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Cholinergic Interneurons in the Feeding System of the Pond Snail Lymnaea stagnalis. III. Pharmacological Dissection of the Feeding Rhythm

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Cholinergic interneurons in the feeding system of the pond snail *Lymnaea stagnalis*. III. Pharmacological dissection of the feeding rhythm

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SUMMARY

The feeding activity of the pond snail *Lymnaea stagnalis* was stimulated by depolarization of a modulatory interneuron (SO) or of a N1 pattern-generating interneuron. The cholinergic antagonists phenyltrimethylammonium (PTMA), methylxylocholine (MeXCh), hexamethonium (HMT) and atropine (ATR) were applied at 0.5 mM in the bath and their effects on the rhythmic feeding pattern were monitored.

Each of the antagonists slowed or blocked the feeding rhythm.

The block was due to interference in the pattern generating network, not to disturbance of modulatory inputs.

The experimental results favour a model in which the alternation of protraction (N1) and retraction (N2) phases occurs by recurrent inhibition. The results would be more difficult to explain on the reciprocal inhibition model.

When all the N1 output was blocked, the N1 neurons fired rhythmic bursts endogenously.

1. INTRODUCTION

This, the third paper of a series on the feeding system of the pond snail, *Lymnaea stagnalis*, is concerned with the pharmacological dissection of the network of interneurons which generate the feeding rhythm. The output of the feeding system comes from the motoneurons, cell types 1–10, which are driven by three classes of feeding pattern generating interneuron, known as N1, N2 and N3 cells. During the feeding rhythm, these three kinds of neuron fire in turn, with the N1 neurons active in the protraction phase, N2 during rasping and the N3 during swallowing (Rose & Benjamin 1981*b*). Burst of spikes in the N1, N2 or N3 neurons were shown to be able to reset the feeding rhythm suggesting that they were indeed the pattern generating interneurons (Elliott & Benjamin 1985*a*). Their synaptic interactions (described in detail below, see figure 8*a*) provided an explanation of the sequence of the feeding rhythm, and it is the model construction from these interactions which will be tested here by pharmacological dissection.

Pharmacological agents could be applied to the isolated central nervous system (CNS), from which spontaneous rhythmic feeding patterns can often be recorded (Benjamin & Rose 1979). However, these occur randomly and so it is hard to know if the effects are due to the agonist or to chance. One possibility is to depolarize one of the N1 pattern-generating interneurons, whose activity can accelerate the normal feeding pattern or if the preparation is quiescent, can initiate the 'fictive feeding' rhythm. In N1-driven rhythms the feeding rate is rarely as fast as that seen in the intact snail (Elliott & Benjamin 1985*b*; Dawkins 1974). The problem of achieving a reliable rhythm may be overcome by depolarization of the SO, a single modulatory interneuron in the buccal ganglia, which can activate fictive feeding rhythm at its normal rate (Rose & Benjamin 1981*a*; Elliott & Benjamin 1985*b*). The SO neuron stimulates the feeding rhythm by a strongly facilitating excitatory synapse with each of the N1 neurons. Because the feeding rate of the SO rhythm is close to that of the intact snail and since SO stimulation evokes fictive feeding reliably, many of the rhythms described in this paper (figures 1 and 3–6) are elicited by SO depolarization.

The *Lymnaea* CNS also contains other modulatory interneurons. Among these are a pair of electrically coupled neurons in the cerebral ganglia whose activity is essential for rhythmic feeding. These are the cerebral giant cells (CGCs) and their axons project to the buccal ganglia (McCrohan & Benjamin 1980*a*)). The CGCs contain serotonin (McCaman *et al.* 1984). Normally, these cells fire tonically in the isolated CNS preparation (McCrohan & Benjamin 1980) at a rate (0.5–1.5 Hz) sufficient to permit the expression of the feeding rhythm. They achieve their modulatory effects by connections with the feeding moto- and interneurons (Benjamin & Elliott 1989).

Thus, in the *Lymnaea* feeding system the modulatory neurons can activate and control the rate of fictive

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feeding produced by the N1, N2 and N3 pattern-generating interneurons. The changeover from protraction (N1 phase) to the first part of retraction (rasping, N2 phase) occurs through recurrent inhibition, with the N1 neurons exciting the N2 cells, which inhibit the N1 cells. The N3 cells are inhibited by both N1 and N2 neurons and fire by post-inhibitory rebound when the N1 and N2 cells have finished firing.

Pharmacological agents could be used to examine the model in two ways. First they could be applied while recording a particular interneuron→interneuron synapse and to look for changes in the identified postsynaptic potentials (PSPs). Secondly, they could be applied during fictive feeding and their effects on the rhythmic pattern assessed. This second technique, which tests the accuracy of the network model, is known as pharmacological dissection (Marder 1987) and is the approach adopted here.

As a foundation to the use of pharmacological dissection as a test of the mechanism of rhythm generation, the preceding two papers (Elliott *et al.* 1992; Elliott & Kemenes 1992) have shown that the N1 interneurons are multiaction, premotor interneurons and that their synaptic effects can be explained on the hypothesis that their main transmitter is acetylcholine (ACh). No evidence was found that the modulatory SO or the N2 or N3 pattern-generating interneurons were likely to be cholinergic (Elliott & Kemenes 1992). This means that the cholinergic agonists or antagonists produce precisely localized lesions of the feeding system, namely, selective blockage of the synaptic output from the N1 interneurons.

