by an equally rapid repolarization and subsequent recovery of spike activity (Fig. 8A). The occurrence of input 3 following RPeD1 activity was

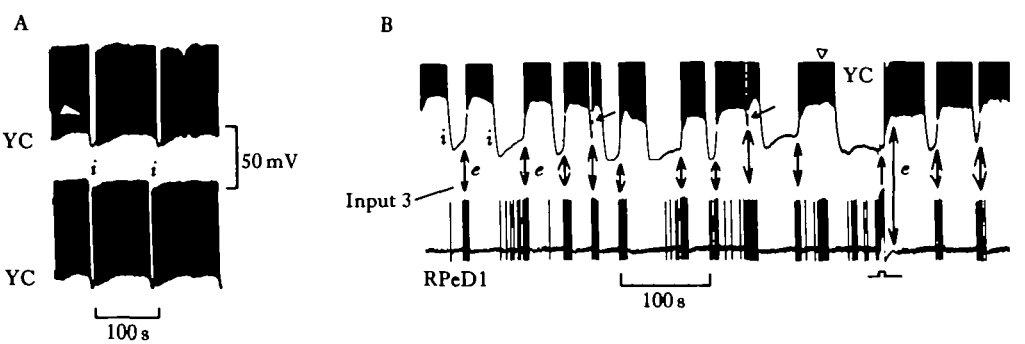
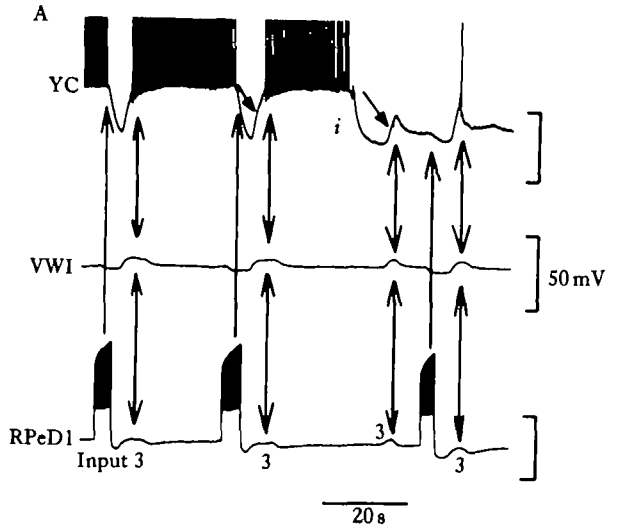


Fig. 7. (A) Synchronous occurrence of the spontaneous inhibitory input on two Yellow Cells (YC) of the visceral Yellow Cell cluster. (B) Long-term recording of RPeD1 and a visceral Yellow Cell cluster.neurone. Spontaneous inhibitory inputs (*i*, only labelled at beginning of records) alternate with input 3 (double-headed arrows and marked as *e*) and this leads to long bursts of spikes in the Yellow Cell and subsequent silent periods. The spontaneous inhibitory input strongly hyperpolarizes the Yellow Cell which remains silent until input 3 triggers the next burst of spikes (monitored as an acceleration of spike activity in RPeD1). Towards the end of the records, after a particularly long interburst interval, input 3 was initiated by a current induced burst of spikes in RPeD1 and this triggered Yellow Cell firing. Single sloping arrows indicate a very short interburst interval when the spontaneous inhibitory input was rapidly followed by input 3. Open arrowhead indicates the only example where there are two consecutive occurrences of input 3.

monitored on the VWI and RPeD1 in Fig. 8A and was also assumed to be occurring on the Yellow Cell. This was seen directly in the later part of the record when the



