THE NEURAL BASIS OF BEHAVIOURAL CHOICE

IN THE SNAIL

LYMNAEA STAGNALIS

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CONTENTS

ABSTRACT

1) A detailed study was carried out on the subthreshold inputs received by, and on the activity of, the cerebral ventral 1 interneuron (CV1), a previously described higher-order modulatory feeding interneuron in the pond snail *Lymnaea stagnalis* (McCrohan, 1984b).

In addition to the cyclical synaptic inputs from the feeding central pattern generator (CPG), CV1 was shown to receive three types of subthreshold inputs - two types of IPSPs (of amplitudes 1 and 4 mV respectively) and EPSPs. The bias of inputs received by CV1 was shown to be important in determining CV1's ability to initiate feeding motor output from the buccal CPG. In the isolated CNS, spontaneous changes in the balance of inputs resulted in different activity "states" of CV1, the exact relevance of which is unknown. A change in inputs received by CV1 was also partly responsible for initiating feeding following food application to the lips, and for modulating the feeding response following previous experience, i.e. satiation and positive conditioning.

2) *Lymnaea's* response to isolated aversive stimuli was shown to consist of a stereotyped avoidance behaviour, which only inhibited ingestive feeding movements for a short time during full withdrawal. Behavioural experiments in an aversive tasting environment, however, showed that spontaneous feeding movements were reduced, but not completely inhibited with *Lymnaea* still occasionally sampling the environment in an attempt to find edible food. If food was encountered, then normal feeding movements were seen.

The inhibition of feeding by aversive stimuli seen in the whole animal was not reflected by electrophysiological recordings from the lip-CNS preparation, which suggests that the CV1 pathway was not involved in inhibiting feeding in response to aversive stimuli.

3) CV1 was proposed to be homologous to cerebral-to-buccal interneurons in several other gastropods, namely the PC_p, CBI-2 and CB_1 of *Pleurobranchaea*, *Aplysia* and *Limax* respectively. This was based on their similar activity and inputs, and ability to initiate and modulate feeding following application of food to the lips and previous experience.

4) A preliminary electron microscope study of the oral area and tentacles demonstrated the presence of several candidate sensory structures which may be involved in sensory signalling of mechanosensory and chemosensory stimuli to the feeding network of *Lymnaea*. These included two types of cilia found both on the oral area and tentacles, and large "microvilli" structures which constitute the lips.

5) Conditioning experiments demonstrated strong appetitive (positive) conditioning to amyl acetate in the whole animal which was retained for up to three weeks. This learning was retained in the lip-CNS preparation, and electrophysiological recording showed that the learned feeding response was at least partly via the CV1 pathway. In contrast, *Lymnaea* showed weak food aversive conditioning, with only a short-lived (<24 hrs) generalised unresponsive state seen after training. This is unusual within the gastropod molluscs.

6) The results are discussed in relation to comparative studies on other gastropod molluscs, and any differences related to the contrasting environments in which the different species have evolved, and thus adapted to.

DECLARATION

I hereby declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

H. A. Whelan, 1994

EDUCATION AND RESEARCH EXPERIENCE

Since obtaining a first class honours degree in Biology from the University of York in 1990, I have worked in the Department of Physiological Sciences (now Biological Sciences) at Manchester University under the supervision of Dr C. R. McCrohan. My work was funded by a S.E.R.C. studentship.

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CHAPTER 1 - GENERAL INTRODUCTION

GENERAL INTRODUCTION

Understanding how the nervous system generates behaviour is one of the most fundamental and interesting problems within neuroscience. Studies of this nature, however, are difficult owing to the obvious flexible nature of animal behaviour, and the complexity of the underlying neural networks. As a result, many studies have been carried out using "simpler" invertebrate systems. There are two main reasons for this: firstly, invertebrate nervous systems consist of relatively small numbers of neurons which are usually constant in their number and location, making it possible to investigate neural networks at the level of the individual, identified cells within the "circuit"; and secondly, early behavioural studies on invertebrates led to the belief that invertebrates showed inflexible stereotyped behaviour (or fixed action patterns) which were mediated by simple neural networks.

A "fixed action pattern" (FAP) can be defined as a stereotyped sequence of movements that is only performed in highly specific situations, and occurs in exactly the same manner every time. This term is now considered to be rather antiquated as it implies a very inflexible behaviour pattern, and does not allow for any modulation of behaviour, e.g. slower sequence speed, or reduced number of sequence cycles. It is now accepted that such variation occurs in many "FAP-like" behaviours (Schleidt, 1974). One classic example is the bee waggle dance, which is actively modified to represent different messages such as the distance and direction of the food source (Von Frish, 1967). Therefore, broader, more appropriate terms such as "motor pattern" are generally in greater use today. Typical examples of invertebrate "motor pattern" behaviours include feeding, walking, flying and swimming, all of which comprise coordinated, repetitive rhythmic movements. Because of their relative simplicity much work has been carried out on these types of behaviour in different invertebrate models,

including swimming in the marine molluscs *Tritonia* (Getting and Dekin, 1985) and *Clione* (Arshavsky, 1986), locust flight (Stevenson and Kutsch, 1987), and feeding in the gastropod molluscs *Lymnaea stagnalis* (Benjamin and Elliott, 1989) and *Pleurobranchaea californica* (Davis *et al.*, 1984).

Studies on invertebrates systems (and also vertebrates) led to the generally accepted view that "motor patterns" or "FAPs" were generated centrally by central pattern generators (CPG; Delcomyn, 1980). A central pattern generator usually consists of several neurons (or populations of neurons) which are connected synaptically and produce a coordinated cyclical output when activated (Friesen and Stent, 1978; Selverston and Moulins, 1985). It was generally found that sensory input was not necessary for central pattern generation (Rose and Benjamin, 1981b; Robertson and Pearson, 1983), and it was probably this observation that led to many early experiments being carried out in either the isolated central nervous system (CNS) or greatly reduced preparations. The lack of sensory input in these experiments resulted in the postulation of inflexible "hard-wired" neural models which are probably far removed from real behaviour.

Many of these "hard-wired" neural models incorporated command neurons, single neurons which were capable of initiating and terminating specific behaviour patterns. The concept of the command neuron was first introduced by Wiersma and Ikeda (1964) who described neurons that could elicit rhythmic movements of swimmerets in the crayfish. During the next decade the concept became very fashionable in invertebrate research, with workers discovering "command neurons" in systems ranging from sea slugs (Gillette *et al.*, 1978) to cockroaches (Pearson and Fourtner, 1975). However, as further research was carried out on more intact preparations, more variations in the output of supposedly "hard-wired" networks were seen. This led to the concept of true command neurons being questioned, since it relied on

very rigid and inflexible systems with little sensory feedback or modulation from other parts of the CNS (Kupfermann and Weiss, 1978). It gradually became clear that invertebrates did not have "hard-wired" nervous systems, and that so-called "command neurons" had varying ability to switch on activity in a network at different times depending on internal and external factors. This "gated" modulation of output from networks is now thought to be the basis for decision making, with higher-order interneurons providing a locus at which internal and external cues converge.

THE USE OF INVERTEBRATE SYSTEMS TODAY

An important justification for using invertebrates in neuroethological studies is that functional similarities have been found between several different invertebrate and vertebrate systems (ranging from molluscs and insects, to fish and humans; Kien *et al.*, 1992). Therefore, it is frequently valid to apply neural "organisational principles" found from studying lower animals, such as invertebrates, to the more complex vertebrate nervous system. Several general "organisational principles" were recently highlighted by Kien *et al.* (1992):

1) Neural networks are composed of many smaller multifunctional networks.

2) Changes in motor pattern can occur if different neurons within the same network are active at different times, or the total number of neurons active is reduced.

3) Generation of behaviour often requires a very large number of neurons, especially when motor output is being modified.

4) Specific behavioural functions cannot be localised to a specific neuron (i.e. a command neuron), but are a result of activity in and between networks of neurons.

If the principles underlying the neural generation of behaviour are truly comparable

in all systems, then the advantage of working with invertebrates is great, as the total number of neurons involved is extremely small compared to that in vertebrate brains, whilst the behaviour is complex enough to be interesting, but not so complex as to make it difficult to analyze. Gastropod molluscs have proved to be extremely good models for neuroethological studies, despite the view of some workers that their behavioural repertoire is too limited to be of any interest (*Aplysia* was described by Hoyle (1984) as a "behaviourally boring glob of squishy protoplasm"!). Despite these doubts, there has been an enormous amount of research carried out on molluscs, resulting in the postulation of many important and fundamental neuroethological theories, including the famous *Aplysia* model for the cellular basis of learning (Kandel and Schwartz, 1982).

The gastropod mollusc *Lymnaea stagnalis* (great pond snail) has been no exception. Numerous behavioural and electrophysiological studies have been carried out into several aspects of its behaviour. These include feeding (Benjamin, 1983; Benjamin and Elliott, 1989; Elliott *et al.* 1988; Tuersley and McCrohan, 1987a,b, 1988, 1989), withdrawal (Ferguson and Benjamin, 1991a,b; Syed and Winlow, 1989a), locomotion (Syed *et al.*, 1988; Syed and Winlow, 1989b; Winlow and Haydon, 1986; Haydon and Winlow, 1986), respiration (Janse *et al.*, 1985; Syed *et al.*, 1990, 1991a,b) and egg-laying (Jansen and Bos, 1984; Jansen and Ter Maat, 1985; Jansen *et al.*, 1985; Ter Maat *et al.*, 1989; Vlieger *et al.*, 1980). There have also been several studies into how these different behaviours inter-relate (Kyriakides and McCrohan, 1988; McCrohan and Winlow, 1985; Syed and Winlow, 1988, 1991; Ter Maat *et al.*, 1982; Winlow *et al.*, 1981). These extensive studies into *Lymnaea*'s different behaviours offer a real advantage to present day workers, as many of the "hard-wired" neural connections have already been described, making the job of describing modulation of behaviour at a neural level easier.

The feeding system of *Lymnaea stagnalis* has been particularly extensively researched and described. The "hard-wired" central pattern generator has been identified, along with several "command-like" neurons. Therefore, feeding in *Lymnaea* is an excellent system in which to study neural and thus behavioural modulation. Furthermore, the factors that could modulate feeding behaviour are relatively easy to anticipate, i.e chemosensory input to the system (different tastes; food), incompatible behaviours (e.g. withdrawal), and previous experience (i.e. satiation or conditioning).

THE FEEDING SYSTEM OF *LYMNAEA*

Behaviour

Lymnaea stagnalis is found mainly in freshwater ponds and very slow moving rivers. Although *Lymnaea* is actually an omnivore, its main source of food comes from algal grazing (Dawkins, 1974).

The consummatory feeding behaviour in *Lymnaea* consists of a stereotyped sequence of rotational movements of the muscular buccal mass, resulting in food being scraped up into the mouth by the radula (toothed strip) and swallowed. This behavioural sequence can be described as a cyclical rhythm, consisting of four different phases: resting, protraction, retraction 1 (rasp) and retraction 2 (swallow) (Rose and Benjamin, 1979; Figure 1.1).

In the first, or resting phase, the buccal mass is inactive, the cartilaginous odontophore and attached radula are withdrawn well within the buccal cavity and the mouth is closed. This phase is usually absent or extremely short in the presence of food. The second phase, protraction, consists of the buccal mass rotating the odontophore which results in the radula being pushed out of the open mouth and into contact with the food substrate. This is followed closely by the third phase, retraction 1 (rasp), where the odontophore is moved in the opposite

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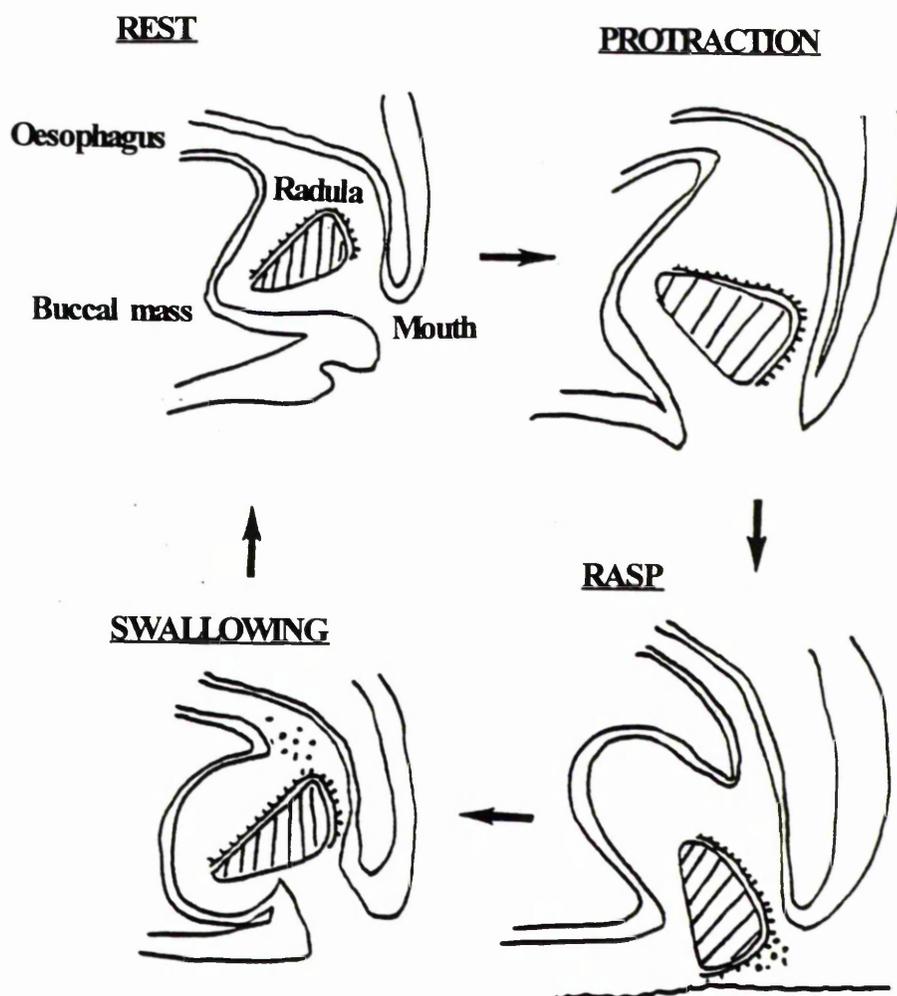
Figure 1.1 Simplified diagram showing the four phases of the feeding cycle. Rest - position of buccal mass and radula between feeding cycles. Protraction - 1st phase of movement when the buccal mass and odontophore rotate forwards and the radula is eventually pressed against the food substratum. Rasp (first stage of retraction) - the buccal mass begins to move backwards and the food is scraped from the substratum by the radula into the buccal cavity. Swallowing (2nd phase of retraction) the radula and odontophore are moved strongly backwards pushing the food into the oesophagus. Adapted from Benjamin and Elliott (1989).

(backwards) direction by the buccal mass, resulting in the radula being moved across the substrate scraping food into the mouth. The final phase, retraction 2 (swallow) is where the radula is brought sharply back into the buccal cavity so forcing food down the oesophagus (Benjamin and Elliott, 1989).

In the presence of food, the feeding cycle is repeated up to several hundred times with each cycle taking about 3-5 seconds (Benjamin and Rose, 1979). Spontaneous feeding cycles also occur in the absence of food stimuli and this has been termed "food search activity" (Tuersley and McCrohan, 1987a). Food search behaviour has also been observed in *Helisoma* (Kater and Rowell, 1973).

The buccal central pattern generator (CPG).

Feeding movements are the result of the innervation of the buccal musculature (Rose and Benjamin, 1979) by identified feeding motoneurons (B1-B10) located on the dorsal surface of the buccal ganglia (Rose and Benjamin, 1981a), and of the lips by motoneurons on the ventral surface of the cerebral ganglia (CV3, 5 and 7; McCrohan, 1984a) (Fig. 1.2). The motoneurons themselves do not generate the feeding rhythm, but are driven by a separate central pattern generator network of interneurons (also located in the buccal ganglia).

The buccal central pattern generator (CPG) consists of three populations of interneurons (Benjamin and Rose, 1980; Elliott and Benjamin, 1985a): 1) the dorsal N1s (n=10), 2) the dorsal (n=3) and ventral N2s (n=2)[1] (Elliott and Benjamin, 1985a; Elliott, pers. comm.), and 3) the dorsal N3s (tonic and phasic; n=3) interneurons. The three populations of interneurons fire consecutively during each feeding cycle, as a result of their synaptic interconnections and the intrinsic properties of the individual neurons (for details see

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[1]A very recent study indicates that there are probably several other, as yet unidentified, N2s located in the buccal ganglia (Kemenes and Elliott, 1994)

Figure 1.2 Diagrammatical representation of the dorsal surface of the buccal ganglia and the anterior surface of the cerebral ganglia of *Lymnæa* showing the location of the motoneurons (unshaded) and interneurons (shaded) of the feeding circuit. See text for details. Adapted from Benjamin and Elliott (1989).

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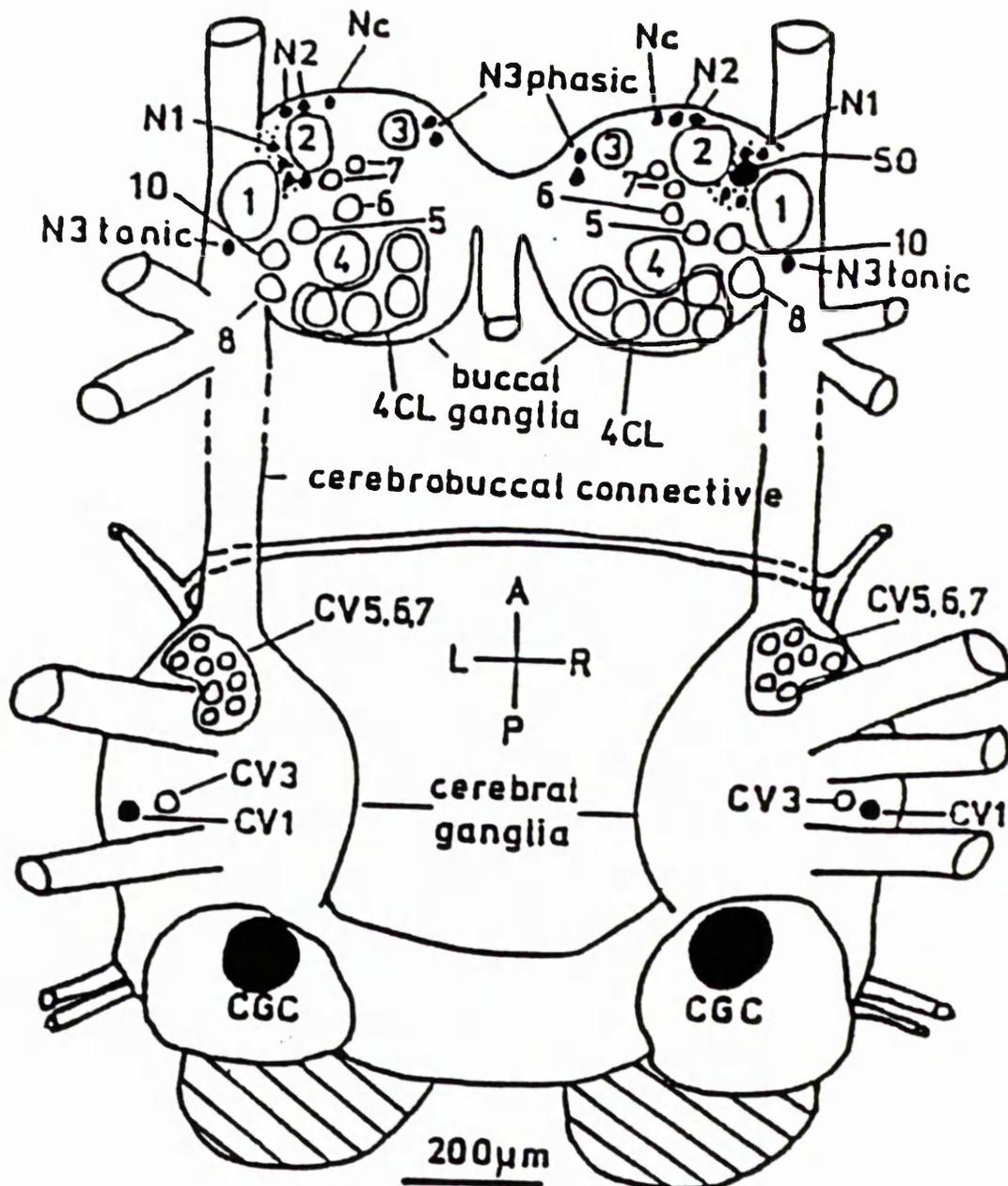
review Benjamin and Elliott, 1989; Elliott, 1992), to drive the buccal motoneurons (Elliott and Benjamin, 1985a). Different combinations of synaptic inputs from the N networks determine the phase of the feeding rhythm in which each motoneuron fires (e.g. the buccal motoneuron B3 fires during the N2 phase as it is inhibited during the N1 phase and excited above threshold by N2 interneurons; Benjamin and Elliott, 1989) (Fig. 1.3). Experiments using a semi-intact preparation in which simultaneous recordings were made from interneurons of the N network and buccal mass muscles, showed that activity in N1, N2 and N3 interneurons coincides with protraction, retraction 1 and retraction 2 respectively in the behaviourally defined feeding cycle (Benjamin and Elliott, 1989). Therefore, the N1-N2-N3 rhythm described by Benjamin and co-workers has been accepted to represent true feeding in the intact animal. Subsequent research, however, has found that the *Lymnaea* feeding CPG is capable of generating subtly different rhythms which may have behavioural significance (Kyriakides, 1988). Examples include the "N2" rhythm (Kyriakides and McCrohan, 1989), which is possibly responsible for rasping in the absence of ingestion of food, and the "N1/N3" rhythm (Tuersley and McCrohan, 1988) a candidate rhythm for egestion or vomiting. Therefore, the feeding CPG does not always produce the inflexible feeding "FAP" (i.e. the N1-N2-N3 rhythm) as originally thought, but is also capable of producing different outputs by employing the second general organisational principle (Kien *et al.*, 1992) outlined earlier in this Chapter.

