Glutamatergic N2v Cells Are Central Pattern Generator Interneurons of the Lymnaea Feeding System: New Model for Rhythm Generation

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Brierley, M. J., M. S. Yeoman, and P. R. Benjamin. Glutamatergic N2v cells are central pattern generator interneurons of the Lymnaea feeding system: new model for rhythm generation. J. Neurophysiol. 78: 3396–3407, 1997. We aimed to show that the paired N2v (N2 ventral) plateauing cells of the buccal ganglia are important central pattern generator (CPG) interneurons of the Lymnaea feeding system. N2v plateauing is phase-locked to the rest of the CPG network in a slow oscillator (SO)-driven fictive feeding rhythm. The phase of the rhythm is reset by artificially evoked N2v bursts, a characteristic of CPG neurons. N2v cells have extensive input and output synaptic connections with the rest of the CPG network and the modulatory SO cell and cerebral giant cells (CGCs). Synaptic input from the protraction phase interneurons N1M (excitatory), N1L (inhibitory), and SO (inhibitory-excitatory) are likely to contribute to a ramp-shaped prepotential that triggers the N2v plateau. The prepotential has a highly complex waveform due to progressive changes in the amplitude of the component synaptic potentials. Most significant is the facilitation of the excitatory component of the SO → N2v monosynaptic connection. None of the other CPG interneurons has the appropriate input synaptic connections to terminate the N2v plateaus. The modulatory function of acetylcholine (ACh), the transmitter of the SO and N1M/N1Ls, was examined. Focal application of ACh (50-ms pulses) onto the N2v cells reproduced the SO → N2v biphasic synaptic response but also induced long-term plateauing (20–60 s). N2d cells show no endogenous ability to plateau, but this can be induced by focal applications of ACh. The N2v cells inhibit the N3 tonic (N3t) but not the N3 phasic (N3p) CPG interneurons. The N2v → N3t inhibitory synaptic connection is important in timing N3t activity. The N3t cells recover from this inhibition and fire during the swallow phase of the feeding pattern. Feedback N2v inhibition to the SO, N1L protraction phase interneurons prevents them firing during the retraction phase of the feeding cycle. The N2v → N1M synaptic connection was weak and only found in 50% of preparations. A weak N2v → CGC inhibitory connection prevents the CGCs firing during the rasp (N2) phase of the feeding cycle. These data allowed a new model for the Lymnaea feeding CPG to be proposed. This emphasizes that each of the six types of CPG interneuron has a unique set of synaptic connections, all of which contribute to the generation of a full CPG pattern.

INTRODUCTION

The N2v cells are rhythmically active plateauing interneurons forming part of the network controlling feeding in the snail, Lymnaea. They have complex synaptic connections with feeding motor neurons (Brierley et al. 1997a) that appear to be mediated by glutamate (Brierley et al. 1997b). Three types of evidence suggest that they may be part of the feeding central pattern generator (CPG) (Brierley et al. 1997a): they are driven by the modulatory interneuron called the slow oscillator (SO) and fire during the N2/rasp phase of the feeding rhythm; they are coupled electrotonically to other retraction phases CPG interneurons the N2d (dorsal) cells; and they have a pattern of synaptic connections with motor neurons, suggesting that, with the N2ds, they control the rasp-swallow phases of the feeding cycle involved in food ingestion movements. The work discussed in this paper will provide further and more conclusive evidence for the role of the N2vs in the Lymnaea feeding CPG by describing their synaptic connections with other interneurons of the feeding network.

Lymnaea feeding interneurons divide into two types, the N cells of the CPG itself and modulatory neurons like the slow oscillator (SO) and the serotoninergic cerebral giant cells (CGCs), equivalent to the metacerebral giant cells of other mollusks (e.g., Weiss et al. 1978). So, far, five types of CPG interneurons have been described, the N1Ms (N1 medials) and N1Ls (N1 laterals), the N2d (dorsals), and the N3t (tonics) and N3p (phasics). We will argue that the N2vs are a sixth member of the CPG network and that they are the principal element active during the rasp phase of the feeding cycle. A new model of the Lymnaea CPG network will be presented to account for activity in all six cell types of CPG interneuron. It will reveal a pattern of synaptic connectivity of remarkable richness with the duration and strength of synaptic connections playing an important role in determining the timing of different phases of the feeding pattern.

METHODS

Previously described methods were used to carry out multiple simultaneous intracellular recordings from interneurons and motor neurons of the Lymnaea feeding network (Brierley et al. 1997a; Yeoman et al. 1994a). The twisted buccal ganglion preparation was used to simultaneously record from N2v on the ventral surface and different combinations of the SO, N1M, N1L, N3t, N3p, and N2d interneurons on the dorsal surface of the buccal ganglia (Fig. 1). The only nonbuccal CNS recorded were the CGCs. These are a symmetrical pair of uniquely identifiable giant neurons (90- to 120-μm diam) located on the medial surfaces of the anterior lobes of the cerebral ganglia (McCrohan and Benjamin 1980). They can be identified easily by visual observation. N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid–buffered normal saline (Benjamin and Winlow 1981) or, alternatively, high Mg2+/high Ca2+ (HiDi) saline to raise the threshold for polysynaptic connections (Elliott and Benjamin 1989), were perfused continuously over the preparation. Acetylcholine was applied focally to the surface of the N2v or N2d cells by pressure pipette while continuously bath perfusing the preparation with normal saline.
other members of the CPG network are also important. These but further synaptic connections that the N2v makes with partly responsible for the resetting ability of the N2v cell, (see further text). This would have been arrow) due to an inhibitory synaptic connection from the spikes caused a hyperpolarization in the SO (upward sloping

