Analysis of the Feeding Motor Pattern in the Pond Snail, *Lymnaea stagnalis*: Photoinactivation of Axonally Stained Pattern-generating Interneurons

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We have photoinactivated identified feeding interneurons known as N1 and N2 neurons. These are pattern-generating neurons that are active in the protraction of the radula and rasping phases, respectively, of the feeding cycle of the pond snail. The N1 or N2 feeding interneurons in the buccal ganglia were filled with the fluorescent dye 5(6)-carboxyfluorescein (5-CF) from the cut end of the nerve that contains their axon. Filling the cerebrobuccal connective ($N = 151$) stained just one N1 cell in the contralateral buccal ganglion. Filling the postbuccal nerve stained neurons symmetrically in both buccal ganglia ($N = 75$): only one labeled cell in each ganglion is an N2 interneuron.

The feeding rhythm was evoked by depolarizing a modulatory neuron, the SO, located in the buccal ganglia. The axonally filled N1 interneuron was irradiated at its axon in the buccal commissure with blue laser light (intensity of $0.5 \text{ MW} \cdot \text{m}^{-2}$). Irradiation of just one N1 completely blocked the feeding rhythm (seven preparations). In seven further preparations, N1 ablation slowed the SO-driven feeding rhythm and weakened the N1 input to the feeding neurons. Irradiation of the cell bodies of both the filled left and right N2 interneurons killed the cells but did not produce any consistent change in the feeding rate (15 preparations). The feeding interneurons and motoneurons still showed the characteristic N2 phase synaptic inputs, so more, as yet unidentified, N2 neurons must be located in other parts of the buccal ganglia.

We conclude that the participation of the identified N1 interneurons is essential for the normal feeding pattern while other, still to be identified N2 neurons must be present and must contribute to the feeding rhythm. We suggest that the extra redundancy of the N2 network may be related to the greater necessity of sensory feedback control during rasping than during protraction of the radula.

[Key words: mollusk, *Lymnaea*, feeding, photoinactivation, interneuron, carboxyfluorescein, axon]

Our understanding of the cellular mechanisms of rhythmicity has mostly come from the analysis of simple systems of neurons and even from analysis of individual neurons. Of particular

importance have been the crustacean stomatogastric ganglia, where the interactions of synaptic and endogenous membrane currents have been well analyzed. The progress has been particularly quick because of the limited number of cells (~ 30) and the ability to photoinactivate cells injected with Lucifer yellow (LY) or 5(6)-carboxyfluorescein (5-CF). However, the neural basis of behavior is best understood in more complex systems, for example, locust flight or bat echolocation. In these systems, we understand well the ethological interactions between the CNS and environment, but know little about the cellular events within the CNS, because of the large number of neurons involved.

Between these two levels of complexity lie the feeding systems of mollusks. Over the last 10 years, a large number of the neurons involved in the sensory, modulatory, pattern-generating, and motor output have been described in a wide range of mollusks (*Aplysia*, *Helix*, *Helisoma*, *Limax*, and *Lymnaea*). The analysis of the generation of the rhythmic feeding pattern is perhaps best understood in *Lymnaea stagnalis*, where the pattern is produced by the buccal ganglia (total neuronal count ~ 300 neurons; Boyle et al., 1983). Many of the neurons have been identified and their role in production of the fictive feeding pattern characterized. Until now their role has only been tested by stimulation experiments, with injection of tonic depolarizing currents or short bursts of spikes for resetting experiments. However, the relative importance of a particular cell or group of cells in the production of the rhythm can only be fully tested by their selective removal from the circuitry. It is the purpose of this article to test our understanding of the cellular basis of the feeding rhythm by extending the photoinactivation method to this more complex system.

The photoinactivation technique was introduced by Miller and Selverston (1979) for the stomatogastric ganglia of crayfish. Initially they used blue light from a quartz bulb to kill neurons injected with LY; more recently blue lasers have been used to kill neurons more quickly. This method has been very useful because many of the stomatogastric cells make electrical synapses and so if simple hyperpolarizing currents are applied the effects spread widely. However, when the filled cells are irradiated they are uncoupled from the remaining electrically coupled network, which remains unaffected (Selverston and Miller, 1980).

The intracellular injection of LY from a pipette is a good approach when there are only a few cells or when neurons can be identified visually. Sadly, in the *Lymnaea* feeding system there are only one or two interneurons (the SO, CGCs) that can be recognized visually; the other feeding interneurons (including the N1, N2, and N3 cells, which generate the rhythm) are too

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small and too like the adjacent motoneurons to be quickly identified by visual criteria. To find these cells we have developed the axonal filling method of Iles and Mulloney (1971) to stain healthy neurons from their cut axons with a fluorescent dye, 5-CF, which can be used later for selective photoinactivation (Kemenes et al., 1991, 1992). We have found that this dye travels up the nerves of *Lymnaea stagnalis* and fills the cell bodies in less than 3 hr, such that the cells still display the normal feeding pattern. Furthermore, it fluoresces well under the blue laser light and the quantity of dye transported is sufficient that a blue laser can be used to kill the stained neurons selectively. Finally, the preparation can be fixed and the staining used to confirm the morphology of the stained cells.

Other techniques of cell ablation, such as injection of proteolytic enzyme (Rosen et al., 1989) or surgical ablation (Syed et al., 1992), have been used successfully in mollusks. However, these methods are not appropriate here. As noted in the preceding paragraph, visual criteria do not suffice for identifying the small feeding interneurons in *Lymnaea*, and some (e.g., N1) do not lie on the surface of the ganglia but in a lower, second layer (Elliott and Kemenes, 1992). With the proteolytic enzyme approach, there is thus a considerable danger that several other cells would be contaminated with the enzyme before impalement of the required interneuron. With surgical removal, there are obvious difficulties in finding the right cell to remove. However, with our axonal filling method, it is possible to remove a single cell, or group of cells, without damage to the adjacent cells because only the filled cells are damaged by the laser irradiation. Also, it is possible to ablate selected neurons without having to impale them to confirm identification, providing, of course, that the neurons are well defined on an anatomical criterion, namely, that these are the only cells in a part of the ganglion with a particular axonal projection. We will show below that N1 and N2 feeding interneurons can be unambiguously identified on this morphological criterion alone and so our new technique is particularly suitable to testing the inactivation of these interneurons on feeding pattern generation.

Our new development of the photoinactivation method also has advantages over testing the role of interneurons by injection of hyperpolarizing current. We have great uncertainty about how far the current spreads through the cells, and this uncertainty is compounded by the fact that some interneurons (including the N1 cells) are in an electrically coupled network of neurons and it may be difficult to know if the injection of current actually alters the firing rate, et cetera, of the hyperpolarized cell. However, we show (see section 2.1) that the effectiveness of irradiation of the axon can be evaluated by somatic recording and shown to be fully effective.

These new results mean that we are now in a position to do selective ablation experiments in mollusks, in which single neurons, or groups of neurons *with axons in a single nerve* can be irradiated and removed from the neural circuit, and we have applied this to the feeding pattern-generating interneurons, N1 and N2.

Overview of the feeding motor system

The pond snail *Lymnaea stagnalis* feeds by the rhythmic protraction and retraction of the radula in response to tactile and chemical stimuli to, for example, the lips or buccal cavity. The movements of the radula are produced by the muscles of the buccal mass, which in turn are supplied by motoneurons in the buccal ganglia. Each feeding cycle is composed of three move-

ments: protraction of the radula, rasping, and swallowing. Feeding cycles may follow each other directly, or may be separated by quiescent periods. Experiments with semi-intact and isolated CNS preparations show that the same fictive feeding pattern can still be recorded from the motoneurons in the absence of any sensory input (Benjamin and Rose, 1979; Rose and Benjamin, 1979). The motoneurons themselves do not seem to contribute to the rhythm; instead, this is produced by three kinds of pattern-generating interneurons, known as N1, N2, and N3 neurons. These are located in the buccal ganglia and were first identified physiologically by Rose and Benjamin (1981b), who showed that the three N-cells were active in turn, with the N1 phase corresponding to protraction of the radula, the N2 phase to rasping, and the N3 phase to swallowing. In electrophysiological experiments Rose and Benjamin also showed that there were several (up to perhaps 10) cells of each N-class within each buccal ganglion. Elliott and Benjamin (1985a) showed that the N-cells could reset the phase of the rhythm, and when they impaled pairs of N-cells, they found fast, short-latency synaptic connections between the three kinds of N-cells that could account for the pattern-generating roles. Thus, so far, the participation of particular neurons in the generation of the pattern has been studied by stimulation experiments alone and no ablation experiments have been performed at the level of individual neurons.

