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Cholinergic Interneurons in the Feeding System of the Pond Snail Lymnaea stagnalis. II. N1 Interneurons make Cholinergic Synapses with Feeding Motoneurons

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Cholinergic interneurons in the feeding system of the pond snail *Lymnaea stagnalis*. II. N1 interneurons make cholinergic synapses with feeding motoneurons

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SUMMARY

The N1 neurons are a population of interneurons active during the protraction phase of the feeding rhythm.

All the N1 neurons are coupled by electrical synapses which persist in a high Mg/low Ca saline which blocks chemical synapses. Individual N1 spikes produce discrete electrotonic postsynaptic potentials (PSPs) in other N1 cells, but the coupling is not strong enough to ensure 1:1 firing.

Bursts of N1 spikes generate compound PSPs in the feeding motoneurons. The sign (excitation or inhibition) of the N1 input corresponds with the synaptic barrage recorded during the protraction phase.

Discrete PSPs are only resolved in a Hi–Di saline. Their variation in latency and number can be explained by variation in electrotonic propagation within the electrically coupled network of N1 cells.

The excitatory postsynaptic potentials (ESPS) in the 1 cell are reduced by 0.5 mM antagonists hexamethonium (HMT), atropine (ATR), curare (d-TC) and by methylxylocholine (MeXCh), all of which block the excitatory cholinergic receptor (Elliott *et al.* (*Phil. Trans. R. Soc. Lond.* **336,** 157–166 (Preceding paper.) (1992)). The 1 cell EPSPs were transiently blocked by phenyltrimethylammonium (PTMA), which is both an agonist and antagonist at the 1 cell excitatory acetylcholine (ACh) receptor (Elliott *et al.* 1992).

The inhibitory postsynaptic potential (IPSP) in the 3 cell is blocked by bath applications of MeXCh and PTMA, which both abolish the response of the 3 cell to ACh (Elliott *et al.* 1992).

The effects of the cholinergic antagonists on the response of 4 cluster and 5 cells to N1 stimulation matches their response to ACh (Elliott *et al.* 1992).

It is concluded that the population of N1 cells are multiaction, premotor cholinergic interneurons.

1. INTRODUCTION

This is the second paper of this series on the feeding system of the pond snail, *Lymnaea stagnalis*. The aim of these experiments is to use the technique of pharmacological dissection to test the model of the pattern generation. In this paper we shall identify which of the feeding interneurons are likely to use ACh as their transmitter and so locate the sites at which cholinergic antagonists will make lesions in the system.

During the feeding rhythm, the buccal motoneurons receive a regular sequence of three phases of synaptic input. The simplest hypothesis, suggested by Rose & Benjamin (1981*a*), is that the synaptic input originates from three kinds of pattern generating neurons. Three types of interneuron were indeed identified and called N1, N2 and N3. These interneurons fire in turn, with the N1 activity corresponding to protraction of the radula, N2 activity to rasping and N3 activity with swallowing.

In many isolated central nervous system (CNS) preparations, the feeding system is slowly active and 'fictive feeding' can be recorded. The rate of feeding may be increased by depolarization of a N1 interneuron. However, to induce feeding at the normal rate, it is normally necessary to stimulate the SO, a single modulatory interneuron in the buccal ganglia (Elliott & Benjamin 1985*b*). This activates feeding by a facilitating excitatory postsynaptic potential (EPSP) to the N1 pattern generating interneurons.

The N1 interneurons are small cells located dorsally in the buccal ganglia (Elliott & Benjamin 1985*a*; see figure 1). Intracellular recordings from the motoneurons show slow changes in membrane potential during the N1 phase of the rhythm (Rose & Benjamin 1981*b*; Elliott & Benjamin 1985*a*), but there are normally no discrete post synaptic potentials (PSPs) in the motoneurons. Stimulation of a N1 interneuron in normal saline also usually leads to smooth changes in membrane potential in the follower motoneuron. Because direct evidence for N1→motoneuron synapses has not been presented before, the first part of this paper (figures 2–6) will test the hypothesis that the N1 neurons do make monosynaptic connections with buccal motoneurons (cell types 1 to 10).

The motoneurons also respond to acetylcholine (ACh) (Elliott *et al.* 1992) and their cholinergic responses always have the same sign (excitation or inhibition) as the PSP recorded during the N1 phase of the feeding rhythm. The simplest hypothesis, tested

here (figures 7–11), is that the N1 neurons are multiaction, premotor interneurons which release ACh at all their terminals.

When the antagonists are applied for pharmacological dissection (paper III, Elliott 1992) it is important to know the precise lesions made by the antagonists. Therefore, in the final part of the paper (figures 11 and 12) it will be shown that the other buccal interneurons (SO, N2 and N3) are unlikely to use acetylcholine (ACh) as their transmitter as their synaptic PSPs survive in cholinergic antagonists.

2. METHODS

The CNS was isolated and motoneurons in the buccal ganglia were penetrated with glass microelectrodes and identified in normal saline as described in the preceding paper (Elliott *et al.* 1992). Electrodes filled with a saturated solution of Lucifer yellow in 1 M lithium sulphate were used to stain N1 neurons (figure 1*a*). Some N1 interneurons were located by probing in the buccal ganglia close to the 1 and 2 cell motoneurons. This method was used to locate the neurons shown in figures 2–6. On average, six electrode tracks in the area known to contain the N1 cells were made before an N1 neuron was penetrated.

A faster technique to find interneurons is axonal filling with 5 (6) carboxyfluorescein (5-CF) (Kemenes & Elliott 1991; Kemenes *et al.* 1991). The nerve known to contain the axon of the desired interneuron was cut, the bath drained of saline and a Vaseline dam quickly constructed around the tip of the cut nerve. A 5% solution of 5-CF (pH 7) was applied to the cut end and normal saline replaced in the rest of the bath. After 2–3 h the 5-CF had travelled up the nerve and cells could be seen under blue light. This was provided by a Schott light source, fitted with a 500 nm interference filter (Ealing optical, part 35–5297) or from a 7 mW helium cadmium laser. The intensity of the laser was set to 10 kW m^{-2}, below the level at which photoinactivation of the cells begins (Elliott & Kleindienst 1990; Kemenes *et al.* 1991). The fluorescing interneurons were impaled with potassium acetate micropipettes under the blue light. To help check that the tip of the electrode was indeed in the cell, a 0.025% solution of 5-CF in 8 M potassium acetate was used in the electrode, so that its tip glowed in the blue light.

Filling the cerebro-buccal connective revealed up to 5 cells in the contralateral buccal ganglia, of which at least one was a N1 interneuron (figure 1*b*). This technique enables the N1 neurons to be found quickly (the N1 interneuron was always impaled on the first electrode track). Equally, if movement occurs and the cell is lost, the same N1 interneuron can be reimpaled. This technique is very important for pharmacological experiments as the N1 neurons are small and without this method can be difficult to hold long enough to perfuse a range of solutions. N2 interneurons were also found by axonally filling the post-buccal nerve which contains one of their axons (Elliott & Benjamin 1985*a*).

To characterize the synaptic potentials, the ganglia were bathed in a Hi–Di saline which increases the spike threshold and so reduces polysynaptic pathways and spontaneous synaptic inputs. To block chemical synapses, the high Mg/low Ca saline was applied. Further details, including the composition of salines are given previously (Elliott *et al.* 1992).

The synaptic potentials were recorded on a digital tape recorder and played back into a chart recorder (Gould, TA550) or into the A/D input of a microcomputer interface (CED1401). Presynaptic active potentials were fed to a comparator and the TTL signal used to trigger the 'peristimulus' data collection program (CED). In figure 6, five to seven samples were collected and displayed on screen or laser printer so that the presynaptic traces overlapped, while the successive postsynaptic traces were shifted vertically to enhance the separation of the individual sweeps.

The cholinergic antagonists atropine (ATR), curare (d-TC), hexamethonium (HMT), methylxylocholine (MeXCh) or phenyltrimethylammonium (PTMA) were added to normal or Hi–Di saline at 0.5 mM. Acetylcholine is abbreviated as ACh.

3. RESULTS

(*a*) *Physiology of the N1 interneurons*

The N1 interneurons are located dorsally in each buccal ganglion (figure 1, see also Elliott & Benjamin

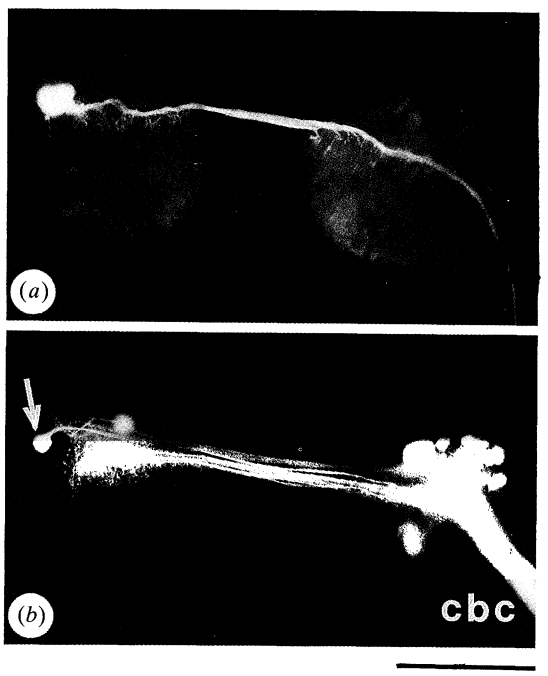

Figure 1. Location of N1 interneurons. (*a*) The cell body of a single N1 interneuron injected with lucifer yellow from a micropipette is located dorsally in the buccal ganglion, and the axon crosses through the contralateral buccal ganglion before emerging in the cerebro-buccal connective. The high concentration of lucifer yellow has caused the cell to swell slightly during filling. (*b*) A 2 h fill of the right cerebro-buccal connective shows 15 cells in focus in the ipsilateral ganglion and two in the contralateral, left, buccal ganglion. The arrowed cell was impaled and found to be a N1 interneuron; cbc, cerebro-buccal connective; anterior is towards the top of the picture and the scale bar is 200 μm.