It was obvious that the "extra" evoked burst of N2v cell occurring in Fig. 2 (a common feature of SO-driven rhythms). This delayed the onset of the next SO-driven fictive feeding was generated by steady depolarization of the N2v. The plateau appeared to be generated endogenously as it persisted when chemical synaptic connections were blocked (Brierley et al. 1997a). During the N3 phase of the cycle (N3 IPSPs on the SO and excitatory postsynaptic potentials (EPSPs) on the B3), there were no clear PSPs on the N2v that corresponded with the N3t (N3 tonic) input to the B3 cell. This suggested that the falling phase of the plateau was due to an intrinsic property of the N2v. This was supported by evidence reported in Brierley et al. (1997a) showing that the N2v plateau, including the falling phase, still occurred when all (chemical) synaptic input was blocked in high Mg2+/zero Ca2+ saline.

These data suggested that the N2vs were hyperpolarized by the SO during the late N3 phase and N1/protraction phase of the fictive feeding cycle. They also were depolarized during the N1 phase, which could be due to N1 and/or SO inputs, but appeared to receive no synaptic input from the N3 swallow phase CPG interneurons. These predicted patterns of synaptic connections were tested by direct paired recordings between the N2vs and the other interneuronal types.

**RESULTS**

**Resetting of the feeding CPG by the N2v cells**

One type of evidence that would indicate that the N2v cells were part of the CPG would be if artificial activation could reset the rhythm in the rest of the CPG network. An example of five such experiments is shown in Fig. 2. Here fictive feeding was generated by steady depolarization of the SO. This produced bursting in the SO itself and phase-locked activity in the N2v cell. Bursts of N2v spikes coincided with large hyperpolarizing inputs on the SO, indicating, on the basis of previous analysis (Rose and Benjamin 1979) that bursts of spikes on the N2v occur during the N2/rasp phase of the feeding cycle. Once the regular feeding pattern was generated, an extra burst of activity was evoked by current injection in the N2v during the interburst interval of the ongoing rhythm. This delayed the onset of the next SO-driven N2v burst and subsequent bursts in both cells (Fig. 2). This sudden delay was greater than would have been predicted by the progressive slowing of the rhythm that also was occurring in Fig. 2 (a common feature of SO-driven rhythms). It was obvious that the "extra" evoked burst of N2v cell spikes caused a hyperpolarization in the SO (upward sloping arrow) due to an inhibitory synaptic connection from the N2v cell to the SO (see further text). This would have been partly responsible for the resetting ability of the N2v cell, but further synaptic connections that the N2v makes with other members of the CPG network are also important. These could include the electrotonic coupling with the N2d cells (Brierley et al. 1997a).

**Synaptic inputs to the N2vs**

Based on a previous analysis of the feeding system (Elliott and Benjamin 1985a,b; Yeoman et al. 1993, 1995), examination of the waveforms and timing of synaptic inputs to the N2v cells enabled predictions to be made about the likely interneurons providing these inputs. During a SO-driven fictive feeding rhythm, the N2v plateaus were triggered by a gradual depolarizing prepotential (Fig. 3). This prepotential was highly complex, consisting of depolarizing and hyperpolarizing components, likely to be originating from several types of presynaptic interneurons.

At the start of SO spike activity (Fig. 3) the N2v received small unitary inhibitory postsynaptic potentials (IPSPs) that appeared to be 1:1 with SO action potentials (confirmed in Fig. 4). At the beginning of N1/P (protraction) phase (shallow smooth hyperpolarizing input on B3) of the cycle, the N2v began to slowly depolarize, and the plateau was triggered at the end of this phase. However, superimposed on the underlying depolarizing waveform (ramp shaped) were unitary IPSPs that were again 1:1 with the SO spikes. Despite their large amplitude, these IPSPs did not prevent the triggering of the plateau in the N2v. The plateau appeared to be generated endogenously as it persisted when chemical synaptic connections were blocked (Brierley et al. 1997a). The SO-driven fictive feeding rhythm, the N2v plateaus were triggered by a gradual depolarizing wave on the SO (upward sloping arrow). This delays the occurrence of subsequent bursts in the both the recorded cells. This delay is greater than would be expected by the gradual slowing of the rhythm that also was occurring.

**FIG. 3**. Current injection into the slow oscillator (SO) drives a fictive feeding rhythm in the N2v cells. Bursts of N2v spikes occur at the same time as large hyperpolarizing waves in the SO. This is defined as the N2/rasp phase of the feeding rhythm based on previous behavioral analysis. A short depolarizing pulse evokes an "extra" burst of spikes in the N2v cell (electrode unbalanced) and a corresponding hyperpolarizing wave in the SO (upward sloping arrows). This delays the occurrence of subsequent bursts in the both the recorded cells. This delay is greater than would be expected by the gradual slowing of the rhythm that also was occurring.