In the isolated CNS, the rhythmic feeding pattern (fictive feeding) normally occurs sporadically. If a snail is starved and then allowed to feed for a few minutes before death, the fictive feeding rate is much increased but the length of successive cycles varies randomly. A more consistent pattern is elicited by depolarization of a modulatory neuron, for example, the SO, a single neuron in either the left or the right buccal ganglion (Rose and Benjamin, 1981a; Elliott and Benjamin, 1985b), or by stimulation of either of the CV1 interneurons in the cerebral ganglia. Normally, the CV1 cells are silent during SO-driven rhythms and vice versa (McCrohan, 1984). We have chosen SO stimulation in our experiments for the following reasons. First, the SO is a buccal interneuron and this makes the impalements less technically difficult. Second, the SO is the only known buccal interneuron that can drive the feeding pattern at the rate seen *in vivo* (about 1 cycle every 4 sec; Dawkins, 1974). Third, the SO is known to be excited by feeding stimuli (e.g., sucrose) applied to the buccal cavity (Kemenes et al., 1986) or lips (G. Kemenes, unpublished observation) and the pattern of the rhythm produced by SO depolarization corresponds with that seen in semi-intact preparations excited to feed by sucrose (Elliott and Andrew, 1991). Thus, studies of the effects of cell ablation on the SO-driven fictive feeding pattern are likely to provide insights into the normal taste-driven feeding in the intact snail. Finally, the result of SO stimulation is very reliable due to the strong reciprocal excitatory connections with the N1 cells (see Fig. 1), such that an excitatory stimulus sufficient to fire 5–10 SO action potentials initiates a full cycle of the feeding pattern. The strong reciprocal excitation also means that steady, prolonged depolarization of the SO results in successive cycles at a stable feeding rate, so valid statistical comparisons can be made between the feeding rate before and after laser treatment.

Occurrence of the feeding pattern does depend, however, on excitatory input from higher-order, modulatory interneurons called the CGCs. These are paired serotonergic neurons in the cerebral ganglia with axons that run into the buccal ganglia (McCrohan and Benjamin, 1980a,b). They normally fire toni-

cally at about 0.5–2 Hz, although in a few preparations they may fire weak bursts. Their activity is needed for SO-driven rhythmic feeding activity to occur (Benjamin and Elliott, 1989; M. Yeoman, personal communication).

Details of the pattern-generating network

The connections between the three kinds of N-cell (N1, N2, N3) described by Elliott and Benjamin (1985a) are shown in Figure 1*B* together with their connections to the SO. The fictive feeding pattern is generated as follows: a depolarizing stimulus to the SO excites the N1 interneurons through their reciprocal excitatory connections. This leads to a burst of N1 spikes of gradually increasing frequency. Since the N1 interneurons excite the N2 interneurons, the N2 interneurons gradually depolarize until they reach threshold and fire action potentials. The inhibitory synapse from the N2 to N1 cells ensures that the N1 activity is then terminated and only the N2 fires in this phase. This recurrent inhibition mechanism is the main switch from N1 (protraction) to N2 (rasping, retraction) phases of the feeding cycle.

Both N1 and N2 interneurons inhibit the N3 cells, which fire through postinhibitory rebound once their input from the N2 cells dies away. The N3 interneurons also inhibit the N1 neurons and prevent any more N1 activity until the N3 PIR effect has declined. However, if the depolarizing stimulus to the N1 cells is maintained, a second cycle of feeding will be eventually initiated when the N3 inhibition of the N1 cells is overcome. In the related pulmonate *Planorbis*, evidence for the same mechanism of pattern generation has been demonstrated (Arshavsky et al., 1988).

We have used axonal filling with 5-CF to overcome three problems with any attempt to use photoablation to test the role of any of the elements in this model. The first problem is a technical one: since the N1 and N2 types of interneurons are difficult to distinguish visually from the surrounding cells, finding them is a time-consuming activity and other adjacent cells might be contaminated with small amounts of LY. During filling, the electrode may block and have to be replaced by a potassium acetate electrode and it is often difficult to reimpale these small interneurons. The second problem is that, although there can be more than one cell of each N-class within the buccal ganglia, probing with a microelectrode can only provide an estimate of the exact number of each type. Since there seem to be up to 10 “replicate” cells of each physiological N-class in a single buccal ganglion, any attempt to impale all the cells would be very difficult and in any experiment it would be impossible to be certain that all members of a particular subclass had been penetrated. The second problem that axonal filling overcomes is that injection of the fluorescent dye LY into the N-cells showed that each kind of N-cell could be separated into subclasses based on consistent differences of morphology. These differences are accompanied by smaller, but still consistent differences in physiology. For example, some N3 cells (called N3 tonic) have axons entirely within the buccal ganglia and fire a large number of spikes in each feeding cycle and display strong PIR. Another type of N3 cell (called N3 phasic) has axons in the ipsi- and contralateral dorsobuccal nerve (dbn), fires few spikes, and has only weak PIR. Evidence for the same kinds of subclasses has been found in the feeding systems of the related mollusks *Planorbis* and *Helisoma* (Arshavsky et al., 1988; Quinlan and Murphy, 1991).

Using axonal filling with 5-CF simultaneously overcomes both the problems outlined above. In the first place, we can stain all

Figure 1. The buccal feeding system of *Lymnaea*. *A*, An overview of the buccal ganglion. The left ganglion shows the position of the buccal neurons, with the 1, 2, 3, 4 cluster (4 CL), and 7 motoneurons as open circles, the N1 and N2 interneurons as solid circles, and the SO shaded. The right ganglion shows the outline morphology of the N1 and N2 interneurons with their main branches. Anterior is toward the bottom. The scale bar is approximate. *cbc*, cerebrobuccal connective; *dbn*, dorsobuccal nerve; *pbn*, postbuccal nerve; *bc*, buccal commissure. *B*, The main connections shown by Elliott and Benjamin (1985a) between the SO and the three types of central pattern-generating interneuron, N1, N2, and N3. The SO and N1 cells are active in the protraction phase and the N2 and N3 cells in the retraction phase.

the cells with axons in a particular nerve and thereby see all the neurons in a particular subtype plus the surrounding cells. We show below (see sections 1.1 and 1.2 of Results) that in the case of the N1 and N2 types of interneurons the relevant cells can be unambiguously identified on morphological criteria from the filling pattern alone. The second advantage of the filling technique follows from this: we can eliminate fluorescing N1 and N2 cells from the neural circuit without need for impalement through the early experiments with impalement, which show that the cells in particular locations are the required N-cells.

The two types of neurons for which this analysis is most suitable are the N1 and N2 types described by Elliott and Benjamin (1985a). We chose the N1 type because, when LY was injected into its soma, the sole axon was found in the contralateral cerebrobuccal connective (cbc). We have so far found no other feeding neuron with an axon in the cbc contralateral to its soma. We chose the N2 type because it has an axon in the postbuccal nerve (pbn), which is situated on the midline and so the cells in the left and right ganglia could simultaneously be filled axonally and then irradiated in turn.

The first section of our results shows that there is, in fact, only one N1 cell and one N2 cell in each buccal ganglion that can be filled through these nerves. We have shown that inactivation of a single N1 neuron slows or stops the SO-driven feeding rhythm, but that inactivation of one or both axonally stained N2 interneurons does not affect the N2 phase of the feeding pattern.

Materials and Methods

Snails (*Lymnaea stagnalis*) were kept in standard conditions as described recently (Elliott et al., 1992). All the snails used in these experiments were raised from the egg in the laboratory on a diet of lettuce

alone, to ensure that their neurons were only weakly pigmented. [These neurons are not affected by blue light from the laser; the neurons of wild-caught snails are heavily pigmented and inactivate in this blue light (Elliott and Kleindienst, 1990).]