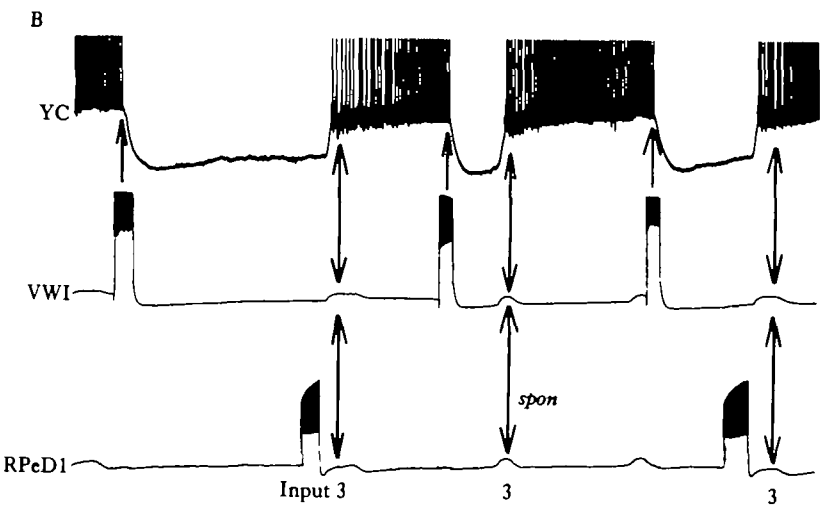


Fig. 8. Interactions between synaptic effects of (A) input 3 and RPeD1 and (B) input 3 and the VWI on Yellow Cells. (A) Bursts of spikes were evoked in RPeD1 by current injection and this caused an inhibition of Yellow Cell (YC) spike activity (single-headed arrow). Input 3 is implicated in the recovery phase of the V-shaped waveform because it is known to occur on Yellow Cells after RPeD1 activation and was monitored at the beginning of the records on the VWI and RPeD1 (double-headed arrows). Later in the record the Yellow Cell was hyperpolarized by the occurrence of the spontaneous inhibitory input (*i*) and then the depolarizing part of the waveform of input 3 could be directly compared with the recovery phase of the Yellow Cell response in the earlier part of the records and is seen to be extremely similar (solid-headed arrows). (B) Single arrows indicate the inhibition of Yellow Cell activity by VWI. Input 3 is evoked by RPeD1 activation or occurred spontaneous (*spon*) indicated in both cases by double-headed arrows. In three examples Yellow Cell activity was inhibited first by VWI and then reinitiated by input 3.

Yellow Cell had been inhibited by the spontaneous inhibitory input (*i*). In the absence of Yellow Cell spike activity the shape of the depolarizing phase of input 3 both its spontaneous and its evoked occurrences was extremely similar to that occurring in the recovery phase of the V-shaped waveform (waveforms indicated by solid-headed arrows in Fig. 8) when the cell was showing normal spike activity, supporting the point that input 3 is mainly responsible for the repolarization phase of the RPeD1-induced inhibition.

Interactions between VWI inhibition and input 3 excitation

The VWI produced more prolonged inhibition of Yellow Cells than RPeD1. A burst of spikes in the VWI lasting a few seconds caused cessation of spiking for 20 s or more (Fig. 8B). Activity in Yellow Cells was reinitiated following VWI inhibition by the spontaneous occurrence of input 3 or following a current injection-induced burst of RPeD1 spikes (Fig. 8B). Several examples are shown in Fig. 8B where a Yellow Cell was first inhibited by the VWI and then subsequently reactivated by input 3 (double-headed arrows).

This experiment emphasizes that the VWI with its powerful inhibitory effect, and input 3, with its equally powerful excitatory effect, are two of the main elements in the circuit controlling spike activity in many Yellow Cells.

Interactions between input 3 excitation and spontaneous inhibitory input

When the VWI was silent, the normal situation in the isolated brain, spike activity in the Yellow Cells of the visceral Yellow Cell cluster was mainly influenced by regular spontaneous occurrences of input 3 and the spontaneous inhibitory input (Fig. 7B). Extra spike activity in RPeD1 due to input 3 caused no obvious inhibition of Yellow Cells (too low frequency) and of much more significance were the excitatory effects of input 3 on the Yellow Cells occurring at the same time. Input 3 and the spontaneous inhibitory input tended to occur in an alternating pattern and this resulted in the type of activity shown in Fig. 7B (the spontaneous occurrence of input 3 in this record caused a burst of spikes in RPeD1 which thus acted as a monitor for input 3 occurrence on both cells). Thus spiking in the Yellow Cell was alternately inhibited and initiated by the occurrence of the spontaneous inhibitory input and input 3 (marked *i* and *e* respectively). Initiation by spontaneous input 3 or induced by RPeD1 activation was equally effective in initiating Yellow Cell spiking (Fig. 7B).

Although input 3 and the spontaneous inhibitory input usually occurred in an alternating pattern (the only exception in Fig. 7B is shown with an open arrowhead) the interval between their occurrences was very variable. If input 3 rapidly followed inhibition (single-headed arrows) a full-sized hyperpolarization was prevented, and spike activity caused in the Yellow Cell for a short time. This alternation between the two spontaneously occurring inputs may indicate some interaction between the interneurones responsible, perhaps of a mutually inhibitory type, but as yet there is no direct evidence for this.