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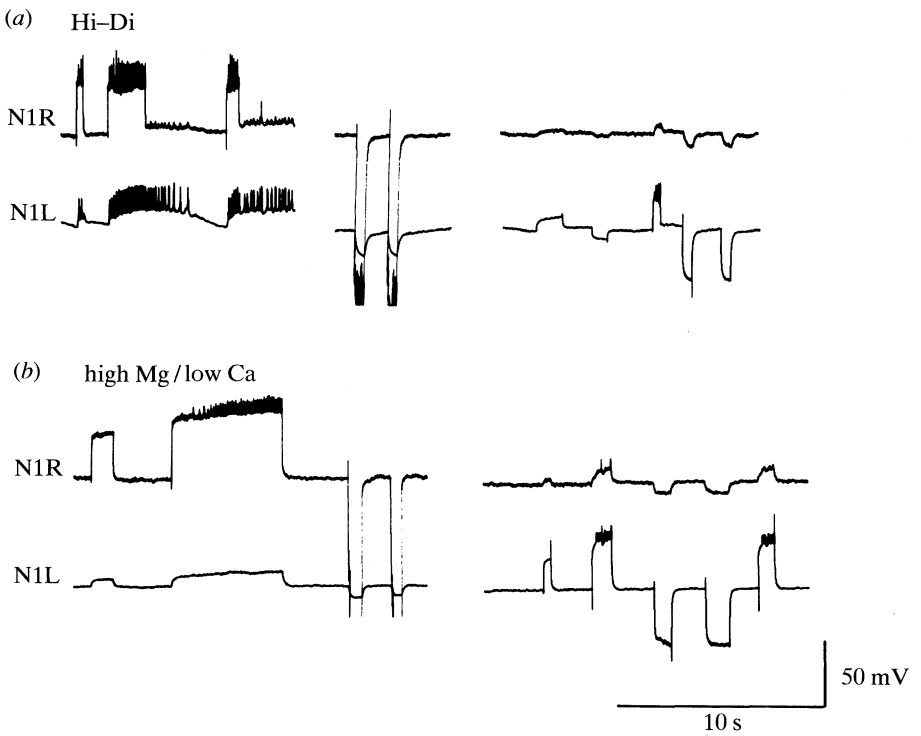

Figure 2. Electrical synapses between contralateral N1 neurons. (*a*) In Hi–Di saline, injecting positive current into the right N1 depolarizes the left cell. N1 activity continues on ('reverberates') after the end of the two longer pulses. The second excerpt show that injecting negative current into N1R hyperpolarizes the left N1 also, while the third excerpt shows that current pulses of either polarity into the left N1 affect the right N1 membrane potential. (*b*) In high Mg/low Ca saline, which blocks chemical synaptic transmission, the electrical coupling persists. In the first excerpt the current injections are into the N1R, in the second excerpt into the left N1. (Same cells as in (*a*).)

(1985*a*)). Their cell body action potentials are small (ranging from 2–40 mV) and gradually decrease in size as their firing rate increases (figure 3). These spikes are most likely to be action potentials which fail to propagate into the soma.

The suggestion (Rose & Benjamin 1981*b*) that the N1 cells are electrically coupled has been tested by applying Hi–Di or High Mg/low Ca saline. Figure 2*a* shows that, in the Hi–Di saline, injection of positive or negative current into either N1 cell depolarizes or hyperpolarizes the contralateral N1. Each spike in the right cell is associated with a spike in the left, while conversely spikes in the right only produce an electrical PSP (elPSP) in the left. In a high Mg/low Ca saline, the electrical coupling persists with responses to injection of positive and negative current (figure 2*b*). This strengthens the evidence that all the N1 cells are electrically coupled and shows that this coupling extends even between the two ganglia. However, in none of these salines is the electrical coupling strong enough to ensure that the N1 cells spike synchronously or even 1:1.

With weak depolarizing current, the N1 neurons will fire a burst of spikes during the stimulus (e.g. figure 2*a*: first stimulus), but with stronger stimuli, N1 activity persists after the end of the depolarizing current. This 'reverberation' is typical of all N1 cells. In some cases, the reverberation consists of a series of small spikes which is a reflection of action potential activity in other N1 cells (e.g. figure 2*a*: small spikes in N1R; full spikes in N1L); in other cases the action potentials continue in the penetrated cell (e.g. figure 3*f*, *g*).

Rose and Benjamin (1981*b*) suggested that the reverberation could arise from positive feedback between the N1 cells as a result of electrical coupling. Alternatively, it could arise from activation of plateau potentials (see Hartline *et al.* (1988) for a review). Figure 2 shows that reverberation is recorded in the Hi–Di saline (as well as normal saline) but no reverberation has been seen in high Mg/low Ca saline. Although the stimulus given to the N1R in figure 2*b* leads to the same firing rate as in Hi–Di, the cell immediately returns to resting potential. Neither did stronger stimuli elicit any reverberatory N1 activity. (The same result can be seen in figure 9; cf. parts *g*, *i* and *h*.)

It is therefore unlikely that the reverberation is due to the electrical coupling. Instead, it could be due to activation of calcium currents, which may be important in altering the membrane impedance.

(*b*) *N1 interneurons make synapses with motoneurons*

Rose & Benjamin (1981*b*) showed that, during feeding, the rhythmic N1 synaptic inputs to the motoneurons correlated with activity in the N1 interneurons. For the N1 cells to qualify as premotor interneurons, it must be shown that they can produce synaptic potentials in the motoneurons and that no other neurons are interposed.

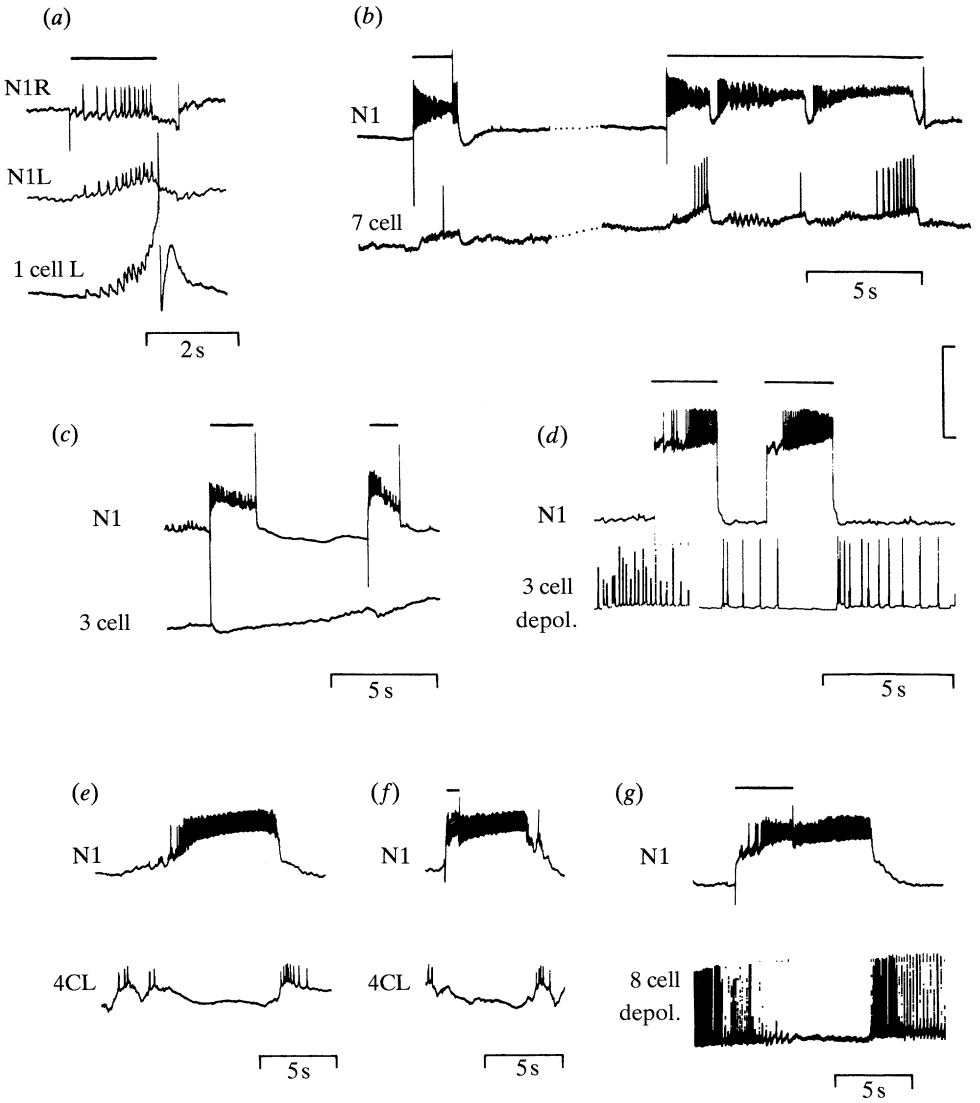

Figure 3. Synaptic effects of the N1 interneurons on motoneurons in normal saline. Depolarizing stimuli are indicated by solid bars. (*a*) Excitation of a 1 cell; note the 1:1 synaptic potentials. Scalebar: N1R, 45 mV; N1L, 90 mV; 1 cell, 13 mV. (*b*) Excitation of a 7 cell. In the first part, the N1 activity produces a depolarization of the 7 cell with a single spike. In the second part, the N1 activity is periodically interrupted by N2 and N3 inhibitory feeding inputs. Each burst in the N1 cells depolarizes the 7 cell. Scalebar: N1 and 7 cell, 45 mV. (*c*) Inhibition of a 3 cell at resting potential. Scalebar: N1, 60 mV; 3 cell, 90 mV. (*d*) Inhibition of a depolarized 3 cell by stimulation of an N1 interneuron (different preparation to (*c*)). Scalebar: N1 and 3 cell, 75 mV. (*e*) and (*f*) Spontaneous (*e*) and stimulated (*f*) N1 bursts hyperpolarize the 4 cluster cell and inhibit the small spikes, which are probably due to full size action potentials in another electrically coupled 4 cluster cell (Benjamin & Rose 1979). (*g*) Inhibition of an 8 cell which was depolarized throughout the excerpt shown. (*e–g*) Scalebar: N1, 4CL and 8 cell all 60 mV.