Axonal filling. The CNS was dissected out and pinned down in a Sylgard dish containing standard snail saline (Elliott et al., 1992). N1 neurons were filled by cutting a single cerebrobuccal connective (cbc) and constructing a dam from white petroleum jelly (Boots) around the lightly dried end of the nerve (Kemenes et al., 1991). The dam was filled with a saturated solution of 5(6)-carboxyfluorescein (Kodak) at pH 7 and covered with more petroleum jelly. The rest of the CNS was covered with saline. The travel of the dye up the nerve was followed using the blue light provided by a Schott fiber optic light source fitted with a 500 nm short-pass blue filter (Oriol Scientific, Leatherhead, UK). Cells in the ipsilateral ganglion could be seen with the Schott light after 1 hr in these room temperature experiments. After 2 hr, the petroleum jelly igloo was removed and the ganglia were washed with fresh saline. To fill N2 cells, the igloo was constructed around the end of the postbuccal nerve (pbn) that contains the axons of left and right N2 neurons (Fig. 1).

Laser illumination. Laser illumination was provided as described recently (Elliott and Kleindienst, 1990; Kemenes et al., 1991). The blue light (wavelength of 440 nm) from a helium-cadmium laser (Omni-chrome, model 439X) was passed through a 20× beam expander and focused through a 120 mm focal length lens to provide a spot of light 100 μm in diameter. The beam was then bounced off a movable mirror, which was adjusted until the desired spot was illuminated. The beam was normally attenuated by two neutral density filters to provide an intensity of 10 $\text{kW}\cdot\text{m}^{-2}$. This had no effect on the cells. Removal of the filter raised the beam intensity to 0.5 $\text{MW}\cdot\text{m}^{-2}$. This led to gradual photoinactivation of the N1 interneuron (see section 2.1 of Results). In all the N1 inactivation experiments described here, the laser beam was pointed at the N1 axon in the buccal commissure (bc; see Fig. 1). Normally, the N2 cells were inactivated by directing the beam at the N2 somata in turn (see section 3.1 of Results). As there are no other stained cells adjacent to the N2 soma (Fig. 2E,F), we can be sure that no other cells will be damaged when the N2 soma is irradiated. In four experiments the beam was pointed on the buccal commissure to irradiate the N2 axons as well; in this case damage to all the neurons with axons in the pbn is certain.

Cell impalement. Cell impalement was performed as described recently (Elliott et al., 1992). The ganglionic sheath was softened with protease and cells impaled with potassium acetate electrodes of tip resistance 20–50 M Ω . The potassium acetate contained 0.025% 5-CF so that the electrode tips could be followed under the blue laser light during impalement.

Identification of neurons. Neurons were identified as follows. Visual criteria suffice for the very large 1, 2, 3, and 4 cluster (4 CL) motoneurons. The SO could be seen in many preparations as a white neuron lying between the 1 and 2 cells or just anterior to the 2 cell. Its identity was confirmed by its ability to initiate fictive feeding when depolarized. N1 cells also initiate feeding activity, but their identity was established by their spikes, which are much smaller and much faster than those of the SO. Furthermore, the N1 neurons often could be identified by their synaptic input to the motoneurons (e.g., excitation of 1 cell). The N2 cells were identified by their typical flat-topped depolarization with a few big spikes during the N2 phase of the feeding rhythm, by their ability to reset the phase of the rhythm, and by their ability to excite the 3 cell and inhibit other motoneuron types (e.g., 4 CL motoneurons). The 5, 7, 8, and 10 cell types, all motoneurons, were identified by their firing pattern in the feeding rhythm.

Lucifer yellow injections. Lucifer yellow (LY) was injected into previously unstained N1 neurons in 16 experiments to compare the success of axonal irradiation of neurons stained with 5-CF from the cerebrobuccal connectives and neurons stained somatically. Neurons were impaled using electrodes drawn on the same puller settings, but filled with a saturated solution of Lucifer yellow. Current pulses of -5 nA lasting 500 msec, repeated at 1 Hz, were used to inject Lucifer yellow for 15 min. After injection the Lucifer yellow electrode was withdrawn and the interneuron reimpaled with a potassium acetate electrode, as the Lucifer yellow electrodes tended to attenuate the size of the action potentials.

Morphology. At the end of the experiment, the buccal ganglia were removed and placed in 4% formaldehyde fixative in 0.1 M phosphate buffer (pH 7.4) for 10 min, followed by 4% formaldehyde in methanol (1 hr). The ganglia were then passed through two changes of ethanol

and one of methyl salicylate (10 min each), before being laid out on a slide in fresh methyl salicylate. Cells were observed and photographed in whole-mounts on a Nikon epifluorescence microscope fitted with an FITC filter set.

Results

1. Axonal filling

As noted in the introductory remarks, the N1 and N2 types of neurons are part of the pattern-generating circuit for feeding. Injection of LY dye into N1 cells showed that each cell had an axon in the contralateral cbc and that the N2 types of neurons each had two axons, one in the pbn and one in the dbn (Elliott and Benjamin, 1985a). The first part of this project was to use the axonal filling technique to determine how many N1 and N2 cells were present in each ganglion.

1.1 Location of N1 neurons

Filling the cbc with 5-CF for 2 hr shows an average of 37 stained cells in the ipsilateral buccal ganglion (Kemenes et al., 1991) and up to five fluorescent cells in the contralateral buccal ganglion. Filling for a longer period does not increase the number of cells visible (Kemenes et al., 1991). In practically every preparation (151 of 152 preparations), one of the contralateral cells is located under or next to the 2 cell motoneuron (usually it is under the 2 cell). In 83 preparations, this cell was illuminated with low-power blue laser light and then impaled with a lightly fluorescent potassium acetate electrode (Fig. 2B). Since both cell and electrode fluoresced, we could be sure that the filled cell had been impaled and from the physiological recording we are sure in each case that it was indeed the N1 cell. This is not surprising, since this is the location of the N1 cells described by Elliott and Benjamin (1985a). The number of other cells visible in the contralateral ganglion is much more variable; they are only clearly seen in the best stained preparations. They are located around the edge of the ganglion; the most frequently seen cells occur on the lateral or anterior edges of the ganglion—one of these can be seen in focus in Figure 2A. Impalement of any of these other cells shows that they are not N1 cells and that they have no synaptic relationships with any feeding motor interneurons. We conclude that there is only one N1 interneuron with this morphology in each buccal ganglion.

It might be that among the brightly stained cells in the ipsilateral ganglion are feeding neurons with axons in the bc that would also be affected by laser irradiation of the bc. However, none of the known feeding interneurons has axons in the bc as well as the cbc ipsilateral to the soma. We also checked with the laser for bipolar cells; one or two have “axonal” branches that project toward the bc but none projected fully into the bc. We also checked the brightly stained ipsilateral cells for association with (or effects on) the feeding rhythm and have found none and so are confident that irradiation of the stained axons in the bc will only remove one neuron from the feeding circuit, namely, an N1 cell.

1.2 Location of N2 neurons

The N2 interneurons were first stained by Elliott and Benjamin (1985a) and found to have axons in the pbn and dbn. We have chosen to stain the pbn (rather than the dbn) because it is situated on the midline, and contains the axons of left and right N2 interneurons. Thus, the cells in both ganglia can be filled at once and in photoinactivation experiments neurons in both left and right sides can be inactivated in turn. When the pbn is filled for 2 hr, an average of 55 ± 6 cells are seen (mean $\pm$ SE, $N =$

Figure 2. Morphology of buccal neurons filled with 5-CF. *A*, A fill of the cerebrobuccal connective (*cbc*) with 5-CF showing the group of about 35 cells in the ipsilateral ganglion and two cells in the contralateral. The *arrowed cell* is an N1; the other fluorescent cell was checked and is not an N1. *B*, Photomicrograph of an N1 filled with 5-CF fluorescing in a spot of 100- μ m-diameter blue laser light and white background illumination. The micrograph was taken while the N1 was impaled with the potassium acetate electrode containing 0.025% 5-CF. The use of the fluorescent dye in the electrode enables us to confirm that the impaled cell is the fluorescent cell. The two other electrodes were used to impale a 3 cell and 4 CL neurons. The micrograph was taken through the GG475 filter to block blue light. *C*, Micrograph of the buccal commissure (*bc*) showing the intact axon (*arrow*) of an unirradiated N1. *D*, As *C*, but after 10 min of irradiation of the *bc* with 0.5 MW $\cdot$ m $^{-2}$ blue laser light. The N1 soma is indicated by the *solid arrow*. Note the faded appearance of the axons (*open arrow*). *E*, A fill of the postbuccal nerve (*pbn*) showing the symmetrical distribution of cells. One fluorescent cell, identified as an N2 physiologically, is *arrowed*. *F*, A higher-power view of a second *pbn* fill, showing the soma (*arrow*) and axon of the N2 (*arrowheads*). Scale bars: *A*, *B*, *D*, and *E*, 200 μ m; *C* and *F*, 100 μ m.