Initiation of feeding motor output from the buccal CPG.

Experiments carried out on the isolated CNS have shown that feeding motor output (FMO, the N1-N2-N3 ingestive feeding rhythm) can occur in the absence of any sensory feedback. Several neurons which can initiate feeding motor output have already been

Figure 1.3 Summary of the feeding central pattern generator. A: Hypothetical "hard-wired" diagram showing the minimum number of connections required to give the pattern thought to represent ingestive feeding shown in B. Filled circles denote inhibitory connections, bars excitatory connections.

B: Output patterns of the CPG subtypes and three motoneurons when the SO is either spontaneously active or depolarised. Arrows indicate synaptic connections: i, inhibitory PSP and e, excitatory PSP. Adapted from Benjamin and Elliott (1989).
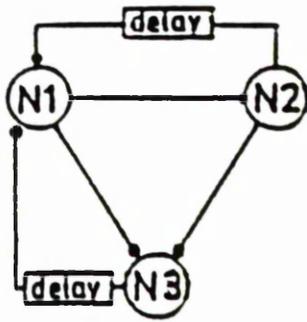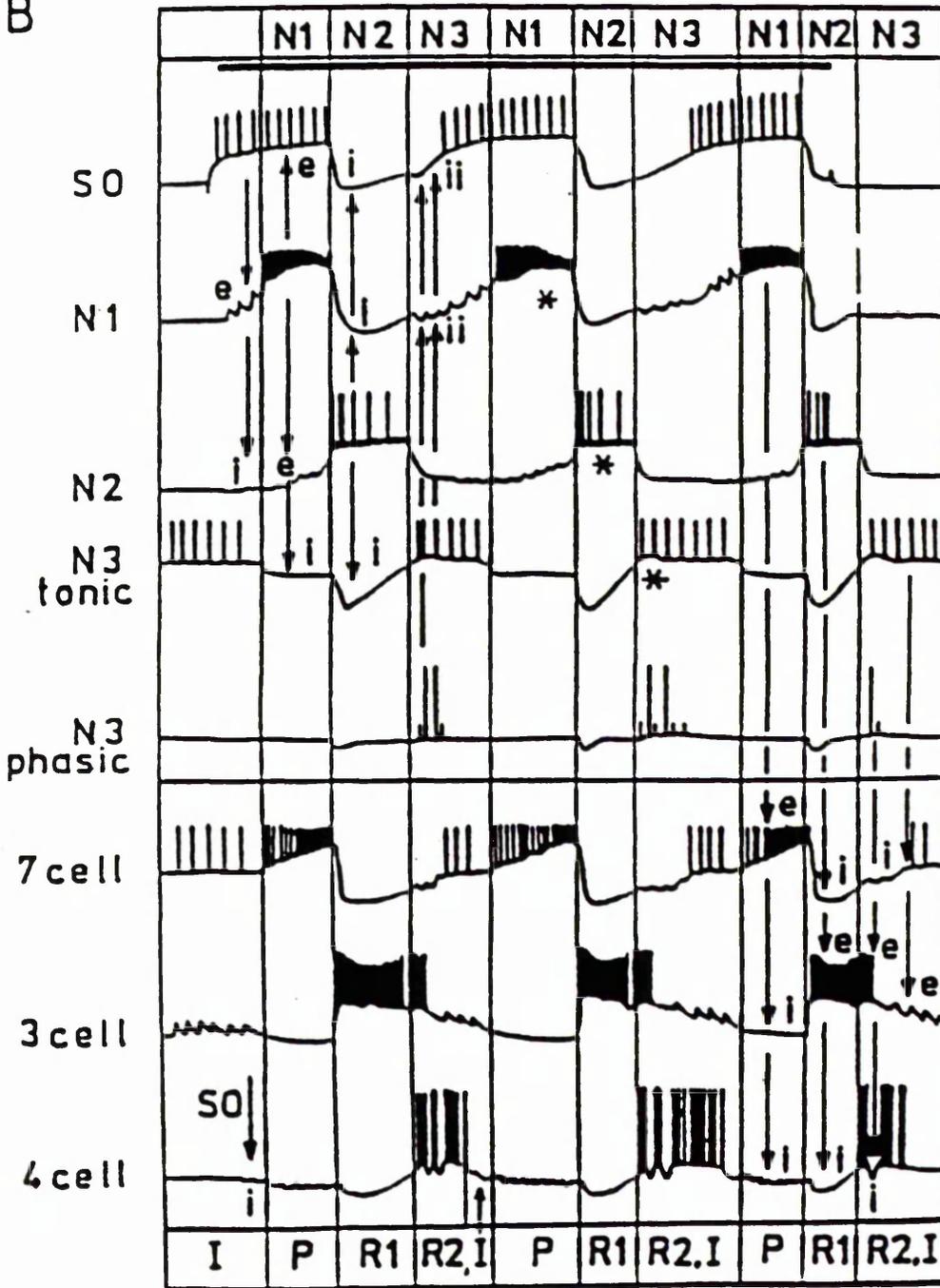
identified (for review; Benjamin *et al.*, 1981; Benjamin and Elliott, 1989).

The first neuron type that can initiate FMO are the N1 interneurons. The N1 neurons are premotor cholinergic interneurons (Elliott and Kemenes, 1992; Elliott *et al.,* 1992), and steady depolarisation of one of more of this interneuron type is sufficient to activate feeding cycles (Benjamin and Elliott, 1989; Elliott, 1992). Experiments indicate that the CPG is able to generate feeding cycles independently of any input from any other neurons, due to the endogenous bursting properties of the N1 interneurons (Rose and Benjamin, 1981b; Elliott, 1992). However, it is generally thought that initiation of feeding cycles is normally due to activity of one or several so-called "higher-order", or command-like interneurons (command-*like* because their activity is sufficient to lead to initiation of the feeding rhythm but is not necessary; Kupfermann and Weiss, 1978). These include the cerebral ventral 1 cells (CV1) (McCrohan, 1984b; McCrohan and Kyriakides, 1989), the slow oscillator neuron (SO) (Rose and Benjamin, 1981a; Elliott and Benjamin, 1985b) and the Nc interneuron (Benjamin and Elliott, 1989). The cerebral giant cell (CGC) (McCrohan and Benjamin, 1980a,b; McCrohan and Audesirk, 1987) is also capable of initiating feeding but its main role is thought to be modulatory.

The cerebral ventral 1 interneurons (CV1) are a population of at least three cells located on the ventral surface of each cerebral ganglion close to the origin of the superior and median lip nerves (McCrohan, 1984b; McCrohan and Kyriakides, 1989). Studies using the fluorescent dye Lucifer Yellow have shown the morphologies of all CV1s to be similar, with neuritic branching in the cerebral ganglia and a single characteristic axonal projection to the buccal ganglia (McCrohan, 1984b; McCrohan and Kyriakides, 1989) where the feeding CPG is located (Rose and Benjamin, 1981b).

Steady depolarisation of one CV1 can result in the initiation and maintenance of rhythmic feeding motor output from the buccal CPG. This activation is due to direct, monosynaptic excitatory connections from CV1 to the N1 interneurons (McCrohan and Kyriakides, 1989). CV1 also receives rhythmic synaptic inputs in phase with feeding activity (excitation during N1 and inhibition during N2), which arise directly or indirectly from the buccal CPG. Two subtypes of CV1 were described by McCrohan and Kyriakides (1989): $CV1_a$, which receives strong feedback from the CPG leading to very phasic bursting activity; and $CV1_b$, which receives weaker feedback, often resulting in continuous firing when depolarised. In addition to the compound inputs originating in phase with activity of the CPG, both subtypes receive apparently unitary inhibitory postsynaptic potentials (IPSPs). These IPSPs are much smaller, and therefore less effective, in $CV1_b$, only becoming visible when the cell is depolarised (McCrohan and Kyriakides, 1989). The identification of different subtypes of CV1 and the identifiable inputs received by them, led to the postulation that CV1 interneurons were homologous to heterogeneous populations of cerebral-to-buccal interneurons found in other gastropod species (*Pleurobranchaea californica*, Gillette *et al.*, 1978; *Limax maximus*, Delaney and Gelperin, 1990a; and *Aplysia californica*, Rosen *et al.*, 1991).

The slow oscillator (SO) is a single, possibly cholinergic (Yeoman *et al.*, 1993), neuron located in either the left or right buccal ganglion. It has extensive neuritic processes in both the right and left buccal ganglia (Elliott and Benjamin, 1985b). It makes monosynaptic connections with the N1 and N2 interneurons of the central pattern generator and the motoneuron B4 (N1 excitatory, and N2 and B4 inhibitory) and in turn is excited by N1 and inhibited by N2 and N3 interneurons (Elliott and Benjamin, 1985b).

Like CV1, steady depolarisation of the SO results in the initiation and maintenance

of feeding motor output from the CPG. This initiation is due to direct excitatory connections between the SO and N1 interneurons, and, like CV1, the SO bursts in phase with feeding inputs since it receives inhibition during the N2 phase of the cycle. However, there are differences between the CV1- and SO-driven rhythms. Progressive increases in the current injected into the SO can lead to an increase in feeding cycle frequency up to a minimum cycle time of about four seconds (the speed at which feeding cycles occur in the intact feeding animal; Dawkins, 1974; Kemenes *et al.*, 1986). Additionally, feeding rhythms driven by SO can only be maintained for a limited time (0.5-2 minutes) before cycles become disrupted. This is in contrast to a rhythm driven by CV1, which is much less variable, i.e. the feeding rhythm's frequency is more-or-less constant irrespective of the amount of current passed, and can be maintained indefinitely with little disruption of cycles (McCrohan and Kyriakides, 1992). Experiments using a semi-intact preparation (lip-CNS preparation) showed that the SO is weakly excited by food stimuli (sucrose) and touch to the lips (Kemenes *et al.*, 1986), and so may be partly involved in modulating feeding motor output in response to food stimuli.

The Nc interneuron is located in the buccal ganglia and has an axonal projection to the cerebral ganglia (Benjamin and Elliott, 1989). The Nc receives weak excitation during the N1 and N2 phases of the cycle (this excitation is thought to be monosynaptic; Benjamin and Elliott, 1989). The function of Nc is still unclear, but it is generally thought to play a role in relaying information about CPG activity to lip motoneurons in the cerebral ganglia and the rest of the brain (C.J.H. Elliott, pers. comm.). In some preparations, if stimulated by high levels of depolarising current, Nc activity can lead to initiation of feeding motor output by exciting the SO via a polysynaptic pathway (Benjamin and Elliott, 1989). However, Nc is not

thought to initiate feeding under normal conditions in the intact animal, as levels of current required are unphysiological and initiation unreliable (C.J.H. Elliott, pers. comm.).

Finally, the cerebral giant cells (CGC) are a pair of wide-acting modulatory interneurons located in each anterior lobe of the cerebral ganglia. They have large, serotonin-containing cell bodies (diameter = c. 100μm) and axonal projections to the buccal ganglia and peripheral structures including the lips and buccal mass (McCrohan and Benjamin, 1980a,b). Apparently homologous cells have been described in several other gastropod molluscs (*Aplysia*, Kupfermann and Weiss, 1982; *Pleurobranchaea*, Gillette and Davis, 1977; *Planorbis*, Berry and Pentreath, 1976; and *Helisoma*, Granzow and Kater, 1977).

In isolated CNS preparations the CGCs usually fire steadily at a rate between 0.5 - 2 spikes per second (McCrohan and Benjamin, 1980a,b), although *in vivo* the firing rate is thought to be lower (M. Yeoman, pers. comm.). The effects of CGC activity on feeding motor output has been found to be variable. In some cases, an increase in firing rate of the CGC causes an increase in intensity of firing of the feeding motoneurons, and in previously non-rhythmic preparations, can even lead to initiation of feeding motor output (McCrohan and Audesirk, 1987). However, in preparations already showing rhythmic activity an increase in CGC activity often leads to cessation of feeding motor output (Tuersley and McCrohan, 1989). Thus, although the CGC can initiate feeding output under some conditions, this initiation is unreliable and usually only occurs after some considerable delay (10 seconds). Therefore, the CGCs are probably better classified as "modulatory" neurons whose wide-acting effects make feeding more or less likely. This is probably achieved by general excitation or depression of the whole feeding system by the levels of serotonin released by the CGC. It is thought that this modulation is at least partly due to the CGCs exciting the N3

interneurons (McCrohan *et al.*, 1989; McCrohan and Kyriakides, 1992). Studies using a semi-intact preparation have shown that the CGCs are weakly excited by food and touch to the lips (Kemenes *et al.*, 1986).

Possible behavioural roles for CV1 and SO.

Of the four higher-order interneuron types, CV1 and SO appear to have similar function, in that they can (upon artificial depolarisation) switch on feeding motor output in a previously non-rhythmic preparation. Studies have shown that there is no synaptic link between CV1 and the SO, and so they represent two independent pathways for initiation of feeding (McCrohan, 1984b). This again indicates that the feeding system of *Lymnaea* is not a simple "hard-wired" network, and that feeding does not occur as a result of activity in one "command neuron" but probably results from activity in a range of modulatory neurons. Thus, the interesting question is, why have many separate pathways by which feeding can be modulated, and in what situation is each pathway employed?

A possible answer to these questions may be suggested by studying the location of the higher-order neurons and the subtle differences in their effects, and also by comparing these *Lymnaea* neurons to apparently homologous neurons in other gastropod species. CV1 is located in the cerebral ganglia close to the lip nerves and is therefore in an appropriate position for receiving sensory information about the presence of food in the external environment. Thus, it is feasible to suggest that CV1 could be responsible for regulating feeding activity in response to sensory input to the lips. In fact, studies on other gastropod species have found this to be true for presumed homologous cells (the PC_p, *Pleurobranchaea californica*, Gillette *et al.*, 1978; CB_1, *Limax maximus*, Delaney and Gelperin, 1990a; and CBI-2, *Aplysia californica*, Rosen *et al.*, 1991; see later for details). The SO, however, is

located in the buccal ganglia from which nerves run into the buccal mass and the oesophagus, providing innervation for feeding movements, but also receiving sensory feedback from these areas. Thus, the SO is in an appropriate position for receiving information about the presence of food in the buccal cavity and alimentary canal. The SO has also been shown to be better at modulating the frequency of feeding than maintaining the feeding rhythm. Therefore, the SO could be responsible for "cycle-by-cycle modulation" of feeding (McCrohan and Kyriakides, 1992); i.e. responsible for increasing feeding activity and swallowing when there is food present in the buccal cavity, or termination of feeding movements when the oesophagus is full. Possible support for this hypothesis includes the discovery of an oesophageal mechanoreceptor neuron (OM) in the buccal ganglia, which is excited by the distension of the oesophagus (Elliott and Benjamin, 1989). The OM has been shown to make inhibitory connections with the SO and all the CPG interneurons. Thus, upon oesophagus distention there is a termination of feeding motor output (Elliott and Benjamin, 1989). In *Pleurobranchaea*, the PCNs (paracerebral neurons; Gillette *et al.*, 1978) and the VWC (visceral white cell; McClellan, 1983) are in similar locations to *Lymnaea's* CV1 and SO respectively, and are both involved in modulating feeding motor output. Studies have shown the PCNs to have an "appetitive" role (initiating feeding in response to food; Gillette *et al.*, 1978), with the VWC having a more "consummatory" role, modulating rate of feeding (Gillette and Gillette, 1983) and possibly initiating regurgitation behaviour (McClellan, 1983; Croll *et al.*, 1985b).

FEEDING SYSTEMS OF OTHER GASTROPOD MOLLUSCS

There has been much work on the feeding systems of other gastropod molluscs, and this research can be used to give further insight into the role of possibly homologous neurons

within the feeding system of *Lymnaea*. The neural elements of the feeding systems of other gastropod molluscs have been shown to be, as in *Lymnaea*, limited to the cerebral and buccal ganglia (*Pleurobranchaea californica*, Gillette *et al.*, 1982a,b, Cohen and Mpitsos, 1983a,b, Croll *et al.*, 1985a-c; *Aplysia californica*, Rosen *et al.*, 1991, Morton and Chiel, 1993b; *Helix pomatia*, Balaban, 1993; *Limax maximus*, Delaney and Gelperin, 1990a-c; and *Achatina fulica*, Yoshida and Kobayaski, 1991, pers. comm.). The research on the buccal central pattern generators in these species, however, is limited compared to *Lymnaea*, with neurons within the network remaining largely unidentified. This is in contrast to research on the populations of cerebral-to-buccal interneurons involved in modulating feeding, which is quite detailed for some species (PCNs of *Pleurobranchaea*, Gillette *et al.*, 1978, 1982b, Kovac and Davis, 1980a, Kovac *et al.*, 1982, 1983a,b, Davis *et al.*, 1983, Croll *et al.*, 1985c; CBs of *Limax*, Delaney and Gelperin, 1990a-c, King *et al.*, 1987; CBIs of *Aplysia*, Rosen *et al.*, 1987, 1988, 1991). Studies on the activity of these cerebral interneurons have shown that several of the interneurons within the population are phase-locked with the feeding rhythm, and capable of initiating feeding motor output following artificial depolarisation in the isolated CNS (Kovac *et al.*, 1983a; Delaney and Gelperin, 1990b; Rosen *et al.*, 1991). This resembles the activity of $CV1_a$ in *Lymnaea* (McCrohan, 1984b). In addition to their activity in the isolated CNS, these cerebral interneurons are excited by the application of food to the lips of a semi-intact preparation, resulting in the initiation of feeding (Gillette *et al.* 1978; Delaney and Gelperin, 1990c; Rosen *et al.*, 1991). This is consistent with their location within the nervous system, i.e. they are close to the origin of the lip nerves, which allows sensory input from the lips to modulate their activity. Since CV1 of *Lymnaea* has very similar activity in the isolated CNS and similar location, it is probable that CV1 is also capable of initiating feeding following application of food to the lips.

The most detailed studies on cerebral feeding interneurons in other species have been on the PCNs of *Pleurobranchaea*. The activity of these neurons (and therefore their ability to initiate feeding) is dependent on the balance of subthreshold excitatory and inhibitory subthreshold inputs received by them. Several classes of inhibitory and excitatory neurons which innervated the PCNs both monosynaptically and polysynaptically were identified (Kovac *et al.*, 1983a,b; London and Gillette, 1984). Experiments on semi-intact preparations showed that food on the lips excited both the excitatory and inhibitory neurons, but since the excitation was predominantly of the excitatory pathways (Davis *et al.*, 1983), the overall bias of inputs to PCN was excitatory. This ultimately caused PCN to become active and thus drive feeding motor output (Fig. 1.4A).

Subsequent experiments on *Pleurobranchaea* showed that the PCN pathway was also involved in modulating feeding following satiation and aversive conditioning to certain foods. These studies showed that both the behavioural and the underlying neural response to food following satiation and aversive conditioning were similar. Behaviourally, *Pleurobranchaea* actively avoided food by showing complete inhibition of feeding, and withdrawal (Davis *et al.*, 1983). The neural mechanism underlying this inhibition of feeding following satiation and aversive conditioning was simply that the bias of inputs received by PCN following food to the lips was altered. Instead of more EPSPs being received than IPSPs (as seen in naive animals, Fig. 1.4A), the opposite occurred, with food application resulting in more IPSPs than EPSPs being received (Davis *et al.*, 1983; Figure 1.4B). The result was that PCN were inhibited and initiation of feeding did not occur. Further studies on the neural mechanisms of food avoidance conditioning in *Pleurobranchaea* showed that this response was due to strong attenuation or abolition of EPSPs at a single identified synapse within one of the polysynaptic excitatory pathways to PCN (Kovac *et al.*, 1985, 1986).

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Figure 1.4 Schematic neural model showing how aversive conditioning inhibits feeding behaviour via the PC_p (phasic subtype of PCN) pathway.

A: Unconditioned; sensory information (Sens.) that elicits feeding follows a predominantly excitatory pathway (Exc.) through the network (Heavy arrows). The cyclical inhibitory neurons (Inh.) are also activated resulting in PC_p firing cyclically in phase with the feeding network.