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SO \rightarrow N2v

Experiments of the type shown in Fig. 4 (n = 6) showed that the SO had striking but variable amplitude biphasic synaptic effects on the N2v cells. The preparation had been bathed in HiDi saline for 20 min to block any polysynaptic pathway. Stimulation of the SO therefore revealed its postsynaptic effects in isolation from any other pathway and produced a unitary, presumably monosynaptic, potential. Examination of the response to a train of SO spikes (Fig. 4A) showed that the biphasic PSP consisted of an initial faster hyperpolarizing component, followed by a slower depolarizing waveform. This second depolarizing component only became prominent after five or more SO spikes (Fig. 4A). The PSPs in the N2v followed 1:1 with a constant latency (10 ms), reinforcing the evidence for a monosynaptic connection (Fig. 4B, i and ii). Both the hyperpolarizing and depolarizing components of the SO \rightarrow N2v PSP facilitated with repetitive firing of the SO (Fig. 4A). During the first five SO spikes, the amplitude of the hyperpolarizing component increased most rapidly (Fig. 4Bi) with little increase in the second depolarizing component obvious until the fifth spike of the SO train. After the fifth or sixth spike, the depolarization became increasingly important, causing an overall depolarization of the N2v. After \sim 12 SO spikes, the depolarizing component reached a maximum amplitude and remained at this level (Fig. 4Bii).

This SO \rightarrow N2v biphasic synaptic connection appeared to contribute significantly to the N2v prepotential during the SO-driven rhythm shown in Fig. 3. It is responsible for the unitary IPSPs seen during the late N3 phase (SO), the large IPSPs, and the gradually depolarizing waveform occurring during the P phase (N1 + SO) of the N2v feeding cycle. The facilitating depolarizing component of the SO \rightarrow N2v response is likely to be particularly important in generating the ramp-shaped depolarization of the N2v prepotential. The progressive increase in this depolarization due to a train of SO spikes is clear in Fig. 4A.

N1M \rightarrow N2v

We tested the hypothesis that the N1M cells provide a depolarizing component of the prepotential that triggers N2v plateaux by recording N1Ms with N2v cells in the twisted preparation (Fig. 5B, n = 6). This was most instructive in preparations where slow spontaneous fictive feeding rhythms occur in the absence of SO activity. In the example shown in Fig. 5A, a slow depolarizing wave (1) preceded in the N2v plateaux in the absence of inhibitory synaptic potentials, indicating that the SO was inactive. This was important because it suggested that the N1Ms may be generating the depolarizing potential in the N2v alone. The record in Fig. 5Bi showed that the depolarization of the N2v started exactly at the moment when the N1M began to fire. In these spontaneous patterns, the duration of N1M firing is much longer...
than in SO-driven rhythms (Elliott and Benjamin 1985b), increasing the period of each feeding cycle. Under these circumstances, fluctuations in firing rate of the N1M often occurred and these were accompanied by corresponding peaks in the depolarizing waveform of the N2v. Eventually the plateau was triggered followed by a cessation of N1M firing. More direct evidence that the N1Ms are connected synaptically to the N2v cells comes from the experiment shown in Fig. 5Bii where driving the N1M by current injection depolarized the N2v. Again fluctuations in firing rate (indicated by a reduction in spike amplitude) caused corresponding changes in the compound PSP waveform. We were never able to see unitary EPSPs in the N2v, relatively few spikes during the N1 phase compared with the N1Ms, they still could be contributing to the complex synaptic input the N2v receives during this protraction phase of the feeding cycle (Fig. 3B). Simultaneous recording of the N1Ls with the N2v cells (n = 4) in the twisted preparation show that they inhibited the N2v cells, the opposite to the N1Ms. Evoking a burst of spikes in the N1L generated an inhibitory wave on the N2v (Fig. 6B). This was followed by a delayed depolarization and bursts of spikes in the N2v. We have no evidence that this excitation is due to direct N1L→N2v effects because detailed examination of the PSPs after each N1L spike never showed a depolarizing component to the response (Fig. 6, C and D). It is more likely to be due to the strong N1L→N1M excitatory synaptic connection described by Yeoman et al. (1995) and therefore an indirect effect due to the N1L→N1M→N2v pathway, where the N1Ms rather than the N1L cells excited the N2v (Fig. 5).

Examining the specific N1L→N2v response, when the N1L was made to fire by current injection at more physiological rates (Fig. 6C), showed that each N1L spike was followed by a unitary IPSP in the N2v. These were of constant latency (5 ms in Fig. 6D) and appeared to be monosynaptically produced. The interesting feature of the IPSPs evoked by a train of N1L spikes was that, after an initial facilitation, they gradually reduced in amplitude as the train progressed (Fig. 6, C3 and D4), indicating synaptic depression. No change in membrane potential (e.g., long-term hyperpolarization) could account for this reduction in IPSP amplitude (dashed line in Fig. 6C, N2v trace). This gradual decrease in IPSP amplitude was the opposite to the facilitatory nature of the SO→N2v biphasic synaptic connection.