8). These are symmetrically distributed in both buccal ganglia (Fig. 2*E*). The N2 interneuron as described by Elliott and Benjamin (1985a) has its cell body located anterior to the 2 cell. In 75 of 84 preparations, a single fluorescent cell was seen in this position; there are no other similar-sized fluorescent cells close by (Fig. 2*F*). In 10 preparations the fluorescent cell was impaled under the low-power laser light and in each of these cases it proved to be an N2 interneuron. Although many of the surrounding cells look superficially similar in size and color to the stained N2 neuron under white light illumination, impalement confirms that they do not meet the physiological criteria for being an N2 interneuron and we have therefore concluded that

there is only one N2 cell located in this part of the buccal ganglion. We cannot exclude the possibility that there may be more N2 cells contained within other regions of the buccal ganglia. It is likely, for example, that there are cells homologous to the N2 cells found by Arshavsky et al. (1988) in *Planorbis* with ventral somata and with axons contained entirely within the buccal ganglia.

2. Photoinactivation of N1 neurons

2.1 Inactivation of individual N1 cells

We inactivated 21 N1 neurons filled with 5-CF by pointing the laser beam on their axon in the *bc*. We chose to irradiate their

Figure 3. Photoinactivation of the axonally stained N1 neuron blocks synaptic transmission. *A*, Before laser irradiation, N1 activation generates the rhythm typical of fictive feeding. After 10 min of illumination of the bc the N1 can no longer activate feeding (normal saline), even though the resting potential is still 44 mV and synaptic and action potentials are seen in the cell body (both N1 stimuli were 0.5 nA). *B*, Analysis in high-divalent (*Hi-Di*) saline of the excitatory synapse from the N1 interneuron to the ipsi- and contralateral 1 cell motoneurons. Before laser irradiation of the bc, the left and right 1 cells are both excited by depolarizing stimulation of the N1 interneuron. After laser irradiation for 10 min, the connection is destroyed and cannot be restored by extra current. *C*, In an unstained N1 in high-divalent saline, laser illumination of the bc has no effect. The resting potential at the end of the experiment was -60 mV.

axons in the bc rather than their cell bodies for three reasons. First, we had found that the N1 interneuron cell body usually lies under the 2 cell, which, being a pigmented giant neuron, might absorb the blue light and so weaken the strength of the beam. Also, the distance between the cut end of the N1 axon in the cbc and the bc is about half the distance from the cbc to the N1 soma, so in the 2 hr filling period more 5-CF will have reached the bc than the soma. Finally, we wanted to be sure that the cell had really been removed entirely from the circuit: if we had first impaled the soma and irradiated it, we could watch the soma die but we could not be sure that the axonal

branches were inactive. However, by irradiating the axon and recording from the soma, we could test if the damage to the axon was sufficient to remove the N1 interneuron from the neural circuit.

We found that, when the axon of a stained N1 interneuron was irradiated, the cell slowly depolarized. After up to 10 min of illumination the membrane potential was 28 ± 5.3 mV (mean $\pm$ SE, $N = 21$). While the membrane potential dropped to less than 15 mV within the 10 min kill period in 7 of the 21 experiments, in the others the membrane potential ranged from 12 to 66 mV. After illumination 35% of the N1 cells still fired action potentials (albeit smaller) and showed synaptic input. The persistence of the soma resting and action potentials did not entirely surprise us, as the site of illumination in the bc was over 200 μ m away from the cell body. Nonetheless, none of the cells could generate any synaptic output. We illustrate the two extremes: Figure 3, *A* and *B*, shows the N1 interneuron that showed the biggest spikes after 10 min of laser irradiation and one of the N1 interneurons in which the membrane potential had declined to 0 mV, respectively. Figure 3*A* shows that before irradiation of the bc the stained N1 could generate rhythmic activity typical of feeding including direct, monosynaptic excitation of the 1 cell. After irradiation, the N1 fires tonically and no inputs are seen on either of the two motoneurons. Figure 3*B* displays a test of the N1 $\rightarrow$ motoneuron connections before and after illumination using a high-divalent saline, which enhances monosynaptic connections and reduces polysynaptic effects (see Elliott and Kemenes, 1992), exhibiting the effects of the N1 cell on the left and right 1 cell, a buccal motoneuron. Before laser illumination, the N1 stimulus evokes an EPSP of gradually increasing size, with a small component starting before the N1 action potentials, probably due to the electrical coupling between N1 interneurons with different thresholds (Elliott and Kemenes, 1992). After laser inactivation of the N1, the same stimulus produces no effect on the 1 cell, nor does a much increased current. Since there is no change of either 1 cell membrane potential, not only does the direct chemical connection appear to be destroyed, but also the electrical connection with other N1 neurons, which could indirectly excite the 1 cells. The abolition of the N1 synaptic output was found in all 17 preparations in which tests were made.

In three unstained preparations, an N1 was impaled with a potassium acetate electrode, so that no dye was present in either electrode or axon. In each of these three control experiments (Fig. 3*C*), the N1 $\rightarrow$ 1 cell synaptic connection persisted after the bc had been irradiated for 10 min.

Figure 2*C* shows a micrograph of a fixed and cleared preparation of the bc showing the axon of an N1 that had been axonally filled with 5-CF. This may be compared with the preparation shown in Figure 2*D*, in which the bc had been illuminated at full laser power for 10 min. Note that the N1 axon has now been broken.

Figure 4 provides more details of the inactivation process. In particular, we compare the inactivation of the N1 filled axonally with 5-CF with inactivation of the contralateral N1 filled by injecting LY into the soma, since this is the most commonly used filling method. During illumination, both cells depolarize, fire action potentials, and die. The action potentials excite the feeding network, so it is usual to see the rhythmic inputs to the feeding motoneurons characteristic of fictive feeding, for example, the small depolarizing inputs on the 1 cell. The LY-filled cell is always quicker to inactivate and its membrane potential

always dropped to be less than 15 mV (average time of 5.1 ± 0.76 min, mean $\pm$ SE, $n = 16$).

In three experiments, either a 5-CF-filled N1 or an LY-filled N1 was impaled with two electrodes in the soma and steady -1 nA current pulses applied through one electrode, with the voltage change recorded using the second electrode. As before, laser irradiation causes a decline in the membrane potential, whether the interneuron is stained with 5-CF or LY (Fig. 4*B,C*). After 5 or 6 min, the membrane potential stabilized at -5 – 15 mV. During irradiation, the cell soma resistance also drops, from about 20 M Ω to 7 M Ω . This is a difference from the somatic kills where the membrane potential and resistance both always decline to zero (Elliott and Kleindienst, 1990; Kemenes et al., 1991, 1992). We also confirmed (Fig. 4*B,C*) that, in control, unfilled N1 neurons, irradiation of the bc had no effect on either membrane potential ($N = 4$) or membrane resistance ($N = 2$) over the entire 10 min period of illumination.

We conclude that, by destroying the axons of N1 cells filled with 5-CF, they can be effectively inactivated. This is due not to the destruction of the cell soma, but to a drop in the resistance of the membrane mainly at the bc. This severely reduces the electrical and chemical synaptic output and this will prevent normal participation in the feeding rhythm. This was borne out in the subsequent experiments in which we looked at the effect of irradiation of single N1 cells on the spontaneous or SO-driven feeding rhythm.