Figure 3, showing the results from quiescent preparations bathed in normal saline, confirms that stimulating a single N1 cell produces synaptic inputs onto the motoneurons. For example, an evoked burst of spikes in a N1 neuron produces a series of depolarizing PSPs in the contralateral 1 cell and these summate to lead to a 1 cell action potential (figure 3*a*). N1 stimulation also excites the 7 and 10 cells (figures 3*b* and 8 respectively). Other motoneurons are inhibited by N1 stimulation. Normally, the 3 cell shows continuous synaptic inputs from the N3 tonic interneurons. Because these interneurons are inhibited by N1 cells (see Elliott & Benjamin 1985), it is often hard to distinguish the direct synaptic effects of the N1 interneuron on the 3 cell from polysynaptic effects mediated by the N3 tonic interneuron. Therefore, the records in figure 3*c*, *d* were chosen as the two records with no apparent N3 tonic inputs and they show a weak inhibition of the 3 cell by N1 interneurons. The 4 cluster cells and 8 cells are also weakly inhibited by N1 activity (figure 3*e*, *f*). In normal saline, N1 stimulation usually produces smooth, compound PSPs. Discrete PSPs are rare. They are most often seen in the 1 cell, but even here they are only seen in about one third of the normal saline preparations (cf. figure 3*a* and 8).

To examine the synaptic connections in more detail, a Hi–Di saline was applied to 22 preparations in which N1 interneurons had been found. This saline raises the spike threshold by 10–15 mV (Elliott &

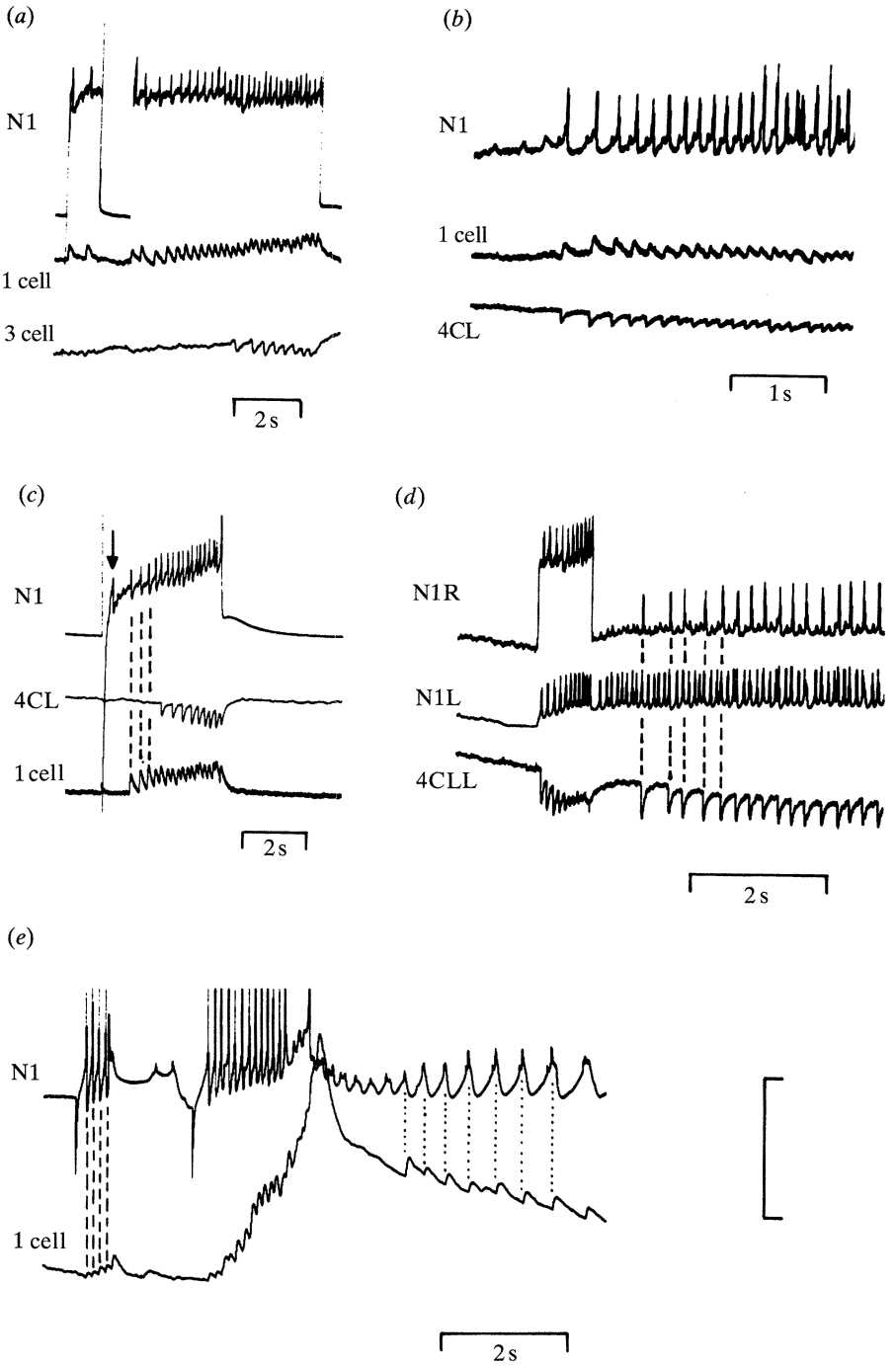

Figure 4. N1 interneurons make discrete synaptic potentials in the Hi–Di saline. (*a*) Records from a N1 cell, 1 and 3 cells showing discrete EPSPs and IPSPs in the 1 and 3 cells respectively. The 3 cell was depolarized by a spontaneous synaptic input. Note that there are many fewer 3 cell IPSPs than N1 spikes. (The decline in size of the 3 cell IPSPs at the end may be the result of the gradual hyperpolarization of the membrane or of summation.) Scalebar: N1, 60 mV; 1 cell, 20 mV; 3 cell, 4 mV. (*b*) Each N1 spike correlates with a 1 cell EPSP and 4 cluster cell (4CL) IPSP. The N1 cell is contralateral to the motoneurons. Scalebar: N1, 55 mV; 1 cell, 25 mV; 4CL, 25 mV. (*c*) Another N1 interneuron and 1 and 4 cluster cells. Note that the first N1 spike (arrow) is not associated with any PSP and that the 4 cluster cell IPSP only begins after the sixth spike. Scalebar: N1, 70 mV; 4CL, 30 mV; 1 cell, 7 mV. (*d*) Recordings from two contralateral N1 neurons with a 4 cluster cell. The 4 cluster cell IPSPs match the contralateral N1 spikes only. Scalebar: N1R and N1L, 65 mV; 4CL, 12 mV. (*e*) Intracellular recordings from a N1 neuron and a 1 cell motoneuron. Each N1 action potential is associated with a 1 cell EPSP (e.g. lines of dashes), but there are more EPSPs than N1 action potentials. The other 1 cell EPSPs correspond to spikes in the N1 cell (e.g. lines of dots). The N1 spikes are likely to be due to action potentials in other, electrically coupled, N1 cells. N1, 30 mV; 1 cell, 8 mV.

Benjamin 1989) and so reduces polysynaptic pathways and spontaneous synaptic inputs. When N1 neurons are stimulated, the feeding rhythm will still cycle (N1→N2→N3→N1 . . .), but more slowly than in normal saline.

Stimulating a N1 neuron in Hi–Di saline still excites the 1 cell and inhibits the 3 and 4 cluster cells (figure 4). In nine out of 17 Hi–Di saline preparations, discrete EPSPs were seen in the 1 cell, and discrete inhibitory postsynaptic potentials (IPSPs) were recorded in four out of 7 N1→4 cluster cell preparations. Discrete IPSPs were less common in the 3 cell with only two out of seven preparations.

In figure 4*b* each N1 spike is followed by a discrete EPSP and IPSP in the contralateral 1 and 4 cluster cells, respectively. However, this is not always the case; the discrete PSPs do not always follow 1:1. In most experiments there are more N1 action potentials than PSPs. For example, in figure 4*a*, *c*, the 3 or 4 cluster cell receives many fewer IPSPs than N1 action potentials. Although the correspondence is better in the 1 cell, even here the first N1 spike is not associated with a 1 cell EPSP (figure 4*c*: arrow).

In a few preparations, there appear to be more PSPs than N1 action potentials. For example, in figure 4*e*, the N1 is twice stimulated and each spike evokes a discrete EPSP in the 1 cell (lines of dashes). Note, however, that in the first N1 burst there are 4 spikes and at least 5 EPSPs. At the end of the record the 1 cell is still receiving EPSPs without full sized action potentials in the N1 cell. The lines of dots show that the EPSPs are correlated with small spikes in the N1 cell, which probably correspond to action potentials from other electrically coupled N1 neurons.

Evidence that this is the correct interpretation is given in figure 4*d*, where intracellular recordings from two (contralateral) N1 neurons are shown, together with a 4 cluster cell. Stimulating the right N1 excites the contralateral N1 and produces discrete IPSPs in the 4 cluster cell. Both N1L spikes and the 4 cluster cell IPSPs follow the N1R cell action potentials 1:1. However, in the reverbatory plateau which follows, the spikes in the left N1 no longer follow 1:1 and the 4 cluster cell IPSPs only match with the contralateral N1.