Effects of the mutually inhibitory connections between VWI and RPeD1 on Yellow Cell firing

It was shown earlier that RPeD1 and the VWI had reciprocal inhibitory connection

(Figs 1A, 3C) so that spike activity in one cell prevented activity in the other. Both these cells in turn inhibited the spiking activity of the Yellow Cells, although inhibition by the VWI was much stronger in its effect than RPeD1. This means that when we are considering the relative functions of the two interneurones in controlling the electrical activity of the Yellow Cells, the prevention of activity in the VWI by RPeD1 is of much more significance than inhibition in the opposite direction.

In experiments examining the relative influence of the two interneurones, the inhibitory effects of activation of the VWI were first established by applying steady suprathreshold depolarizing currents. This produced an inhibition or lowering of frequency of single spiking Yellow Cells (Fig. 9A) or, in cells capable of doubletting, a change in the type of firing (in Fig. 9C, from doubletting to 'low frequency' single spiking as defined by Benjamin, 1978). Also most spike activity in RPeD1 was prevented. Intervention of RPeD1 inhibited the VWI despite it being injected with depolarizing current and this in turn released the VWIs' inhibitory effects on the Yellow Cells, returning them to their pattern of firing prior to interneuronal activity. The duration of these effects depended on how long RPeD1 was made to fire (Fig. 9A,B) and even quite low frequency firing of RPeD1 was effective in preventing VWI spiking (Fig. 9A). There were some weak direct inhibitory effects on Yellow Cell firing when RPeD1 spikes were evoked and this was seen as a period of silence in Yellow Cell activity even though the cell was depolarizing due to coincident removal of VWI inhibition (Fig. 9A). One further complication was that activation of RPeD1 also induced delayed input 3 on RPeD1 itself and on the VWI and the Yellow Cells (Fig. 9A–C, double-headed arrows). This had little effect on RPeD1 but strongly inhibited the VWI and excited the Yellow Cells. Thus in Fig. 9A a strong burst of spikes occurred in the Yellow Cells and in Fig. 9C doublets (a sign of increased depolarization) during input 3.

Distribution of synaptic inputs to the Yellow Cells and Yellow-green Cells in relation to interneuronal morphology

It was important to determine the distribution of the four synaptic inputs to the Yellow Cells and Yellow-green Cells and whether the cells occurring in various locations were equivalent in the inputs they received. In a number of experiments where RPeD1 and the VWI were recorded together, a third electrode was used to sample as many neurosecretory cells as possible in order to ascertain the distribution of synaptic inputs. In the CNS from one animal it was possible to record 18 Yellow Cells and Yellow-green Cells (about one-third of the total population in the *Lymnaea* CNS) in all five ganglia where they occur. Results from this experiment confirmed the data from several hundred neurosecretory cells accumulated from the whole study. It showed that the VWI inhibited Yellow Cells and Yellow-green Cells in all five ganglia. Randomly selected cells in left and right pleural ganglia, anterior right parietal, left parietal and visceral ganglia (visceral Yellow Cell cluster cells and others close to the visceral-left parietal connective) were all inhibited by the VWI.

Synaptic responses from RPeD1 and input 3 occurrence were more restricted in distribution than those from VWI. Responses to excitation of RPeD1 only occurred in Yellow Cells and Yellow-green Cells of the visceral ganglion and the two ganglia to the right of this – the right parietal and right pleural ganglia (see Fig. 10 for the