2.2 Effect of N1 inactivation on the spontaneous feeding rhythm

As noted above, fictive feeding often occurs spontaneously in the isolated CNS. We found that, of 18 preparations in which the cbc had been filled for 2 hr, 10 showed spontaneous fictive feeding. In these 18 preparations, we irradiated N1 neurons on their axons, and during inactivation rhythmic activity was seen in nearly all (17) preparations. After completion of the laser inactivation, and a 10 min wash, only four preparations showed rhythmic activity. The transient increase in fictive feeding is not hard to explain since electrical depolarization of an N1 interneurons evokes rhythmic feeding pattern (Rose and Benjamin, 1981b) and we know that axonal irradiation causes depolarization and action potentials in N1 cells (see above). Although the decrease in spontaneous fictive feeding is significant [χ^2 (1 df) = 0.016], it might occur by fatigue and so we must be cautious before concluding that this is a real difference. However, as a comparison, we show below that in experiments to inactivate the N2 (where the same amount of time elapsed between the pre- and postlaser tests), of 10 preparations that showed spontaneous feeding before irradiation, all 10 still demonstrated fictive feeding after photoinactivation.

2.3 Effect of N1 inactivation on the SO-driven feeding rhythm

The SO is a single interneuron in the buccal ganglia (either in the left or right ganglion) that can initiate a constant rate of fictive feeding (Rose and Benjamin, 1981a; Elliott and Benjamin, 1985b). It is the only buccal neuron that, when depolarized electrically, can initiate the feeding rhythm at the rate seen *in vivo* (0.2 – 0.3 Hz; Dawkins, 1974) even in quiescent preparations. Therefore, we used SO stimulation to provide a reliable, known stimulus to test the effects of N1 irradiation on fictive feeding.

In two experiments we succeeded in filling one N1 axonally, filling the contralateral N1 with LY, and finding the SO. Irra-

Figure 4. Comparison of axonal irradiation to kill Lucifer yellow-filled (LY) and 5-CF-filled N1 interneurons. In each case the 5-CF was applied from the cut end of the cbc and the LY by intrasomatic injection. *A*, Electrical recording from an N1 filled with 5-CF, the contralateral homolog filled with LY, and a 1 cell motoneuron. All three neurons have axons in the bc but only the filled neurons are affected. Note that the LY-filled cell membrane potential drops quickly to 0 mV but the 5-CF-filled N1 declines more slowly and only reaches -15 mV. *B* and *C*, Results from three preparations in which N1 neurons were filled with LY or 5-CF, or remained unstained (control), and were impaled with two electrodes. Current pulses (-1 nA, 500 msec at 1 Hz) were passed through one electrode and the voltage changes recorded with the second electrode. Only the filled cells are killed, and in neither case does the somatically recorded resistance drop to zero.

diation of the bc destroyed both left and right N1 interneurons. As before (Fig. 4), the somatically stained cell died quicker than the axonally stained cell. After irradiation, the rate of SO-driven fictive feeding was slowed. We have not attempted a more detailed analysis of the effects of inactivating the left and right N1

Figure 5. Inactivation of an axonally stained N1 interneuron blocks the SO-stimulated feeding rhythm. *A*, Prelaser control, 0.4 nA stimulus elicits the normal feeding pattern with rhythmic inputs to the SO, N1, 1 cell, and 4 CL neurons. The inhibitory inputs to the SO come mainly from the N2 interneurons, while the N1 inputs excite the 1 cell and inhibit the 4 CL neuron. *B*, At 3 min into the irradiation, the N1 has depolarized, but the N1 and N2 rhythmic feeding inputs are still visible on all the neurons when the SO is stimulated with the same current. *C* and *D*, At 6.5 and 8 min, the SO stimuli produce slower rhythms. *E* and *F*, After a 5 min wash, the SO with the same current (0.4 nA, *E*) or with more (0.7 nA, *F*) cannot evoke any rhythmic activity, although a weak inhibitory input onto the 4 CL neuron is still present after each SO stimulus. This weak input indicates that other N1 interneurons are still active. *G*, The irradiation of the bc kills the N1 cell over about 3 min, but does not affect the other cells, even though they have axons in the bc (but not in the cbc, where the 5-CF was applied). The result of inactivating this single N1 cell can be seen by comparing *A* with *B–F*.

neurons since probing for the contralateral N1 interneuron to fill with LY is a time-consuming experiment and the ganglia may deteriorate when a long search period follows the 2 hr filling time.

Instead we have concentrated on the effects of ablating a single, axonally filled N1 on the SO-driven feeding rhythm. In 14 experiments we found the SO evoked control feeding pattern, and then irradiated the bc containing the fluorescent axon of the N1 interneuron. After a 10 min wash, the same stimulus was applied to the SO. In half (seven) of the preparations, we found that no rhythm could be evoked, even with increased SO stimulation. In six preparations the SO evoked a slower rhythm,

Figure 6. Laser photoinactivation of an N1 that has been axonally stained blocks the feeding rhythm. *A*, Before laser treatment, a 0.8 nA stimulus to the SO evokes the normal rhythm with small excitatory inputs to the 1 cell (from N1 interneurons) and a combination of inhibitory and excitatory inputs to the 3 cell (N1 and N2 inputs). Note that the 3 cell receives a barrage of N3 inputs before the feeding rhythm is activated. *B*, After the laser irradiation of the bc for 10 min, the same SO stimulus evokes only a tonic firing of the SO and no effect on the 3 cell. *C*, The electrode was withdrawn from the 1 cell and the contralateral (unstained) N1 interneuron impaled. The same stimulus to the SO evokes the expected small facilitating EPSPs on the N1, of which a detail is seen in *D*. *E*, with the N1 depolarized by 1.4 nA, it fires weakly and tonically until the SO is depolarized. The N1 then fires rhythmic bursts, but these do not appear to be strong enough to elicit the feeding pattern: note the 3 cell only shows one small input and the SO shows none of the expected rhythmic activation. Voltage calibrations are all 20 mV except for the N1 in *D*.

while in the remaining preparation no significant difference in feeding rate was found.

Figures 5 and 6 show records from the preparations in which N1 ablation halted the feeding rhythm. In Figure 5, the SO was stimulated before, during, and after laser inactivation of an N1. In this experiment, the N1 was also impaled so we can compare the time taken to block the feeding rhythm and the time taken to depolarize the N1 cell. In the prelaser control, SO depolarization (0.4 nA) evokes the typical pattern of fictive feeding, with bursts of activity in the SO and N1 interneurons. Records from two motoneurons are also shown; the 1 cell shows small depolarizations produced by each N1 burst while the 4 CL mo-

Figure 7. The reduction in feeding rate in SO-driven rhythms in the seven (of 14) preparations that still showed fictive feeding after ablation of an N1. *A*, Percentage change in SO-driven feeding rate in the seven replicates and the mean ($\pm$ SE) change. The difference is significant [t test, t (6 df) = -3.44 , $P = 0.014$]. *B*, The change in the size of the N1 input to the 1 cell (in mV) in three preparations together with mean + SE. The change is significant at the 5% level. *C*, Results from one preparation where replicate stimuli (each 0.5 nA) were given to the SO to produce 5 cycles of feeding activity before, during, and after laser irradiation and where the feeding rate was monitored for each stimulus. Note the drop during the kill of the N1 cell. *D*, The relationship of feeding rate to SO stimulus size before and after laser irradiation of the N1. At least two replicates were made at each stimulus size and the lines join up the mean feeding rate. Note that 0.1 nA failed to evoke any response in either situation. The difference between initial and postlaser stimuli is biggest with small SO stimuli.

toneuron shows the typical N1 inhibition followed by a biphasic input from the N2 neurons (inhibition followed by excitation) (Rose and Benjamin, 1981b; Elliott and Kemenes, 1992). With 3 min of laser irradiation, when the N1 membrane potential is nearly zero (Fig. 5*G*) and the 1 cell is in consequence noticeably depolarized, the SO drives a faster rhythm. The N1 cell is almost dead by 6.5 min of irradiation, but the recording, if played back on a higher gain, still shows a small synaptic ripple in its membrane potential and a strong feeding rhythm can still be evoked. By 8 min, the N1 is inactive and the feeding rhythm is much slower. After a 5 min wash, to remove any transmitter(s) released by the dying N1, the same current to the SO cannot evoke a rhythm: there is only a very small initial hyperpolarization of the 4 CL motoneuron and a very weak input depolarizing the 1 cell. In these "stopped preparations," the blockage of rhythmic activity cannot be overcome by increased current, for example, in Figure 5*F*, where an increase in the current from 0.4 to 0.7 nA still does not evoke any rhythmic activity. This result was replicated in each of the three preparations in which the test was applied.