The N1 interneurons also inhibit the 5 cells and, in the Hi–Di saline, discrete synaptic potentials were also recorded in these cells (four preparations). Two different kinds of 5 cell were distinguished by the use of this saline: (i) the 5eli cells which there is an electrical synapse with the N1 neurons as well as an inhibitory chemical synapse and (ii) the 5i cells in which the connection was purely an inhibitory chemical synapse. Recordings from these two kinds of 5 cell in Hi–Di saline are shown in figure 5. Part (*a*) shows that injection of current of either polarity affects the membrane potential of both cells. In the last excerpt of figure 5a, the 5eli cell is already depolarized so that when the N1 interneuron fired for a longer period the 5eli firing rate slows and, after 2 s, stops firing. This leaves small electrically conducted PSPs in the 5eli cell.

The recordings in the figure 5*b–d* are from another type of 5 cell (5i) in which there is no sign of these small electrotonic spikes or of electrical coupling. Activity in the N1 cell, whether evoked by depolarizing current (figure 5*b*) or spontaneously (figure 5*c*), inhibits the 5 cell. The expanded record shows that each N1 action potential is accompanied by an IPSP (figure 5*d*: lines of dashes), but 'extra' IPSPs are recorded for each small N1 spike (lines of dots). As expected for a chemical synapse, hyperpolarizing the 5 cell reverses the IPSPs, (figure 5*e*) and there is still no sign of an electrotonic PSP.

The latencies of the discrete chemical PSPs revealed in Hi–Di saline are not constant (figure 6*a–c*). This is the case with the 1 cell EPSP, with the 4 cluster cell IPSP, and with the 5i cell reversed IPSP. The latency from the peak of the N1 action potential to the start of the PSP varies by up to 10 ms. Note that, in the case of the N1→5i cell IPSP, not only does the latency vary, but the start of the top three IPSPs is recorded 5–10 ms before the spike in the interneuron.

Figure 6*d–f* shows that the latencies for electrotonic synapses vary also. In figure 6*d* the latency of the electrotonic PSP in the 5eli cell varies by about 5 ms (compare, for example, the lower two traces). Figure 6*e*, *f* shows the PSP between the same two contralateral N1 interneurons, first in Hi–Di saline (figure 6*e*) and then in high Mg/low Ca (figure 6*f*). In both cases, the latency varies (by up to 10 ms), and some PSPs begin before the N1 action potential. The variation seen in recordings from electrically coupled cells is thus of the same order of magnitude as the variation in latency the N1→motoneuron chemical synapses.

These experiments with the Hi–Di saline show that the N1 neurons often produce short-latency, discrete synaptic potentials in the motoneurons. The variation in latency in electrical as well as chemical synapses suggests that the variation is not sufficient to indicate the presence of polysynaptic pathways.

(*c*) *Synaptic pharmacology of the N1→motoneuron synapse*

To test the hypothesis that the N1 neurons are cholinergic, intracellular recordings were made from N1 interneurons and motoneurons while the blocking agents shown to be effective by Elliott *et al.* (1992) were perfused.

The N1 interneuron excites the 1 cell (see figure 3 and 4) and this motoneuron was also excited by acetylcholine (ACh). The effect of ACh was blocked by hexamethonium (HMT), atropine (ATR), curare (d-TC) and by methylxylocholine (MeXCh). However, phenyltrimethylammonium (PTMA), is both an agonist and antagonist at the 1 cell excitatory ACh receptor (Elliott *et al.* 1992). Therefore, in eight preparations the N1 interneuron and 1 cell were recorded together and antagonists passed through the bath at 0.5 mM.

The N1→1 cell synaptic potentials are diminished by all the five cholinergic antagonists tested (figures 7, 9 and 11). A persistent block was found with HMT (seven preparations), MeXCh (six preparations), ATR (three preparations) and with d-TC (3 preparations). Recovery is complete within 5 min. The examples chosen all show discrete EPSPs which disap-

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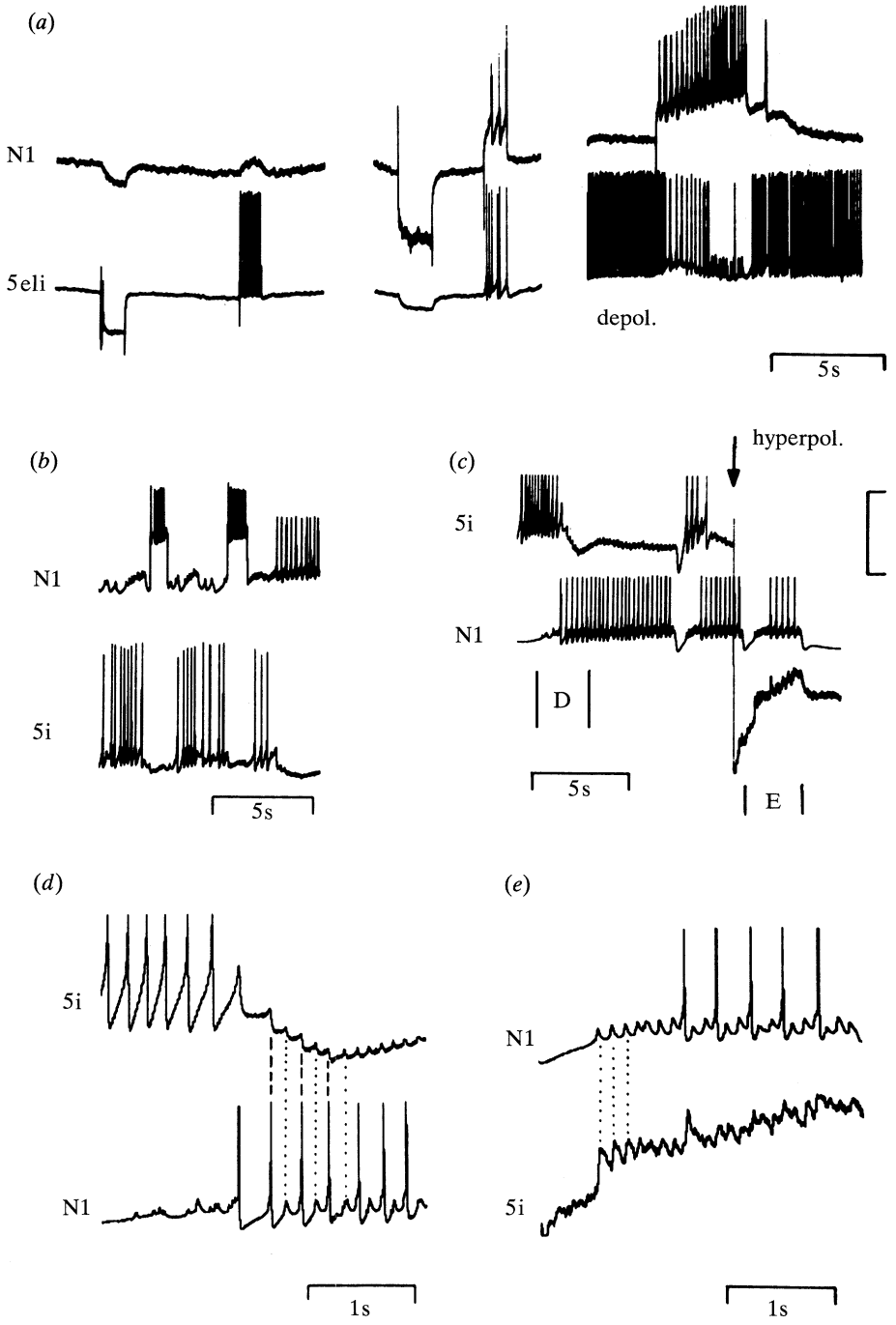

Figure 5. Connections of the N1 interneurons with 5 cell motoneurons. Some 5 cells (labelled 5eli in (*a*), cf. figure 9) are electrically coupled to N1 cells. All 5 cells are inhibited by the N1 neurons. (*a*) The first excerpt shows that negative and positive current pulses into the 5 cell affect the N1 neuron, while the reverse is also true (second excerpt). The last excerpt shows that, when the 5eli cell is already depolarized, a longer N1 stimulus inhibits the 5eli cell. Scalebars: N1 and 5eli, 50 mV. (*b–e*) Recordings from a 5i cell which is not electrically coupled to the N1 cell. N1 activity, whether induced by current injection (*b*) or spontaneous (*c*) inhibits the 5i cell. In (*c*) the 5i cell was hyperpolarized (arrow). (*d*) and (*e*) show that discrete PSPs are present, which correspond to either N1 action potentials (lines of dashes) or smaller N1 spikes (lines of dots). Hyperpolarizing the 5i cell reverses the IPSPs (*e*). Scalebars: (*b*) N1 and 5i, 60 mV; (*c*) N1, 60 mV; 5i, 30 mV; (*d*) and (*e*) N1, 30 mV 5i, 15 mV.

pear in these antagonists, but the antagonists also block transmission in three preparations in which the N1 interneuron produced a smooth depolarization in the 1 cell and where no discrete EPSP was visible.