The production of pharmacological lesions provides two kinds of tests of the network model: (i) are the synaptic connections in the pattern-generating network correctly identified? Suppose, for example, that all the synaptic potentials produced on the feeding interneurons by the N1 interneurons were inhibitory and not a range of excitiatory and inhibitory. Then antagonists like hexamethonium (HMT) and atropine (ATR) which block the excitatory postsynaptic potentials (EPSPs) only would not affect pattern generation and this would show that the synaptic potentials had not been correctly identified. (ii) Have all the relevant cells been located? Suppose that some N1-like neurons had not been located and used a different transmitter (e.g. dopamine). Then rhythmic activity might be expected to persist with application of cholinergic antagonists.

In the experiments described below, those cholinergic agonists and antagonists shown to be effective in the feeding system of *Lymnaea* (Elliott *et al.* 1992; Elliott & Kemenes 1992) are perfused through the bath at their effective concentration (0.5 mM). This will produce a selective lesion, blocking the output from the N1 cells and this will test the Elliott & Benjamin (1985*a*) model of the feeding pattern generator. The results support the principal features of the model, including recurrent inhibition and can be explained without recourse to new cell types.

A brief account of these findings has appeared previously (Elliott 1989).

2. METHODS

For these experiments, snails were starved overnight and allowed to begin feeding on lettuce before the dissection was begun. This increased the proportion of preparations in which spontaneous rhythmic feeding activity was seen up to 80% (Elliott & Benjamin 1989; Elliott & Andrew 1991).

Intracellular recordings were made from identified neurons in the buccal ganglia as described before (Elliott & Benjamin 1989; Elliott *et al.* 1992). Normally the whole CNS was dissected out and left intact, but in those experiments in which records were made from the CGC cells in the cerebral ganglia, the cerebral commissure was cut and the cerebral ganglia turned back to expose the CGCs clearly.

Throughout the experiments in this paper, the ganglia were bathed in standard snail saline (composition given in Elliott *et al*, 1992), to which the antagonists atropine (ATR), hexamethonium (HMT), methylxylocholine (MeXCh) or phenyltrimethylammonium (PTMA) were added at 0.5 mM. Only preparations in which the N1→1 cell synapse was effectively blocked by the antagonist were chosen for analysis. This was found in 21 preparations.

3. RESULTS

Spontaneous feeding activity is seen in 80% of these *Lymnaea* feeding preparations. In six, 'fictive feeding' activity was blocked by passing 0.5 mM cholinergic antagonists through the bath. However, such an experiment is rather uninformative, because the feeding rate varies at random (Elliott & Andrew 1991). Much more powerful tests are presented below, in experiments in which the fictive feeding was driven by activating a modulatory interneuron (SO) or by stimulating an N1 pattern generating neuron.

When the SO, (a single modulatory interneuron in the buccal ganglia) is depolarized, it soon elicits the rhythmic feeding activity with normal timing (Rose & Benjamin 1981*a*; cf. the top panels of figures 1, 4 and 6). Alternatively, the feeding sequence may be evoked by depolarizing an N1 interneuron, but then the feeding rate is never as fast as that seen in vivo (Elliott & Benjamin 1985*b*). In the experiments shown below, the results of SO or N1 stimulation in the presence of cholinergic antagonists were compared with the undisturbed rhythm.

(*a*) *PTMA*

In *Lymnaea*, 0.5 mM PTMA (phenyltrimethylammonium) acts as a weak agonist at two of the ACh receptors, but it also attenuates the cholinergic response of all three types of receptor (Elliott *et al.* 1992). With PTMA perfusion, the main effect was a transient synaptic blockade and, during this time, termination of the feeding rhythm. (With prolonged PTMA perfusion, the synaptic potentials and the rhythm always returned.)

PTMA blocked the ability of the SO to evoke fictive feeding in five preparations. The recordings of the SO

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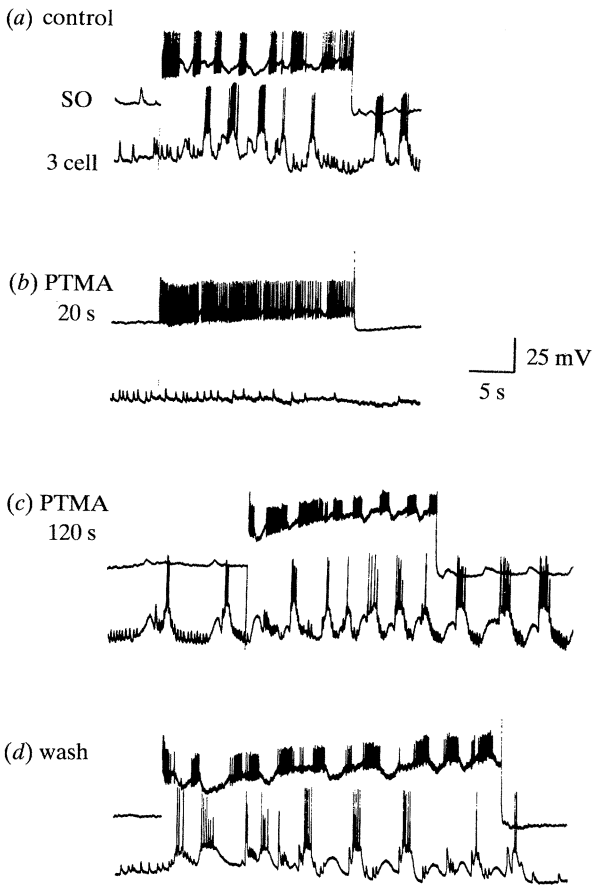