It has proved technically impossible to record N2v, N1L, and SO (all three small interneurons) at the same time to produce a SO-driven rhythm, so the likely effects of the N1L→N2v synaptic input only could be assessed indirectly on the basis of their firing pattern and synaptic potentials recorded on the N2v. The N1Ls typically fired three to five spikes in a SO-driven rhythm (Fig. 6A) and were likely to contribute, with the SO, to the IPSPs occurring during the P (protraction) phase of complex synaptic input received by the N2v and shown in Fig. 3.

It seemed possible that the N1Ls were providing a second inhibitory component to the N2v cells during the N1/protraction phase so that the complex waveform of the prepotentials (ramp shaped), which triggered the N2v plateau (Fig. 3), was presumably due to a combination of excitatory (N1M), inhibitory (N1L), and inhibitory/excitatory (SO) synaptic inputs. Despite the inhibitory component of the prepotential, the combined excitatory effects of the SO and
of the SO in this example increased the amplitude of the plateau with associated spiking.

The lack of activity of other components of the CPG in the preparation shown in Fig. 7, A and B, suggested that ACh was capable of producing these long-term modulatory effects on the N2v without effects on other elements of the CPG. All other CPG neurons or modulatory elements such as the SO are on the opposite dorsal surfaces of the buccal ganglia and could not have been affected by focal application of the ACh onto the ventrally located N2vs. The pipette concentration of ACh used was high (10^{-2} M) and so was probably producing an exaggerated effect, but the actual concentration of ACh reaching the receptors was thought to be lower (probably 1–2 orders of magnitude less) under the constant perfusion conditions used here (Walden et al. 1988).

It was interesting that focal application of 10^{-4} M ACh produced similar biphasic (hyperpolarizing followed by depolarizing) responses on the other type of N2 cell, the N2d cells (Fig. 7D, n = 3). Again prolonged depolarizing responses were seen (Fig. 7D) with plateau potentials repeatedly triggered, like the N2vs (Fig. 7C). As these experiments were carried out in high Mg^{2+}/zero Ca^{2+} saline to block chemical synapses, the effects of ACh must be a direct effect on the N2d cells. It appeared that ACh is capable of producing modulatory effects on both types of N2 cells and

Mimicking the SO/N1M synaptic inputs to the N2v by acetylcholine application produced long-lasting modulatory effects

Considerable evidence indicated that acetylcholine (ACh) was the main transmitter of both the SO (Yeoman et al. 1993) and the N1 cells (N1Ms: Elliott and Kemenes 1992; N1Ls: Vehovszky and Elliott 1995). These data predict that application of ACh onto the N2vs should evoke biphasic (hyperpolarizing/depolarizing) responses to mimic the post-synaptic effects of both the SO and the N1M/N1L cells on the N2vs. This was shown to be true in all five experiments where it was tried. In the example shown in Fig. 7A, a 50-ms focal application of 10^{-2} M ACh onto the surface of a N2v evoked an initial hyperpolarization followed by a depolarization. However, the depolarization lasted for longer than the SO → N2v synaptic response, 20 s compared with ~50 ms, despite the continuous perfusion of the preparation with normal saline. At higher pipette concentrations (10^{-2} M) (Fig. 7B), the long-lasting depolarizing component of the ACh response evoked plateau in the N2v and EPSPs on the B3 motoneuron due to the N2v → B3 synaptic connection (Brierley et al. 1997a). ACh (10^{-2} M) also could evoke long periods of lower amplitude plateaus without spike activity (Fig. 7C) in another preparation. Additional activation
that this could contribute to the long-term oscillatory activity in the feeding CPG network.

**Lack of N3t and N3p \( \rightarrow \) N2v synaptic connections**

Recording of the N2vs in a SO-driven rhythm suggested that the termination of the N2v plateau was *not* due to the activity of the N3 interneurons (Fig. 3). These fire immediately after the N2vs (see Fig. 14) and could have been terminating the plateau by feedback due to inhibitory synaptic connections. This lack of N3 \( \rightarrow \) N2v connectivity was tested directly by the experiments shown in Fig. 8. No synaptic response was recorded on the N2v cell as a result of the evoked spikes in the N3p cell recorded at the same time (Fig. 8A). The membrane potential of the N2vs was varied but still no synaptic response was seen. Similar recordings with the N3t cells showed no synaptic responses on the N2v after evoked action potentials in the N3t cells (Fig. 8). The N3t \( \rightarrow \) B3 synaptic connection produced the expected unitary EPSPs on the B3 recorded at the same time (Fig. 8B, i and ii). This suggested that the N2v plateaus were terminated by an endogenous mechanism, confirming the data from Brierley et al. (1997a) where the falling phase of the N2v plateau potentials remained unchanged when all chemical synapses had been blocked in high Mg\(^{2+}\)/low Ca\(^{2+}\) saline.