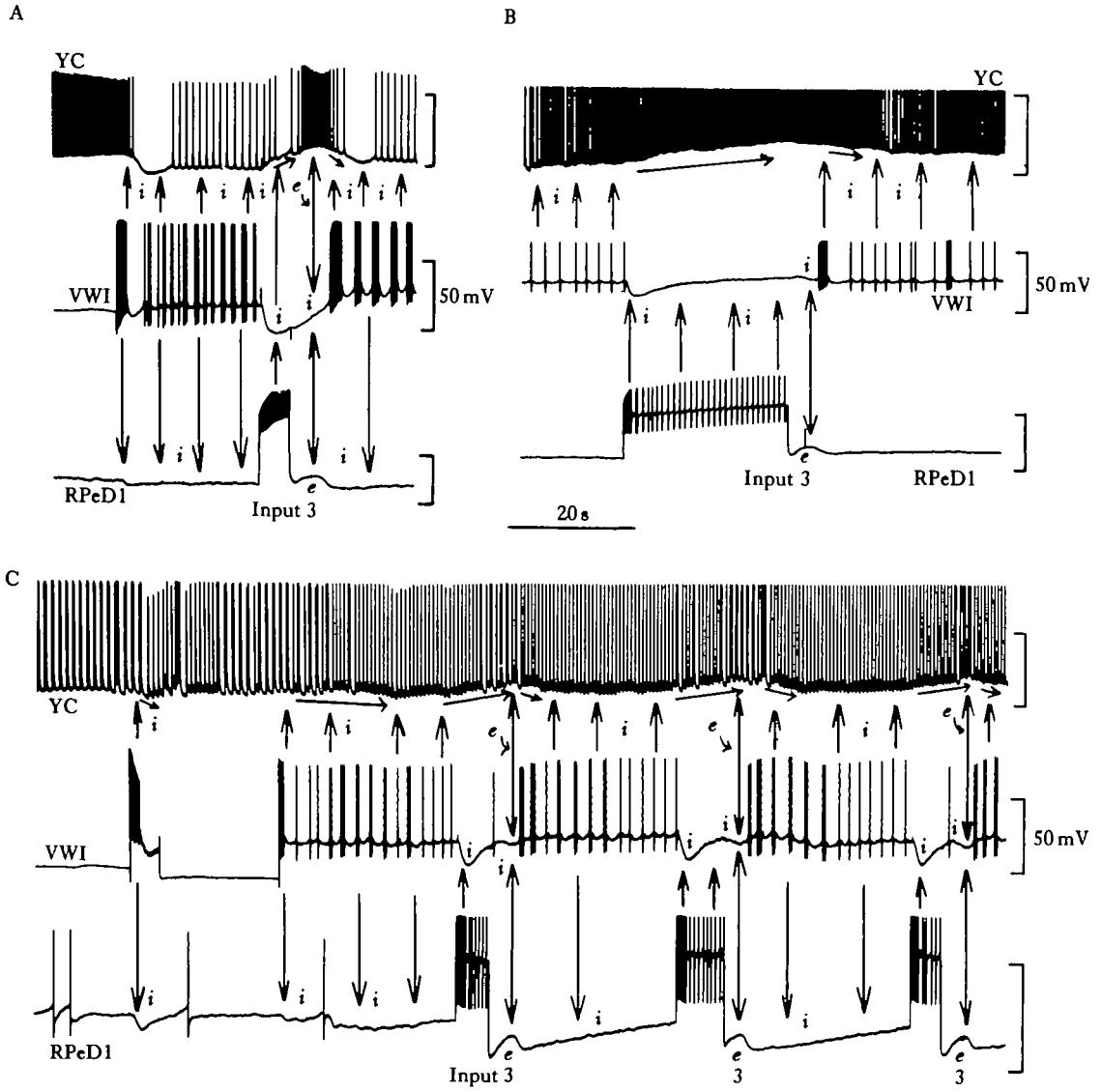


Fig. 9. Effects of the mutually inhibitory connections between the VWI and RPeD1 on Yellow Cell firing patterns. (A) Interruption of VWI inhibitory effects on a single spiking Yellow Cell (YC) by a burst of spikes in RPeD1. (B) Same as A but a different preparation when activity of RPeD1 was tonically maintained for a longer period. (C) Inhibitory effects of RPeD1 on VWI cause change in firing patterns of a doubletting Yellow Cell. Single-headed arrows in (A–C) indicate direct synaptic effects from one cell to another which are all inhibitory (*i*). Double-headed arrows indicate common input 3 inputs occurring as compound EPSPs (*e*) or IPSPs (*i*). Small arrows under Yellow Cell traces indicate the direction of membrane potential change. More detailed evidence for the direct synaptic effects assumed in this figure are given in Figs 1 and 3 and occurrences and effects of input 3 in Fig. 6. See text for further interpretation of these records.

arrangement of ganglia), and not in the left parietal and left pleural ganglia. Input 3 was confined to Yellow Cells and was absent from the Yellow-green Cell population. Even in the Yellow Cells it only occurred regularly in the visceral Yellow Cell cluster, and in cells located on the left side of the right parietal ganglion. Occasionally it was