However, the effect of the antagonist PTMA was to produce a transient reduction in the N1-evoked EPSPs. The recording shown in figure 8, shows that, 120 s after PTMA arrived, the 1 cell and 10 cell EPSPs were both reduced, with no spikes in the 10 cell. But with 300 s of PTMA perfusion, the EPSPs had grown. Note, for example, that the 10 cell is again spiking during N1 stimulation. With washout, the enlarged PSPs remain. This was confirmed in three other preparations in which discrete N1→1 cell EPSPs in Hi–Di

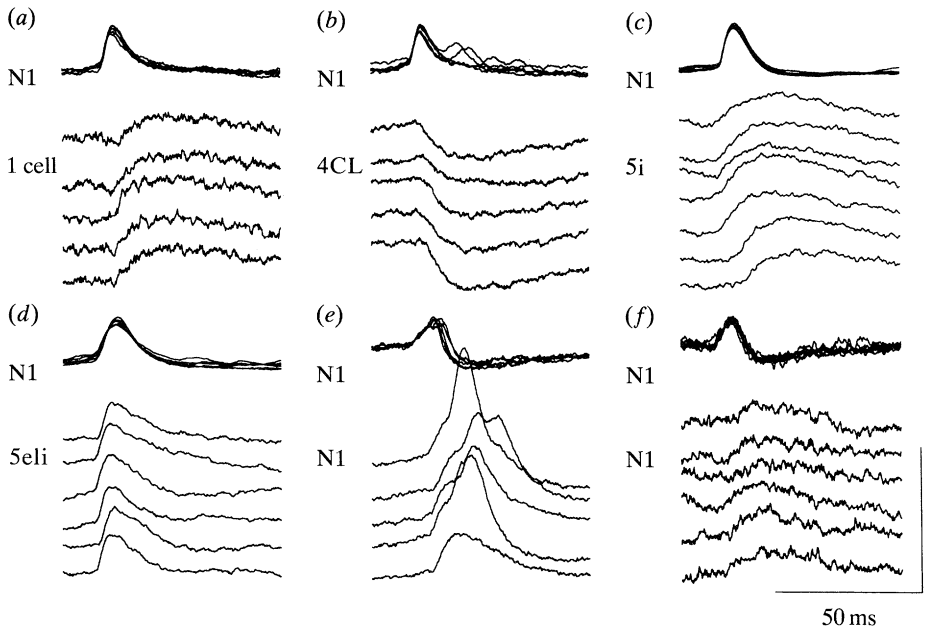

Figure 6. Variation in latency of discrete synaptic potentials evoked by N1 neurons. (*a–e*) in Hi–Di saline. (*a*) N1→1 cell EPSP; (*b*) N1→4 cluster cell IPSP; (*c*) N1→5i cell reversed IPSP; (*d*) N1→5eli cell electrical PSP; (*e*) N1→contralateral N1 electrical PSP; (*f*) N1→contralateral N1 electrical PSP in high Mg/low Ca saline which blocks chemical synapses. The pre and postsynaptic activity was captured using a microcomputer and CED1401 interface. The membrane potential of successive PSPs was shifted upwards before plotting on a laser printer. Scalebar: (*a*) N1, 60 mV; 1 cell, 20 mV; (*b*) N1, 50 mV; 4CL, 20 mV; (*c*) N1, 90 mV; 5i, 15 mV; (*d*) N1, 75 mV; 5eli, 30 mV; (*e*) N1 upper, 100 mV; N1 lower, 30 mV; (*f*) N1 upper, 33 mV; N1 lower, 3 mV.

saline were transiently reduced by PTMA perfusion. The transient phase of one experiment is shown in figure 7.

In most preparations the N1→3 cell IPSPs are too small to see clearly, probably because the resting and reversal potentials are very close; but in one of the seven Hi–Di preparations the resting potential was sufficiently negative that the N1→3 cell IPSPs had reversed and were seen as small, depolarizing events. Both the antagonists MeXCh and PTMA reduced the N1→3 cell IPSP (figure 7). At the 3 cell, these were only effective antagonists of ACh application.

Four cluster and 5 cells have two kinds of ACh receptors and only PTMA reduces the response produced by ACh irrespective of membrane potential (Elliott *et al.* 1992). The cholinergic antagonists have been tested in ten preparations with paired recordings from 4 cluster or 5 cells and a N1 interneuron.

Figure 9 shows simultaneous records from an N1 interneuron, a 5eli cell and a 1 cell. In pure Hi–Di saline, the spontaneous burst of activity in the N1 neuron inhibits the 5eli cell which also shows the usual electrical PSPs superimposed, while discrete EPSPs are recorded from the 1 cell. With HMT, d-TC or MeXCh, the EPSP in the 1 cell is much reduced (and so provides a control for the effectiveness of the antagonist), but the 5eli cell inhibition persists (figure 9*b*, *d*, *f*). With MeXCh the 5eli cell membrane potential declines more slowly than in the controls or with the other antagonists, when it reaches a plateau sooner.

In figure 9*h*, the same ganglion was perfused with a

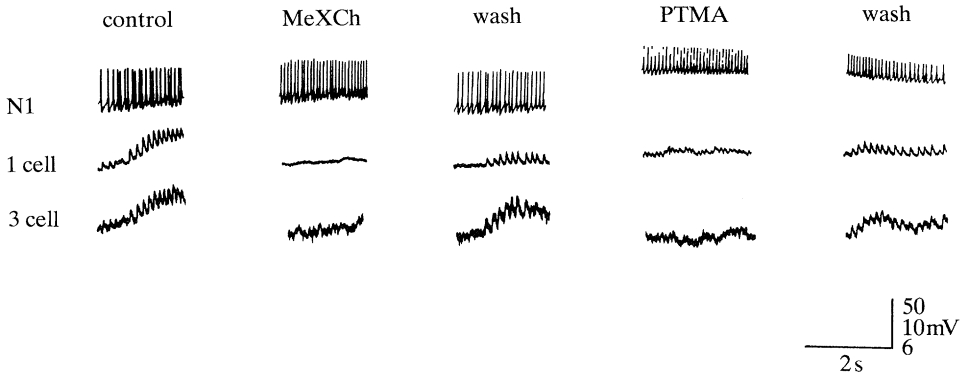

Figure 7. Pharmacology of N1→1 and 3 cell motoneuron synapses. Both MeXCh and PTMA (0.5 mM in Hi–Di saline) block the discrete synaptic potentials produced by the N1 interneuron in the 1 cell (EPSPs) and 3 cell (reversed IPSPs). Before, between and after the antagonists the preparation was washed with Hi–Di saline. The N1 was located by axonal filling with 5-CF.

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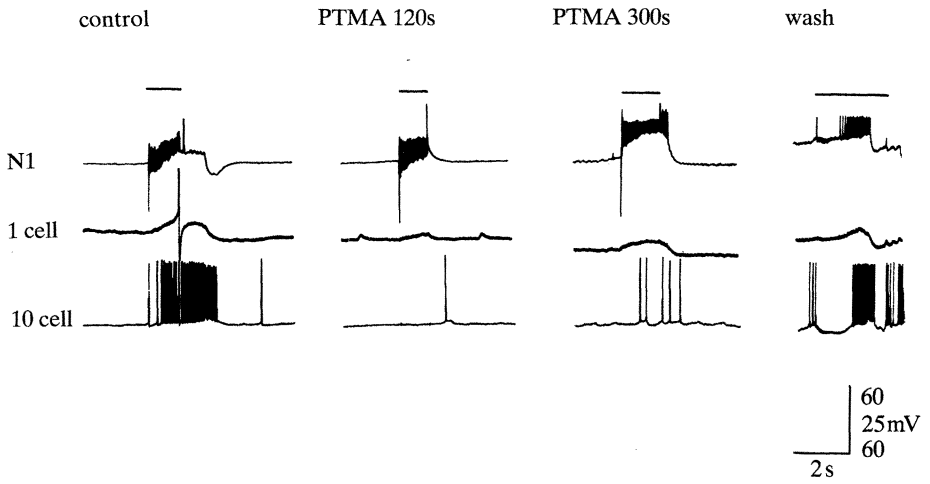

Figure 8. Transient reduction of N1-evoked EPSPs by PTMA. Intracellular recordings from a N1 interneuron, 1 and 10 cell motoneurons. In this experiment, the PTMA was dissolved at 0.5 mM in normal saline. N1 stimulated with depolarizing current as indicated by the solid bars. In the initial control panel, the N1 stimulation excites both 1 and 10 cell, but this effect is blocked by 120 s perfusion of the PTMA (second excerpt). The N1-induced excitation of the 1 and 10 cells returns with longer (300 s) perfusion of PTMA (third excerpt) and remains with wash of normal saline (last panel).

high Mg/low Ca saline to block all chemical synapses. Depolarizing the N1 neuron still produces a quick weak depolarization of the 5eli cell. As the current continues to be injected into the N1, a burst of small spikes and then larger action potentials are recorded in the 5eli cell. There is no longer any sign of the inhibitory input, or of an EPSP in the 1 cell, although these return with the wash in pure Hi–Di saline (figure 9*i*). This confirms the electrical connection between the N1 and 5eli cells and that the N1→1 cell connection is chemical and has no electrical component.

Note that, at the end of the control N1 bursts (figure 9 *a*, *c*, *e*, *g*, *i*), there is a strong, 2 s long hyperpolarization of the N1 and 5eli cells. This is typical of N2 input and it is altered in each antagonist. Then, the N1 activity seems to be terminated by shorter lasting inhibitory inputs, which are present in the 5eli cell as well as the N1 neuron. These inputs are typical of the N3 inputs (see Elliott & Benjamin 1985; Rose & Benjamin 1981*a*, *b*).

In another preparation, an N1 interneuron and a 4 cluster cell were impaled and PTMA passed. This reduced the size of the N1→4 cluster cell IPSP, but did not block it completely (figure 10). The interpretation of this result is difficult, as the PTMA has a direct hyperpolarizing effect on the 4 cluster cell and this may account for some of the reduction. The PTMA reduction of the 4 cluster IPSP was transient, as with the 1 cell.

The results in this section show that the antagonists HMT, ATR, d-TC, MeXCh and PTMA all affect the N1→motoneuron synapses as would be expected if this interneuron releases ACh as its transmitter.

(*d*) *Synaptic pharmacology of the SO, N2 and N3 interneurons*

As with the N1 experiments described above, cholinergic antagonists were perfused while recording from these three kinds of buccal interneurons and/or recording their PSPs in follower cells.

Figure 11 shows recordings from the SO, the N1 and a 1 cell in Hi–Di saline to demonstrate the effect of perfusing the antagonists HMT, MeXCh and ATR on the SO→N1 excitatory synapse. Initially, activation of the SO leads to EPSPs in the N1 cell and 1 cell. Note that the SO→N1 synaptic potentials facilitate strongly (as they do in standard saline, Rose & Benjamin (1981)). The 1 cell EPSPs do not arise directly from the SO, but rather indirectly via the network of electrically coupled N1 interneurons excited by the SO (circuit shown in figure 11*d*). As in figure 4*e*, more EPSPs are recorded in the 1 cell follower than there are spikes in the monitored N1 (or, indeed, in the SO). This emphasizes the polysynaptic nature of the SO→1 cell connection.