Figure 1. 0.5 mM PTMA transiently abolishes the SO-stimulated feeding pattern in the isolated CNS. Simultaneous intracellular recordings from the SO interneuron and a motoneuron, the 3 cell. (*a*) Before application of PTMA, SO stimulation evokes rhythmic activity typical of fictive feeding. (*b*) Twenty seconds after the application of PTMA, the SO is again stimulated with the same current, but no rhythmic activity is seen. (Note that PTMA has no effect on the 3 cell ACh receptors, Elliott *et al.* (1992)). (*c*) After 120 s, rhythmic activity is seen even without SO stimulation. The activation of the SO accelerated the rhythm, as in normal preparations. (*d*) After washing out, the SO can still initiate the rhythmic feeding pattern.

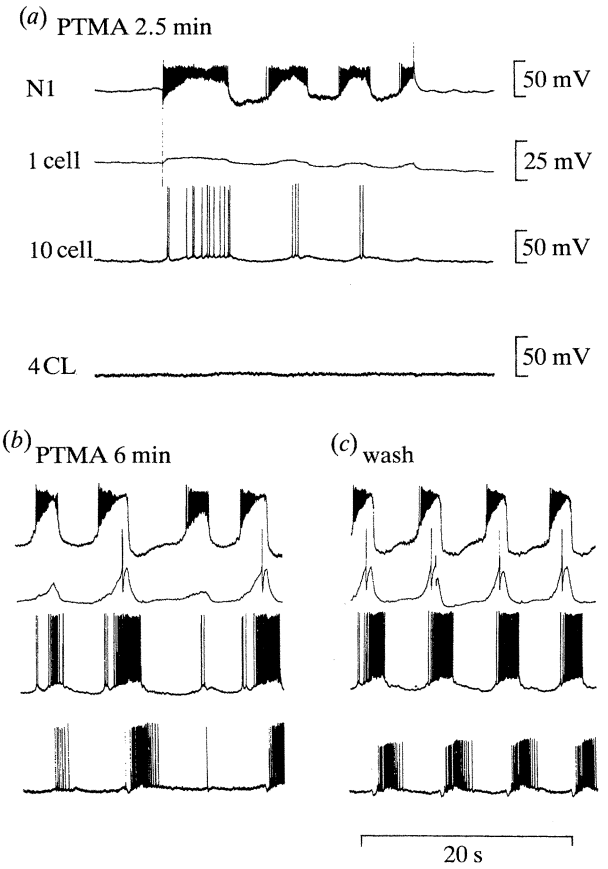

Figure 2. PTMA blocks a N1 driven-feeding rhythm. (*a*) After 2.5 min of 0.5 mM PTMA, as the synaptic blockade began to wane, an N1 interneuron was stimulated with depolarizing current. The N1 fires rhythmic bursts, with the greatest firing frequency in the middle of the burst. The simultaneous intracellular records from the 1 and 10 cell show the expected, partially blocked N1 compound EPSP, which leads to spikes in the 10 cell. Note that the 10 cell does not spike between N1 bursts and that there is no sign of any synaptic input in the 4 cluster cell. This shows that there is no activation of the N2 interneurons. (*b*) After 6 min PTMA, spontaneous rhythmic activity was recorded: the normal feeding rhythm has nearly returned. Some cycles now show evidence for N2 inputs: for example, the 10 cell bursts continue after the N1 has ended and the 4 cluster cell show its characteristic inhibition followed by excitation. (*c*) After washing the normal rhythm is restored: the N1 is still spontaneously active.

and 3 cell (figure 1) was selected because the 3 cell is the only motoneuron in the feeding system which has only the slow inhibitory type of acetylcholine (ACh) receptors (see paper I, Elliott *et al.* 1992) which are insensitive to PTMA. Hence the 3 cell membrane potential and resistance will not be directly affected by PTMA. In the initial control period (figure 1*a*). SO depolarization evokes rhythmic activity, with bursts of spikes alternating between the 3 cell and the SO. As usual, the activity continues after SO stimulation. The SO was again depolarized 0.3 min after the arrival of PTMA, but this time no rhythmic activity was seen, even though the SO firing rate over the first 1.5 s was the same in both cases (10 Hz, see figure 1*b*). Increasing the current supplied to the SO did not lead to feeding (recordings not shown). However, 100 s into the PTMA perfusion, rhythmic activity recovered even without any SO stimulation. The SO did not participate in this rhythm. PTMA activation of the rhythm is neither atypical nor surprising, because the PTMA excites the N1 cells through their excitatory ACh receptors, paper I. The excerpt shown in figure 1*c*, was taken 120 s after the PTMA arrival, and shows part of the spontaneous rhythmic activity. It also shows that SO stimulation increased the feeding rate from 0.17 Hz (mean of three cycles before and after) to 0.31 Hz (mean of six cycles). With washout of PTMA, the spontaneous activity gradually declined, but the ability of the SO to evoke rhythmic feeding remained (figure 1*d*).