**Synaptic connections from the N2v cells to other feeding interneurons**

The above experiments showed that the N2v cells received the appropriate input synaptic connections to be an integral part of the CPG network. This entrained their endogenous plateauing activity to the rest of the CPG network and ensured that they fired during the correct rasp/N2 phase of the feeding cycle. The following results will indicate that the N2v cells also possess important output synaptic connections with other known members of the CPG network and the modulatory SO and CGC interneurons.

**N2v \( \rightarrow \) N1M, N1L**

Both types of N1 cells were hyperpolarized strongly during the N2/rasp phase of the feeding cycle (Figs. 5A and 6A), and the N2vs fired appropriately to provide this inhibitory feedback. Paired recordings of the N1 cells and the N2v (\( n = 10 \)) were carried out to test the hypothesis that the N2v provide the main inhibitory synaptic input to the N1M/N1L cells.

The N2vs inhibited both types of N1 cells (Fig. 9), but the response was much stronger for the N1Ls compared with the N1Ms. In the record shown in Fig. 9A, spontaneous bursts of spikes in the N2v were accompanied by inhibition of the N1L as expected. Evoking bursts of N2v spikes by current injection generated a similar strong hyperpolarization in the N1L at firing levels of membrane potential providing more direct evidence for the N2v \( \rightarrow \) N1L synaptic connection. This experiment was carried out in HiDi saline suggesting a monosynaptic connection. However, episodes of weaker hyperpolarization in the N1Ls were seen when the recorded N2vs were not active and only showed subthreshold plateau potentials (i in Fig. 9A). IPSPs were reduced in amplitude compared with inputs where the N2v was active but the residual hyperpolarizations still have to be explained. It was possible that the unrecorded contralateral N2v was active, but this seems unlikely as left and right N2vs normally fired together due to strong electrotonic coupling (Brierley et al. 1997a). More likely was that it was due to N2d activity through the monosynaptic N2d \( \rightarrow \) N1L synaptic connection reported by Yeoman et al. (1995). An interesting difference was seen on the B3 motoneuron compared with the N1L in Fig. 9A. Lack of N2v spikes meant a complete loss of EPSP synaptic input to the B3, suggesting that the N2v \( \rightarrow \) B3 connection was predominant in providing N2 input.

The N2v \( \rightarrow \) N1M inhibitory connection was much weaker than that for the N1Ls and only occurred in 50% of the cells tested. Evoked bursts of spikes only moderately slowed N1M activity, although a clear (reversed) IPSP could be seen when the N1Ms were inactive (arrowed in Fig. 9B). The other type of N2, the N2d also is known to inhibit the N1Ms (Elliott and Benjamin 1985a). These can stop N1M firing and appear to be more important than the N2vs.

**N2v \( \rightarrow \) N3t**

N3t (tonics) are inhibited throughout the N2/rasp phase of the feeding cycle (Elliott and Benjamin 1985a) (summa-
whereas the N3ts are inhibited for much longer due to the long-duration N2v burst and recover and fire at the beginning of the N3/swallow. The N2v/N2d cells both had similar differential effects on retraction phase motoneurons (Brierley et al. 1997a), and this was crucial for producing the sequence of motoneuron firing required for the rasp/swallow phases of the behavioral feeding cycle.

N2v → SO

Like the N1 cells, the SO was hyperpolarized strongly throughout the N2/rasp phase of the feeding cycle (e.g., Figs. 2 and 3), and the N2v cells are strong candidates for providing this inhibition. Evidence for this is shown in Fig. 11, where evoked bursts of spikes in the N2v strongly hyperpolarized the SO both in normal (Fig. 11A) and HiDi saline (Fig. 11B). The latter suggests a monosynaptic connection.

N2 → CGCs

The CGCs fired in a slow pattern in both intact (Yeoman et al. 1994a) and isolated ganglia (McCrohan and Benjamin 1980) but their activity was modulated weakly by synaptic input to make them fire mainly in the protraction phase of the feeding cycle rather than during the retraction phase. McCrohan and Benjamin (1980) showed that they appeared to be excited during the N1 protraction phase and weakly inhibited during N2 rasp phase to account for this pattern of activity. Figure 12 showed that the inhibition occurred at the same time as spontaneous and evoked bursts of N2v spikes, suggesting that these cells were responsible for the CGC inhibitory input during the rasp phase of the feeding cycle. There was no statistical difference ($P < 0.05$, Student’s $t$-test) in the numbers of spontaneous [15.9 ± 3.1 (mean ± SE)] spikes in N2v burst compared with current driven (14.4 ± 2.1; $n = 14$). The N2v → CGC connection may be monosynaptic as the experiment was carried out in HiDi saline. Because the axonal and neuritic processes of the N2vs are restricted to the buccal ganglia and postbuccal nerve (Brierley et al. 1997a), the CGC → N2v synapse must be located in the same structures.

DISCUSSION

N2v cells are part of the Lymnaea feeding CPG

A variety of evidence strongly supported the hypothesis that the N2v cells are important retraction phase interneurons of the Lymnaea feeding CPG.
N2v cells are part of the feeding CPG

These mainly occurred in the N1/protration phase of the feeding cycle and trigger the N2v plateau. The effects of CGC excitation also contributes to excitation during protrusion, but the long-term effects of CGC firing may occur across the whole feeding cycle (Yeoman et al. 1996). Once the plateau is triggered, no further significant synaptic inputs appear to be involved in terminating the N2v plateaus. Any inhibitory input might have been expected to come from the N3 CPG interneurons. However, direct recordings showed

1) Their activity is phase locked to the rest of the CPG network in a SO-driven fictive feeding rhythm (Fig. 2).