B: Aversive conditioned; excitatory pathways are de-emphasized and existing inhibitory pathways predominate (Heavy arrows). This suppresses feeding. See text for details. Adapted from Davis et al., 1983.
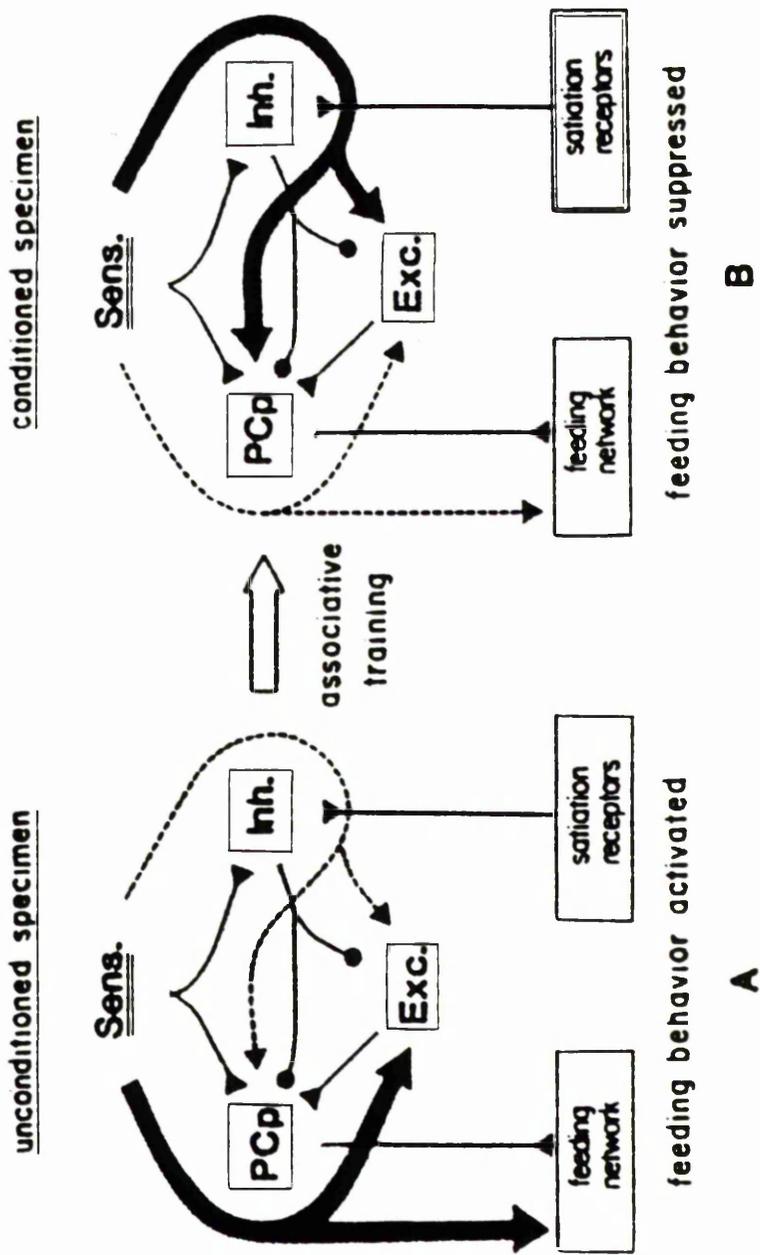
Studies on the CBs and CBIs of *Limax* and *Aplysia* were not as detailed as those on *Pleurobranchaea*. However, they also showed that the subthreshold inputs received by these neurons were important in determining the likelihood of initiation of feeding. Like *Pleurobranchaea*, food application to the lips in a semi-intact preparation resulted in the excitation of the CBs and CBIs and thus initiation of feeding (Delaney and Gelperin, 1990c; Rosen *et al.*, 1991). Although the effect of associative aversive conditioning was not studied in these species, the effect of a non-associative inhibition of feeding was studied in *Limax* (Delaney and Gelperin, 1990c). Behavioural experiments showed that an electric shock to the foot in the whole animal caused withdrawal and reduced responsiveness to food for several minutes. Subsequent experiments carried out in semi-intact preparations where the lips and foot tissue were left attached to the CNS, demonstrated that an electric shock to the foot resulted in an increase in IPSPs received by CBs, and thus reduced responsiveness of CB to food extracts and no initiation of feeding for up to 10 minutes (Delaney and Gelperin, 1990c). It was postulated that since the CBs had a very similar role to the PCN of *Pleurobranchaea* in the initiation of feeding under normal conditions, and non-associative experience could inhibit feeding via the CB pathway, the CBs were probably, like the PCNs, the locus for learning-induced changes which affected the feeding response following associative conditioning (Delaney and Gelperin, 1990c).

FACTORS THAT AFFECT THE FEEDING RESPONSE

The research on putative homologues of CV1 in other species has shown that they are at least partly responsible for initiating feeding following food application to the lips, and that their ability to do this is influenced by several internal and external factors (see last section). These populations of neurons are therefore important in determining whether the decision to

feed is made or not. Since the main aim of this thesis is to understand how *Lymnaea* makes the decision to feed, specifically looking at the role CV1 has in this "decision making process", the different factors which could influence this decision are now considered.

Sensory input from the lips

There have been many studies into chemoreception of gastropods and it has been demonstrated that the specific "taste" of potential food is important to most gastropods when making the decision to feed or not. Most gastropods show clear and strong discrimination between different food types (for review, see Croll, 1983; Kuslansky *et al.,* 1987). Studies on *Lymnaea*, however, have shown it to be an exception, with little discrimination between food compared to other gastropods. It feeds on vegetable and animal matter and has little preference for certain "tasting" foods (Bovbjerg, 1968; Croll, 1983). This is probably a function of *Lymnaea* being a grazing omnivore (Dawkins, 1974) rather than it not having the chemosensory ability to discriminate between foods. This is borne out by more specific studies which have shown some subtle discriminatory behaviour between attractive tastes, e.g. *Lymnaea* shows slightly different feeding responses to different concentrations of the two sugars, sucrose and maltose (Kemenes *et al.,* 1986).

The effect of aversive tastes on the feeding behaviour of gastropods however, is much more universal, with bitter tastes such as quinine or quinidine causing complete inhibition of feeding and withdrawal in all gastropods studied. These include *Pleurobranchaea* (Mpitsos and Collins, 1975), *Limax* (Delaney and Gelperin, 1990c), *Helix* (Maksimova and Balaban, 1984) and *Lymnaea* (Kemenes *et al.,* 1986)

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Incompatible behaviours

At any given time an animal may receive sensory cues for two or more different behaviours. If these behaviours are incompatible then one will have to take precedence over the others, whilst if they are compatible the different behaviours will need to be coordinated. This will influence the decision making process.

An example of incompatible behaviours in gastropods is withdrawal and feeding. Withdrawal is a protective reflex and takes precedence over all other behaviours (Winlow *et al.*, 1992). Studies on several gastropod species including *Pleurobranchaea* and *Aplysia*, however, have shown that although withdrawal will inhibit feeding if the noxious stimulus is strong enough, the feeding-withdrawal interaction is in fact based on reciprocal inhibition, i.e. feeding is suppressed by withdrawal *and* withdrawal is suppressed in strongly feeding animals (Kovac and Davis, 1980b; Teyke *et al.*,1989). In contrast, locomotion and feeding behaviours are considered to be mutually compatible in *Lymnaea* since they can occur at the same time. Close examination of these behaviours show that they do not simply occur independently of each other but are coordinated. Rasps never occur at the same time that the snail changes head direction (Dawkins, 1974), presumably because a rasp cannot be effective unless the head is held still over the substrate. Electrophysiological recordings indicate a neural substrate for this interaction; locomotory motoneurons receive inhibition from the feeding central pattern generator during the N2 (rasp) phase of feeding (Kyriakides and McCrohan, 1988).

Satiation

The level of satiation of the animal is an important factor affecting the strength of feeding response. Satiation has been studied in several gastropod molluscs including

Pleurobranchoea (Davis *et al.*, 1977; Davis and Gillette, 1978; Davis *et al.*, 1983; Kovac *et al.*, 1985; Croll *et al.*, 1987), *Helix* (Balaban and Maksimova, 1988; Balaban , 1993), *Aplysia* (Susswein and Kupfermann, 1975; Susswein *et al.*, 1978; Kuslansky *et al.*, 1987) and *Lymnaea* (Tuersley and McCrohan, 1987a; Elliott and Benjamin, 1989). These studies showed that the behavioural response to satiation differed between species, and this was thought to be a consequence of the different environments they had adapted to (Tuersley and McCrohan, 1987a). *Pleurobranchoea, Aplysia* and *Helix* all became less active when satiated, and showed reduced responsiveness to, or even withdrawal from food (Davis *et al.*, 1983; Susswein *et al.*,1978; Balaban, 1993). *Lymnaea's* response to satiation is quite different; instead of becoming inactive and withdrawing from food, *Lymnaea* simply stops its "food search activity", the spontaneous rasping seen in the absence of food (Tuersley and McCrohan, 1987a). This mechanism of dealing with satiation is thought to be possible since *Lymnaea* cannot detect food unless the environment is sampled (Tuersley and McCrohan, 1987a) which is consistent with a study by Bovbjerg (1968) showing that *Lymnaea* has no ability to detect vegetable matter at a distance.

Despite differences in how different species deal with satiation there are several common mechanisms which are thought to signal satiation. Distention of the gut has been shown to have an important role in signalling satiation in *Pleurobranchoea* and *Aplysia* (Croll *et al.*, 1987; Kuslansky *et al.*, 1987). This may also be important in *Lymnaea*, since buccal oesophageal mechanoreceptors (OM) which are excited by gut distention and inhibit buccal motoneurons, have been identified (Elliott and Benjamin, 1989). Neurohormonal factors in the haemolymph are also thought to be important in signalling satiation in *Helix* (Balaban and Maksimova, 1988).

Learning

There has been a huge amount of research into the learning capabilities of molluscs. This research has demonstrated that molluscs are capable of many types of learning ranging from simple non-associative processes, e.g. habituation and sensitization in *Aplysia* (Kandel and Schwartz, 1982), through to complex social learning e.g. observational learning in the octopus (Fiorito and Scotto, 1992). Since this thesis tries to understand how different factors affect *Lymnaea's* decision to feed, this section will only cover associative conditioning of gastropod molluscs, concentrating mainly on paradigms that affect feeding behaviour, i.e. positive (appetitive) conditioning and food avoidance conditioning.

Research on the learning capabilities of gastropod molluscs such as *Pleurobranchaea*, *Limax, Helix* and *Aplysia* has shown their learning abilities to be well developed and also, in some cases, quite sophisticated. There have been several studies on *Pleurobranchaea* showing food avoidance conditioning to be rapid (seen after only a few training trials), and "selective", i.e. *Pleurobranchaea* only withdraws from the food which had been paired with the aversive stimulus (Mpitsos and Davis, 1973; Mpitsos *et al.*, 1978; Davis *et al.*, 1980). *Pleurobranchaea* is also capable of "differential" avoidance conditioning (Mpitsos and Cohen, 1986a-c, Mpitsos *et al.*, 1988). Other studies showed that food avoidance learning is retained in a semi-intact preparation, which has enabled the neural mechanisms underlying the food avoidance behaviour to be examined (Davis *et al.*, 1983; Kovac *et al.*, 1985, 1986). Appetitive conditioning, however, is less well developed in *Pleurobranchaea* with learning only taking place after very many training trials (Mpitsos and Davis, 1973).

Similar research into *Limax's* learning capabilities has shown it to be rather sophisticated. Not only has food avoidance conditioning been shown to be "specific" (Gelperin, 1975), but *Limax* has also been shown to be capable of second-order food

avoidance conditioning, and to demonstrate "blocking" and "US pre-exposure" effects (Sahley *et al.*, 1981). Food avoidance learning is also, as in *Pleurobranchaea*, retained in the semi-intact brain after dissection (Gelperin and Culligan, 1984), and additional research showed that the semi-intact preparation itself can show food avoidance learning (Chang and Gelperin, 1980; Culligan and Gelperin, 1983; Gelperin, 1986). *Limax* also demonstrates strong positive appetitive conditioning, which can be both "specific" and "differential". Appetitive conditioning, however, is slower than food avoidance conditioning taking several training trials before learning occurs (Sahley *et al.*, 1990, 1992). Similar to *Pleurobranchaea* and *Limax*, food avoidance conditioning in *Helix* is very well developed and "specific" (Maksimova and Balaban, 1984; Balaban, 1993). Learning can occur in an isolated semi-intact brain preparation as well as in the whole animal (Balaban *et al.*, 1987). In addition to this, *Helix* shows two-way conditioning, i.e. following training with an unconditioned stimulus (US)= noxious prod and a conditioned stimlus (CS) = food, the CS results in the conditioned response of withdrawal as expected, but a weak US results in weak exploratory feeding which is not seen before training (Balaban, 1993). This indicates that *Helix*, like *Limax*, has quite sophisticated learning capabilities. This is supported by the fact that *Helix* also associates stimuli with environmental cues (Balaban, 1993; Balaban and Bravarenko, 1993). Environmental or contextual cues have also been shown to be important for learning in *Aplysia* (Colwill *et al.*, 1988a,b). Considering the amount of research into learning of *Aplysia*, the amount of work specifically looking at the conditioning of the feeding response is relatively small. One interesting study, however, showed that following food avoidance training, application of the food CS resulted not only in the expected "conditioned response" of withdrawal, but also to a central defensive "fear-like state". This "fear-like state" was characterised by a facilitation of siphon withdrawal, inking and escape locomotion and a

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reduction in responsiveness to food, i.e. the CS prepared *Aplysia* for imminent danger (Carew *et al.*, 1981; Walters *et al.*, 1981).

The amount research on *Lymnaea's* learning abilities is limited compared to that of other gastropods. The majority of studies have been on appetitive positive conditioning. *Lymnaea* was shown to exhibit strong conditioned feeding and long term memory (several weeks) following two separate paradigms; 1) pairing a gentle mechanical stimulus (lip touch) with sucrose (Kemenes and Benjamin, 1989); and, 2) pairing a novel odour/taste with sucrose (Audesirk *et al.*, 1982; Alexander *et al.*, 1982). The strength of learning was found to be affected by the age of the snail (older snails showed weaker learning) and also by the frequency of the conditioning trials (Audesirk *et al.*, 1982; Alexander *et al.*, 1982). Aging has also been found to affect learning capabilities of *Aplysia* (Bailey *et al.*, 1983), and *Helix* (Balaban, 1993). With sufficient food deprivation, *Lymnaea* showed strong appetitive learning after only one training trial (Alexander *et al.*, 1984). One trial conditioning is not unusual in aversive conditioning paradigms of other gastropods; *Pleurobranchaea*, *Limax* and *Aplysia* have all been shown to be able to learn after one aversive trial (Mpitsos *et al.*, 1988; Gelperin, 1975; Carew *et al.*, 1983). However, one trial learning following appetitive conditioning is unusual, and this indicates that learning to recognise new potential food sources must be very important for *Lymnaea*. This is thought to be a consequence of *Lymnaea* being a wide-ranging grazing omnivore (Alexander *et al.*, 1984). In contrast, published work on any type of aversive conditioning of *Lymnaea* is extremely limited, and even this work is not conclusive, with no, or only weak learning seen even after many training trials (Buytendijk, 1921; Fishel, 1931, as quoted by Hyman, 1967).

AIMS OF THIS THESIS

The main aims of this thesis were to investigate what factors influence *Lymnaea's* decision to feed, and what was the role of the CV1 population in making this decision.

These questions were particularly interesting since several studies on the behaviour and feeding systems of other gastropod molluscs, including *Pleurobranchaea, Limax* and *Aplysia,* showed that populations of cerebral-to-buccal neurons, possibly homologous to the CV1 population in *Lymnaea,* had a key role in making the decision to feed.

With this comparative research in mind, another aim of this thesis was to relate any difference in behaviour and/or neural mechanisms to the contrasting environments that different species had evolved in. This was considered to be an important part of the thesis since Tuersley and McCrohan (1987a) had already implied that the difference in behavioural response to satiation shown by *Lymnaea* compared to other gastropods, was due to the different environments that the species had adapted to.

In an attempt to accomplish these aims the following areas were studied:

i)   CV1's activity and inputs in the isolated CNS.

ii) CV1's response to attractive and aversive stimuli to the lips

iii) The effect of incompatible behaviours such as withdrawal on both the behavioural and neural elements of the feeding response.

iv) The effect of previous experience on the behavioural and neural elements of the feeding response: both non associative paradigms (satiation) and associative paradigms (positive and aversive conditioning) were studied.

v) A preliminary study of the morphology of the lips and putative chemosensory structures, to attempt to identify structures which are responsible for signalling the presence of food or

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aversive stimuli in the external environment.

All the results are discussed in relation to comparative research on other gastropod species.

CHAPTER 2 -
MATERIALS AND METHODS

MATERIALS AND METHODS

THE EXPERIMENTAL ANIMALS

Lymnaea stagnalis, weighing 1.5-6 g were obtained from animal suppliers (Blades Biological, Kent), and were maintained in continuously aerated standard snail water (Elliott and Benjamin, 1989; Table 2.1) at room temperature (approximately 20°C). Unless stated, animals used for all behavioural and electrophysiological recordings had been kept in a sub-satiated state (fed only twice a week) on a diet of lettuce and Tetramin fish food.

Table 2.1 - COMPOSITION OF STANDARD SNAIL WATER.

	mM/L	mg/40L dist H_2O
$KHCO_3$	0.04	176
KNO_3	0.05	202
$MgSO_4.7H_2O$	0.08	387.2
$NaHCO_3$	0.7	235.2
$CaCl_2.2H_2O$	2	11360

THE ELECTROPHYSIOLOGICAL PREPARATIONS

Animals selected for electrophysiological recordings were of a large size (4-6 g). Experiments were carried out using one of two preparations:

1) Isolated CNS preparation - All the major ganglia and nerves were dissected out from the animal. No other tissue was left attached to the CNS except for a small part of the pro-oesophagus (left attached to the dorsobuccal nerves) which aided the pinning out of the preparation. The CNS was pinned by its nerves to a Sylgard-lined dish, so that the ventral surface of the cerebral ganglia and the dorsal surfaces of all other ganglia were exposed.

2) Semi-intact preparation - The CNS was carefully dissected out from the animal whilst leaving the lips, tentacles and some of the foot tissue attached via the lip nerves. To aid pinning of the CNS, the attached tissue was then cut down the midline, resulting in a "split-lip" preparation with both the right and left sets of lip nerves being left attached to a section of tissue consisting of the right or left tentacle respectively and half the lip tissue. Part of the pro-oesophagus was also left attached (as above). The semi-intact preparation was then placed in a compartmented Sylgard dish. The CNS was pinned down in one chamber with the ventral surface of the cerebral ganglia and dorsal surfaces of all other ganglia exposed, and the lip tissue pinned in the other chamber. To ensure that the lip tissue was completely isolated from the CNS and that chemosensory stimuli applied to the lips did not effect the CNS directly, the gap between the two compartments was sealed with vaseline. For experiments where recordings were made from the cerebral A cluster neurons (Ferguson and Benjamin, 1991a,b), the cerebral commissure was cut. This did not affect the physiological properties of the cells.

Throughout all experimental procedures, CNS preparations were maintained in MOPS-buffered saline (Table 2.2; Brown, 1990). Prior to recording, the parts of the CNS from which intracellular recordings were to be made had the outer periganglionic sheath removed using fine forceps. The CNS was then treated with protease (type XIV, Sigma) for 1-2 minutes (to soften the inner sheath and aid microelectrode penetration) and washed thoroughly with saline.

INTRACELLULAR RECORDINGS

Intracellular recordings were made using glass microelectrodes filled with 4M potassium acetate, filtered through a 0.22 μm Millipore filter (67120 Molsheim, France). Electrode resistances ranged from 10-15 MΩ (thick-walled glass) for larger neurons (i.e. buccal motoneurons) to 25-35 MΩ (thin-walled glass) for the smaller, more delicate neurons (i.e. CV1). Prior to use, microelectrodes were dipped in waterproof black drawing ink

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(Rotring drawing ink K, etching, 17 black) to aid viewing of the electrode tip under the microscope.

Table 2.2 - COMPOSITION OF MOPS BUFFERED SALINE

	FINAL mM IN SALINE	STOCK SOLUTION	mL STOCK IN 1L DIST H_2O
NaCl	60	2M	30
KCl	1.6	1M	1.6
MgCl$_2$	1.5	1M	1.5
CaCl$_2$	4	1M	4
MOPS	10	250mM	40

N.B. Final pH adjusted to pH 7.5 using 1M NaOH.
MOPS = 3-(N-Morpholino) propane-sulphonic acid (Sigma)

Recorded signals were preamplified by a probe and conveyed to a x10 amplifier incorporating a bridge balance circuit. Signals were displayed using an oscilloscope and recorded on video tape using a two channel pulse code modulating system (Medical System Corps) feeding a domestic video recorder. Permanent records were made using a 2 channel ink jet recorder (Gould 2200). Mains interference (50 Hz) was eliminated by enclosing the preparation area within a 5 mm wire mesh Faraday cage. The preparation was lit using a fibre optic cold light source.

Chemostimuli / neutral solutions (0.1 ml) were applied to the lip tissue of the semi-intact preparation using a mounted graduated syringe. Noxious mechanical prods were applied by hand using a long handled blunt probe.