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In another preparation, recordings were made from a N1 interneuron and 1, 10 and 4 cluster cell motoneurons. Initially, the preparation was quiet and no activity was seen, unless the N1 cell was stimulated, in which case weak N1 bursts took place and were followed by N2 activity. As the synaptic blockade began to wane 2.5 min after application of PTMA, the N1 cell was again excited with depolarizing current and began to burst rhythmically (figure 2*a*). Each N1 burst produced a weak EPSP in the 1 cell and a small EPSP in the 10 cell with a few spikes superimposed, but there is no change in 4 cluster cell membrane potential. This shows that the PTMA has much reduced, but not entirely blocked, the expected synaptic output from the N1 cell. Note (i) that the 10 cell depolarization does not continue after the N1 bursts, and (ii) that there is no change in 4 cluster cell membrane potential following the N1 activity. These two observations show that there is no evidence for activation of N2 interneurons.

After 6 min PTMA, spontaneous bursting activity was recorded (figure 2*b*). As before, this may be due to the PTMA directly stiumulating the N1 cell's own excitatory ACh receptors (Elliott *et al.* 1992). The transient block of N1 transmitter release has begun to pass and each N1 burst now gave rise to a larger 1 cell EPSP (sometimes with spikes), whereas the 10 cell also shows a stronger burst of spikes. Note that some N1 bursts (e.g. the second one shown) are followed by N2 activity, because the 10 cell continues to fire after the end of the N1 burst and the 4 cluster cell shows a quick inhibition, followed by a burst of spikes. After washing out the PTMA for 2 min, N2 interneurons were active in every cycle (figure 2*c*).

These two experiments (figures 1 and 2) show that the effect of PTMA was twofold: reducing the synaptic potentials produced by the N1 neurons and blocking the normal feeding rhythm.

(*b*) *MeXCh*

The antagonist methylxylocholine (MeXCh) selectively blocks the excitatory and slow inhibitory receptors (Elliott & Kemenes 1992; Elliott *et al* 1992). In the experiment shown in figure 3, intracellular recordings were made from the SO, a 1 cell and an 8 cell. In the presence of MeXCh, the SO was stimulated strongly, so that it fired at a high frequency and eventually the electrode became unbalanced. No synaptic input was seen in the 1 cell, but the SO fired in short rhythmic bursts and the 8 cell received waves of synaptic input, a few of which led to spikes. (Because the 8 cell has no direct input from the SO, this shows that the SO neurons are exciting the N1 cells which in turn produce the 8 cell compound synaptic potential through the unblocked fast inhibitory receptors.)

The record in the lower panel of figure 3 begins 10 s after the wash with normal saline began. About 5 s later, the stimulating current was gradually increased, until the SO was firing slowly. This is soon followed by a depolarizing (N1) input to the 1 and 8 cells. After 20 s the SO, 1 and 8 cells all receive a strong

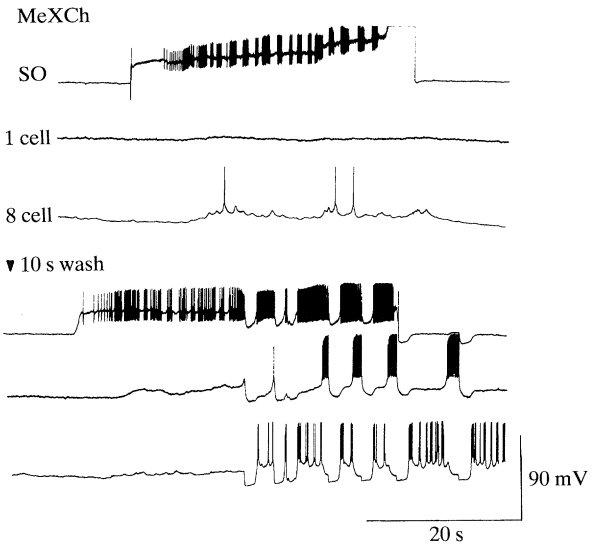

Figure 3. MeXCh blocks the SO stimulated rhythm. Simultaneous intracellular recordings from the SO, a 1 cell and an 8 cell. (The 8 cells are electrically coupled to the 4 cluster cells and differ mainly in the longer duration of the N2 synaptic input.) Upper panel: in 0.5 mM MeXCh strong stimulation of the SO fails to elicit any feeding rhythm and eventually the electrode becomes unbalanced. Lower panel: this begins 10 s after the start of the wash. Weaker stimulation of the SO soon elicits the full sequence of feeding activity. The N1 inputs excite the 1 cell and produce a gradual inhibition of the 8 cell. The N2 phase is marked by strong inhibition of SO and 8 cell, while the N3 inputs cause the short, quick acting, IPSPs within the 8 cell bursts.

hyperpolarization, typical of the N2 phase. This is followed by full rhythmic activity, each cycle consists of N1, N2 and N3 inputs, as shown by the burst of 1 cell spikes, the deep hyperpolarization of the 8 cell (N2) and the weaker N3 hyperpolarizations which break up the 8 cell bursts. Even after the end of SO stimulation, the rhythm continues, albeit more slowly. This experiment shows that MeXCh not only blocked the N1 synaptic output, e.g. to the 1 cell, but also stopped the rhythm, even though the SO was stimulated to an adequate spike rate. As MeXCh blocks the slow inhibitory and excitatory output from the N1 neurons, this experiment shows that just the fast inhibitory output from the N1 neurons is not sufficient to stimulate the feeding rhythm. MeXCh blocked the feeding rhythm in three preparations and slowed the rhythm in a further preparation.