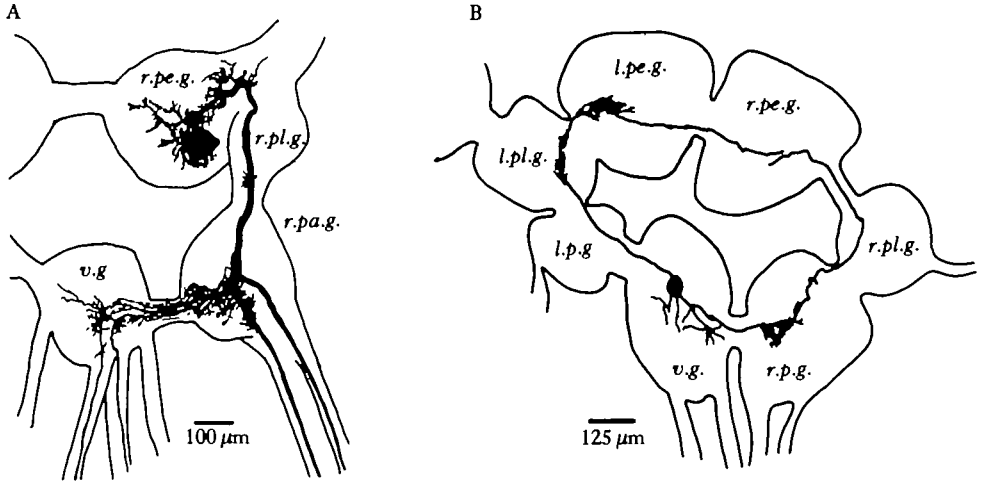


Fig. 10. Drawings traced from photographs of whole mounts where (A) RPeD1 and (B) the VWI were filled with Lucifer Yellow. Both drawings underestimate the amount of fine dendritic branching and this is particularly true of the fine terminal branching of the VWI in the right pedal ganglion. *pe.g.*, pedal ganglion; *pl.g.*, pleural ganglion; *pa.g.*, parietal ganglion; *v.g.*, visceral ganglion; *r.*, right; *l.*, left; *p.g.*, pedal ganglion. Lucifer Yellow fills carried out by P. Allison (A) and P. Modabber Moghadam (B).

found in Yellow Cells from other areas of the visceral ganglion and cells in the anterior part of the right parietal ganglion. Overall about half the total Yellow Cell population receive input 3.

The distribution of the spontaneously-occurring inhibitory input was not examined in detail and was only observed in Yellow Cells from the visceral Yellow Cell cluster, where it probably occurred on all (8–10) cells.

The location of neurosecretory cells postsynaptic to VWI and RPeD1 accorded remarkably well with the distribution of the axonal processes of the two cells determined in a separate study using Lucifer Yellow injections (Fig. 10 but see also Haydon & Winlow, 1981, for morphology of RPeD1). Thus RPeD1's axonal processes and dendrites were restricted to right pleural, right parietal and visceral ganglia (Fig. 10A) which were the only ganglia where postsynaptic responses could be recorded in electrophysiological experiments. On the other hand, the extensive and ring-like morphology of the VWI (Fig. 10B) corresponded with the occurrence of synaptic connections in all five posterior ganglia of the brain where Yellow Cells and Yellow-green Cells occurred. Overall the anatomical data was consistent with the possibility that the interneurones form monosynaptic connections with the two types of neurosecretory cells but detailed electrophysiological experiments need to be carried out to confirm this.

DISCUSSION

Interpretation of the interneuronal network as a functional system

Fig. 11A summarizes all the synaptic connections revealed by the present study. For simplicity it assumes that all the connections are monosynaptic and that input 3 consists of a single interneurone (although the work of Benjamin & Winlow, 1981