When any of the cholinergic antagonists is passed, the discrete 1 cell EPSPs vanish, but SO stimulation still depolarizes the N1. With HMT and MeXCh the SO takes longer to depolarize the N1 cell: this is probably because the SO is firing less quickly and the SO→N1 synapse facilitates less at lower firing rates. Because the antagonists all block the N1→1 cell connection, but not the SO→N1 connection, it seems unlikely that the SO is cholinergic. The failure of these antagonists to attenuate the SO→N1 synapse, while blocking the N1→SO synapse, was confirmed in four other preparations.

The excitatory response of the 3 cell to the N2 or N3 interneurons has been used to test the possibility that these interneurons may also be cholinergic. In four preparations, 5 min perfusion of either 0.5 mM HMT, MeXCh or ATR failed to block the excitatory inputs from the N2 and N3 interneuron to the 3 cell. Figure 12 shows results from one preparation, in which a N2 interneuron was stimulated in control Hi–Di saline and in saline containing HMT and MeXCh. This preparation was chosen for illustration because its 3 cell also shows the discrete EPSPs made by the N3

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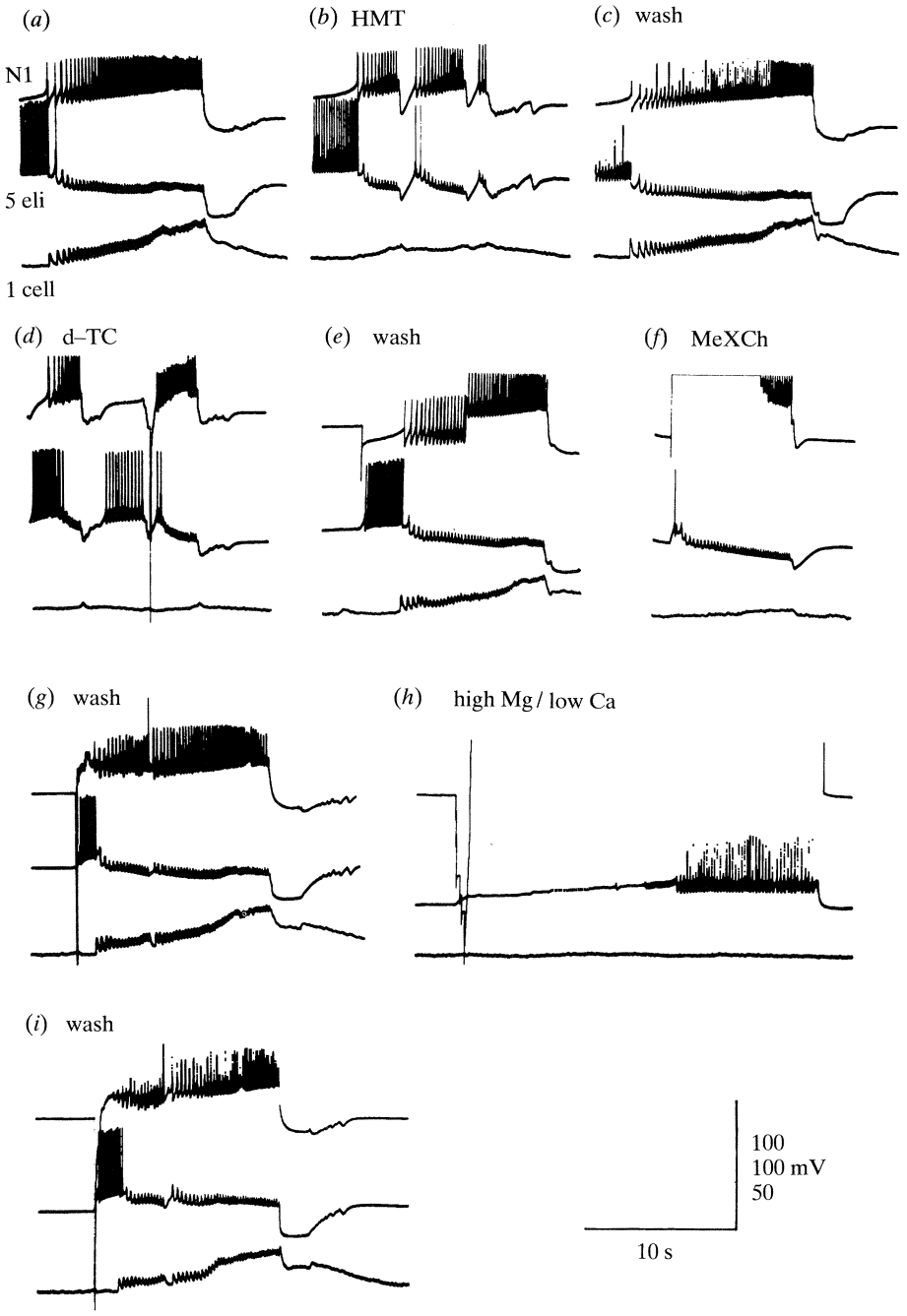


Figure 9. Pharmacology of N1→1 and 5eli cell motoneuron synapses. Records (*a*), (*c*), (*e*), (*g*), and (*i*) from Hi–Di saline. The antagonists HMT (*b*), d-TC (*d*) and MeXCh (*f*) (all at 0.5 mM) block the 1 cell EPSP but the 5eli cell is still inhibited in each cholinergic antagonist. The high Mg/low Ca saline (*h*) also blocks the 1 cell EPSP and the inhibition of the 5eli cell, but the electrical, depolarizing connection remains. The N1 activity is spontaneous or evoked by injection of depolarizing current. Throughout this experiment the DC offset was not adjusted, so the start of the 1 cell trace provides a constant reference for the membrane potential of the N1 and 5 cells.

interneuron. Both N2 and N3 EPSPs are present even in the cholinergic antagonists.

4. DISCUSSION

(*a*) *Physiology of the N1 cells*

The N1 neurons of *Lymnaea* are part of the feeding pattern generator, and are only active in the protraction phase of the feeding rhythm (Elliott & Benjamin 1985; Rose & Benjamin 1981*a*). In the cell body the spike size is less than 40 mV and decreases with firing rate. Thus the N1 cells resemble the cells B31 and B32 of *Aplysia*, (Susswein & Byrne 1988) and 'group 1' cells of *Planorbis* (Arshavsky *et al.* 1988*a*) with small action potentials. Probably, the action potential fails to propagate from its site of initiation into the ganglion and so only an electrotonic remnant is recorded in the soma. As the firing rate increases, so there is less time for the potassium channels to close before the next spike and so the impedance of the membrane drops, reducing the size of the remnant.

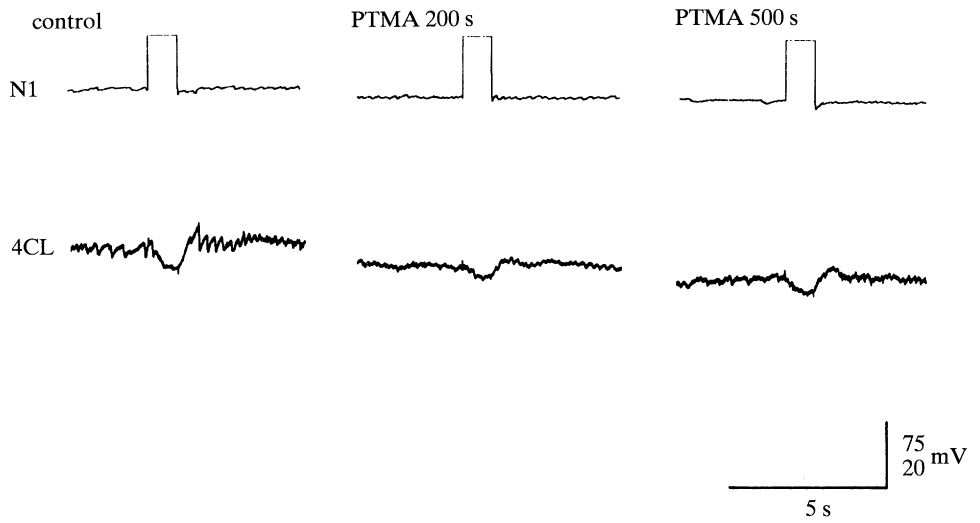
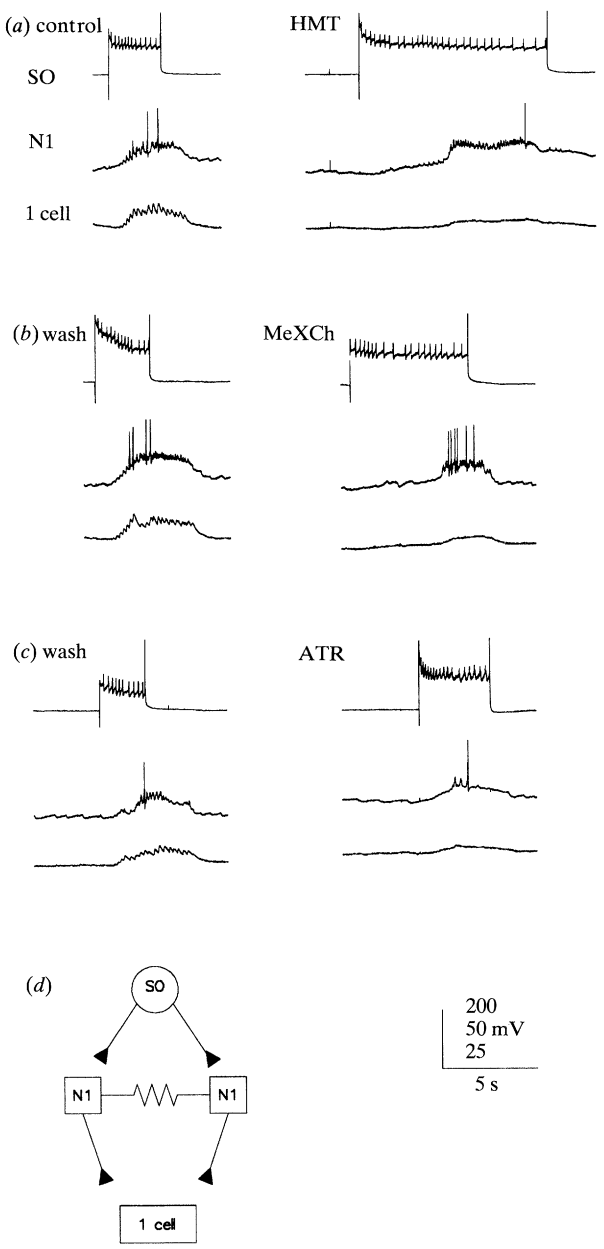


Figure 10. PTMA transiently reduces the IPSP made by the N1 interneuron on the 4 cluster motoneuron. In the control and section, Hi–Di saline was perfused, whereas the PTMA was dissolved at 0.5 mM in Hi–Di saline. The PTMA excerpts were taken 200 and 500 s after the arrival of the PTMA.