In another experiment (Fig. 6), the SO was stimulated with 0.8 nA before and after irradiating the right N1 interneuron. Beforehand, the feeding pattern was evoked as shown by the rhythmic activity of the 1 and 3 cells and the regular interruptions of the SO firing. After removing the filled (right) N1 from the feeding circuit, the same stimulus to the SO created no rhythmic pattern (Fig. 6*B*), nor did increased current (leading to an increased SO firing rate) evoke fictive feeding (not shown). However, we were then fortunate to impale the left N1, which was unstained. The SO still makes an excitatory connection to

this N1 (Fig. 7*C,D*) and individual facilitating EPSPs can be seen on the higher scale. Although we managed this direct pairwise impalement in only one experiment, evidence from recordings like those of Figure 5, *E* and *F*, shows weak N1 inputs on the feeding motoneurons after ablating the stained N1, implying that the SO $\rightarrow$ N1 (unstained) synapse persists. In the experiment of Figure 6*E*, we applied a depolarizing current to the unstained N1 and this resulted in slow tonic firing. Now when the SO is stimulated the N1 is excited and begins rhythmic firing. However, this pattern is not seen in the SO or 3 cell and it is clearly not the full N1 $\rightarrow$ N2 $\rightarrow$ N3 $\rightarrow$ N1... sequence typical of feeding. Thus, in the stopped preparations, N1 ablation prevents SO-driven feeding and this cannot be restarted by increasing the current or by applying a compensatory current to the surviving N1.

Figure 7 summarizes the results from the seven preparations in which rhythmic fictive feeding was observed after N1 ablation. On average the rate of fictive feeding dropped by 44%, but the percentage change ranged widely: one preparation showed a 99% decrease in feeding rate while another showed a 3% increase. (In this preparation we are convinced that the N1 was killed, as it was stained very brightly, but the unusual result may arise from the fact that the preparation was very active and showed a very high level of spontaneous fictive feeding without any SO stimulation throughout the experiment.)

The drop in feeding rate is accompanied by a drop in the amplitude of the rhythmic N1 input to the motoneurons. We analyzed data from three experiments in which the synaptic inputs to the 1 cell were recorded before and after laser irradiation of the N1, as the only synaptic inputs that the 1 cell

Figure 8. Photoinactivation of an N2 interneuron. Note that the first SO stimulus evokes a burst of activity in the N2 neuron indirectly by activating just a single cycle of the feeding pattern, but that the SO test at the end of the sampled period shows no effect in the N2 cell; neither the direct inhibition (Fig. 1; see Elliott and Benjamin, 1985b) nor the polysynaptic excitation is seen despite the SO activating several feeding cycles.

receives in the feeding cycle are EPSPs from the N1 interneurons. In each of our three experiments the amplitude of the N1 EPSP was reduced (Fig. 7B), with an average reduction of 52%, which is significant at the 5% level.

The drop in feeding rate usually takes place during the laser treatment (Fig. 5C). Before irradiation, each depolarizing stimulus of 0.5 nA to the SO led to an SO-driven rhythm with a constant rate (mean of 0.30 Hz). At the start of the laser illumination the feeding rate increased transiently. There can be little doubt that when the N1 is slightly depolarized it is easier for the SO to excite the N1 neuron and so start up the rhythm. However, by the end of the 10 min irradiation, with the N1 dead and its transmitter washed out, the same stimulus to the SO results in a much slower rhythm and this lower rate persists afterward for at least 14 min (mean rate of 0.17 Hz).

We examined the relationship between SO stimulus size and evoked feeding rate before and after the N1 cell is removed from the feeding circuit (Fig. 5D). The SO was depolarized tonically with currents from 0.1 to 0.6 nA. At 0.1 nA the SO just fires tonically and cannot excite fictive feeding because the SO is not firing fast enough for the SO → N1 EPSP to facilitate and lead to N1 action potentials. With a larger stimulus (0.2 nA), the fictive feeding starts up and the feeding rate is already near its maximum (0.3 Hz), because of the strong reciprocal connections between the SO and N1 (Fig. 1). Further increase in stimulus current does not produce any increase in feeding rate. After laser irradiation of the bc to ablate the N1 cell, 0.2 nA is still the threshold current for fictive feeding, but the feeding rate is much slower, just over 0.2 Hz. Only with 0.6 nA stimuli does the SO feeding rate after laser treatment equal that beforehand. Thus, the main difference is that N1 ablation prevents weak SO stimuli from exciting full fictive feeding; with strong stimuli to the SO N1 ablation is less effective. This result was confirmed in a second preparation.

We conclude that the main effect of removing a single N1 cell from the feeding network is to slow the feeding rhythm. In half the preparations this was sufficient to block any rhythmic activity; in the others it serves simply to slow the rate of fictive feeding.

3. Photoinactivation of N2 neurons

3.1 Inactivation of individual cells

The N2 interneurons were filled with 5-CF by cutting the pbn. As noted above this fills the left and right N2 cells because both have axons in this midline nerve. The N2 neurons were inactivated by applying the laser onto the soma rather than the axons in the bc, because this contains the axons of many neurons that have not been identified. Moreover, axonal irradiation would kill both the left and right N2 interneurons, so the effect of removing a single N2 interneuron could not be tested. However, the immediate area around the soma is free of other stained neurons and so by pointing the beam at the soma the N2 can be selectively ablated.

The results of one of the six N2 kills are shown in Figure 8, in which the N2 and the SO were impaled. Initially, the SO stimulation activates the feeding circuit and this leads to action potentials in the N2 interneuron. When the laser is pointed at full power onto the N2 cell body, its membrane potential gradually declines with a burst of action potentials of progressively decreasing size. Eventually the N2 membrane potential reaches zero. This compares closely to the typical pattern of motoneuron somatic inactivation recorded earlier (Elliott and Kleindienst, 1990; Kemenes et al., 1991), the main difference being that killing the N2 interneurons has a consistent effect on the other buccal interneurons. For example, as the burst of N2 spikes begins, the SO receives IPSPs as is expected from their known reciprocal inhibitory connections (Elliott and Benjamin, 1985b). The same is true of the feeding motoneurons; for example, in four of five N2 ablation experiments in which a 3 cell was impaled, during the irradiation a depolarization of the 3 cell was seen during the inactivation. This is entirely as would be expected since the N2 neuron synaptically excites the 3 cell motoneurons. Another record is shown by Kemenes et al. (1992), in which an N2 and a 4 CL motoneuron were recorded: in that case the laser-induced burst of spikes in the N2 inhibited the motoneuron, as was expected from their monosynaptic connections. As the N2 membrane potential declines further, the action potentials disappear and eventually the membrane po-

tential declines to less than 15 mV. The mean time to kill the N2 cells was 6.7 ± 1.5 min, about twice as long as was required to kill the larger motoneurons (3.5 min; Kemenes et al., 1991).

3.2 Effect of N2 inactivation on spontaneous feeding rhythm

As noted above, many preparations are spontaneously active. Although their feeding rate varies randomly, the N2 phase is very constant in duration and in the amplitude of the inputs to the motoneurons (Elliott and Andrew, 1991). We have therefore examined in detail the effects of N2 removal on the spontaneous fictive feeding pattern as well as on SO-driven rhythms. In 10 spontaneously feeding preparations the left and right N2 neurons were irradiated. In each case the feeding rhythm persisted after laser illumination of either one or both interneurons and in each the N2 phase synaptic input was still present on the motoneurons. In Figure 9A, an example is shown of a preparation with records from three motoneurons: the 3 cell, 7 cell, and 4 CL neurons. The N2 input is clear on all three cells (dashed rectangles): it provides a smooth depolarization to the 3 cell, a strong inhibition of the 7 cell, and a biphasic (inhibitory then excitatory) effect on the 4 CL neuron. We measured the duration of the N2 burst in each preparation and found that there was no consistent change in the duration of the N2 phase of the feeding rhythm: in four preparations the N2 duration decreased and in two it increased; the other showed only a very small difference (Fig. 9B). Comparing the duration of the N2 phase before laser treatment and after killing both N2 interneurons, the mean change was 0.16 sec [$t(9 \text{ df}) = 1.18$, $P = 0.27$, paired t test]. In case killing the soma had left some part of the N2 intact, in two preparations we irradiated the axons of the filled cells in the bc, but still found that the N2 inputs were present on the feeding motoneurons. We conclude that inactivating the N2 neurons does not significantly affect the spontaneous fictive feeding pattern.