ANATOMICAL STUDIES USING LUCIFER YELLOW DYE

Anatomical characterisation of some neurons was obtained after recording by the

injection of the fluorescent dye Lucifer Yellow by iontophoresis. Prior to recording, electrode tips were filled with a saturated solution of Lucifer Yellow (Sigma) by capillarity. The shank of the electrode was filled with 1M lithium acetate (potassium acetate was not used owing to precipitation effects) leaving a small air gap between the two liquids, so reducing the dilution of the Lucifer Yellow, but still allowing for good electrical contact with the probe. Neurons were injected with 2-4 nA using 500 msec hyperpolarising pulses at 1 Hz for up to 10 minutes. However, with small neurons current injection for 1-2 minutes was usually sufficient to fill the neuron. The occasional reversal of current polarisation (depolarising) for one cycle helped to prevent or reduce electrode blockage. The preparation was then pinned to a piece of Sylgard (after all lip tissue was removed) and fixed for at least two hours in 4% paraformaldehyde in 0.1 M phosphate buffer, at pH 7.4. The preparation was then dehydrated through an alcohol series (30, 50, 70, 90, 100, 100% ethanol - 40 minutes each) and cleared in methyl salicylate for two hours. Whole mounts were viewed either in methyl salicylate or fluorolite using a Nikon epifluorescence compound microscope with ultra violet light (excitation filter 495 nm, barrier filter 515 nm), and drawn with the aid of a *camera lucida.*

ELECTRON MICROSCOPY

Scanning electron microscopy. Prior to dissection, the snails were anaesthetized by submerging them in a saturated aqueous solution of menthol in snail water (Pantin, 1948; Murphy and Kater, 1980; Haydon *et al.*, 1982) for 15 minutes. This relaxed the lip and tentacle area. Parts of the lip, tentacle and radula were then dissected from the snail and fixed in 2% glutaraldehyde + 2% Osmium Tetroxide in 0.1 M NaCacodylate buffer + 0.1 M sucrose, pH 7.4, for 2 hours at 4°C. The specimens were then washed three times in 0.2 M NaCacodylate buffer + 0.1 M sucrose for 20 minutes, and dehydrated through an alcohol series (50-70-90% ethanol - each for 20 minutes, followed by 100% ethanol - three times,

each for 1 hour). After fixation, the specimens were critical-point dried using a Poloron critical point dryer, adhered to specimen stubs using electroconductive cement/silver DAG, and left to dry. Specimens were then sputter coated with gold-palladium and examined in a Cambridge 320 SEM.

Transmission electron microscopy (TEM). For TEM small parts of the lip tissue were obtained and fixed as for the SEM. The specimens were then placed in 50:50 Spurr resin to ethanol (Spurr, 1969), and left on a rotary mixer overnight. Specimens were then transferred to 70:30 resin to ethanol for one hour, then placed in three changes of 100% resin for 1 hour, and finally embedded in resin overnight at 60°C to polymerise. Thin sections were taken (60 nm) using a Reichert Ultracut and glass knife, and collected on 200 mesh copper grids. Sections were stained on grids with Uranyl Acetate and Lead citrate, allowed to dry and examined in a Hitachi H600 transmission electron microscope.

BEHAVIOURAL ANALYSIS

Much of this thesis is dedicated to comparing whole animal behaviour to neural activity in the brain, and so there are many sets of behavioural experiments, each differing slightly. For this reason, exact details of experimental protocol are described in "mini methods" sections at the beginning of each relevant section of Results. The general principles, however, are outlined below.

Assessing the effect of mechano- and chemosensory stimuli on the behaviour of *Lymnaea*

Snails selected for each behavioural experiment were of similar size and from the same delivery batch (i.e. at similar stages of development). Unless otherwise stated, snails selected had also been kept under the same conditions for at least two weeks prior to the experiment, i.e. sub-satiated at 20°C in the home tanks. Where possible, experimental groups were used as their own controls to minimise individual differences between snails. Individual

snails were labelled using "Pink Charm" nail varnish, which had no adverse effect on their behaviour (but made them look rather attractive).

Experimental apparatus consisted of a large glass Petri dish (diameter 10 cm) containing a 0.5 cm depth of standard snail water, raised on a tripod stand. A mirror positioned underneath the Petri dish enabled observation of the snail's head-foot complex and radula movements; therefore all withdrawal, locomotion and feeding behaviours could be scored. Individual snails were placed in the Petri dish and allowed 5 minutes to recover from the handling before any recordings were made (after this time period snails were usually in the alert locomotory state; Tuersley, 1988). Approximately 0.1 ml of experimental solution was then dropped near to the snails head using a mounted syringe and the subsequent response noted. Time of withdrawal and locomotion was noted, and any other observations of resultant behaviour noted in qualitative form. The feeding response was recorded in two forms; latency to bite after applying the solution and total number of bites in two minutes. Behavioural results were scored using a BBC microcomputer programmed as an event recorder (Azzopardi, 1986).

Non-parametric statistical tests, Wilcoxon ranked pairs test and Mann-Whitney U test (Colquhoun, 1971; Zar, 1984), were used, as data could not be assumed to be of normal distribution, e.g. arbitrary times for latency to bite were awarded to unresponsive snails.

Classical conditioning

Both positive and negative (aversive) conditioning was carried out. However, the general protocol was the same for all experiments. Usually snails were randomly assigned to one of two groups, the experimental conditioning and random presentation control. A random presentation control is generally considered to be the most powerful control group (Rescorla, 1967), as it ensures there can be no association between the unconditioned stimulus and the

conditioned stimulus. In some experiments more extensive control groups were run to enable fuller understanding of the association being made; details of these are discussed in the relevant Results section. The experimental animals usually received between 15-25 training trials over 3 to 5 days: 5 trials per day, with an inter-trial period of 90 minutes. This time separation between training trials was used as it was found to produce the most effective learning in previous studies on *Lymnaea* (Audesirk *et al.*, 1982; Kemenes and Benjamin, 1989). As with all classical conditioning paradigms, the experimental animals received the conditioned stimulus (CS) followed closely by the unconditioned stimulus (US) (i.e. the CS-US pairing interval was not more than a few seconds). To ensure the random presentation controls received the same exposure to the CS and US, control animals received a neutral stimulus-US pairing every time the experimental group received a CS-US pairing, and then at a random time in the inter-trial period received a CS-neutral stimulus pairing. To equalise THE amount of handling between groups throughout the experiment the experimental animals also received a neutral stimulus-neutral stimulus pairing at this random time. The neutral stimulus used was snail water. This was because it mimicked the mechanical effects of applying the CS (usually a liquid to the animals lips) without having any chemosensory effect. Experimental testing was carried out at times ranging from a few hours to several weeks after the last conditioning trial, depending on which learning paradigm was being carried out. Testing data was obtained using the same scoring methods as described above and the extent of learning assessed using non parametric tests, since data could not be assumed to be normally distributed.

CHAPTER 3 - RESULTS

THE ROLE OF CV1 IN THE CONTROL OF FEEDING

THE ROLE OF CV1 IN THE CONTROL OF FEEDING.

INTRODUCTION

Several studies on different species of gastropod mollusc have demonstrated the existence of populations of cerebral-to-buccal interneurons which can initiate feeding motor output in response to application of food to the lips (Gillette *et al.*, 1978, 1982b; Delaney and Gelperin, 1990a-c; Rosen *et al.*, 1991). In 1989, McCrohan and Kyriakides postulated that the CV1 population of *Lymnaea* was homologous with the cerebral-to-buccal interneuron populations described in other species, based on their similar locations, morphology and activity in the isolated CNS.

Since all previous work on CV1 in *Lymnaea* has been carried out on the isolated central nervous system (McCrohan, 1984b; McCrohan and Kyriakides, 1989), knowledge about CV1's true role in relation to the animal's behaviour is limited. This Chapter expands on previous research by investigating CV1's role in controlling feeding in both the absence and presence of sensory input (food stimuli), by using isolated and semi-intact CNS preparations.

The results in this Chapter are divided into two parts. The first part investigates the activity of CV1 and its role in the isolated CNS (i.e. in the absence of sensory input). The second part investigates the modulatory role of CV1 following both sensory stimuli to the lips and satiation.

RESULTS A - ISOLATED CNS

The first aim of this Chapter was to reassess CV1's activity in the isolated CNS, both in terms of the synaptic inputs it receives, and its overall firing activity and effect on the feeding CPG. In doing this, the previously described "command-like" role of CV1, and also

several of the old criteria by which CV1 was identified (McCrohan, 1984b; McCrohan and Kyriakides, 1989) were questioned. All recordings were made from the phasic subtype $CV1_a$ (McCrohan and Kyriakides, 1989), henceforward referred to as "CV1". $CV1_a$ was used over $CV1_b$ for two reasons: 1) the phasic subtype $CV1_a$ had very similar activity to a possible homologous interneuron type in *Pleurobranchaea*, the phasic paracerebral cell PC_p (Gillette *et al.*, 1982b; Kovac *et al.*, 1983a) on which most research had been carried out studying the effects of previous experience (Davis *et al.*, 1983); 2) $CV1_a$ was more easily identified than $CV1_b$, owing to its comparatively large size and constant location within the cerebral ganglia.

For all electrophysiological recordings CV1 was identified by: i) its position within the ganglion (Fig 3.1A); ii) receipt of characteristic subthreshold synaptic inputs; iii) strong phasic burst of actions potentials, which were usually, but not always in phase with rhythmic activity in the buccal ganglia; and iv) morphology (Fig. 3.1B). Iontophoretic injection of the fluorescent dye Lucifer Yellow showed that all cells that conformed to criteria (i), (ii) and (iii) above had the correct morphology for CV1 (as described by McCrohan, 1984b) (Fig. 3.1B). These criteria are modified from those outlined for identifying CV1 in previous studies, and reasons for this will be discussed in detail in the Discussion at the end of this Chapter.

Synaptic inputs to CV1

Previous work on CV1 demonstrated that, in addition to the compound synaptic inputs occurring in phase with the activity of the CPG, CV1 received a series of repeated (possibly unitary) IPSPs at frequencies and amplitudes of up to 8 s^1 and 8 mV respectively (McCrohan, 1984b; McCrohan and Kyriakides, 1989). In this study, experiments carried out in the isolated CNS led to further characterisation of these inputs and demonstrated that the IPSPs could be divided into two distinct populations (Fig. 3.2A). This division of the IPSPs into two amplitude groups is shown in the form of a histogram (Fig. 3.2B) which presents the number

Figure 3.1 A) Schematic diagram of the ventral surface of the cerebral ganglia, showing the location of CV1 in relation to other identified neurons. SLN, superior lip nerve; MLN, median lip nerve; CBC, cerebrobuccal connective; CGC, cerebral giant cell; CV3, cerebral ventral 3 cell (McCrohan, 1984a); (A), identified "white" cerebro-buccal interneuron (McCrohan and Croll, 1991).

B) Morphology of two CV1s as found by the injection of Lucifer yellow. The cell bodies are located between the origin of the superior and median lip nerves (SLN, MLN). The axon projects from the cerebral ganglia to the ipsi- and contralateral buccal ganglia. Fine neuritic branching is seen in the cerebral and buccal ganglia. NB: The neural projection towards the cerebral commissure (CC) is characteristic of $CV1_a$-type only. RCG; right cerebral ganglion, BG; buccal ganglia, CBC; cerebral-buccal connective, DB; dorsal body. (From McCrohan and Kyriakides, 1989)

NB: $CV1_b$ has a similar location and morphology to $CV1_a$, however, it does not have the characteristic neural projection towards the cerebral commissure.
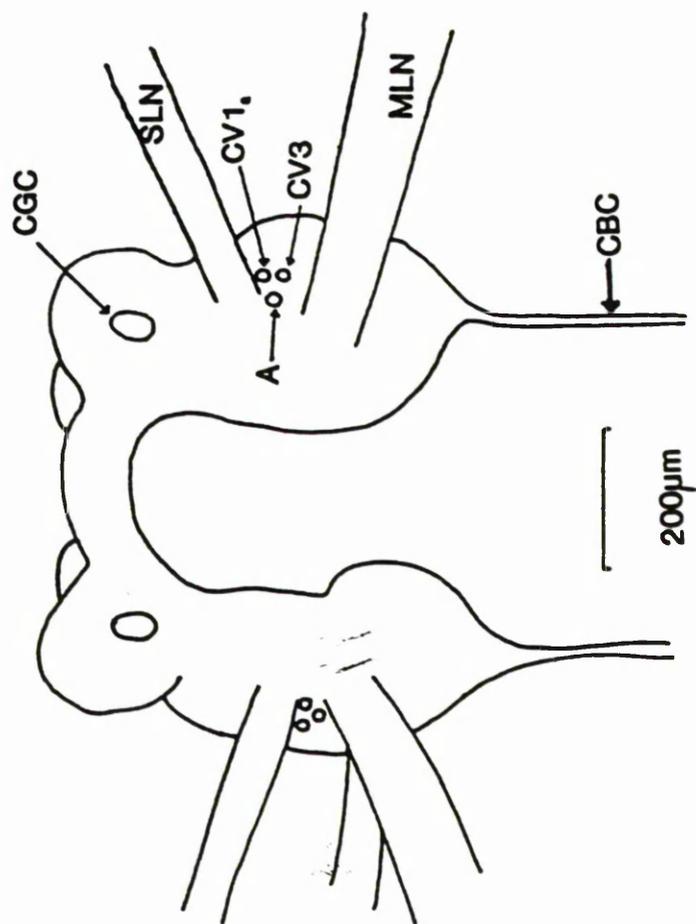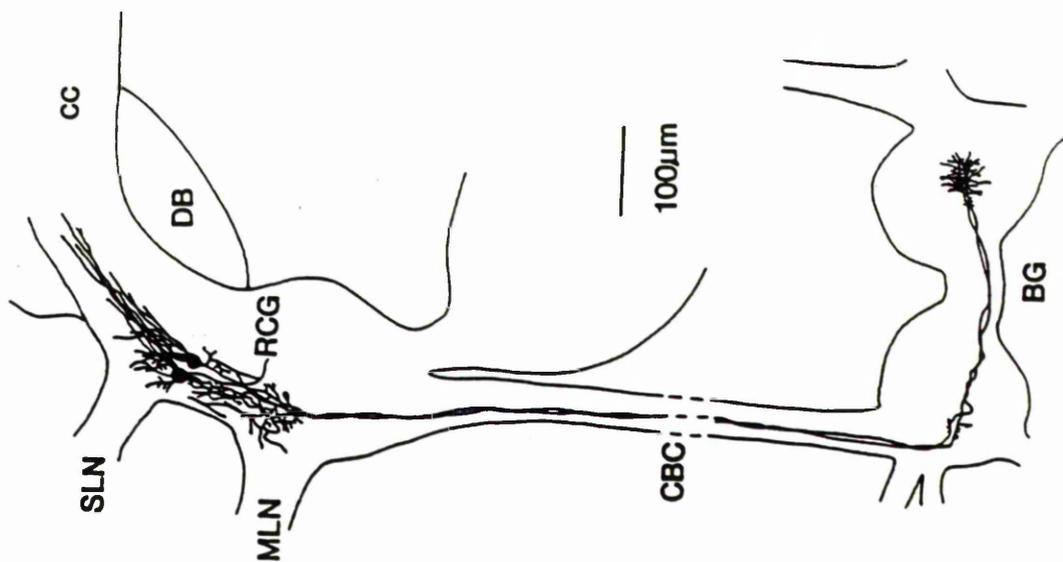
of different amplitude IPSPs present over a 1 minute period in 25 separate CV1 recordings. The study also demonstrated the presence of spontaneous, subthreshold excitatory inputs (EPSPs) to CV1 (Fig. 3.2A).

At resting potential (-63 ± 6 mV; n=18) the amplitudes of the two types of IPSP were approximately 1mV and 4mV, and of the EPSPs 2mV, although the apparent amplitude of EPSPs varied owing to blocked (axonal) spikes being superimposed on some of them. The small amplitude IPSPs occurred more-or-less continuously, whilst the large amplitude IPSPs occurred in more discrete groupings and were usually most frequent following a burst of action potentials in CV1 (Fig. 3.2A). It is possible, therefore, that the latter contribute towards termination of bursts. The large amplitude IPSPs sometimes masked the smaller IPSPs, with the two types (when they occurred simultaneously) summing to produce larger amplitude compound IPSPs, especially after a burst of action potentials (Fig. 3.2A). The EPSPs, again apparently unitary, were seen predominantly preceding each burst, and may provide excitation contributing to the burst itself (Fig. 3.2A).

Both the small IPSPs and the EPSPs were seen in all preparations (except for a very few exceptions, e.g. Figure 3.7B). The presence of the large IPSPs, however, was much more variable. In 25% of preparations (n=25) large IPSPs were not present, and in the remaining preparations they occurred mainly following CV1's burst. However, in some cases, large IPSPs were seen occurring almost continuously with frequencies of up to $4s^1$ (e.g. Fig. 3.7B).

Following surgical isolation of the cerebral ganglia from the remainder of the CNS (n=8), the three types of subthreshold input were still present (Fig. 3.3B), indicating that the presynaptic inhibitory and excitatory neurons which supply these inputs to CV1 are themselves located in the cerebral ganglia. Also, CV1 still showed strong rhythmic bursts of action potentials, similar to those seen in the intact CNS (except for the absence of the deep

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Figure 3.2 A) Identifiable inputs received by CV1. Cyclical activity in phase with activity in the feeding CPG is seen as bursts of action potentials superimposed on compound N1-phase excitation. Small and large amplitude IPSPs ($IPSP_s$, $IPSP_L$), and EPSPs are labelled.
Partial summation of the two types of IPSP are also indicated.
B) Histogram showing the frequency of different amplitude IPSP over a 1 minute period (pooled data from 25 separate CV1 recordings). Two peaks are seen around 1 and 4 mV, suggesting two separate populations of IPSPs. Values are slightly scattered around these two values possibly owing to differing resting potentials between preparations.

A
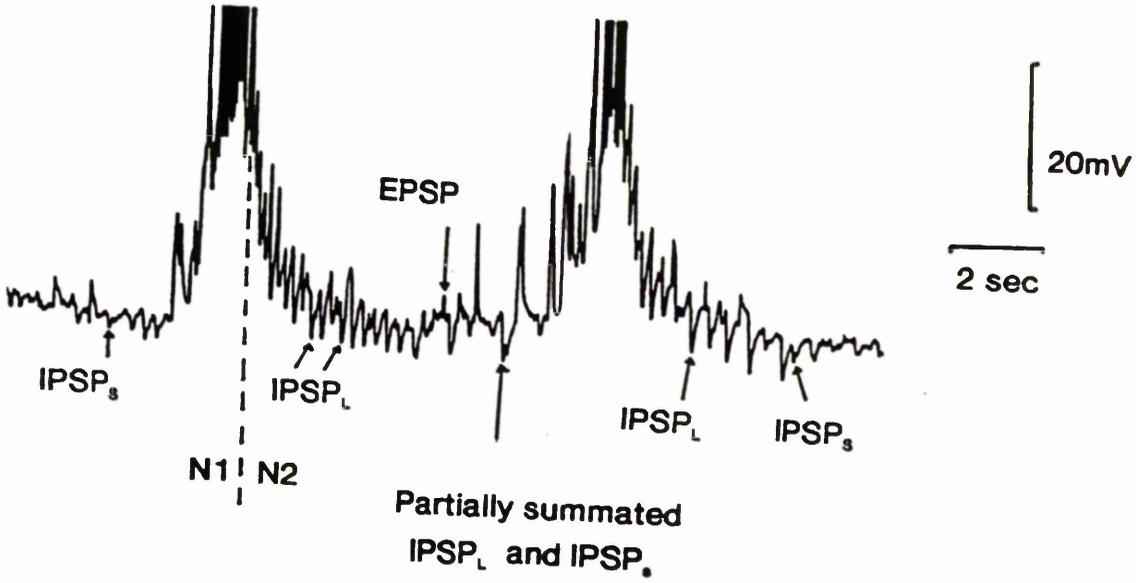
B
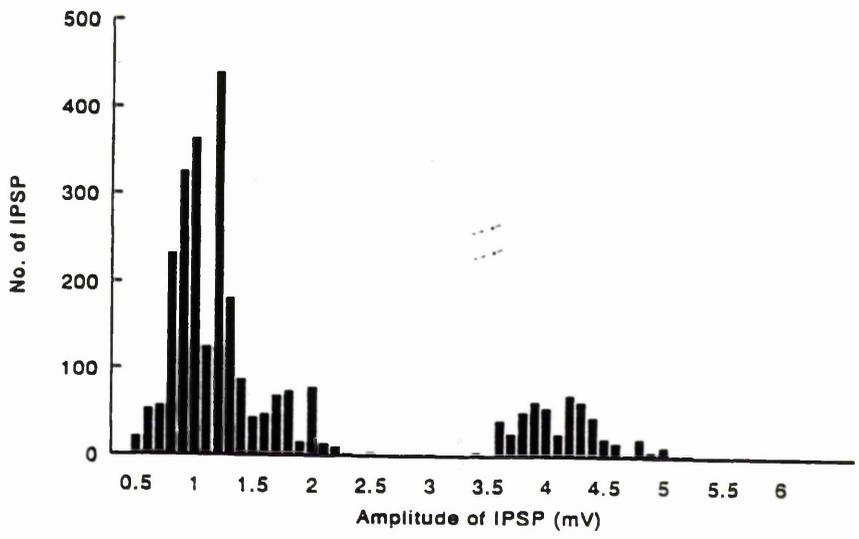
N2-phase inhibition) (Fig. 3.3A,B). Strong phasic bursting of CV1 was previously described in isolated brain (i.e. nerve ring minus buccal ganglia) by McCrohan (1984b). The present study, using isolated cerebral ganglia, showed that non-buccal rhythmic inputs to CV1 (resulting in phasic bursting) must originate specifically from neurons located in the cerebral ganglia.

Activity of CV1 in relation to the feeding rhythm

In the intact CNS, phasic excitation and inhibition of CV1, in phase with buccal feeding motor output, were shown to occur in two ways.

i "Follower" activity. This type of activity only occurred during ongoing feeding motor output from the CPG. Bursts of action potentials in CV1 were initiated at the same time as, or just after, the onset of the N1 phase, with spikes superimposed on a compound excitatory input (Fig. 3.4A), presumably originating from the buccal ganglia. Few or no EPSPs were seen preceding the burst, and there was little or no acceleration of spike frequency during the burst. Termination of the burst was either at the onset of, or during, the N2 phase. Thus, CV1 appeared to be "phase-locked" with CPG activity, i.e. passively following ongoing feeding motor output.

ii "Command-like" activity. In this case initiation of a burst in CV1 occurred prior to the N1 phase and was preceded by EPSPs (Fig. 3.4B). Spike frequency accelerated throughout the burst, reaching its peak during the N1 phase, presumably due to excitatory synaptic feedback

Figure 3.3 A) Activity and inputs received by CV1 in the intact CNS. All types of subthreshold inputs are present and CV1 is showing spontaneous phasic bursting activity. Note the deep N2 inhibition phase originating from the buccal central pattern generator which is absent from Fig. 3.3B.