The membrane impedance may also decrease as a result of activating calcium channels since, in low calcium saline, reverberation in the N1 network is blocked (figures 2 and 9*h*) suggesting their importance in the N1 membrane. The failure of the action potential to propagate into the soma is shown below to have serious consequences for synaptic transmission from the N1 interneurons.

When a single N1 is stimulated, then the whole network of N1 cells is excited by the electrical coupling (figure 2). If the stimulus is sufficiently long, the activity will persist (or 'reverberate') after the end of the stimulus. The electrical coupling persists in Hi–Di and high Mg/low Ca salines which raise the spike threshold and block chemical synapses respectively (figure 2). However, the reverberation only persists in Hi–Di saline and not in high Mg/low Ca (figures 2 and 9*h*). This suggests that the reverberation may depend on the activation of Ca currents rather than just the electrical coupling between cells. This would be expected if the N1 neurons of *Lymnaea*, like the cyberchron cells of *Helisoma* (Merickel & Gray 1980), or group 1 neurons of *Planorbis* (Arshavsky *et al.* 1988*b*) are endogenous bursters or if they show plateau potentials.

Figure 11. The effect of cholinergic antagonists on the SO→N1 synapse, recorded with a 1 cell motoneuron in Hi–Di saline. (*a–c*) The effect of passing HMT, MeXCh or ATR at 0.5 mM is to block to discrete EPSPs in the 1 cell but leave the SO→N1 synaptic connection. In HMT and MeXCh the SO fires more slowly than in the control panels; this reduces the size of the SO→N1 EPSP because it is strongly facilitating, and so slows the activation of the N1 interneuron. All records from the same preparation, in which the N1 interneuron was located by axonial filling (Kemenes *et al.* 1991). (*d*) Diagram to show the excitatory connections between the (single) SO, the electrically coupled network of N1 cells and a 1 cell motoneuron. The filled 'synaptic triangles' represent excitatory synapses. (A weak inhibitory connection from the N1 to SO is not included.)

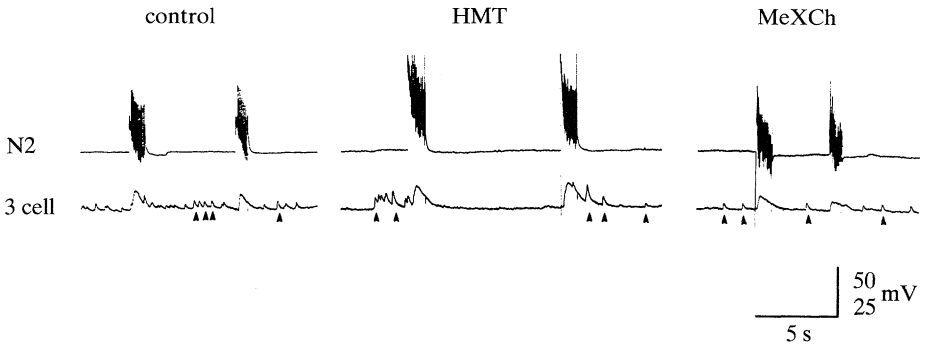


Figure 12. Cholinergic antagonists fail to block the N2 and N3 excitatory inputs to the 3 cell. In the initial, control, excerpt, the ganglion was bathed in Hi–Di saline; subsequently HMT or MeXCh (0.5 mM in Hi–Di saline) was perfused. Recordings are shown after 5 min in each saline, with a 5 min wash between the two antagonists. Twice in each panel the N2 interneuron is stimulated; this produces a slow summating EPSP in the ipsilateral 3 cell. The discrete EPSPs (some marked with an arrowhead) come from the N3 tonic interneuron described by Elliott & Benjamin (1985*a*). The persistence of N2 and N3 EPSPs in the cholinergic antagonists makes it seem unlikely that the N2 or N3 interneurons release ACh as their transmitter.

(*b*) *Physiology of the N1→motoneuron synapse*

The N1 interneurons excite some motoneurons (1, 7 and 10 cells: figures 3*a*, *b* and 8) and inhibit others (3, 4 cluster and 5 cells: figures 3*c*, *d* and 9). The sign of the N1 PSPs correspond exactly with the predictions from synaptic inputs seen during the protraction phase of the feeding rhythm. In normal saline, the synaptic potentials are usually compound PSPs and individual, discrete PSPs are rarely seen. The only exception to this is the 1 cell, where discrete EPSPs are present in about half the preparations.

In Hi–Di saline, these same synaptic inputs persist, but discrete PSPs are more common (figure 4). The Hi–Di saline raises spike thresholds by 10–15 mV and this is likely to co-ordinate firing in the N1 neurons, so that all N1 neurons, so that all N1 neurons will release their transmitter together. However, for pairs of cells in the same ganglion, the number of discrete motoneuronal PSPs does not usually match the number of N1 action potentials; the match is much better for pairs of contralateral cells (figure 4).

The discrete synaptic potentials often start during the rising phase of the action potential (figure 6*a*, *b*), although transmitter release should be greatest in the falling phase (Llinas *et al.* 1981). Furthermore, some excerpts (figure 6*c*, *d*) show that the synaptic potential can start up to 10 ms before the action potential reaches the soma. While the short latency (less than 10 ms) is of the same order as that seen in other buccal cells known to be monosynaptically connected (e.g. OM→motoneuron, Elliott & Benjamin (1989); *Aplysia*, Gardner & Kandel (1972)), the N1→motoneuron latency is much more variable (figure 6*a–c*) than in the chemical synapses seen in other buccal ganglia. A similar variation exists in the latency of the electrical synapses made by N1 cells, both in the Hi–Di and high Mg/low Ca salines (figure 6*d–f*).

These three discrepancies (lack of 1:1 PSPs; short-or negative- latency to PSP; variation in latency) can be explained as follows: the axon of the N1 cells leaves the cell body and crosses through the opposite buccal ganglion before ascending in the contralateral cerebro-buccal connective (figure 1). If the impulse is initiated at the start of this connective, then it is not surprising that 1:1 connections are strongest with contralateral cells, or that contralateral PSPs can be recorded before the spike has propagated to the N1 soma, or that the latency varies with changes in the electrotonic properties of the intraganglionic axon.

(*c*) *Pharmacology of the N1→motoneuron synapse*

The hypothesis examined in this section is that the N1 neurons release ACh as their transmitter. In figures 7, 9 and 11 the discrete EPSPs in the 1 cell were substantially reduced by four cholinergic antagonists: hexamethonium (HMT), curare (d-TC), methylxylocholine (MeXCh), atropine (ATR) exactly as would be expected from their blocking effect on ACh iontophoresis onto this cell (Elliott *et al.* 1992). At the excitatory ACh receptor, PTMA is both an agonist and antagonist (Elliott *et al.* 1992) and so, if the N1 cells release ACh it is not surprising that the compound excitatory synaptic potentials produced by the N1 interneurons in the 1 and 10 cell are only transiently reduced by PTMA (figure 8).

At the 3 cell, PTMA and MeXCh both block the effects of ACh and in this case, without any agonist effects. At the N1→3 cell synapse, both these antagonists block the IPSPs (figure 5).

The other cell where the effect of cholinergic antagonists has been investigated is the 5 cell. All 5 cells have two kinds of inhibitory cholinergic receptor (fast, d-TC sensitive and slow MeXCh sensitive; see Elliott *et al.* 1992). It might be that the N1 neurons make contact with only one of these kinds of receptor. However, application of either d-TC or MeXCh fails to completely block the N1-induced inhibition of the 5eli cell (figure 9*d*, *f*) suggesting that the N1 neurons make contact with both kinds of receptor. (This result was also found with the 5i cell.) Because neither kind of inhibitory receptor is affected by HMT (Elliott *et al.* 1992), it is not surprising that this antagonist does not block the N1→5 cell inhibition.

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The administration of cholinergic antagonists reduced and virtually eliminated the discrete 1 cell EPSP (figures 7, 9 and 11), suggesting that co-transmission is not involved in the discrete EPSPs. However, each of these antagonists left a small, graded residual excitatory component in the 1 cell. This component was not seen when chemical synaptic transmission was completely blocked by high Mg/low Ca saline (figure 9*h*). The residue may be due to only partial blockage of ACh release from uncoordinated firing in other N1 cells, but it could also be due to release of a co-transmitter.

(*d*) *Are the N1 premotor, multiaction, cholinergic interneurons?*

The first three experiments presented in this paper aim to approach the question: are the N1 interneurons sufficient to account for all the synaptic input to the motoneurons in the protraction phase? The evidence shows that, when the N1 activity is synchronized, by applying the Hi–Di saline, discrete PSPs are recorded in all the motoneurons. Their sign (excitation or inhibition) corresponds with that of the cholinergic responses as well as the synaptic inputs seen in the protraction phase of the feeding rhythm. No interposed interneuron (NX) is required to account for the variation in latency of the discrete potentials, because the latency of even the electrical PSPs varies just as much as the EPSPs and IPSPs. Indeed, such a NX neuron can really be eliminated by the observation that some PSPs begin before the spike reaches the N1 soma. An alternative possibility, that the N1 neurons are merely followers of other cholinergic neurons (N0), was suggested by the negative latencies and the observation that N1 cells are excited by ACh (Elliott *et al.* 1992). However, this hypothesis can also be eliminated because stimulation of N1 neurons produces the same cholinergic PSPs: any N0 neuron would have to be electrically connected to the N1 cells.