2) The phase of the CPG rhythm was reset by artificially inducing extra bursts of N2v spikes, a characteristic of all CPG neurons (Fig. 2).

3) N2vs have inhibitory and excitatory synaptic connections with retraction phase motoneurons, and these are necessary (with the N2ds) to produce the rasp-swallow phases of the motor neuron firing pattern (Brierley et al. 1997a).

4) N2vs have extensive input and output synaptic connections with the rest of the CPG network (summarized in Fig. 13A) and provide the main component of N2/rasp phase inhibitory input to several of these (N1L, Fig. 9; N3t, Fig. 10) as well as driving the N2d cells via a strong electrotonic synapse (Brierley et al. 1997a).

5) N2vs have reciprocal synaptic connections with the modulatory SO (Figs. 4 and 11) and CGC (Fig. 12) (Yeoman et al. 1996) interneurons. These are important in the cycle-by-cycle control of the feeding oscillator, but also long-term modulatory effects on the N2vs are present (Yeoman et al. 1996).

We believe that the N2vs are the most significant type of retraction phase interneuron cell so far discovered because they fire throughout the N2/rasp of the feeding cycle (Brierley et al. 1997a), unlike the N2ds, which only fire briefly at the beginning of this phase (Elliott and Benjamin 1985a). Their ability to generate plateau potentials by an endogenous mechanism (Brierley et al. 1997a) is also likely to be significant in providing oscillatory ‘‘drive’’ to the whole CPG rhythm.

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We believe that the N2vs are the most significant type of retraction phase interneuron cell so far discovered because they fire throughout the N2/rasp of the feeding cycle (Brierley et al. 1997a), unlike the N2ds, which only fire briefly at the beginning of this phase (Elliott and Benjamin 1985a). Their ability to generate plateau potentials by an endogenous mechanism (Brierley et al. 1997a) is also likely to be significant in providing oscillatory ‘‘drive’’ to the whole CPG rhythm.

1) Their activity is phase locked to the rest of the CPG network in a SO-driven fictive feeding rhythm (Fig. 2).

2) The phase of the CPG rhythm was reset by artificially inducing extra bursts of N2v spikes, a characteristic of all CPG neurons (Fig. 2).

3) N2vs have inhibitory and excitatory synaptic connections with retraction phase motoneurons, and these are necessary (with the N2ds) to produce the rasp-swallow phases of the motor neuron firing pattern (Brierley et al. 1997a).

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that no feedback connection occurred from the N3t or N3p cells to the N2v cells.

The SO, N1M, and N1L are the three main types of interneurons that provided the N1/protraction phase synaptic input to the N2v (Fig. 13 B). The final waveform of the potential changes were highly complex and not only determined by the number of presynaptic cells involved but also by activity-dependent changes in the amplitude of particular synaptic responses. The SO is active during the late N3 phase, and this provided a purely inhibitory synaptic input. However, during N1/protraction, the delayed depolarizing component of the SO → N2v biphasic PSP (Fig. 13 A) became progressively larger and thus increasingly important in determining the overall depolarizing ramp-shaped waveform occurring during this phase of the cycle. However, the IPSP component of the SO → N2v response still persisted, and the large IPSPs obvious during N1/protraction were also due to the SO. The other main depolarizing input to the N2v was due to the N1M → N2v excitatory synaptic connection (Fig. 13 A). The N1Ms accelerate their spike activity toward the end of protraction, and their excitatory effects must be particularly important in the steeper part of ramp. The N1Ms form an electrotonically coupled subnetwork (~4 cells on each side) (Rose and Benjamin 1981a) of N1M and were capable of driving N2v plateau alone (Fig. 5 B). However, the N1M rhythm was weak and irregular, and a fast fictive feeding rhythm required the SO (depolarizing) input to the N2v cells. The final cell type providing input to the N2v cells are the N1L cells (Fig. 13 A). These cells fired four or five spikes during the N1/protraction phase of the cycle, and we were surprised to find that these cells inhibited the N2v. This was the opposite to the N1Ms. However, the inhibitory effect of the N1Ls became weaker with successive spikes, and so we presume it was less important in the steeper parts of ramp just before plateau triggering.

In summary (Fig. 13 B), the shape of ramp-like N2v prepotential occurring during N1/protraction initially was determined by SO/N1L inhibitory inputs plus some excitatory component from the SO and the N1Ms. The latter became dominant toward the end of the ramp, finally leading to the triggering of the N2v plateau.