(*c*) *HMT and ATR*

In *Lymnaea*, at 0.5 mM, these antagonists are selective for the excitatory ACh receptor (Elliott *et al.* 1992).

Figure 4 shows recordings of the SO, 1 cell and a 4 cluster cell before, during and after 0.5 mM HMT. Without HMT, the preparation is rhythmically active and SO stimulation increases the feeding rate. Note the N1 depolarizations (not big enough to reach the spike threshold) of the 1 cell occur at the end of each SO burst and the coordinated N2 inhibitions of SO and 4 cluster cell.

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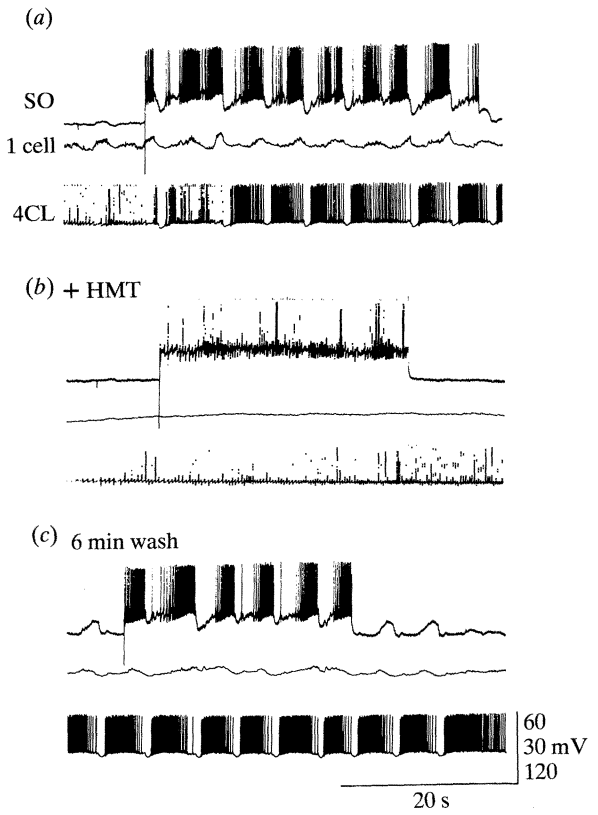

Figure 4. HMT blocks the feeding rhythm. Intracellular recordings from the SO, 1 cell and a 4 cluster cell. (*a*) Normal saline: stimulation of the SO leads to rhythmic activity in all these feeding cells. (*b*) In 0.5 mM HMT, depolarization of the SO with the same current as (*a*), fails to elicit any rhythmic activity. The SO and 4 cluster cell fire tonically, while the 1 cell remains at its resting potential. (*c*) After 6 min wash in normal saline, the rhythmic SO-driven activity is restored, although the N1 phase excitation of the 1 cell is still reduced. Note that the feeding pattern also occurs spontaneously before and after SO stimulation.

With HMT, (figure 4, central panel) the spontaneous synaptic input to all the cells is much reduced; the 1 cell and SO are silent and the 4 cluster cell fires tonically. Activation of the SO (same current as before) now fails to elicit any rhythmic activity.

The lower panel shows that 6 min wash is sufficient to restore SO-driven and spontaneous feeding inputs, although the amplitude of the N1 inputs to the 1 cell have not yet been restored.

Figure 5 shows records from another preparation in which the SO, a 10, 9 and 5 cells were recorded. Throughout the entire record the SO was stimulated with a constant depolarizing current. For all the top panel, and for the first part of the lower panel, the preparation was perfused with 0.5 mM HMT and rhythmic activity proceeds very slowly. Note that, even in HMT, each strong N2 input (when the 10 and 9 cells are excited and the SO and 5 cell are inhibited), is preceded by a weak burst of spikes (superimposed on a N1 compound EPSP) in the 10 cell, so that the excitatory output from the N1 cells has not been completely blocked by the HMT. In this experiment, there is no evidence that the rhythm runs without a N1 phase. A similar slowing of the rhythm was found