3.3 Effect of N2 inactivation on SO-driven feeding rhythm

The SO was depolarized with a constant current and used to drive fictive feeding in a further five preparations, before and after irradiating the left and right N2 neurons. In each case the N2 input persisted on the buccal interneurons and motoneurons. In the example shown in Figure 10A, the N2 inputs can be recognized as the strong inhibition of the SO and the smooth depolarization, with spikes on top, of the 3 cell. Note that these inputs can be still be seen after inactivation of both N2 interneurons. In two preparations, we irradiated both the somata and the axons in the bc, and found that the N2 inputs on the SO and on the motoneurons still persisted.

The details of the quantitative analysis of five preparations are shown in Figure 10, B and C. After killing the left and right N2 interneurons, the mean difference in duration of the N2 inputs is -0.51 sec, but this is not significant [paired t test, $t(4 \text{ df}) = 1.86$, $P = 0.07$]. Most of this change arises from two preparations (Fig. 10B, replicates 1 and 2), but the other preparations showed little change. It may be significant that the preparations that showed the largest drops had initial N2 durations that were much longer than normal (their initial N2 duration is longer than 2 sec but the mean duration of our 15 preparations was 1.46 sec). Nor did inactivation of the N2 neurons affect the fictive feeding rate (mean change is a nonsignificant increase of 23%, much of which can be accounted for by the drop in N2 duration in preparations 1 and 2; see Fig. 10C).

In summary, inactivating both the known N2 cells does not

Figure 9. Spontaneous N2 inputs persist in the feeding motoneurons after ablation of the two identified N2 neurons. *A*, A sample from one preparation showing excitation of the 3 cell, inhibition of the 7 cell, and a biphasic (inhibitory then excitatory) input the 4 CL neuron. Each N2 phase is enclosed by a dashed rectangle. In this recording three different 4 CL neurons were impaled, one for each section. Voltage calibration is 20 mV for all except the middle 4 CL recording, where it is 12 mV. *B*, Analysis of the effects of inactivating the two stained N2 interneurons in turn in 10 spontaneously active preparations. All 10 preparations were spontaneously active before, during, and after killing the N2 cells. The mean duration of the N2 phase is not significantly changed, the error bars being ± 1 SE.

abolish the N2 phase of the feeding rhythm; in fact, the synaptic inputs continue and have same duration whether fictive feeding is spontaneous or induced by SO stimulation. Nor do our measurements show any consistent change in the SO-driven feeding rate.

Discussion

We have ablated N1 or N2 feeding neurons that had been axonally filled with 5-CF by illuminating them with blue laser light. The axonal filling tests showed that, in each ganglion, there was just one N1 neuron and just one N2 neuron with the known morphology in each of the buccal ganglia (Fig. 2). Thus, our lesions to the CNS are very specific: we have killed either only one N1 and one or two (left and right) N2 interneurons. Killing a single N1 cell consistently slows down the SO-driven feeding rate and in 50% of the preparations this is sufficient to stop feeding altogether (Figs. 5, 6). In preparations in which the fictive feeding occurs after N1 ablation, the feeding rate is slower and the N1 input to the motoneurons, for example, the 1 cell, is

Figure 10. During SO-driven fictive feeding, N2 inputs persist in the buccal motoneurons after ablation of both of the two identified N2 neurons. *A*, Recording from one preparation in which the SO, an N2, and a 3 cell motoneuron were impaled. The N2 inputs to the SO and 3 cell can be recognized by the strong inhibition of the SO and the excitation of the 3 cell, though the 3 cell also receives smaller, discrete EPSPs from the N3 interneuron (Elliott and Benjamin, 1985a). After killing both left and right N2 interneurons, the N2 inputs persist. Quantitative analysis shows that there is no statistically significant change in the N2 duration (*B*) or SO-driven feeding rate (*C*) when the two N2 neurons are ablated. Means are shown $\pm$ 1 SE.

reduced (Fig. 7). Killing the N2 neurons, on the other hand, has little effect on either the spontaneous or the SO-driven fictive feeding, with no significant change in feeding rate or N2 duration (Figs. 9, 10).

We shall discuss these results to assess first the efficiency of the killing technique, second the possible redundancy in the feeding system, and third their usefulness in verifying the feeding model.

1.1 Axonal filling and killing

Our first concern is with the efficiency and selectivity of our developments to the axonal filling and killing techniques. While Kemenes et al. (1991) showed that the motoneurons could be filled reliably and consistently killed when irradiated on the soma, filling N1 and N2 interneurons presents a more selective challenge. It is clear that, with 5-CF, we have been able to fill N1 and N2 neurons in the parts of the buccal ganglia in which we expected to find their cell bodies from Elliott and Benjamin's (1985a) LY injections. These neurons were seen reliably, in 99% and 90% of the preparations, respectively. We found little variation in the number of filled cells, nor was there any indication that the replicates filled different subclasses of the total population, and so we feel confident that the method is consistent and does not stain a proportion of the cells. We have not found any preparation in which two N1 cells or two N2 cells were visible and we therefore conclude that these interneurons are unique in each ganglion. This conclusion was strengthened in each case by a search of the adjacent cells that failed to show any neurons with the same morphological and physiological properties.

We can also be confident that both the axonal and somatic photoinactivation methods work reliably because 100% of the filled neurons were inactivated within 10 min, respectively. At the same time, since unstained cells are not affected by 10 min of irradiation (Figs. 3, 4, 8; see also Elliott and Kleindienst, 1990; Kemenes et al., 1991), we can be sure that the lesion we make is very precisely confined to those cells that have been both stained and illuminated. We have also demonstrated, with N1 cells, that the axonal irradiation is sufficient to remove the entire cell from the network. Irradiating the axon does not just lead to an anatomical destruction of the fibers (Fig. 2D); it also causes a drop in the membrane impedance such that any inputs are ineffective in generating synaptic output (either chemical or electrical; Fig. 3). This is the case whether the soma can still show resting and action potentials or whether it has been depolarized to zero.

Our other concern is with the specificity of the kill. With N2 somatic kills, we know that only the N2 soma will be in the center of the beam, as there are no other stained cells nearby (Fig. 2E,F), and so our irradiation is quite specific. This is not so in axonal kills of the N1 neurons filled from the contralateral cbc in which the bc is illuminated, since up to five other cells show up in the ganglion. Undoubtedly, these cells will be killed along with the N1 interneuron, so we have checked carefully that these cells do not participate in (or modulate) the feeding pattern. In a few experiments we did fill N2 neurons from the pbn and then irradiated the bc. In this scenario, we were certainly killing most of the 55 neurons that have axons in the pbn, many of which may be involved in feeding, but we still did not find a change in the SO-driven feeding pattern.

1.2 Redundancy

Although we have only found one N1 and one N2 interneuron in each buccal ganglion, it is clear that there are more homologous cells present. For example, Rose and Benjamin (1981b) suggested that there were up to 10 N1 cells in the ganglion that were electrically coupled. However, only one of these N1 cells appears to have an axon in the contralateral cbc; others may be confined to the buccal ganglia. We have found that the N1 with an axon in the cbc is essential for the SO-driven feeding rhythm,

since ablation of a single N1 of this type blocks the SO-evoked pattern in half the preparations and slows the rhythm significantly in the others. Photoinactivation of the N1 also reduces spontaneous rhythmic feeding activity. This neuron makes strong cholinergic connections with the feeding motoneurons and with the SO (Elliott and Kemenes, 1992), and its axon running into the cerebral ganglion suggests that it may play a role in coordinating feeding activity in the two ganglia. However, we have not tested its role in the cerebral ganglion because the cbc was always cut to fill the axon. We speculate that the different types of N1 interneuronal morphology may be related to different types of physiology and pharmacology and some may resemble the dopaminergic neuron of *Helisoma* with an axon solely within the buccal ganglia (McLean et al., 1989).