This study emphasized the complexity of the synaptic control of the protraction phase of the feeding cycle. Changing the slope of the N1/protraction phase ramp of the N2v cells would determine the duration of protraction phase of the CPG network and the whole feeding cycle. The steeper the slope the more rapid the N2v plateau would be triggered, resulting in a reversal of the cycle from protraction to retraction (see the overall CPG pattern in Fig. 14). Previous authors also have emphasized that changes in the duration of the protraction phase was a major target for control of frequency of the fictive feeding rhythm (e.g., Elliott and Benjamin 1985a). The SO → N1M excitatory synaptic connection was considered to be the main site of control, but recently it was found that the CGCs also could control the frequency of the feeding oscillator by influencing the duration of the N1/protraction phase of the cycle. The CGCs excited both the SO and N1M cells (Yeoman et al. 1996) and so added to the excitatory drive on these cells that normally would come from food in the intact animal (Kemenes et al. 1986).

Again this reduced the duration of the N1/protraction phase of the feeding cycle (Yeoman et al. 1996).

The final type of input synaptic connection to be considered is the reciprocal electrotonic connection between the N2vs and the N2ds (Fig. 13 A) (Brierley et al. 1997a). This mainly was considered to play a role in the N2vs driving the N2ds (see further) but during the brief firing of the N2ds there could be a minor N2d → N2v excitatory effect.

**Modulatory effects of ACh**

ACh was applied focally to the surface of the N2v to see if it could mimic the biphasic (i.e.) synaptic effect of SO neuronal stimulation. Previously published data (Yeoman et al. 1993) showed that ACh was the likely transmitter of the SO. This was successful in that an initial hyperpolarizing...
followed by a longer lasting depolarizing response was seen. However a short (50 ms) application of ACh, in a preparation continuously perfused with normal saline, produced a long-lasting depolarization lasting for =1 min (10^{-2} M ACh). Superimposed on the depolarizing waveform were sequences of plateau potentials. This suggested that ACh, as well as being involved in short-term, cycle-by-cycle synaptic transmission, could cause long-term potentiation of plateauing in the N2v cells. This may well be involved in maintaining the feeding pattern and could account for the observation (Rose and Benjamin 1981a) that the CPG continued to oscillate for a number of cycles after the end of SO activation. For comparison, a similar experiment was carried out on the other type of N2 cell type, N2d, and again long-term activation was seen (Fig. 7D). It should be noted that plateau in the N2vs can be generated by current injection in quiescent preparations where the SO was inactive, showing that ACh was not necessary for the basic plateauing. In contrast, current injection in quiescent preparations cannot induce plateaus in the N2d cells (Brierley et al. 1997a), and so ACh may play an important role in inducing long-term plateauing in this cell type. This would modify the conclusion in Brierley et al. (1997a) that the plateaus in the N2ds were entirely due to electrotonic potentials arising from the N2v. We cannot completely rule out the possibility that focal application of ACh to the N2d cells also may have activated adjacent cells (N1Ms or N1Ls) occurring on the same surface of the buccal ganglia, so more detailed evidence is required. This is unlikely to be the case for ACh effects on the N2v cells as these are on the opposite ventral surface away from the main CPG population. The ability of modulatory substances to initiate plateauing in CPG cells was seen in the other preparations (Ramirez and Pearson 1991; Sigvardt et al. 1985) and appears to be a common feature of oscillatory systems.

**N2v output synaptic connections**

N2v cells had significant output synaptic connections with other CPG interneurons and the SO and CGC modulatory cells. These were all inhibitory (Fig. 13A) apart from the N2d where electrotonic coupling, N2v → N2d, provided a strong excitatory drive (Brierley et al. 1997a).

The N2vs provided strong feedback inhibition to the N1Ls, SO, and CGC cells, preventing them firing during the N2/retraction phase of the feeding cycle (Fig. 14). N2vs only had weak inhibitory feedback connections to the N1M cells, so the strong hyperpolarizing synaptic input that terminates N1M firing must be provided by the N2ds or some unknown type of N2 cell. The only other important connection the N2v made was with the N3t cells. The N3t were strongly inhibited by the N2v throughout the N2/rasp phase of the feeding cycle (Fig. 14) and recovered and fired by postinhibitory rebound (PIR) (detailed evidence in Elliott and Benjamin 1985a). There was no connection between the N2v cells and the N3p CPG interneurons. These are inhibited briefly during the N2/rasp phase of the feeding cycle and so presumably must receive inhibitory input from the N2ds alone.

The discovery of the differential inhibitory effects of the N2 cells, N2v → N3t N2d → N3p, was important because it allowed the N3ps to recover and fire earlier in the cycle than the N3t cells (Fig. 14). This is crucial in the feedback role of the N3 cells on the N1M/N1L and SO cells. The N3p inhibited these three cell types immediately after the end of N2 inhibition (Fig. 14), and this was continued by the overlapping N3t induced inhibition until the start of the next feeding cycle. Thus three phases of sequential feedback inhibition, N2v → N3p → N3t, prevented activity in the protraction phase CPG interneurons N1M/N1L and SO during the rasp and swallow phases of the feeding cycle.

We conclude that the output synaptic connections of the N2v are essential for producing the pattern of activity in the rest of the Lymnaea interneuronal network.