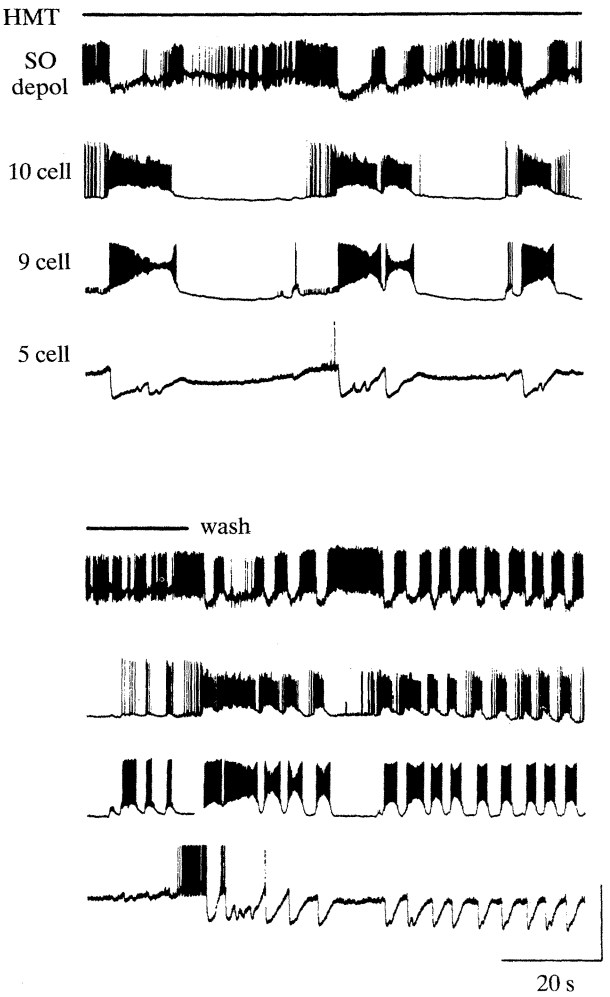

Figure 5. HMT shows the feeding rhythm. Simultaneous recordings from the SO (depolarised throughout with a constant current), 10 cell, 9 cell and 5 cell motoneurons. Throughout the upper panel, 0.5 mM HMT in normal saline was perfused. Note the slow rhythm with strong N2 inputs (i to SO and 5 cell; e to 10 cell and e to the 9 cell). The perfusion was switched to normal saline 20 s into the lower panel: the feeding rhythm accelerated rapidly to its normal rate. The two panels are continuous excerpts. Scalebar: SO, 5 and 10 cells, 50 mV; 9 cell, 100 mV.

in a second preparation, while complete block by HMT was obtained in four preparations.

Within 20 s of the start of the wash with HMT-free saline, the rhythmic activity begins to accelerate; by the end of the excerpt the feeding sequence and rate are indistinguishable from undisturbed preparations.

ATR like HMT, may prevent SO activation of the feeding rhythm. The records shown in figure 6 are from the same preparation as figure 4, but now the SO, 1 and 4 cluster cells are accompanied by an N1 interneuron. Shortly after the start of the excerpt, SO stimulation increases the feeding rate; note the N1 activity and, in the 1 cell, the N1-phase compound PSP, while the SO and 4 cluster recordings are dominated by the inhibitory N2 inputs. With 0.5 mM ATR, SO stimulation fails to evoke any feeding rhythm, although the N1 cell fires tonically throughout the excerpt. After the end of the SO stimulus, the N1 cells stops firing. This break is not likely to be the

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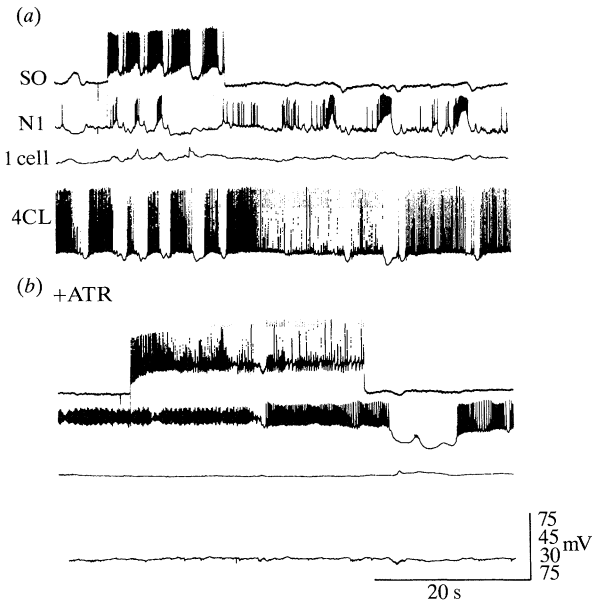

Figure 6. ATR blocks the feeding rhythm. Intracellular recordings from the SO, an N1 interneuron, a 1 cell and 4 cluster cell motoneurons. (*a*) In normal saline, SO stimulation leads to an increase in feeding rate. The prominent inhibitions of the SO and 4 cluster cells are produced by the N2 neurons. (*b*) In 0.5 mM ATR in normal saline stimulation of the SO with the same current does not lead to any rhythmic activity: the 1 and 4 cluster cells are silent and the SO fires nearly tonically.

consequence of synaptic input from N2 or N3 interneurons, because these cells should produce an inhibitory then excitatory synaptic input to the 4 cluster cell (see, for example, figure 2*b*). It is more likely that the break in N1 firing is endogenous due to the N1 rhythmic activity. ATR blocked the rhythm in three preparations and showed the rhythm in a fourth.

(*d*) *The effects of the cholinergic antagonists on the CGC cells*

The pair of CGC cells is located in the cerebral ganglia and are strongly electrically coupled, so that they always fire 1:1. In the normal saline, these cells fire tonically, (usually at about 1 Hz) and this is essential for maintenance of the normal feeding rhythm (Benjamin & Elliott 1989). When ATR or HMT were perfused in normal saline, the CGC firing rate was unchanged (figure 7), but when PTMA was applied the firing rate increased significantly (figure 7). However, with PTMA, the normally regular firing was interrupted by occasional inhibitory inputs (not shown). The results from these four snails show that the cholinergic block of the feeding rhythm is not due to cessation of the firing rate of the modulatory CGC cells.

DISCUSSION

(*a*) *Cholinergic antagonists block the feeding rhythm*

The results shown above demonstrate that the cholinergic agents can block the feeding rhythm, as would be expected if cholinergic interneurons are part of the feeding pattern generator. PTMA acts as an agonist as well as a weak antagonist at the *Lymnaea* receptors (Elliott *et al.* 1992) and so it is not surprising that its effects alter with time.