B) Isolated cerebral ganglia. All three types of subthreshold input are present, indicating that presynaptic excitatory and inhibitory neurons are located within the cerebral ganglia. CV1 shows phasic bursts of action potentials suggesting that within the cerebral ganglia is a separate rhythm generator.
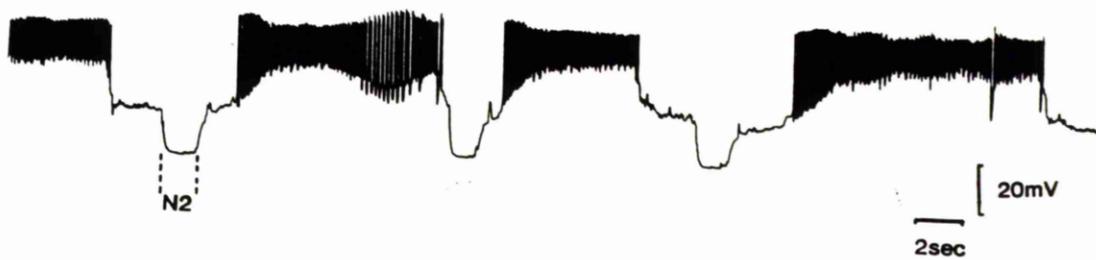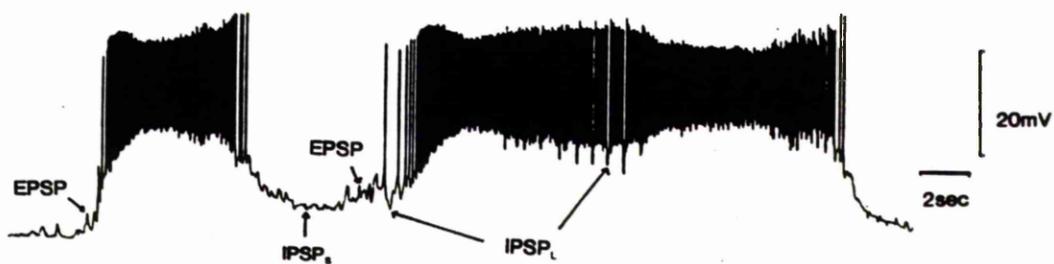
to CV1 from the N1 interneurons. Termination of the burst occurred either at the beginning of, or during, the N2 phase (as with "follower" activity; Fig. 3.4A). Excitation and activity in CV1, therefore, did not follow CPG activity but preceded it, and presumably drove it, owing to its known excitatory connections with N1 interneurons (McCrohan and Kyriakides, 1989).

Figure 3.5 further shows that these two activities were not mutually exclusive. In preparations where there was ongoing rhythmic motor output, CV1 could switch spontaneously from "follower" to *"Command-like"* activity, showing that the different activities were not a result of recording from different subtypes of CV1.

## Ability of CV1 to initiate feeding motor output in the isolated CNS.

Previous studies on CV1 stated that two of the criteria for identification were: (i) its ability to initiate or modulate feeding motor output, and (ii) its receipt of rhythmic synaptic inputs in phase with activity in the buccal ganglia (McCrohan, 1984b; McCrohan and Kyriakides, 1989). However, this study showed that CV1's ability to initiate feeding motor output, or even to receive input from the CPG, appeared to be dependent on the "state" the feeding system was in. Three different states were identified at the beginning of each set of recordings.

i "Command-like" state. This state was seen in 9/30 preparations. In a previously quiescent preparation, injection of steady depolarising current led to initiation of a true feeding rhythm, all phases (N1, N2 and N3) being present (Fig. 3.6). This was the state described by McCrohan (1984b) and McCrohan and Kyriakides (1989).

ii "Disrupted" state. This was seen in 10/30 preparations. Steady depolarisation of CV1 could lead to bursting activity in phase with the feeding CPG, but this activity was not typical (i.e. as in *"Command-like"* state) (Fig. 3.7A). Bursts tended to be prolonged with many interruptions by large amplitude IPSPs; in extreme cases, a single burst of action potentials could last for

Figure 3.4 Spontaneous bursting activity of CV1 with respect to activity of the feeding CPG. All three activity phases of the CPG (N1, N2, N3) are clearly identified as different synaptic inputs to the motoneuron B3. B3 receives inhibition during the N1 phase (shown here reversed as an apparent depolarising wave), excitation during N2 (with a superimposed burst of spikes), and high frequency brief excitatory inputs from N3 interneurons (Benjamin and Elliott, 1989). A: "Follower" activity. CV1 passively follows CPG activity, being excited at the onset of, or during, the N1 phase, seen with spikes superimposed on a compound excitation. The burst then terminates during the N2 phase. B: *"Command-like"* activity. Initiation of CV1's burst is preceded by EPSPs and occurs prior to the onset of the N1 phase. Firing frequency accelerates during the burst and peaks during N1, presumably due to recurrent excitation from the N1 interneurons. (McCrohan and Kyriakides, 1989).

A

B3

N1 N2

N3

"FOLLOWER"

B

N1 N2

N3

B3

20mV

2sec
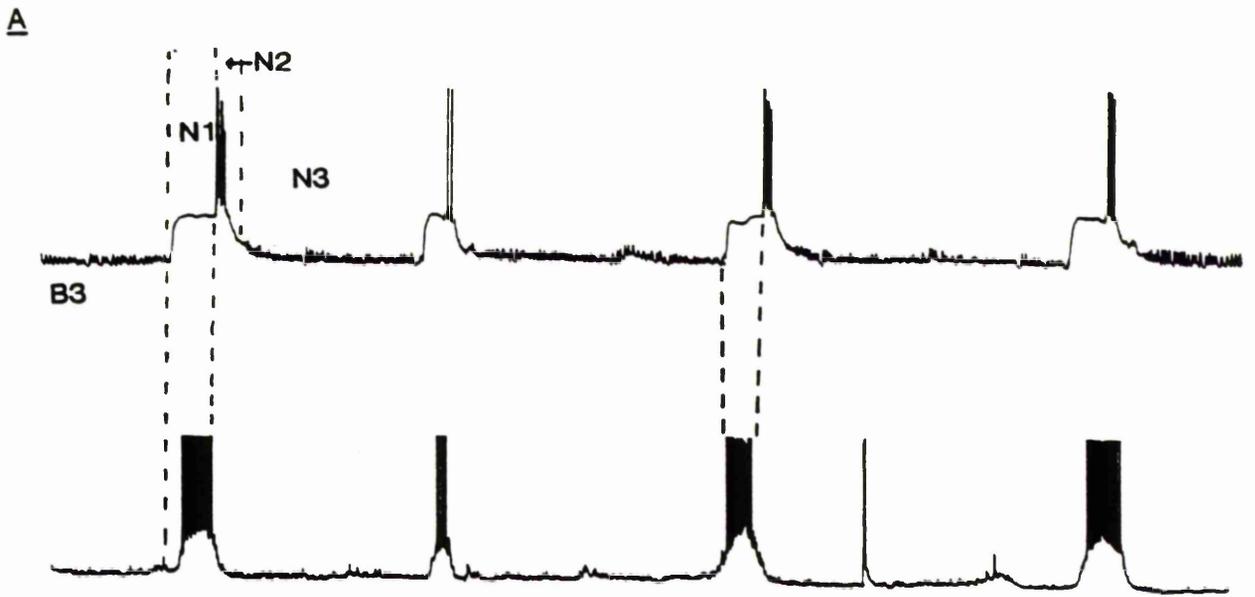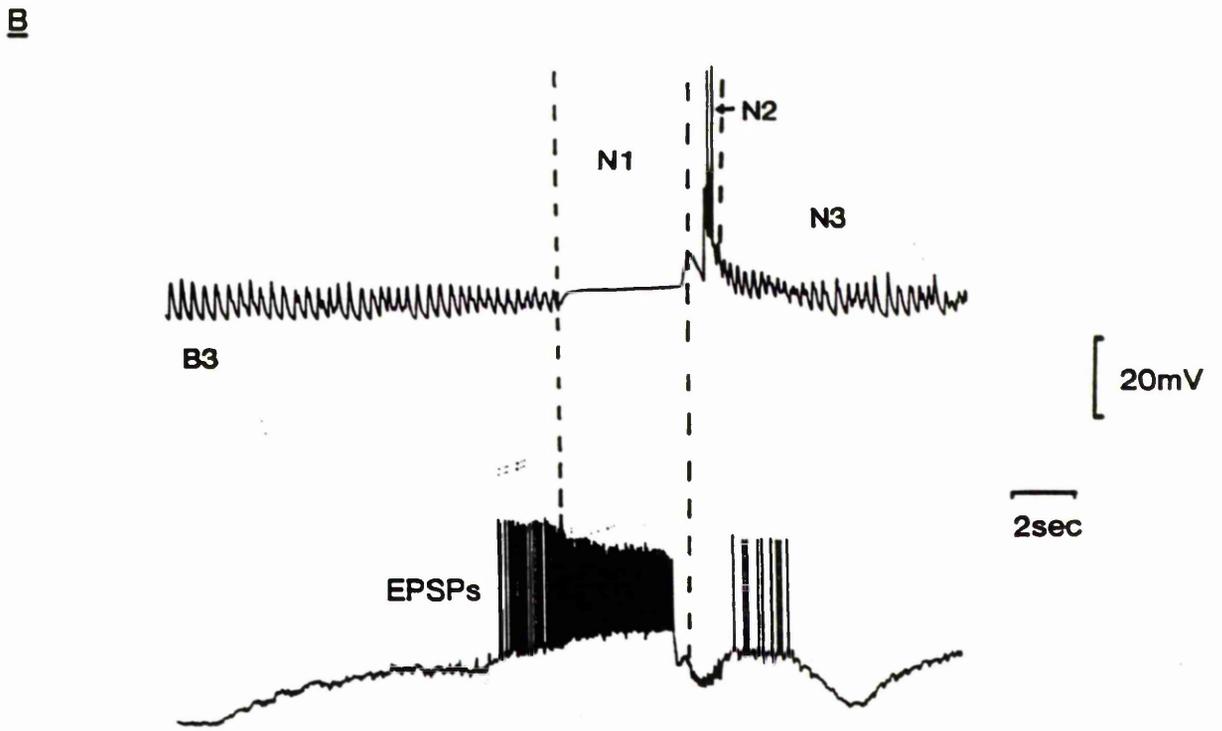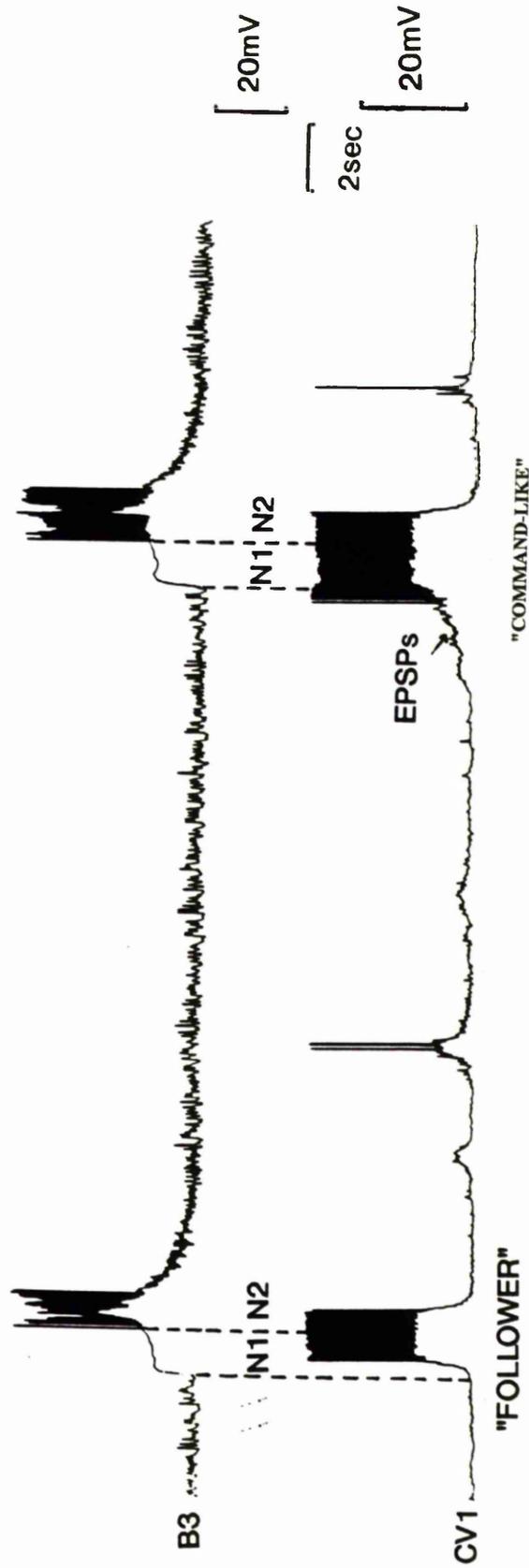
EPSPs

"COMMAND-LIKE"

Figure 3.5 Spontaneous change from "follower" activity to *"Command-like"* activity in CV1. Note the lack of EPSPs preceding the burst in follower activity of CV1 compared to command activity.

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25 seconds or longer (Fig. 3.7B). Thus, the presence of many large amplitude IPSPs appeared to disrupt the timing of bursting in CV1 resulting in either slowing, or complete inhibition, of feeding motor output.

Disruption of bursting in CV1 by large amplitude IPSPs appeared to be correlated with N3 interneuronal input to buccal motoneurons (Fig. 3.7A). The N3 phase of CPG activity was clearly identified as large subthreshold unitary EPSPs in the B3 motoneuron. These arise directly from N3 interneurons (Rose and Benjamin, 1979, 1981b). This suggested a possible link between the large amplitude IPSPs in CV1 and activity in the N3 interneurons of the CPG. Previous studies have shown that the CGCs modulate feeding motor output at least in part by excitation of the N3 interneurons (McCrohan *et al.*, 1989; McCrohan and Kyriakides, 1992). Therefore, the possibility that the CGCs influence initiation of feeding motor output by directly or indirectly causing changes in the frequency of large IPSPs in CV1 was investigated.

Experiments were carried out on both the intact CNS and isolated nerve ring (buccal ganglia removed). Depolarisation of the CGC did not induce any changes in the frequency of IPSPs in CV1, even with the buccal ganglia intact (Fig. 3.8). This suggests that the presence of IPSPs is not related to the level of activity of the CGCs, even by an indirect pathway via the buccal ganglia, and any link between these inputs and N3 activity must be independent of CGC activity.

iii "Dissociated" state. In the remaining 11 of the 30 preparations, activity in CV1 was dissociated from that in the rest of the feeding system. Any phasic bursting seen was not accompanied by rhythmic activity in the feeding CPG (monitored from buccal motoneurons) and, in a quiescent preparation, injection of depolarising current into CV1 led only to tonic firing, with no evidence of synaptic feedback from the CPG, and no initiation of feeding

Figure 3.6 *"Command-like"* state: Initiation of a true rhythmic feeding motor output by CV1. Steady depolarisation of CV1 (Dep) leads to rhythmic activity in a buccal motoneuron, B3, and in CV1 itself. The three phases of the feeding cycle, N1, N2 and N3, are indicated. CV1 receives excitatory input during N1 and inhibition during N2.
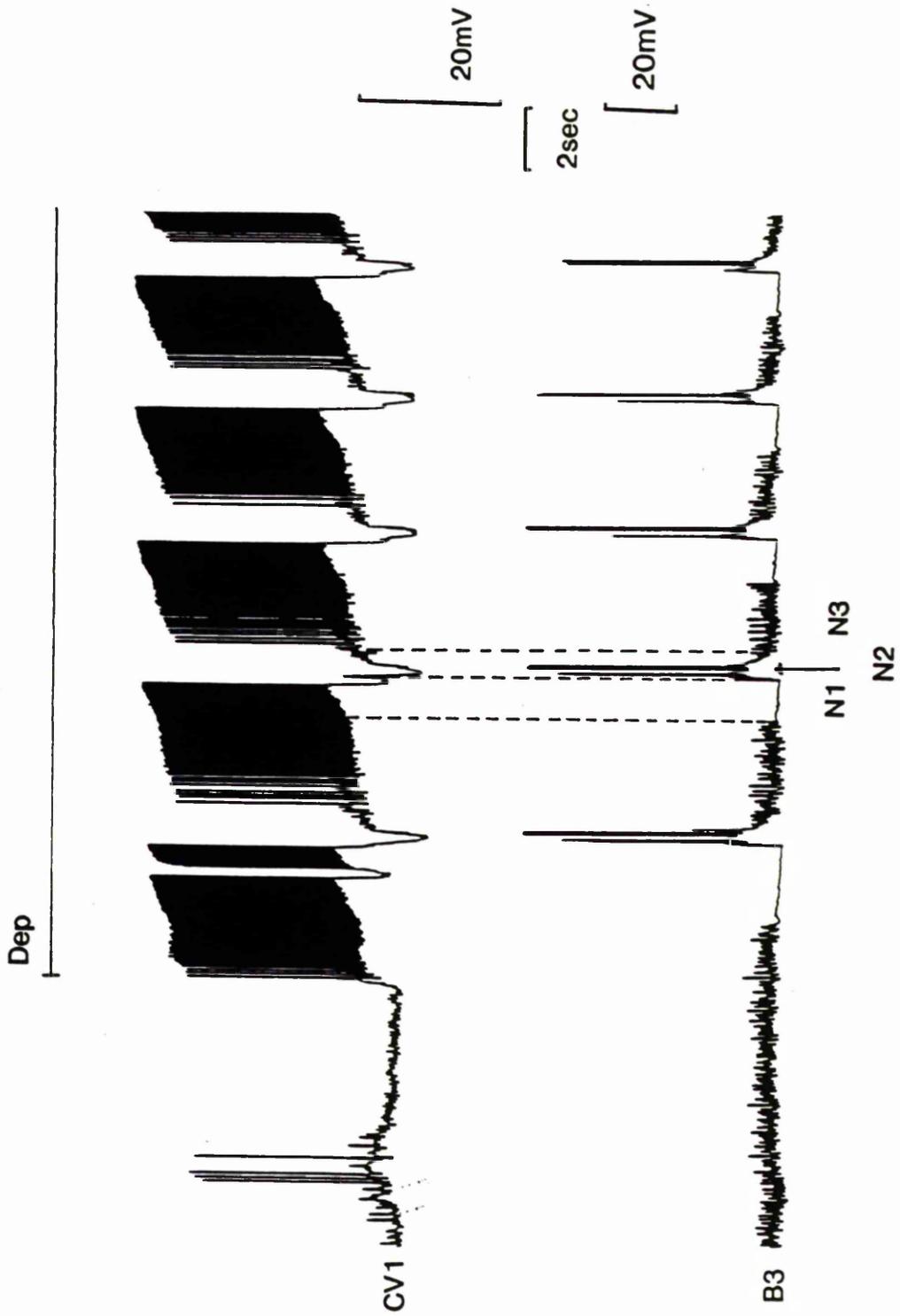
Figure 3.7 "Disrupted" state. A) CV1 is held depolarised throughout the recording. The phases of the feeding cycle can be seen clearly as differing synaptic inputs to buccal motoneuron B3. The presence of large amplitude IPSPs appears to prolong CV1's bursts, and the timing of feeding motor output (as monitored on B3) is slowed. The large IPSPs ($IPSP_L$) in CV1 are grouped together and occur at the same time as periods of N3 input to B3.

B) Extreme example of "disrupted" state; CV1 is depolarised throughout the recording. Interruption of CV1's bursts by large amplitude IPSPs ($IPSP_L$) leads to an extremely prolonged long-lasting burst (over 20 seconds) with the buccal feeding rhythm also being interrupted. Two feeding cycles, monitored on CV1 and buccal motoneuron B4, are indicated.