The principle that any one cell can only respond to a neurotransmitter in one way (Segal & Koester 1982), combined with the observation that the N1 neurons are cholinergic means that the other pattern-generating neurons are unlikely to be cholinergic. For the actions of the N2 and N3 interneurons on 3 and 7 cells (for example) are opposite to the cholinergic action (table 3 of Elliott *et al.* 1992). The same argument applies to the modulatory OM and SO cells, whose actions oppose at least some of the N1 inputs (Elliott & Benjamin 1985, 1989). Figures 11 and 12 substantiate this hypothesis, as the SO→N1, N2→3 cell and N3→3 cell excitatory synaptic potentials remain when cholinergic antagonists known to be effective in *Lymnaea* are passed through the bath.

The conclusion that the N1 interneurons are indeed premotor, multiaction interneurons, as well as part of the pattern generating network, means that predictions can be made as to the effect of pharmacological dissection of the central pattern generator. For example, the cholinergic antagonists HMT and ATR should block excitatory output from the N1 cells to other neurons within the pattern generator (e.g. N1→N2, see Elliott & Benjamin 1985), which might lead to alternation of N1 and N3 phases. Again, PTMA should block the inhibitory output (N1→N3) as well as the excitatory output and so confine the rhythm to N1 cells. These predictions will be tested in the next paper (Elliott 1992).

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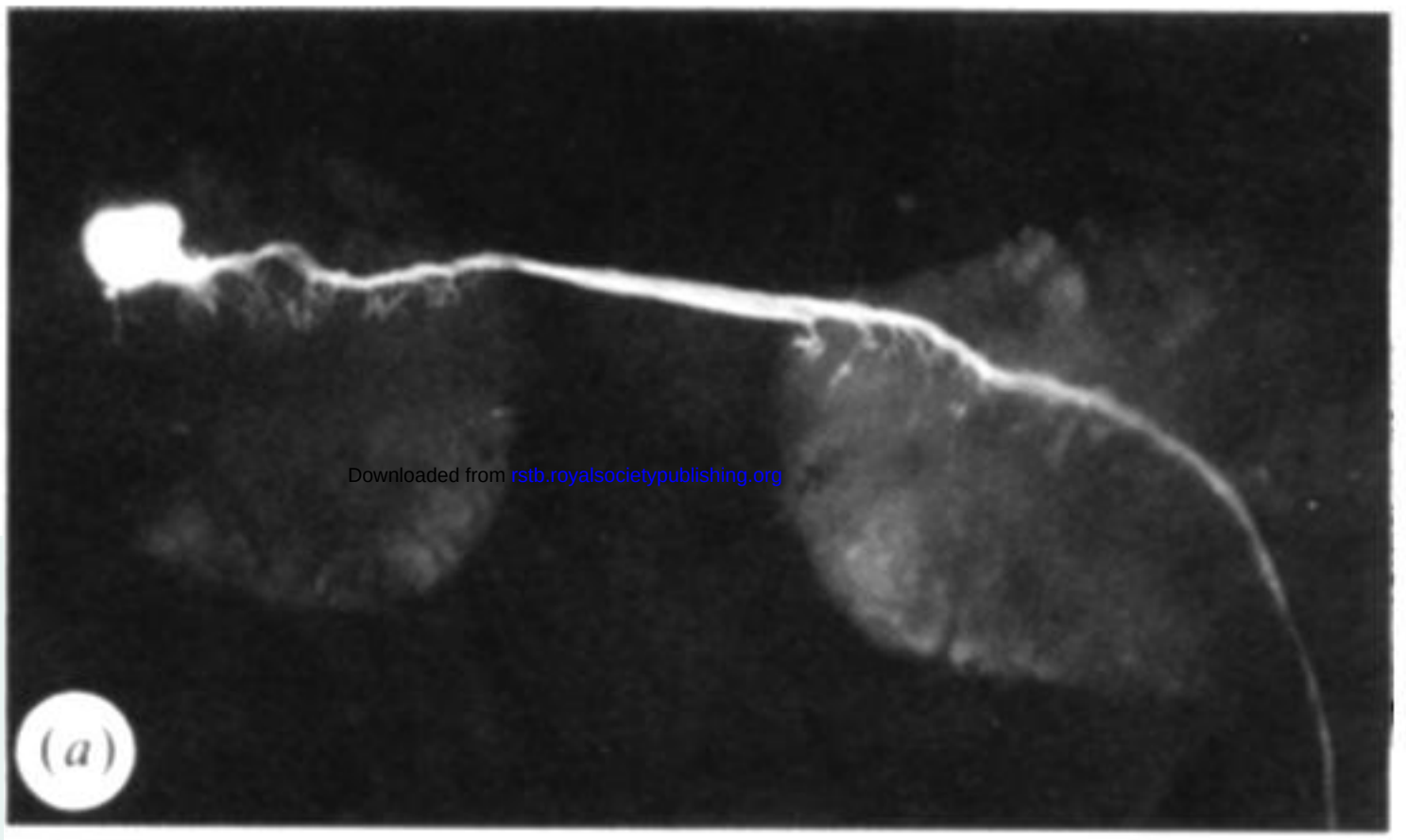

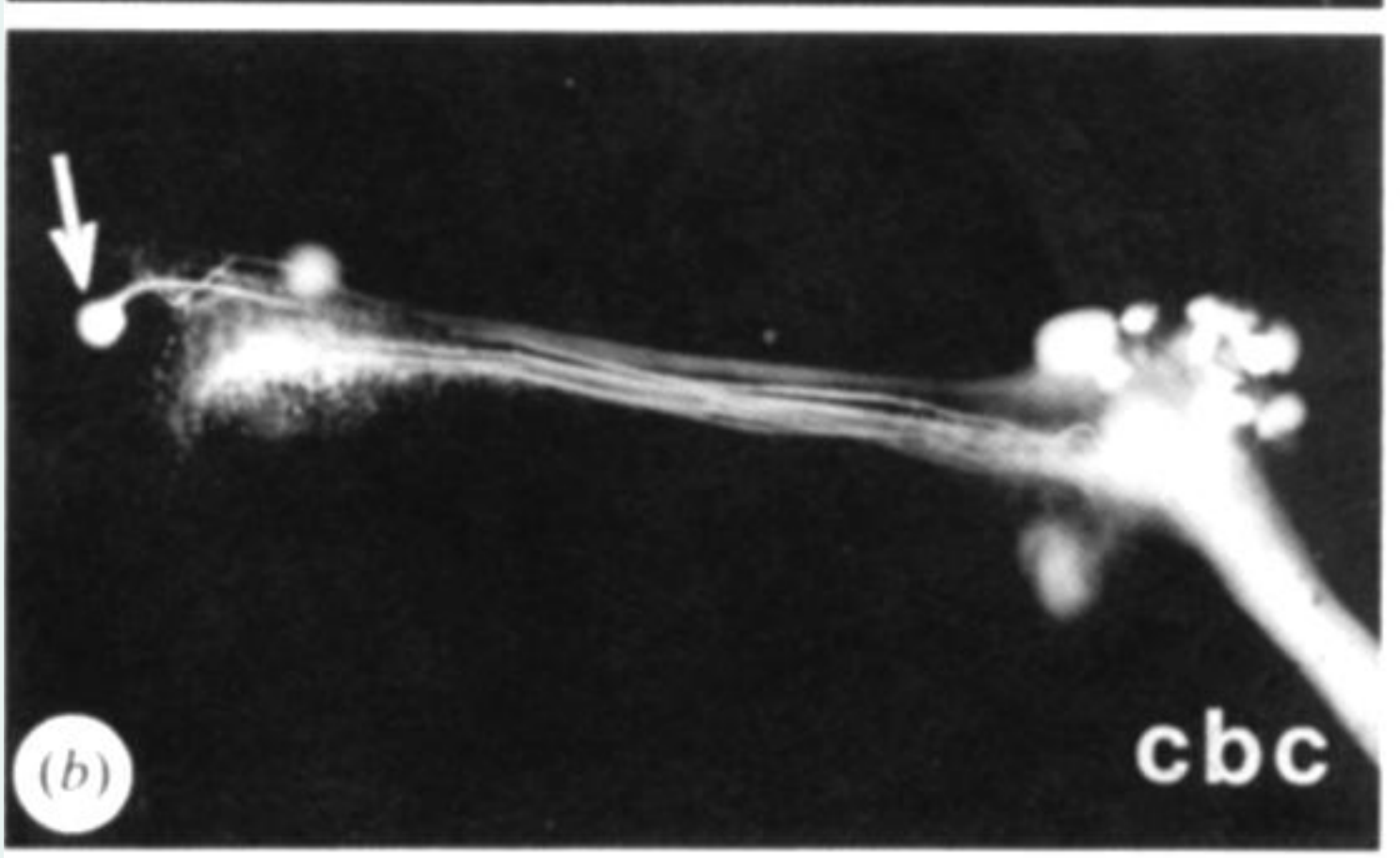

gure 1. Location of N1 interneurons. (*a*) The cell body of a gle N1 interneuron injected with lucifer yellow from a cropipette is located dorsally in the buccal ganglion, and : axon crosses through the contralateral buccal ganglion fore emerging in the cerebro-buccal connective. The high ncentration of lucifer yellow has caused the cell to swell ghtly during filling. (*b*) A 2 h fill of the right cerebro-ccal connective shows 15 cells in focus in the ipsilateral nglion and two in the contralateral, left, buccal ganglion. ie arrowed cell was impaled and found to be a N1 erneuron; cbc, cerebro-buccal connective; anterior is vards the top of the picture and the scale bar is 200 μm.