The other three pharmacological agents (MeXCh, HMT and ATR) are all pure antagonists. In some cases (figure 5) the application of 0.5 mM antagonist does not completely block the N1 synaptic output and also fails to eliminate the rhythm, which runs more slowly (figure 5). In the other experiments figure 3, 4 and 6) both the synaptic output produced by the N1

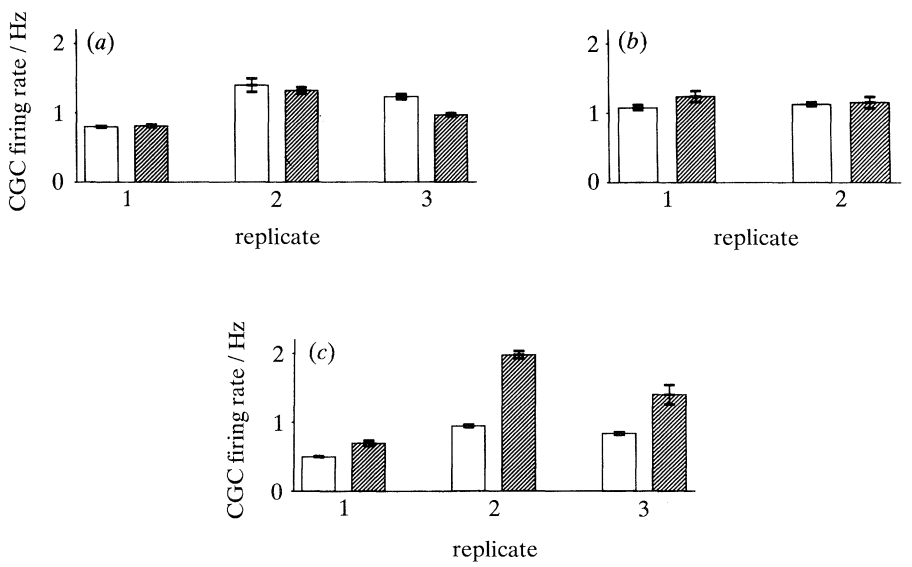

Figure 7. Firing rate of the modulatory CGC cells in (*a*) 0.5 mM HMT, (*b*) 0.5 mM PTMA and (*c*) 0.5 mM ATR. The firing rate was measured in the minute immediately before and 90 s after adding the antagonist. The bar chart compares the mean (± 1 standard error) firing rate in normal saline (open bars) and in saline containing 0.5 mM antagonist (shaded bars). Only PTMA causes a change (significant at the 1% level) in the CGC firing rate.

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interneurons and the feeding rhythm are blocked. These differences between preparations are most probably due to differences in the barrier provided by the sheath around the ganglia, which prevents full access to the neuronal receptors (Ger & Zeimal 1977; Koike *et al.* 1974; Sigvardt *et al.* 1986).

(*b*) *The N1 cells can burst endogenously*

When the feeding rhythm is blocked by cholinergic antagonists, the N1 neurons fail to make their normal output. With PTMA, (figure 2*a*) the N1 neuron fires in bursts without any synaptic inputs from other feeding interneurons, so that the bursting is endogenous to the N1 network. No endogenous bursting was obsrved when synaptic output was blocked by high Mg/low Ca (figure 1 of Elliott & Kemenes 1992), the difference suggesting that the endogenous bursting depends on the ionic composition of the saline.

The 'PTMA-isolated' firing pattern differs from the normal feeding pattern, in which the N1 firing rate increases steadily throughout the N1 burst and is terminated by a strong N2 input (figure 2*c*). In the PTMA-isolated N1 (figure 2*a*) the N1 firing rate is fastest in mid-burst, when the size of the action potentials is smallest. In this respect, the endogenous bursting resembles that of the parabolic bursting neurons like R15 (Benson & Adams 1989), but these neurons fire overshooting action potentials, whereas the N1 action potentials fail to propagate as more than electrotonic remnants. It would appear that somatic action potentials are not necessary for the endogenous rhythm to proceed.

(*c*) *Is the block at modulatory or pattern-generating levels?*

The interneurons in the model of the feeding system have been divided into pattern-generating neurons (N1, N2 and N3) and modulatory neurons (SO, CGC and CV1 cells). It could be that the block shown with cholinergic antagonists acts on either the modulatory or pattern-generating cells, or even on both types.

The first modulatory interneuron to be identified was the CGC cell, one in each cerebral ganglion, which contain serotonin (McCaman *et al.* 1984). Tonic activity in the CGCs is essential for rhythmic activity to continue (Benjamin & Elliott 1989). However, the firing rate remains unaffected by perfusion of the cholinergic antagonists ATR and HMT (which block excitatory receptors) and increased with the agonistic effects of PTMA (figure 7). This latter result is not surprising, because the CGCs were excited by bath ACh in normal saline (Kyriakides & McCrohan 1989). Furthermore, the cholinergic antagonists employed in this paper are not known to block serotonergic synaptic transmission in gastropod molluscs, although the same is not true of d-TC (S.-Rózsa 1984; Walker 1986). The conclusion must be that the cessation of the feeding rhythm is not due to interruption of the CGC modulation.