A)

CV1 Dep

IPSP$_L$

N3

B3

20mV

2sec

B)

CV1 Dep

IPSP$_L$

N1 N2

B4
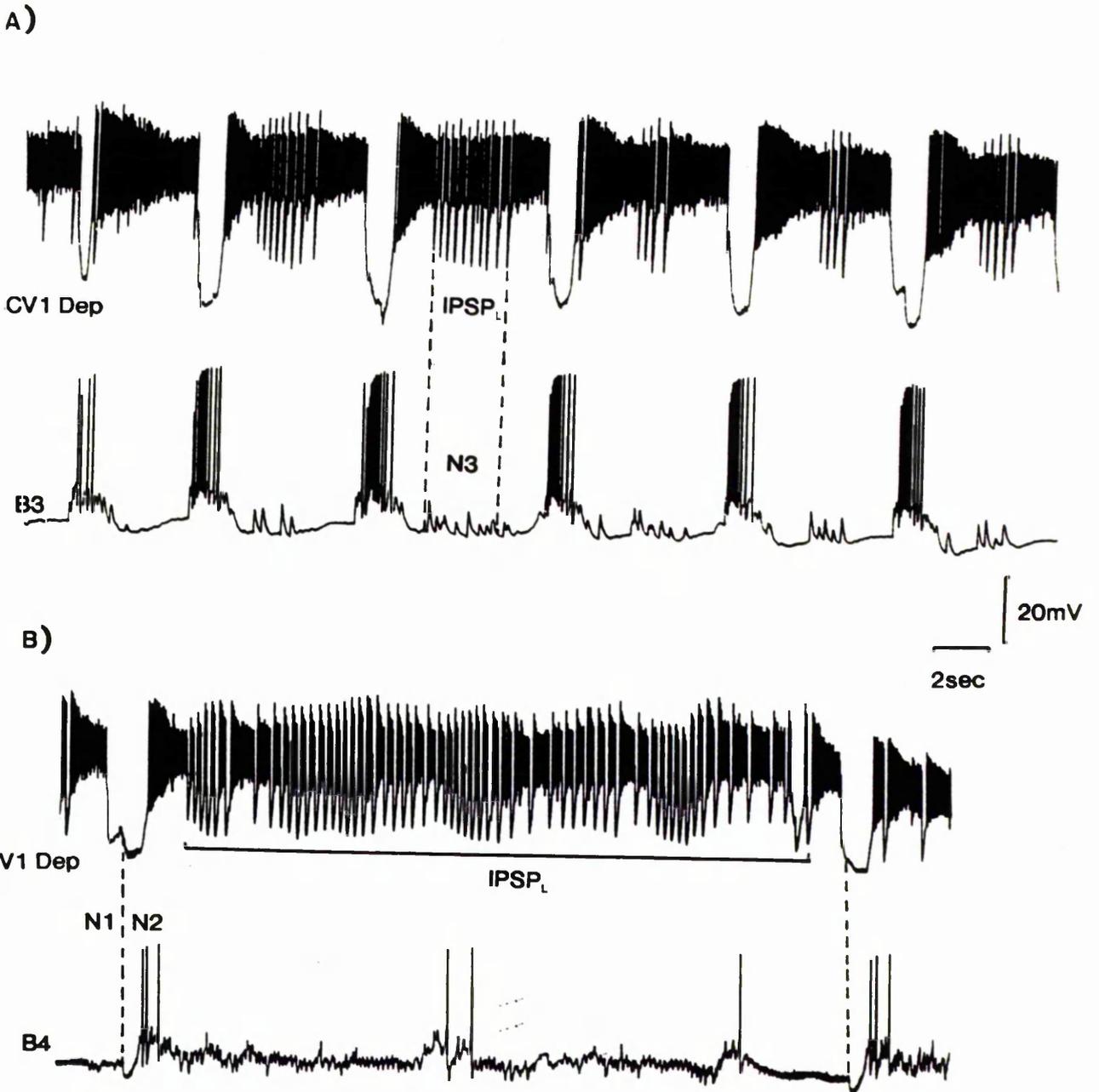
Figure 3.8 Depolarisation of the CGC (dep), leading to increased firing frequency, does not lead to CV1's bursts becoming more interrupted. Depolarisation of CGC does however (in this preparation) lead to an increase in frequency of the feeding rhythm, seen as shorter cycle period in both CV1 and the buccal motoneuron B3. (Note: electrode used for recording and current injection into CGC was out of balance).

CGC

Dep

CV1

B3

20mV

20mV

20mV

5sec

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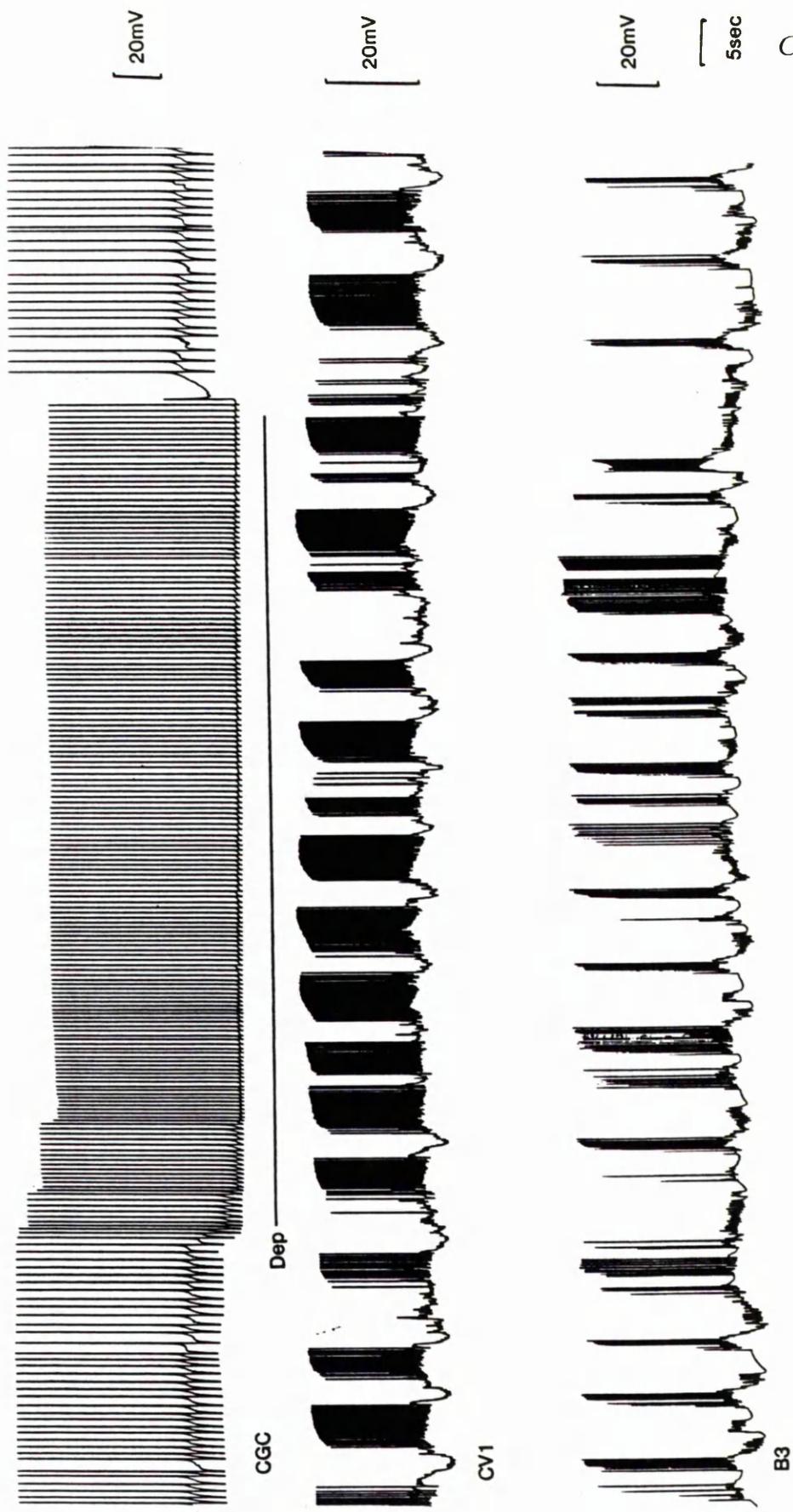
motor output (Fig. 3.9A). The tonic firing of CV1 in this state was often interrupted by large amplitude IPSPs (Fig. 3.9A).

In four of these 11 preparations CV1's activity was seen to switch from the "dissociated" state to a *"Command-like"* state in which depolarisation did lead to activation of the CPG, with all three phases of interneuronal activity being present (Fig. 3.9B). This shows that, at least in these preparations, the "dissociated" state is a true state and was not due to damage to the preparation during dissection (e.g. disruption of the cerebrobuccal connectives).

RESULTS B - SEMI-INTACT PREPARATION

Behavioural analysis of reliability of sucrose as a feeding stimulant.

Several different studies have used sucrose to stimulate feeding in *Lymnaea*, both in the whole animal and in semi-intact preparations (Goldschmeding and Jager, 1973; Kemenes *et al.*, 1986; Tuersley, 1986; Tuersley and McCrohan, 1987a). To enable some comparison to be made with previous behavioural and neural studies, all experiments in this section used a 10^{-3} M sucrose solution to the lips to stimulate feeding (cf. Kemenes *et al.*, 1986).

Previous studies stated that sucrose is a "reliable" feeding stimulant (Kemenes *et al.*, 1986). However, exactly *how* reliable was not studied in detail. As this thesis requires that neural activity is related to whole animal behaviour, the behavioural responses to a 10^3 M sucrose solution had to be understood in detail before any electrophysiological recordings could be made. This meant that all environmental factors affecting responsiveness had to be understood and kept to a minimum. Factors such as room and snail water temperature, level of satiation, and time of year were constant for each experimental set. However, the importance of the time of day at which the experiment was carried out had to be ascertained.

Figure 3.9 "Dissociated" state. A) Steady depolarisation of CV1 (Dep) leads to continuous firing with no synaptic feedback from the CPG, and does not initiate cyclic feeding motor output. Large amplitude IPSPs (IPSP$_L$) are apparent in CV1. Motoneuron B3 shows continuous N3 input.

B) Spontaneous change to *"Command-like"* state (same preparation as (A) three minutes later). Depolarisation of CV1 (Dep) leads to initiation of a feeding cycle in B3 (all phases, N1, N2 and N3, are present).
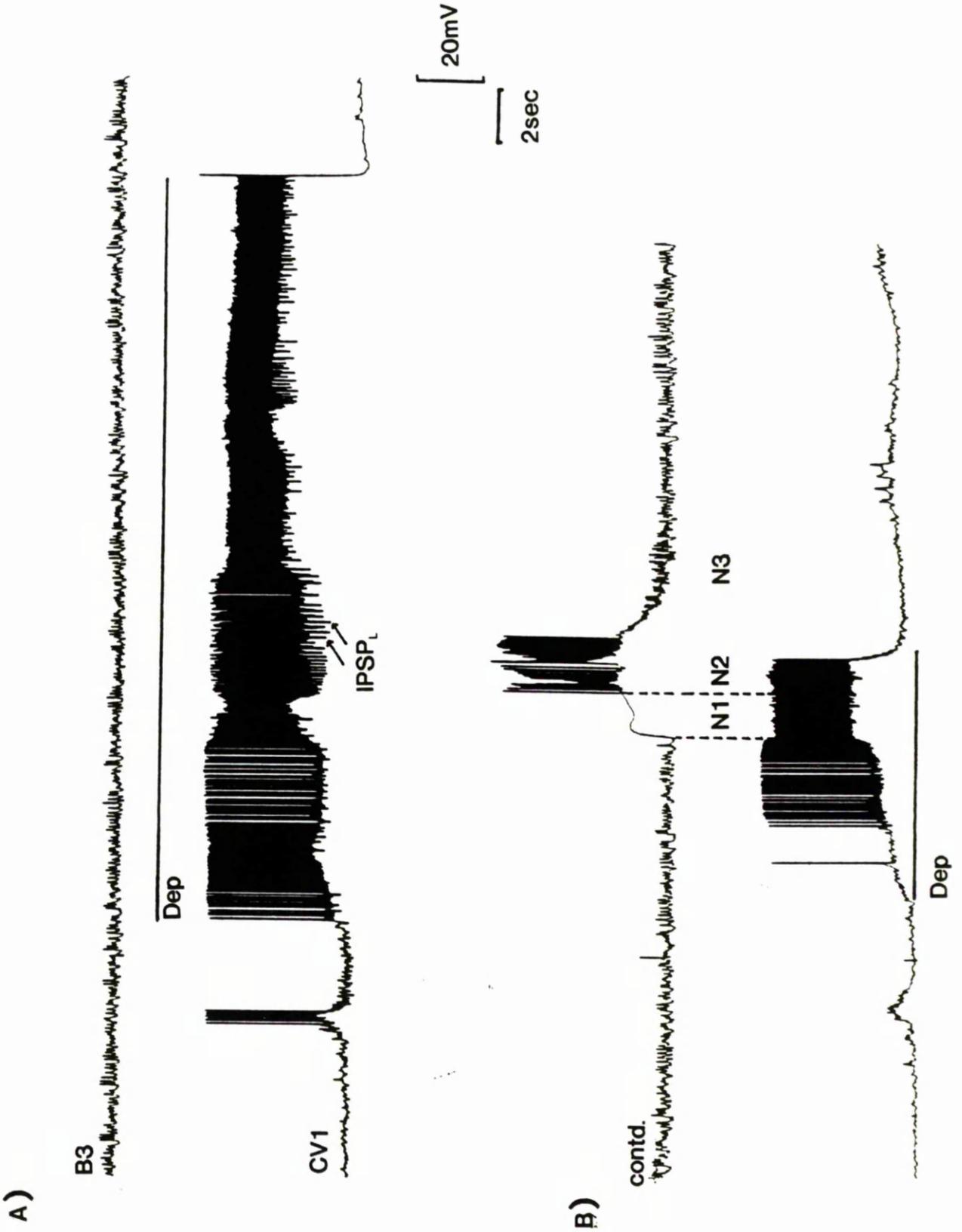
Also, it was important to note any fluctuations in responsiveness to sucrose in individual snails due to factors not understood by, and beyond the control of, the experimenter (e.g. the equivalent of "mood changes", possibly due to the level of 5HT internally, cf. *Pleurobranchaea*, Lee and Pavlocik, 1976) or other transmitters or modulators.

Methods - Twenty snails were tested for their responsiveness to sucrose at 5 separate times during the day: 0900, 1100, 1300, 1500 and 1700 hrs. At each time of testing, snails were placed individually into a Petri dish containing a small depth of snail water (approximately 0.5 cm) and allowed 5 minutes to acclimatise to the new environment (snails were deemed to be fully acclimatised when seen to be in an alert state, i.e. the head-foot complex fully extended and showing locomotion; Tuersley, 1986). 0.1 ml of 10^3 M sucrose was then dripped near to the lips, and the snail's response noted qualitatively as either "response" or "no response". A "response" was noted if biting activity was initiated within two minutes of the sucrose solution being applied. Conversely, "no response" was noted if the snail showed no biting activity within two minutes of the sucrose application to the lips.

Results - The percentage of snails responding to sucrose at different times of day is shown in Figure 3.10. The responsiveness to sucrose was relatively unchanged from 0900-1500 hrs, with sucrose being a very reliable feeding stimulus, initiating feeding in 90-95% of animals. At 1700 hrs, however, the feeding response dropped to 75%, which, although still high, indicated that electrophysiological experiments should be carried out earlier in the day to ensure that sucrose was acting as reliable feeding stimulus.

Figure 3.10 gives no indication of which individuals were responsive at the different times of day. Table 3.1 lists the individual snails (numbered 1-20) that were unresponsive at the five times of day.
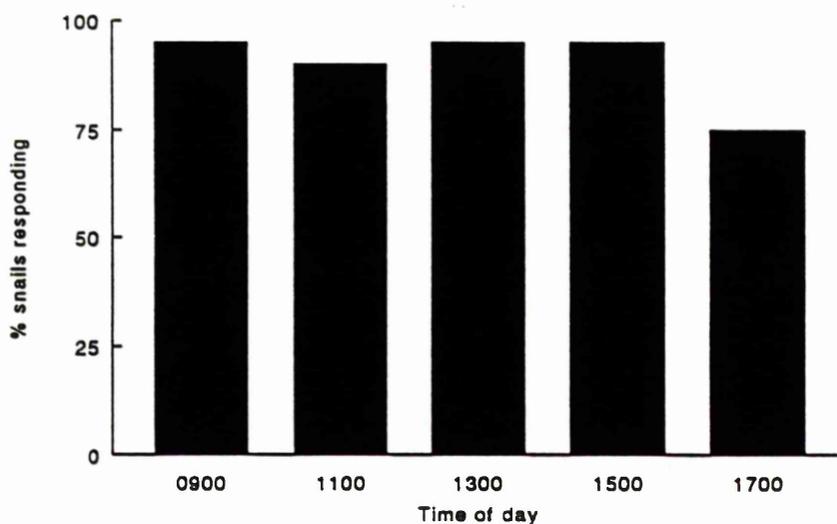
Figure 3.10 Percentage response to sucrose of 20 snails at successive two hour intervals (from 0900 - 1700 hours). From 0900 to 1500 hours, 90-95% of snails respond to sucrose by biting within two minutes of application of 10^3 M sucrose to the lips. However, at 1700 the percentage response is reduced to 75%.

Table 3.1

TIME OF DAY (24 HR CLOCK)	UNRESPONSIVE SNAIL (NO.)
0900	8
1100	4, 15
1300	15
1500	7
1700	3, 7, 11, 18, 20

Table 3.1 The individual snails showing no response to 10^3 M sucrose at five different times of day are shown. The individual unresponsive snails are seen to differ throughout the day, indicating that *Lymnaea* do show short term fluctuations in responsiveness to sucrose. The cause of these fluctuations is unknown.
Table shows representative data from one day only. n=20.

Although the percentage of unresponsive snails stayed relatively constant until 1700 hrs, Table 3.1 shows that the individual snails that failed to respond differed, indicating that the sucrose-unresponsive state was reversible and not due to individual snails being unresponsive throughout. One interesting observation was that the snails that did not respond to sucrose usually appeared to be in a general unresponsive state, with very little locomotion or orientation behaviour seen. This state is probably equivalent to the "still" behavioural state described by Tuersley (1986), which he found to be associated with lower food arousal scores.

The cause of this short-lived unresponsive state cannot be explained, as all the snails were kept under the same conditions prior to, and throughout the experiment. However, it was important to be aware of these changes in responsiveness or "mood" when considering the electrophysiological results in the next section.

<u>**Electrophysiological recordings**</u>

In 9/26 semi-intact preparations, application of 10^3 M sucrose to lip tissue in a previously quiescent preparation led to a cessation or marked reduction of IPSPs, depolarisation of the membrane potential by approximately 4 mV and rhythmic bursting in CV1 (Fig. 3.11A). The IPSPs often returned, disrupting CV1's bursts, after approximately 15-25 seconds (Fig. 3.11A). The underlying changes in synaptic input to CV1 which led to this activity were partially masked by the resulting bursts of action potentials.

In a further 11/26 preparations, application of sucrose did not cause rhythmic bursting, but resulted in a clear change in the nature of subthreshold inputs received by CV1 and a tonic depolarisation of the membrane potential, again by about 4 mV. Prior to sucrose application, inputs received by CV1 were predominantly inhibitory (Fig. 3.11B(i)). However, upon sucrose application there was a cessation of IPSPs and an increase in frequency of EPSPs, accompanied by some irregular spontaneous firing (Fig. 3.11B(ii)). The depolarisation of membrane potential and spontaneous firing were presumably a consequence of these changes in the balance of excitatory and inhibitory inputs to CV1.

Experiments using an isolated cerebral ganglia-lip preparation (n=5) showed similar changes in inputs to CV1 upon sucrose application (Fig. 3.11C), indicating that the sensory pathway affecting activity in CV1 following food stimuli to the lips is confined to the cerebral ganglia and is not via the buccal ganglia. The example shown (Fig. 3.11C) is unusual in that CV1 was extremely quiet and did not receive any EPSPs. However, it clearly shows the cessation of IPSPs (an important observation since this does not occur in presumed homologous cells of other species; see Discussion). IPSPs returned approximately 30 seconds after sucrose application.

In the remaining 6/26 CNS-lip preparations, application of sucrose to the lips resulted in no change in activity of, or inputs to, CV1. A change to a sucrose-sensitive state was never

seen in these preparations, so this lack of response may have been due to damage of the lip nerves during dissection. However, the possibility that this insensitivity may in some cases be the neural equivalent to the sucrose-unresponsive state described in the previous behavioural section, cannot be ruled out.

Control experiments, in which 0.1 ml of snail saline (n=10) or deionised water (n=5), was applied to lip tissue resulted in no changes in inputs to, or gross activity of, CV1 (Fig. 3.12A,B). This indicates that CV1 only becomes active in response to chemosensory cues rather than the mechanical stimulus of application of solution to the lip tissue.

During the course of these experiments recordings were made from several unidentified CV interneurons located in a similar region to CV1 (i.e. between the roots of the lip nerves). A small number of these cells also responded to sucrose with immediate initiation of rhythmic bursting in previously quiescent preparations (Fig. 3.12C). This suggests that either: initiation of rhythmic activity following food stimuli is mediated by a number of neurons in this region (not only by the $CV1_a$-type neuron), or that these cells are synaptically connected to CV1, acting as follower cells, and do not actively initiate feeding following food application to the lips.

Effect of satiation

In *Pleurobranchaea* the putative homologue of CV1 (the PC_p) was shown to be involved in modulating feeding following satiation (Davis *et al.*, 1983). In this section, the possibility that CV1 is involved in the mechanism of satiation in *Lymnaea* is investigated. *Methods* - Three days before the experiment, 20 snails (of medium to large size, 2.5-3.5 g) were taken from the home tank and randomly assigned to two groups, satiated and starved. The two groups were placed in identical scrubbed clean tanks containing aerated filtered snail water (this ensured that there were no algae in the tanks that the snails could feed on). The

Figure 3.11 Effect of 0.1 ml 10^{-3} M sucrose application to lip tissue on activity in CV1 in a semi-intact preparation.
A) Sucrose application results in cessation of IPSPs, depolarisation of membrane potential by approximately 4 mV and rhythmic bursting.

B) (i) Control (absence of sucrose). Inputs received by CV1 are mostly inhibitory with no spontaneous firing (firing is only seen when CV1 is depolarised; Dep). (ii) 5 seconds after sucrose application to the lips. Inputs received are mainly excitatory, with few IPSPs present. The membrane potential is depolarised and isolated spontaneous spikes are seen.
C) Isolated cerebral ganglia-lips preparation. The first application of sucrose leads to a slowing in frequency of IPSPs and second application causes complete cessation of inputs. IPSPs return after approximately 30 seconds.