Although the N1 neuron we have been able to kill is not redundant, the same does not appear to be true of the N2 neurons. Both the left and right neurons can be removed without affecting the feeding rhythm, as judged by the lack of any consistent change in the duration of the N2 phase or in feeding rate. There is no change in either spontaneous or SO-driven fictive feeding. At least in these situations our type of N2 neuron appears redundant and other N2-type interneurons must dominate the rhythm generation. Again, a difference in function between different groups of N2 cells may be important; the N2 neurons we have ablated fire far fewer action potentials than the kind of N2 described in *Planorbis* by Arshavsky et al. (1988). Despite our failure to show that the N2 neurons have an essential role in feeding by photoinactivation, we still think that they are part of the pattern generator because of their ability to reset the rhythm (Elliott and Benjamin, 1985a). However, the existence of N2 axons in the pbn may indicate that their main role may be in pattern distribution rather than pattern generation.

1.3 Testing the feeding model

The main difference between the ablation of N1 and N2 neurons is that removal of one N1 has a strong slowing effect on fictive feeding, but inactivation of even two N2 interneurons has little effect. This is a major new difference in the organization of the feeding system. It would fit well with the results of Elliott and Andrew (1991), who found that the N2 phase of the feeding rhythm was far more stable than the N1 phase. They showed that the firing rate of neurons active in the N2 phase was constant irrespective of feeding rate, whereas the faster the feeding rhythm the faster the N1 neurons fired and the shorter the N1 duration. Elliott and Andrew (1991) speculated that this difference arose because the N2 phase duration was controlled by an endogenous driver potential that terminated the N2 phase of the rhythm whereas the N1 activity ended when the N1 EPSP to the N2 reached the N2 threshold. This hypothesis would explain why removal of one N1 has an effect on spontaneous feeding rhythms: if the N1 is no longer exciting the N2, then the N2 neuron will no longer be able to reach threshold and so the rhythm will get stuck in the N1 phase. With SO rhythms, the main effect may be on the SO $\leftrightarrow$ N1 reciprocal excitatory pathways. We have seen after ablation of a single N1 that the SO can weakly activate the remaining N1 neurons, but does not lead to strong firing in the N1. This is shown by the direct recording of Figure 6 and by the reduced N1 input seen in the 1 cell (Fig. 7).

An alternative hypothesis is that the greater stability of the N2 phase than the N1 phase results from a larger number of yet to be identified N2 than N1 neurons. In this case, inactivating just a small percentage of N2 cells would be expected to have

little effect, whereas inactivating one out of a few (perhaps four) significant N1 neurons would result in a big change to the N1 network. At present we rather prefer the endogenous driver hypothesis, since Elliott and Benjamin (1985a) provided some preliminary evidence for the occurrence of driver or plateau-like potentials in the N2 network.

References

- Arshavsky Yu I, Deliagina TG, Orlovsky GN, Panchin Yu V (1988) Control of feeding movements in the freshwater snail *Planorbis corneus*. III. Organisation of the feeding rhythm generator. *Exp Brain Res* 70:332–341.
- Benjamin PR, Elliott CJH (1989) Snail feeding oscillator: the central pattern generator and its control by modulatory interneurons. In: *Neuronal and cellular oscillation* (Jacklet JW, ed), pp 173–214. New York: Dekker.
- Benjamin PR, Rose RM (1979) Central generation of bursting in the feeding system of the snail *Lymnaea stagnalis*. *J Exp Biol* 80:93–118.
- Boyle MB, Cohen LB, Macagno ER, Orbach H (1983) The number and size of neurons in the gastropod CNS and their suitability for optical recording of activity. *Brain Res* 266:305–317.
- Dawkins M (1974) Behavioural analysis of coordinated feeding movements in the gastropod *Lymnaea stagnalis*. *J Comp Physiol* 92:255–271.
- Elliott CJH, Andrew T (1991) Temporal analysis of snail feeding rhythms: a three phase relaxation oscillator. *J Exp Biol* 157:391–408.
- Elliott CJH, Benjamin PR (1985a) Interactions of pattern generating interneurons controlling feeding in *Lymnaea stagnalis*. *J Neurophysiol* 54:1396–1411.
- Elliott CJH, Benjamin PR (1985b) Interactions of the slow oscillator interneuron with feeding pattern generating interneurons in *Lymnaea stagnalis*. *J Neurophysiol* 54:1412–1421.
- Elliott CJH, Kemenes G (1992) Cholinergic interneurons in the feeding system of the pond snail *Lymnaea stagnalis*. II. N1 interneurons make cholinergic synapses with feeding motoneurons. *Philos Trans R Soc Lond [Biol]* 336:167–180.
- Elliott CJH, Kleindienst H-U (1990) Photoinactivation of neurones in the pond snail *Lymnaea stagnalis*: estimation of a safety factor. *Brain Res* 524:149–152.
- Elliott CJH, Stow RA, Hastwell C (1992) Cholinergic interneurons in the feeding system of the pond snail *Lymnaea stagnalis*. I. Cholinergic receptors on feeding neurons. *Philos Trans R Soc Lond [Biol]* 336:157–166.
- Iles JF, Mulloney B (1971) Procion yellow staining of cockroach motor neurones without the use of microelectrodes. *Brain Res* 30:397–400.
- Kemenes G, Elliott CJH, Benjamin PR (1986) Chemical and tactile inputs to the *Lymnaea* feeding system: effects on behaviour and neural circuitry. *J Exp Biol* 122:113–137.
- Kemenes G, Daykin K, Elliott CJH (1991) Photoinactivation of neurons axonally filled with the fluorescent dye 5(6) carboxyfluorescein in the pond snail *Lymnaea stagnalis*. *J Neurosci Methods* 39:207–216.
- Kemenes G, Daykin K, Elliott CJH (1992) Vital staining with 5(6) carboxyfluorescein in the feeding system of *Lymnaea* and the selective photoinactivation of the filled neurones. *Acta Biol Acad Sci Hung* 43:351–360.
- McCrohan CR (1984) Initiation of feeding motor output by an identified interneurone in the snail *Lymnaea stagnalis*. *J Exp Biol* 113:351–366.
- McCrohan CR, Benjamin PR (1980a) Patterns of activity and axonal projections of the cerebral giant cells of the snail *Lymnaea stagnalis*. *J Exp Biol* 85:149–168.
- McCrohan CR, Benjamin PR (1980b) Synaptic relationships of the cerebral giant cells with motoneurons in the feeding system of *Lymnaea stagnalis*. *J Exp Biol* 85:169–186.
- McLean EM, Behjatnia B, Murphy AD (1989) An identified “dopaminergic” buccal interneuron activates patterned motor activity in *Helisoma*. *Soc Neurosci Abstr* 15:739.
- Miller JP, Selverston AI (1979) Rapid killing of single neurons by irradiation of injected dye. *Science* 206:702–704.
- Quinlan EM, Murphy AD (1991) Glutamate as a putative neurotransmitter in the buccal central pattern generator of *Helisoma trivolvis*. *J Neurophysiol* 66:1264–1271.
- Rose RM, Benjamin PR (1979) The relationship of the central motor

- pattern to the feeding cycle of *Lymnaea stagnalis*. J Exp Biol 80:137–163.
- Rose RM, Benjamin PR (1981a) Interneuronal control of feeding in the pond snail *Lymnaea stagnalis*. I. Initiation of feeding cycles by a single buccal interneurone. J Exp Biol 92:187–201.
- Rose RM, Benjamin PR (1981b) Interneuronal control of feeding in the pond snail *Lymnaea stagnalis*. II. The interneuronal mechanism generating feeding cycles. J Exp Biol 92:203–228.
- Rosen SC, Weiss KR, Goldstein RS, Kupfermann I (1989) The role of a modulatory neuron in feeding and satiation in *Aplysia*: effects of lesioning of the serotonergic metacerebral cells. J Neurosci 9:1562–1578.
- Selverston AI, Miller JP (1980) Mechanisms underlying pattern generation in lobster stomatogastric ganglion as determined by selective inactivation of identified neurons. 1. Pyloric system. J Neurophysiol 44:1102–1121.
- Syed NI, Ridgway RL, Lukowiak K, Bulloch AGM (1992) Transplantation and functional integration of an identified respiratory interneuron in *Lymnaea stagnalis*. Neuron 8:767–774.