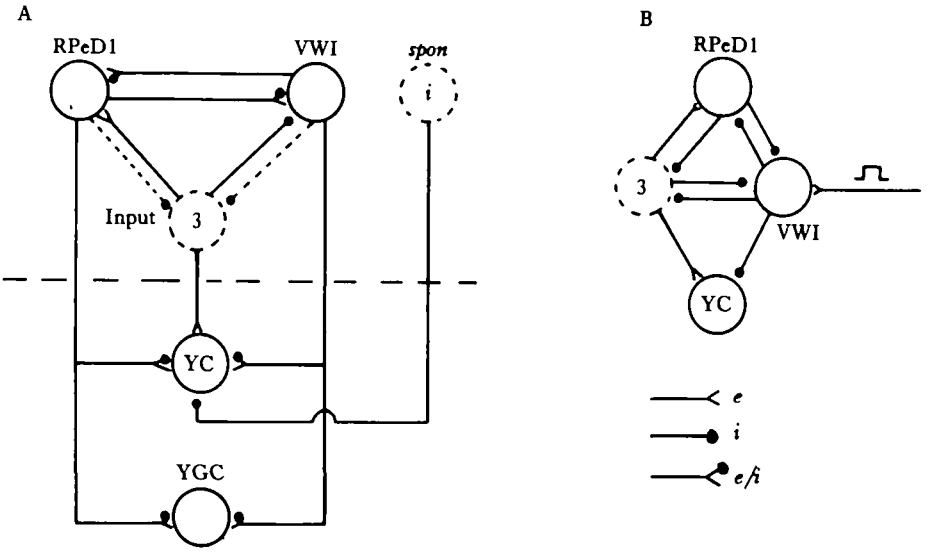


Fig. 11. Circuit diagrams summarizing the synaptic connection revealed in the present study. (A) Connections between interneurones are shown above and those between interneurones and neurosecretory cells below the horizontal dashed line. Solid lines are connections between cells shown by direct recording. Dashed lines are connections shown by indirect methods. Solid circles are neurones which were directly recorded by intracellular electrodes. Dashed circles show cells whose presence was inferred by their synaptic inputs to other cells. The Yellow Cell (YC) illustrated is one from the visceral Yellow Cell cluster. Yellow Cells from other locations have fewer interneuronal inputs. *spon i*, spontaneous inhibitory input; YGC, Yellow-green Cell. (B) Functional interpretations of circuit with only main connections shown. The circuit is seen to operate in two alternative modes when Yellow Cells are either excited by input 3 interneurones or inhibited by the VWI (see text for further explanation).

suggests that at least two interneurones are involved). Interneurones and connections between interneurones are shown in the top part of the figure and the connections to the neurosecretory cells in the bottom part. The Yellow Cells shown are those of the visceral Yellow cluster which receive four synaptic inputs, three of which are functionally inhibitory (although two are nominally biphasic), and only one (input 3) is excitatory. The Yellow-green Cells only receive synaptic input from the two identified interneurones which are both inhibitory so in the absence of other excitatory input it must be assumed that their continuous activity, like that of the bursting Yellow Cells (Benjamin, 1978) is due to some intrinsic pacemaker.

No evidence was obtained for neurosecretory cells having any synaptic effects on the interneurones, but the interneurones themselves are connected by functional inhibitory and excitatory synapses. Reciprocal inhibitory connections occur between the VWI and RPeD1 and this was shown in the present paper by direct recording. Both these interneurones appear to inhibit the interneurones responsible for input 3 (evidence from the present paper and Winlow *et al.* 1981). Conversely, input 3 interneurones inhibit the VWI but excite RPeD1. Although the evidence indicated an inhibitory connection between RPeD1 and input 3 interneurones, the most dramatic effect of RPeD1 spike activity was to cause a delayed activation of input 3 probably by post-inhibitory rebound (see Winlow *et al.* 1981, for evidence).

Although Fig. 11A gives a complete summary of all the connections shown in the

present study it does not allow the circuitry to be interpreted in a very functional way, unlike Fig. 11B which only shows the main synaptic connections and how they finally affect the activity of the Yellow Cells. In Fig. 11B the Yellow Cells are shown to be acted upon by two types of input one of which is inhibitory (from the VWI) and the other excitatory (input 3). The rest of the circuit is seen as a mechanism for allowing either input 3 excitation or the VWI inhibition to dominate the activity of the Yellow Cells and this will be explained later. The inhibitory effect of RPeD1 on the Yellow Cells is too weak to be effective at normal firing levels and so is left out. Because no direct evidence exists for a link between the spontaneous inhibitory input and any of the other interneurones, this has also been excluded from Fig. 11B for the purposes of the present model.