The CV1 cells are also located in the cerebral ganglia and modulate the feeding rhythm through excitatory connections to the N1 neurons (McCrohan 1984; McCrohan & Kyriakides 1989). Because the CV1 cells are not required for SO-driven rhythmic activity to occur, nor are they excited by N1 interneurons (McCrohan & Kyriakides 1989), it is unlikely that the cholinergic block of SO- and N1-driven rhythms are mediated by disruption of CV1 modulation.

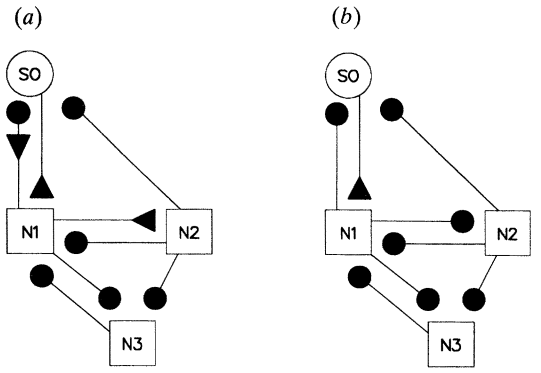

Figure 8. Comparison of the synaptic models of pattern generation in the *Lymnaea feeding system;* —●, inhibitory; —◀, excitatory. (*a*) After Elliott & Benjamin (1985*a*); (*b*) after Rose & Benjamin (1981). In each case a weak inhibitory connection from the N3 interneurons to the SO has been omitted. The pattern is generated by the interactions of the N1, N2 and N3 neurons and is modulated by input from the SO. The N1 neurons are cholinergic (Elliott & Kemenes 1992) and their excitatory output will be blocked by HMT and ATR; their inhibitory and excitatory output by PTMA (Elliott & Kemenes 1992). The experimental results favour model (*a*).

The final modulatory interneuron is the SO, which activates the feeding rhythm by excitatory connections to the N1 cells (figure 8; see Elliott & Benjamin 1985*a*). This cell has been used to drive the feeding rhythm in figures 1 and 3–6 and so the block could occur between the SO and N1 cells. In paper II, Hi–Di saline was used to show that transmission between the SO and N1 neurons is not affected by HMT, MeXCh and ATR (Elliott & Kemenes 1992). The suggestion that the SO is not cholinergic is also confirmed in figure 3, where SO stimulation produces an N1 input onto the 8 cell, so that, even though the SO does activate N1 neurons, the rhythm does not start up. It might also be possible that the antagonists block the feeding rhythm is due to the reciprocal pathway (N1→SO) which has a mixture of excitation and inhibition. However, the excitatory part of this pathway is unlikely to be very significant, because, in N1 driven rhythms there is at most one spike in the SO; the inhibitory pathway may be important in slowing the SO firing rate.

The final evidence that the block is within the pattern-generating network is that the antagonists effectively block N1 driven rhythms, in which the SO plays no role (figures 2 and 6).

(*d*) *Dissection of the pattern generator*

The discussion so far has shown that the block occurs at the level of the central pattern generator. The pharmacological dissection provides no evidence

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for additional neurons and so it is now necessary to test the hypothesis of synaptic interactions.

In the model suggested by Elliott & Benjamin (1985*a*) the alternation of protraction (N1) and retraction (N2) phases was achieved by recurrent inhibition between the N1 and N2 interneurons, so that the N1 neurons excited the N2 cells, which gradually depolarize, eventually firing a short burst and so inhibited the N1 neurons (figure 8*a*). An alternative possibility, that the N1 and N2 neurons make reciprocal inhibitory contacts was suggested by Rose & Benjamin (1981*b*) (figure 8*b*). In both models, the N1→N2 switchover should be blocked by PTMA, as this blocks inhibitory as well as excitatory cholinergic synapses. This prediction is indeed fulfilled (figures 1 and 2).

HMT and ATR, however, only block the excitatory cholinergic synapses and so if the reciprocal inhibition model is correct the feeding pattern should not be affected by these antagonists. On the other hand, if, as suggested by Elliott & Benjamin (1985*a*), the feeding sequence depends on the N1 cells exciting the N2 neurons, the prediction is different. In this case, HMT and ATR should block the feeding rhythm. This is indeed the case (figures 4–6) and figure 6 suggests that with the ATR present the N1 neuron fires for much of the time; the rhythm appears to have stopped in the N1 phase. The evidence thus favours recurrent inhibition rather than reciprocal inhibition.

When ATR or HMT is applied, it might be expected that the normal N1→N2→N3→N1 . . . sequence would be replaced by N1→N3→N1 . . ., because in both models the connection between the N1 and N3 neurons is believed to be by reciprocal inhibition (figure 8) (Elliott & Benjamin 1985*a*; Rose & Benjamin 1981*b*). This has not been observed, probably because the N1 inhibition of the N3 cells is not as strong as the N2 input (Elliott & Benjamin 1985*a*, figure 5) and may not be strong enough to elicit the post-inhibitory rebound in the N3 neurons.

These pharmacological dissection experiments have not revealed any extra neurons but have confirmed that the *Lymnaea* feeding system is constructed so that the changeover from protraction to retraction takes place by recurrent inhibition. This method of neural organization may be quite widespread among grazing molluscs, since Arshavsky *et al.* (1988) have found evidence for recurrent inhibition between the protraction and retraction phase neurons of the pulmonate snail *Planorbis corneus*. However, in the predatory pteropod, *Clione limacina*, the protractor and retractor interneurons seem to be connected by reciprocal inhibition (Arshavsky *et al.* 1989).

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