A)

IPSP

SUCROSE

20mV

2sec

B) (i) control   (ii) 5 sec after sucrose

Dep

IPSP

EPSP

c)

SUCROSE   SUCROSE   20mV

2sec

30 sec later

IPSP return
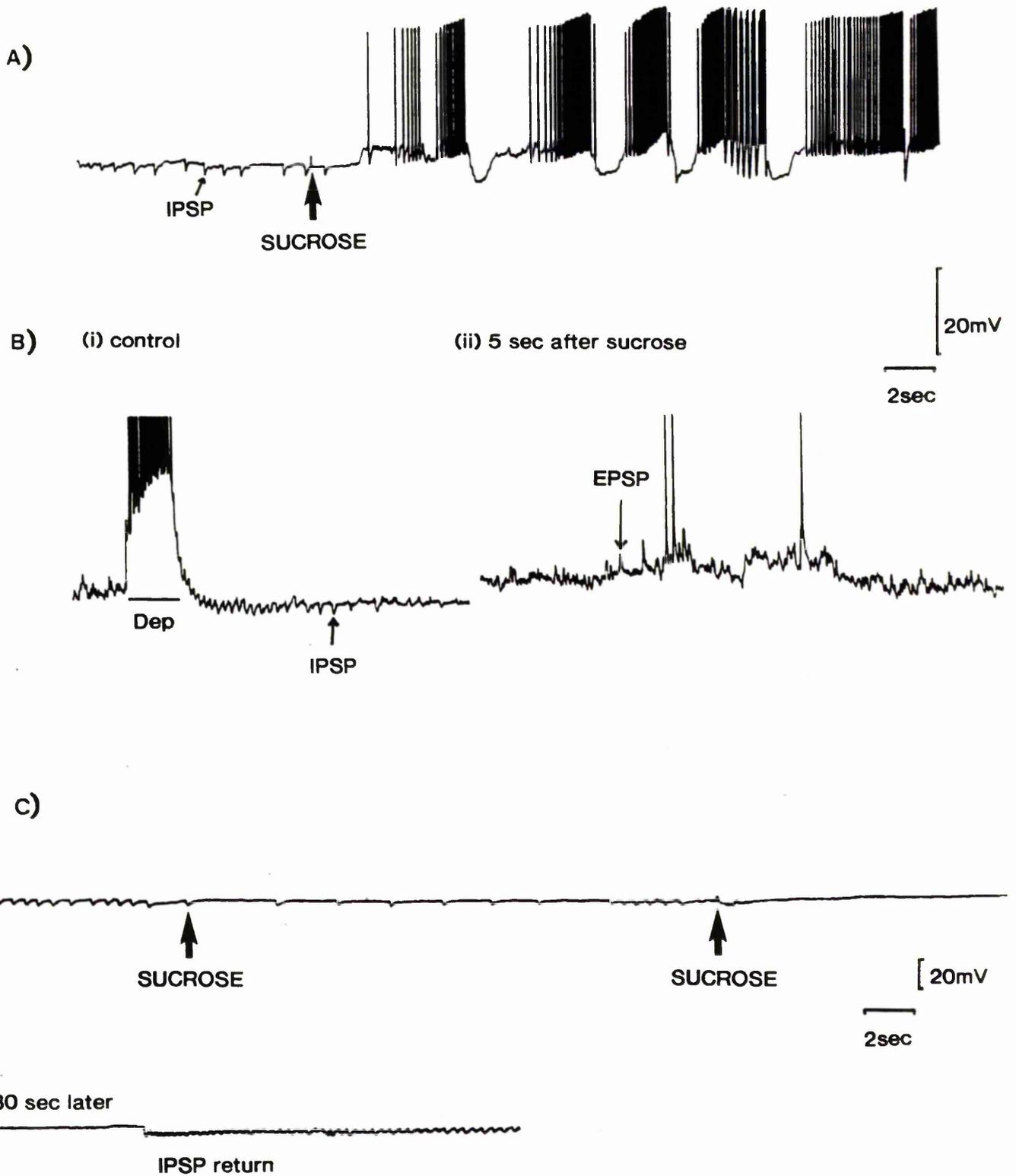
Figure 3.12 Effect of application of neutral feeding stimuli to the lip tissue on activity and inputs of CV1. A) Application of 0.1 ml snail saline results in no change in inputs to or activity in CV1.

B) Application of 0.1 ml deionised water similarly has no effect on the inputs received by, or the activity of, CV1.

C) Unidentified neuron in the vicinity of CV1. Note the neuron receives many EPSPs, but no IPSPs. Application of 0.1 ml of 10^3 M sucrose results in a slight depolarisation of the membrane potential and bursts of action potentials.
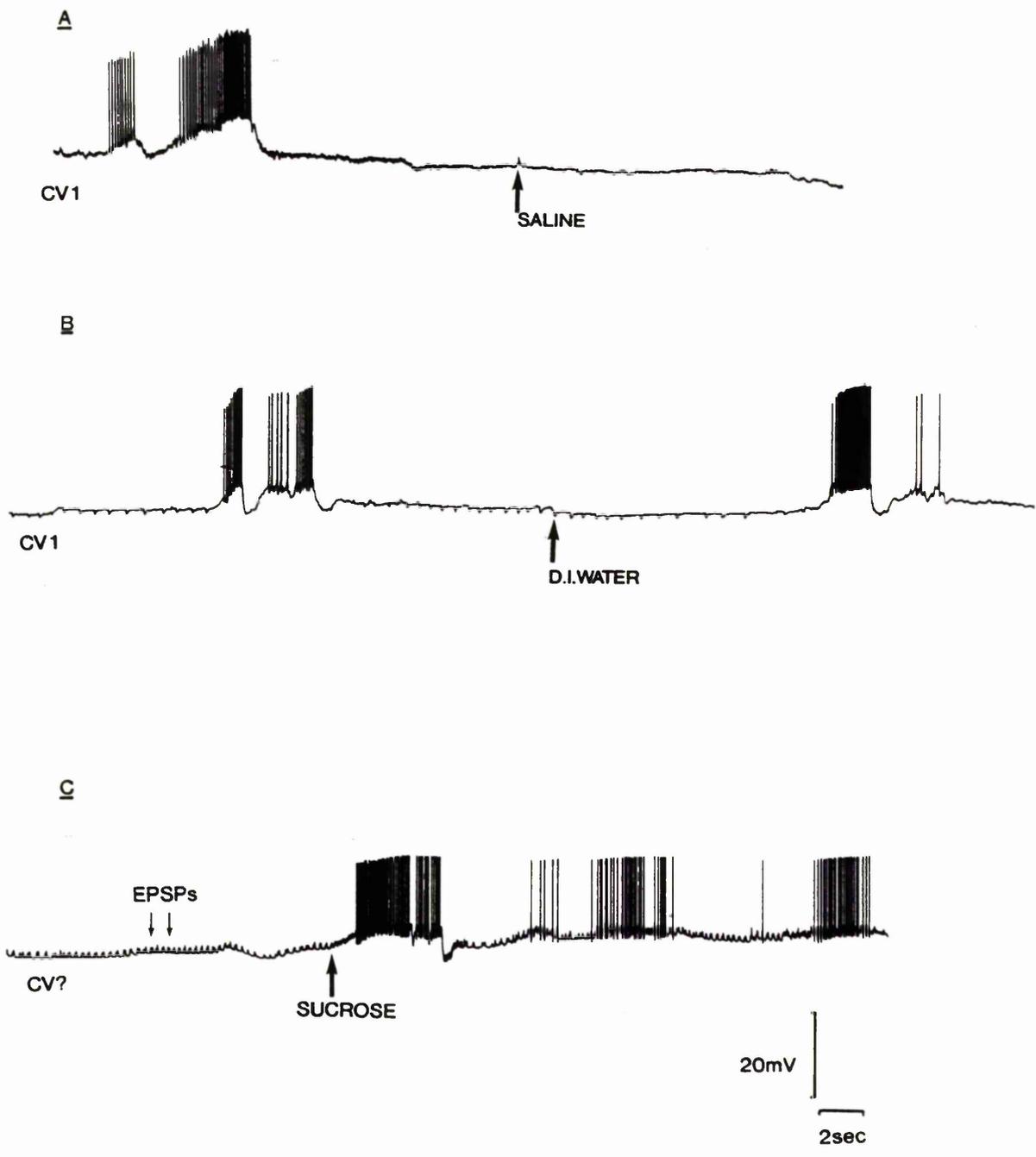
satiated group were then fed *ad libitum* on lettuce, and the starved group starved for three days.

Results - Behavioural experiments comparing the feeding response to 10^3 M sucrose applied to the lips showed no significant difference between the two groups in either latency to first bite (Fig. 3.13A) or number of bites in 2 minutes (Fig. 3.13B) (Mann-Whitney U test). This is in agreement with Tuersley (1986) who found that there was no significant difference in response to food between starved and satiated individuals.

Subsequent electrophysiological recordings of spontaneous activity in CV1 in the isolated CNS (i.e. in the absence of any food stimuli) demonstrated that the occurrence of large amplitude IPSPs differed markedly between the two groups (n=10, each group). In CV1 taken from starved individuals, large amplitude IPSPs occurred only infrequently. In satiated individuals, CV1 received significantly more large IPSPs (Fig. 3.14). The frequency of small IPSPs was not significantly different in the two groups, and any apparent reduction in small IPSPs in the satiated individuals was probably due to the large IPSPs masking the presence of the small IPSPs. The presence of frequent large IPSPs was accompanied by interrupted firing activity in CV1, similar to the "disrupted" state (Fig. 3.14B. cf. Figure 3.7A,B). Bursts of activity in CV1 from starved individuals were uninterrupted (Fig. 3.14C).

Experiments using a semi-intact preparation from both satiated and starved individuals (n=4, each group), demonstrated a very similar response to application of 0.1 ml 10^3 M sucrose to the lips in both "fed" and "starved" CV1s, with sucrose being excitatory to both groups. This excitatory effect was seen as either a change in the inputs received by CV1 (i.e. a cessation of IPSPs and an increase in EPSPs usually leading to bursts of action potentials, Fig. 3.15), or an increase in frequency and intensity of ongoing feeding cycles (not shown). This is in agreement with the behavioural experiments carried out on the same animals prior to electrophysiological recording, which showed no significant difference in the response to

sucrose in fed or starved individuals (see Fig. 3.13).
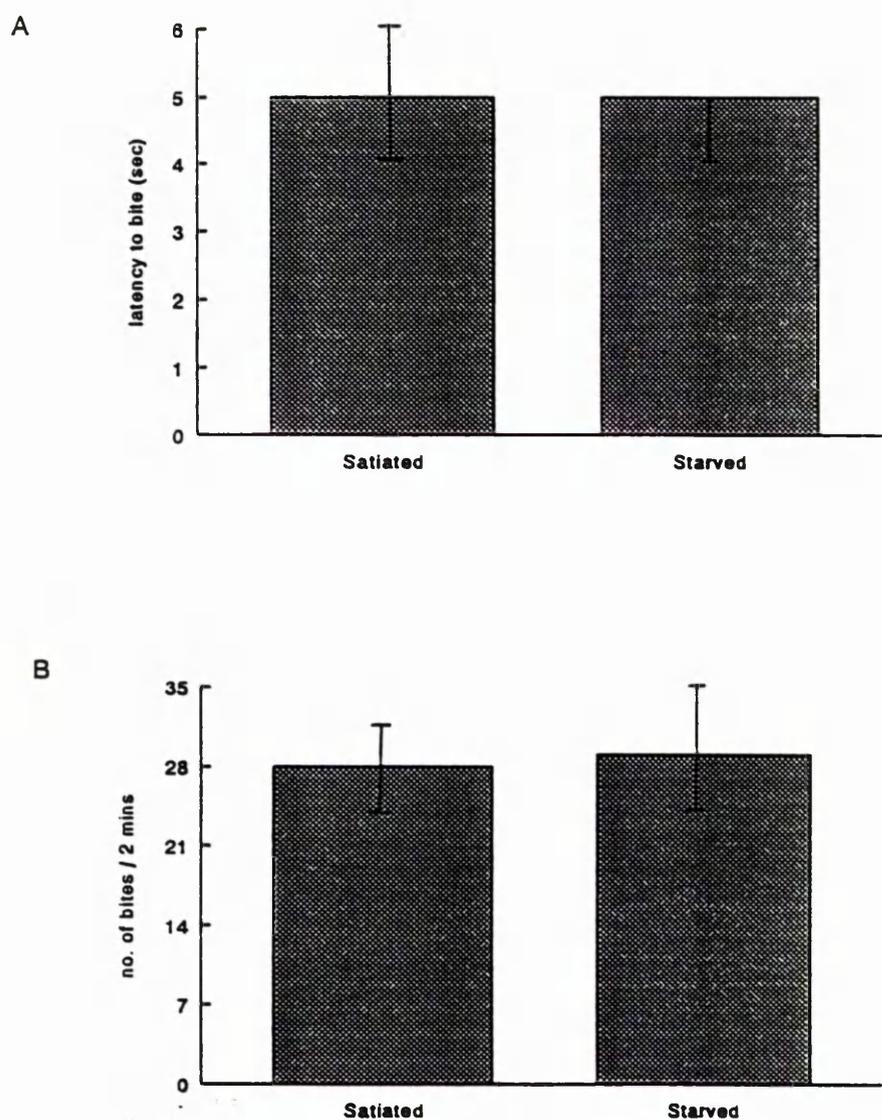
**Figure 3.13** Comparison of feeding response to 10^3 M sucrose in whole animals which were satiated (n=10) or three day starved (n=10). Data are not assumed to be normal and so are presented as medians and interquartile ranges. There is no significant difference between groups in either A) latency to bite or B) number of bites in two minutes (Mann-Whitney U test).

Figure 3.14 A) Occurrence of large and small amplitude IPSPs in CV1 over a 1 minute period (2 minutes after electrode penetration) in the isolated CNS taken from satiated (n=10) versus 3-day starved individuals (n=10). Data were not of normal distribution and are therefore presented as medians and interquartile ranges. The frequency of large IPSPs is significantly greater in satiated compared to starved individuals (P<0.001, Mann-Whitney U test). Frequencies of small IPSPs are not significantly different.

B) Activity recorded from CV1 taken from a satiated individual. Bursts of spikes are interrupted by bouts of large amplitude IPSPs, disrupting bursting activity in CV1.

C) Activity of CV1 taken from a starved individual. CV1 receives very few large IPSPs and its activity is relatively uninterrupted.

A.

B.

sat.　　　　IPSP$_L$

$\left[\rule{0pt}{1.5em}\right.$ 20mV

2sec

C.

star.

85
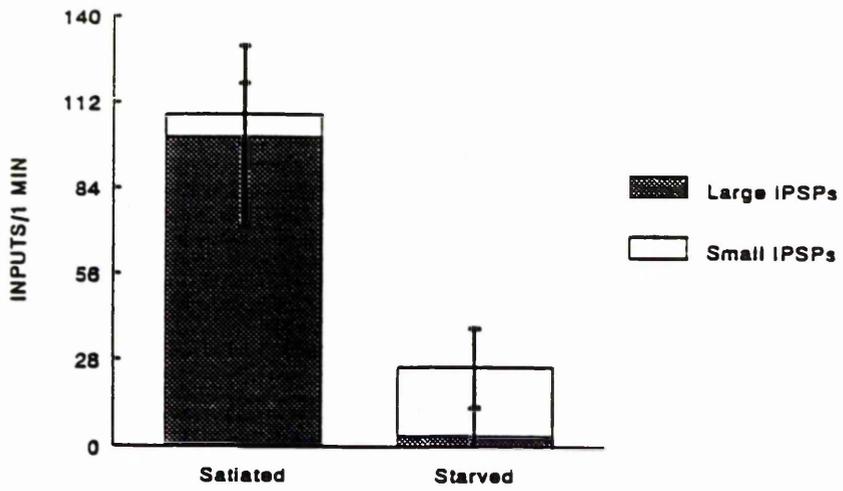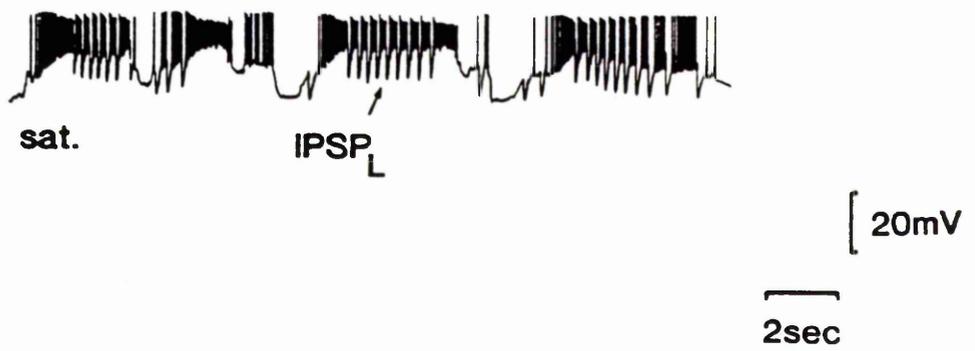
Figure 3.15 Excitatory effect on CV1 following application of 0.1 ml 10^3 M sucrose to the lips of a semi-intact preparation taken from both starved and satiated animals. A) Satiated: CV1 is receiving many large IPSPs. Application of 10^{-3} M sucrose causes a depolarisation of the membrane potential (after initial dip in trace possibly due to mechanical movement of application of the sucrose), a reduction in the frequency of IPSPs and an increase in EPSPs. Bursts of action potentials are seen approximately 10 seconds after sucrose application, presumably due to the change in inputs to CV1. IPSPs return after about 15 seconds.

B) Starved: CV1 only receives small amplitude IPSPs. Application of 10^3 M sucrose causes an increase in EPSPs, depolarisation of the membrane potential and rhythmic bursts of action potentials. This activity is accompanied by the initiation of feeding motor output, as seen by the rhythmic bursting in the identified motoneuron B4, and CV1.
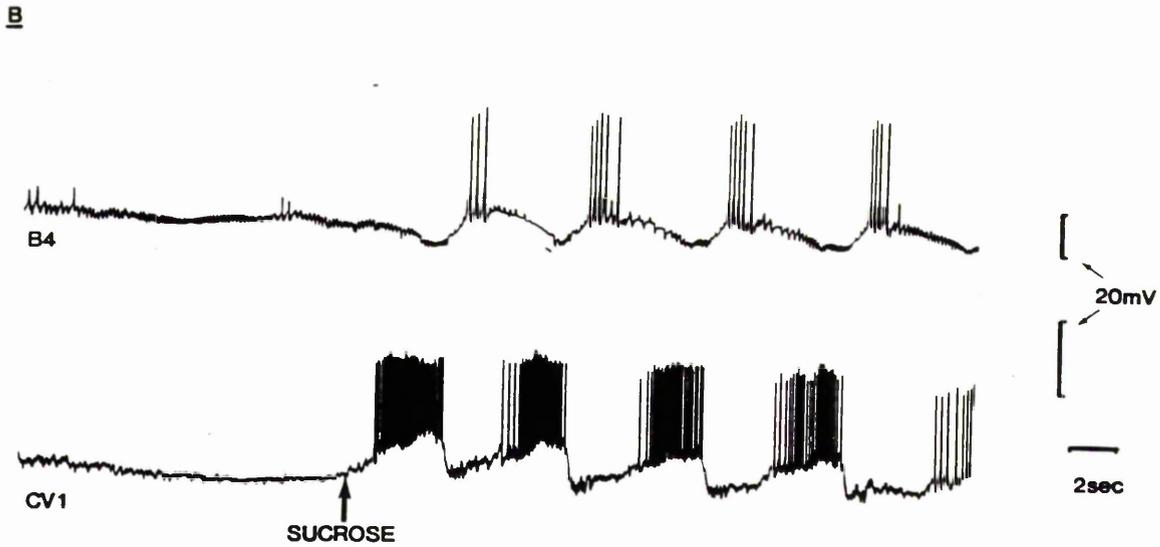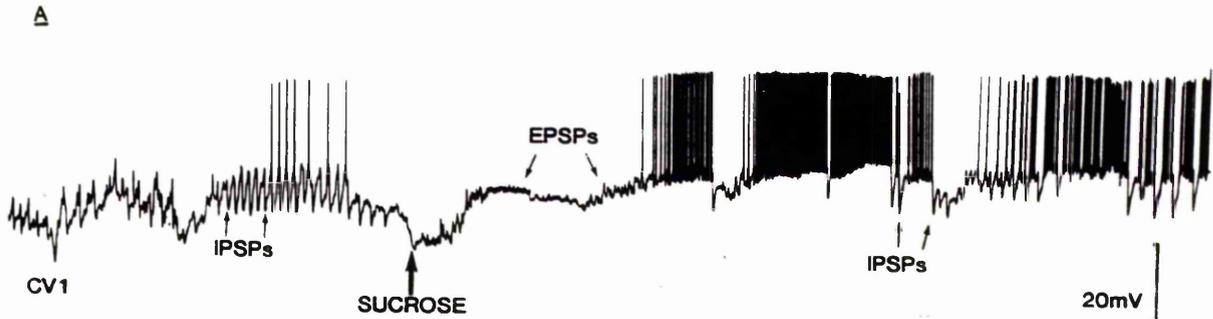
DISCUSSION

Criteria for the identification of CV1.

The criteria for identification of CV1 in previous work (McCrohan, 1984b; McCrohan and Kyriakides, 1989) were: i) ability to initiate and modulate rhythmic feeding motor output; (ii) receipt of synaptic inputs in phase with activity in the buccal CPG; and (iii) morphology. However, results obtained in this Chapter which described the activity and inputs received by CV1, meant that these criteria had to be reassessed.