In one mode of activity of the circuit shown in Fig. 11B, the Yellow Cell's spike activity is dominated by the occurrence of input 3 excitatory input. Input 3 occurs periodically in many preparations at intervals of between 6 and 40 s (Benjamin, 1978) and can accelerate on-going spike activity or trigger off long bursts of spikes in the Yellow Cells. Under these conditions inhibitory inputs from the VWI on the Yellow Cells are normally suppressed due to the high resting potential of the cell but also because input 3 and RPeD1 (itself excited by input 3) together inhibit its activity. Inhibition of the VWI is seen to be the main function of RPeD1 which effectively prevents firing in the VWI even when firing at frequencies below 1 spike s^{-1} (Fig. 9B).

An alternative mode of activity of the circuit shown in Fig. 11B would occur if the VWI fired continuously. In this circumstance the main effect on the Yellow Cells would be inhibition direct from the VWI. Subsidiary effects would be inhibition of input 3 interneurones (Fig. 1D,E) and RPeD1 (Fig. 1A) which would prevent their own reciprocal inhibitory effect on VWI and remove the main source of excitation (input 3) to the Yellow Cells. Thus activation of the VWI would switch the main synaptic influence on Yellow Cells from excitation due to input 3, to inhibition due to VWI. The circumstances under which the alternative mode of operation would occur are unclear because at present nothing is known of the natural inputs that excite the VWI. In isolated brains it is silent but whether this is also the case in more intact preparations needs to be investigated. Furthermore the VWI may not simply prevent Yellow Cell firing but often just reduce the frequency of firing or change the type of firing pattern shown by the Yellow Cells (Fig. 4).

Synaptic effects of the identified interneurones

All the synaptic effects of the identified interneurones were biphasic with an initial depolarizing wave followed by a long duration hyperpolarizing one. Unitary responses to single spikes in the interneurones were rare although sometimes 1 : 1 EPSPs could be seen on RPeD1 following spikes in the VWI. Usually it was necessary to evoke a burst of spikes in an interneurone to see a clear postsynaptic response. It should be noted that even brief depolarizing pulses applied to the VWI caused a strong burst of spikes, rather than a single spike, which often outlasted the stimulus and that high frequency bursts of spikes were the usual pattern of firing with even weak suprathreshold tonic depolarization. Certainly a burst of spikes in RPeD1 was absolutely necessary to see an inhibitory response in the neurosecretory cells and accounts for the failure in a previous study of RPeD1 to find postsynaptic responses in the Yellow Cells

(Benjamin & Winlow, 1981) because much weaker activation of RPeD1 was used. Whether the responses seen in the present study were monosynaptic has yet to be determined but the fact that the distribution of follower cells to both interneurones corresponds closely and is limited by the axonal and dendritic projections of the interneurones makes monosynaptic connections possible. It should be pointed out that the other follower cells of RPeD1 in the visceral and right parietal ganglia all appear to have monosynaptic connections from RPeD1 (Winlow *et al.* 1981).

The postsynaptic responses in the Yellow Cells and Yellow-green Cells are due to conventional conductance increase mechanisms and this is also true for VWIs' and RPeD1s' effects on each other (P. R. Benjamin, unpublished observations). The reversal potential for the hyperpolarizing phase of the RPeD1 BPSP on the neurosecretory cells is between -60 and -80 mV and this suggests that an increase in membrane conductance to K^+ is involved. Increases in K^+ conductance certainly appear to underlie long duration inhibitory PSPs caused by dopamine in other molluscs (e.g. Ascher, 1972). Of particular interest are the K^+-mediated IPSP responses to the giant dopamine-containing cell in *Planorbis* (Berry & Cottrell, 1979) which is probably homologous to RPeD1 in *Lymnaea*. The inability to reverse the hyperpolarizing phase of the VWI BPSP in the neurosecretory cells may be due to the distal location of the synapse involved although other explanations are possible. The depolarizing phase of the BPSPs from both interneurones occurred less reliably than the delayed hyperpolarization and was often lost with repeated activation of the interneurones.

Both RPeD1 and VWI are wide-acting interneurones and previously published data on RPeD1 show that it has many other follower cells in the visceral and right parietal ganglia (Benjamin & Winlow, 1981; Winlow *et al.* 1981). It is also a multi-action interneurone having inhibitory, excitatory and biphasic effects on different postsynaptic cells (Benjamin & Winlow, 1981). Future publications will show that the VWI also has many followers in the *Lymnaea* brain other than the neurosecretory cells described here, some of which are shared by RPeD1, some of which are not. This indicates that both interneurones are parts of much bigger neuronal networks in the snail brain.

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