**A new model for the Lymnaea feeding CPG**

A full summary of the Lymnaea CPG is given in Fig. 14. It includes the firing patterns and synaptic connections of the SO and six CPG interneuronal types. It assumes that the SO is driving the rhythm although recent evidence showed that the natural food stimulus in the semi-intact preparation produced a similar fictive feeding rhythm (Yeoman et al. 1995). Direct multicell recordings have been made from all these cell types and evidence provided in most cases for monosynaptic connections (Elliott and Benjamin 1985a,b; Rose and Benjamin 1981a,b; Yeoman et al. 1995). Further data are known for the CV1s (McCrohan and Kyriakides 1989) and the CGCs (Yeoman et al. 1996), but these are not included in Fig. 14 for simplicity. It is now known that full activation of the feeding rhythm by food (sucrose) requires coactivation of the SO, CGCs, and CPG interneurons (Yeoman et al. 1995). In the SO-driven rhythm of Fig. 14, the gating input from CGCs is assumed to be present. In the isolated preparation, they fired spontaneously at tonic levels sufficient to allow the SO to drive the pattern. In the intact animal, food is required to activate the CGCs, SO, and CPG interneurons (Kemenes et al. 1986; Yeoman et al. 1995). The transmitters for some of these neurons are known. This is acetylcholine for the SO, N1M, and N1L cells (Elliott and Kemenes 1992; Vehovszky and Elliott 1995; Yeoman et al. 1993) and provisionally glutamate for the N2vs (Brierley et al. 1997b). Neuropeptides also are known to be colocalized in some of the cells but their function is unknown. The CGCs and SO contain peptides related to myomodulin and the N2vs myomodulin and buccalin-like peptides. Buccalin also may be present in one of the N1M cells (Santana et al. 1994).

The sequence of activity summarized in Fig. 14 begins with the SO. This drives the N1L and N1M by electrotonic coupling (N1Ls) or a strongly facilitating excitatory (e) synaptic connection (N1Ms). A strong chemical synaptic connection N1L → N1M also helps to excite the N1Ms during protraction. The activity in the SO is reinforced by reciprocal N1L → SO electrotonic connection and by a weaker N1M → SO excitatory chemical synaptic connection. All three protraction phase interneurons provide synaptic inputs to the N2v cells during N1/protraction. The N2vs initially are inhibited (i) by the SO but the SO → N2v biphasic (i–e) synaptic response facilitates so that the delayed e component gradually becomes more important toward the end of N1/protraction. A second component of excitation comes from
the N1M → N2v. The N1Ls inhibit the N2v. This is likely to be important at the beginning of N1/protraction, but as the N1L → N2v inhibitory response progressively weakens, it is not significant by the end of the protraction phase. The N2d cells have similar inputs from the SO and N1M cells, but unlike the N2vs, the N1L input is excitatory, adding to the input from the other two protraction phase interneurons.

At the end of the N1/protration phase, a plateau is triggered in the N2v and this simultaneously activates the N2d via the strong N2v → N2d electrotonic synapse. ACh released from the SO and N1M also may contribute to the N2d plateauing. The N2v fires throughout the N2/rasp phase of the cycle, whereas the N2d only fires briefly at the beginning of N2. Both cells provide inhibitory feedback to the N1L/N1Ms. This is strong for the N2v → N1L connection but weak for N2v → N1M. The N2d cells inhibit both the N1Ls and N1Ms equally but only can be important at the beginning of the N2/rasp phase of the cycle, as they only fire one or two spikes. This suggests that another type of N2 may exist to produce the prolonged inhibition of the N1Ms during the N2/rasp phase of the cycle. Alternatively the N3ps may be most important. They fire, earlier than previously was thought (Yeoman et al. 1995), during the end of N2 phase of the feeding cycle. The N3t and N3p cells are inhibited by both the N1M and N1L cells during the N1/protration phase of the cycle, and the inhibition is maintained by a second phase of inhibition from the N2 cells during N2/rasp. This is differential, with the N3t being inhibited solely by the N2v cells and the N3p by the N2d cells. Both cells recover from this inhibition to fire by PIR, N3ps earlier than N3ts. Both types of N3 cells provide inhibitory feedback to the N1 cells (both N1Ms and N1Ls) and weaker input to the SO. Unitary IPSPs can be seen first, 1:1 with N3p spikes followed by a smoother hyperpolarizing input from the N3t cells. This inhibition is superimposed on a basically depolarizing waveform driven by the SO, leading to the protraction phase of the next cycle of activity.

Feeding CPGs of other mollusks

The feeding systems of other mollusks have similar multiple types of interneurons forming part of the CPG. The system from the related snail Planorbis (Arshavsky et al. 1988a–c) has cells of the N1 and N2 subtypes with similar synaptic connectivity to Lynnaea but apparently no N3s. Cells equivalent to N1–N3 may occur in Helisoma (Quinlan and Murphy 1991), but how closely they resemble the Lynnaea cells is not yet clear except that the B2 CPG interneuron (Quinlan et al. 1995), being glutamatergic, may be similar to N2d cells of Lynnaea (Brierley et al. 1997a). The B31 cells of Aplysia show plateauing properties similar to the N2v cells of Lynnaea (Susswein and Byrne 1988), but whether they play a similar role needs to be further investigated, given that Aplysia has a more flexible feeding system than Lynnaea (Benjamin 1983).

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