Morphology obviously still remains a criterion for identification. However, the first two criteria are inadequate, as they only allow for the identification of CV1 whilst it is in the *"Command-like"* state. Previous work on CV1 (McCrohan, 1984b; McCrohan and Kyriakides, 1989) resulted in the hypothesis that CV1 was a "command-like" interneuron, since steady depolarisation of CV1 was shown to initiate feeding motor output and uninterrupted bursting activity in phase with activity in the buccal CPG. Results presented in this Chapter now show this to be an oversimplification. CV1 can exist in several interchangeable states, which govern whether it is able to initiate and/or modulate feeding motor output, or even simply follow ongoing rhythmic output from the CPG. Therefore, if the old identification criteria were used, over two-thirds of preparations would be disregarded, since CV1 is only in the *"Command-like"* state" in approximately 30% of preparations, the remaining 70% being in either the "disrupted" or "dissociated" state. More rigorous criteria for identification of $CV1_a$ are therefore proposed: (i) position of the soma on the surface of the ganglion; (ii) morphology; (iii) receipt of newly identified subthreshold, possibly unitary, synaptic inputs (large and small IPSPs, and EPSPs); and (iv) strong phasic bursts of action potentials usually, but not always, in phase with activity in the buccal CPG.

Possible homology of CV population with cerebral-to-buccal interneurons of other species.

McCrohan and Kyriakides (1989) hypothesised that the CV1 population was homologous with cerebral-to-buccal interneurons found in the feeding systems of *Pleurobranchaea californica* (the paracerebral interneurons; Gillette *et al.*, 1978, 1982b) and *Limax maximus* (cerebral-buccal neurons; Delaney and Gelperin, 1990a-c). This homology was suggested since these interneurons have similar morphologies and locations to the CV1 population, and also the ability to elicit rhythmic feeding motor output from the buccal ganglia. Since that time, similar populations of cerebral-to-buccal interneurons have also been described in *Aplysia californica* (the CBIs; Rosen *et al.*, 1987, 1988, 1991), the giant land snail *Achatina fulica* (the C1; M. Yoshida, pers. comm.), and *Helix pomatia* (Kemenes, 1992). The more detailed studies on *Pleurobranchaea, Limax* and *Aplysia* showed that, although several of the neurons within the population were capable of initiating feeding, the neurons were not homogeneous, with each proposed to have a slightly different role in controlling feeding motor output (Kovac *et al.*, 1983a,b; Davis *et al.*, 1983; Delaney and Gelperin, 1990a-c; Rosen *et al.*, 1991). A similar arrangement is proposed for *Lymnaea*. There are three pieces of evidence that support this hypothesis.

a) Whilst recording from the isolated CNS, $CV1_a$ showed two types of behaviour, "follower" activity (passively following feeding motor output in the buccal ganglia) and *"command-like"* activity (preceding buccal feeding cycles, and possibly actively driving feeding motor output). This could be a consequence of $CV1_a$ only being responsible for driving feeding motor output at certain times.

b) Several unidentified neurons in the CV1 area responded to application of sucrose to the lips, i.e. $CV1_a$ may not be the only cerebral-to-buccal neuron responsible for initiating feeding in response to food.

c) The input patterns and activity of $CV1_a$ and its surrounding cells (other neurons were

recorded from whilst trying to locate $CV1_a$) were found to be similar to those of the cerebral-to-buccal cells of *Pleurobranchaea* (Kovac *et al.*, 1983a,b), *Limax* (Delaney and Gelperin, 1990ab), and *Aplysia* (Rosen *et al.*, 1991).

$CV1_a$ is now proposed to be homologous to PC_p, CB_1, and CBI-2 of *Pleurobranchaea*, *Limax*, and *Aplysia* respectively (Kovac *et al.*, 1983a,b; Delaney and Gelperin, 1990a-c, Rosen *et al.*, 1991). This is because all these interneurons show, i) strong phasic activity in phase with buccal feeding motor output (FMO), ii) identifiable IPSPs and EPSPs, which have a critical role in determining the activity of the neuron (see below), and iii) excitation following chemostimuli to the lips resulting in initiation of feeding motor output (this thesis; Kovac *et al.*, 1983a,b; Delaney and Gelperin, 1990a-c; Rosen *et al.*, 1991; M. Yoshida, pers. comm.). Rosen *et al.* (1991) also suggested this homology, in addition to $CV1_b$ being homologous to CBI-4 in *Aplysia*.

The role of the subthreshold inputs to $CV1_a$ and its homologues.

The subthreshold synaptic inputs to CV1 ($CV1_a$) and its putative homologues are an important factor in the modulation of these interneurons' activity. In this Chapter it was shown that application of sucrose to the lips resulted in depolarisation of CV1's membrane potential and bursts of action potentials, accompanied by an increase in EPSPs and a cessation of IPSPs. A possible mechanism for how feeding is initiated in *Lymnaea* is proposed in a hypothetical circuit diagram (Fig. 3.16A). The model is very simplified and only shows excitatory and inhibitory *pathways* presynaptic to CV1 supplying the EPSPs and IPSPs, since individual interneurons presynaptic to CV1 have not been identified. In this model, food to the lips causes an excitation of the excitatory pathways resulting in more EPSPs to CV1, and inhibits the two inhibitory pathways (supplying the large and small amplitude IPSPs) reducing the number of IPSPs received by CV1. This change in inputs increases the probability of CV1

becoming active, and thus initiating feeding motor output (FMO) from the buccal CPG.

The results in this Chapter also suggested that modulation of feeding behaviour following satiation was a consequence of a change in inputs to CV1. Behavioural experiments by Tuersley and McCrohan (1987a) showed that when *Lymnaea* was satiated, its response was simply to stop searching for food (i.e. there was an inhibition of "food search" activity; the spontaneous rasping shown by *Lymnaea* in the absence of food). Recordings made from the isolated CNS in this Chapter show that "satiated" CV1s received a significantly greater number of large amplitude IPSP compared to "starved" CV1s. This increased number of large IPSP disrupted CV1's activity, prolonging its bursts, thus inhibiting or slowing the generation of spontaneous feeding motor output from the buccal CPG. Since these recordings were made from the isolated CNS, the increase in IPSPs to CV1 following satiation was totally independent of sensory input from the lips[1]. The changes in neural pathways following satiation are shown in the modified hypothetical circuit diagram (Fig. 3.16B). The sensory pathways from the lips remain unchanged from Figure 3.16A since food application results in initiation of feeding in both satiated and starved snails. However, the inhibitory pathway supplying the large amplitude IPSPs is bolder, denoting the increased number of spontaneous large IPSPs received by CV1. An additional pathway exciting the large IPSP pathway is also included, denoting the central excitation of this pathway in satiated snails in the absence of sensory inputs. The underlying cause of this central excitation is, as yet, unknown. Possible causes could be: 1) excitation from gut mechanoreceptors signalling distention (possibly the oesophageal mechanoreceptor in the buccal ganglia described by Elliott and Benjamin, 1989),

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[1] In fact, sensory inputs (food application to the lips) had exactly the same effect on satiated and starved snails in both behavioural and electrophysiological experiments i.e. it caused an initiation of feeding. This initiation of feeding in response to food stimuli in satiated snails is proposed to occur due to food to the lips inhibiting the spontaneous large amplitude IPSPs, therefore allowing CV1 to show uninterrupted bursting activity and thus drive feeding motor output at normal frequencies (Fig. 3.16B).

Figure 3.16 Hypothetical circuit diagram, summarising possible mechanisms by which feeding motor output is modulated by the CV1 pathway in *Lymnaea*. Synaptic connections indicated are not necessarily monosynaptic. Closed circles represent inhibitory connections and triangles represent excitatory connections. E, I_s, and I_L represent neurons (or populations of neurons) not yet identified, which supply subthreshold EPSPs and small and large amplitude IPSPs to CV1.

A) Subsatiated or starved snails. Initiation of feeding following food to the lips. Food application causes inhibition of inhibitory neurons and excitation of excitatory neurons, resulting in activation of CV1, and thence feeding motor output from the buccal central pattern generator (CPG).

B) Satiated snails. Central excitation of the I_L neurons from an unknown source (see text for details) results in CV1 receiving many large amplitude IPSPs in the absence of sensory input from the lips. CV1's activity is therefore disrupted and output from the buccal CPG does not occur. This results in spontaneous feeding movements being inhibited. Application of food to the lips still results in initiation of feeding, since I_L is inhibited by food, lifting the disruptive effect of large amplitude IPSPs to CV1.

A
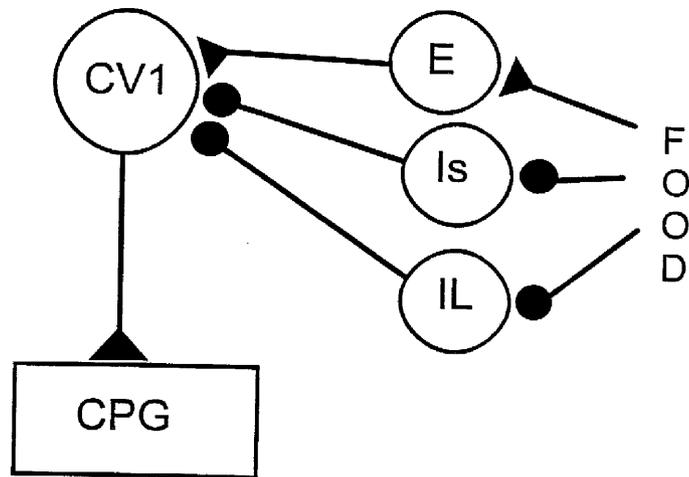
B
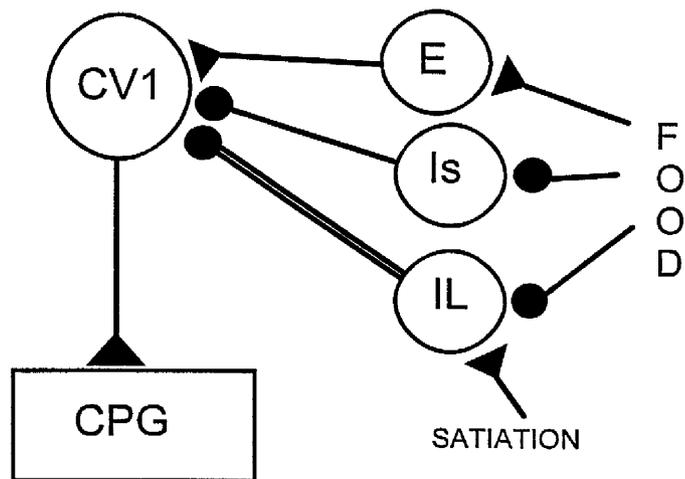
2) raised haemolymph sugar levels, and/or 3) haemolymph-borne factors (haemolymph-borne factors have been shown to at least partly underlie satiation in *Helix* (Balaban and Maksimova, 1988)).

Studies on the cerebral-to-buccal cells homologous to CV1 in *Pleurobranchaea*, *Aplysia* and *Limax* (PC_p, CBI-2 and the CB_1; Davis *et al.*, 1983; Rosen *et al.*, 1991; Delaney and Gelperin, 1990c) showed that the initiation and modulation of feeding was, as in *Lymnaea*, a consequence of changes in the balance of subthreshold inputs received by them. However, there were subtle differences.

In all other species tactile stimuli to the lips could result in an excitation of CV1-homologues, which is in contrast to *Lymnaea* where tactile input (application of saline) had no effect on CV1's activity. In *Aplysia*, the bias of inputs to the CBI-2s altered depending on where the food stimulus was applied, i.e. to the tentacle it caused inhibition, but caused excitation when applied to the inside of the lips (Rosen *et al.*, 1991). This may also occur in *Lymnaea*, however the gross application of food over all chemosensory areas used in this Chapter could not have demonstrated this. Similar studies on *Limax* by Delaney and Gelperin (1990) showed that the bias of subthreshold input to the CB_1 following food application to the lips was altered by surgically removing the buccal, and/or pleural ganglia, causing food application to the lips becoming more excitatory. This again is in contrast to *Lymnaea* where removal of the buccal ganglia had little effect on the subthreshold inputs received following food application to the lips.

The most rigorous and detailed studies of CV1 homologues were on the PC_ps of *Pleurobranchaea*, and therefore can be used to give the most detailed comparisons. Similar to *Lymnaea*, the excitatory and inhibitory subthreshold inputs received by PC_p were shown to be responsible for, 1) the ability of the PC_p to initiate feeding following sensory input to

the lips, and 2) inhibition of feeding following satiation. The neurons presynaptic to PC_p which supply these inputs were located in the cerebropleural ganglion suggesting that they are equivalent to those located in the cerebral ganglia of *Lymnaea*. Several different classes of these excitatory and inhibitory neurons (>10) were identified (Kovac *et al.*, 1983a,b), indicating that the *Pleurobranchaea* PC_p pathway may be more complex than the CV1 pathway in *Lymnaea*. Despite this complexity, a small group of inhibitory neurons, the cyclical inhibitory network (CIN) were demonstrated to have a key role in modulating PC_p's activity; underlying both the cyclical inhibition of the PC_ps during feeding (Gillette *et al.*, 1982b; Kovac *et al.*, 1983b), and the complete inhibition of the PC_p activity following satiation (Davis *et al.*, 1983; Davis, 1983, 1984). The inhibitory inputs to CV1 in *Lymnaea* were proposed to be homologous to the CIN in *Pleurobranchaea* by McCrohan and Kyriakides (1989) due to evidence that the N2-phase compound inhibitory inputs to CV1 during feeding motor output were a summation of the unitary IPSPs received by CV1. This homology is further supported by the results in this Chapter; since surgical isolation of the cerebral ganglia in *Lymnaea* indicated that the inhibitory neurons presynaptic to CV1 were located in the cerebral ganglia, and increased frequency of large amplitude IPSPs received by CV1 following satiation appeared to play a crucial role in modulating feeding behaviour. However, despite the strong evidence for homology between PC_p and CV1 and their inputs, the results also highlighted some fundamental differences in how these neurons modulate feeding behaviour. These differences are attributed to the contrasting feeding behaviour shown by the two species.

Behavioural experiments on *Pleurobranchaea* demonstrated that food application to the lips of hungry animals, like *Lymnaea*, resulted in strong feeding (Davis *et al.* 1977). However, the behavioural response of *Pleurobranchaea* to satiation was totally different FROM that shown by *Lymnaea*. Food application to the lips of satiated *Pleurobranchaea* did not result in feeding,

but in active withdrawal and total inhibition of feeding responses (Davis *et al.* 1977). This is because *Pleurobranchaea* does not deal with satiation by not looking for food like *Lymnaea*, but by withdrawing from food if encountered. Accordingly the neural mechanisms underlying the modulation of feeding are also different from *Lymnaea*, in that they involve modifying the feeding response to sensory input. The neural mechanism proposed by Davis *et al.* (1983) is shown in Figure 3.17. In starved *Pleurobranchaea* food application to the lips does not lead to an increase in EPSPs and inhibition of IPSPs to PC_p, as with CV1, but leads to an increase in both EPSPs *and* IPSPs. The overall effect on PC_p, however, is still excitatory due to the overall increase of EPSPs being greater than the increase in IPSPs (Davis *et al.*, 1983; Fig 3.17A). Following satiation the PC_ps (in contrast to CV1) receive normal levels of spontaneous subthreshold inputs in the absence of food. However, on application of food the bias of the inputs received by PC_ps changes, with the IPSPs being activated more than the EPSPs, thus inhibiting any activity of the PC_ps and therefore any feeding motor output (Davis *et al.*, 1983; Fig 3.17B). Analysis of the IPSPs received by the PC_ps showed that they originated from a strong tonic excitation of the CIN (Davis *et al.*, 1983).

Therefore, although an increase in IPSPs to CV1 and PC_p is fundamental in inhibiting feeding following satiation in both *Lymnaea* and *Pleurobranchaea*, the mechanism by which this is achieved is very different. This difference is proposed to be due to the different environment the two species have evolved in. *Lymnaea* lives in an environment where there is an abundance of potential food. Because of this *Lymnaea* has become a grazer, eating small amounts of food regularly. This could have influenced the way *Lymnaea* has evolved to deal with satiation has in one of two ways: 1) because *Lymnaea* is a grazer it may rarely reach a state of total satiation, meaning that a reduction of the search for food is sufficient to stop "overloading", making total withdrawal from food unnecessary, or 2) because *Lymnaea* lives in an environment where many of the surfaces that it moves across are covered in algae (its

Figure 3.17 Simplified circuit diagram summarising the mechanism by which feeding motor output is modulated by PC_p in *Pleurobranchaea* (Modified from Davis *et al.*, 1983). Closed circles represent inhibitory connections and triangles represent excitatory connections. E and I represent populations of neurons which supply subthreshold EPSPs and IPSPs to PC_p.

A) Subsatiated or starved animals. Application of food to the lips results in an excitation of both the excitatory and inhibitory neurons. However, the excitatory neurons are excited more than the inhibitory neurons (denoted by thicker connection), resulting in the overall bias of inputs received by PC_p being excitatory. This results in activation of PC_p and thence, feeding motor output from the buccal central pattern generator (CPG).

B) Starved animals. Following food to the lips, the bias of subthreshold inputs received by PC_p is inhibitory, since the inhibitory neurons are excited more than the excitatory neurons. This results in PC_p being inhibited and thus feeding motor output being inhibited. NB: The inhibition of PC_p is in response to food to the lips and does not occur centrally in the absence of sensory input (contrast *Lymnaea* Fig. 16B)
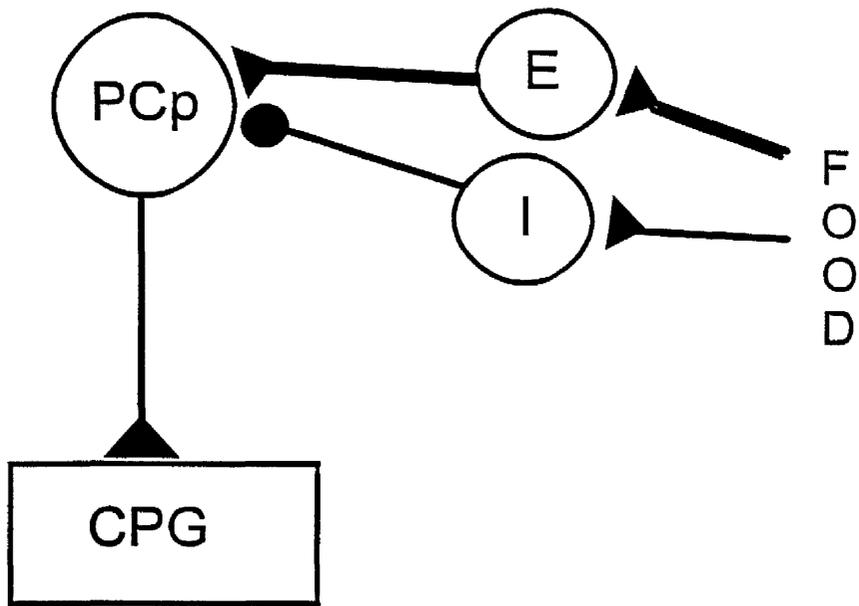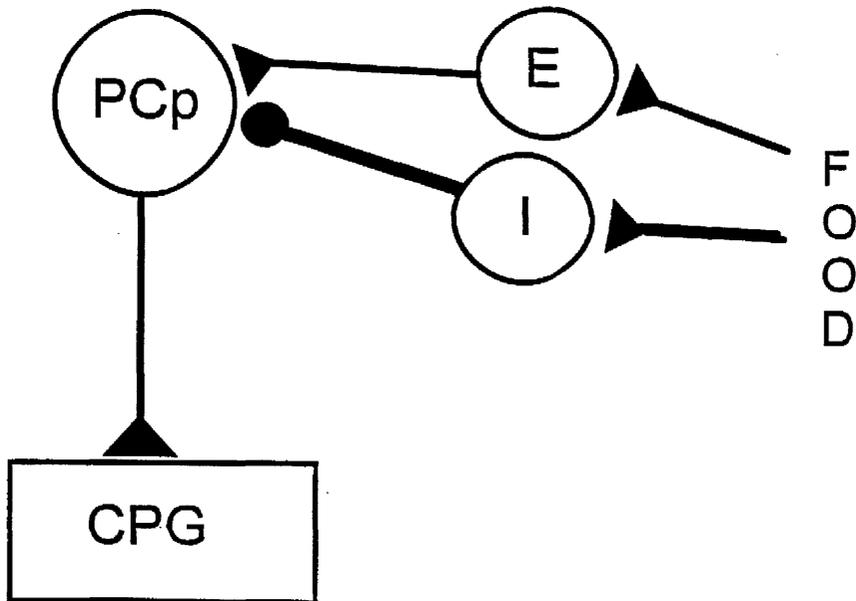
main food source), it may not be practical to withdraw from food when satiated, as it could mean long periods of inactivity whilst other behaviours such as respiration or mating could occur. In contrast, *Pleurobranchaea* lives in an environment where there is a there is a scant food source. Therefore it feeds sporadically, taking large discrete meals when it comes across an isolated prey item. *Pleurobranchaea* when satiated, unlike *Lymnaea*, would need to avoid eating to stop possible rupturing of the crop. Furthermore, since potential food is scarce and rarely encountered, withdrawal from food would be less frequent and would not interfere with other behaviours.

One interesting difference between the experiments on *Pleurobranchaea* and *Lymnaea* is that the increased number of IPSPs to the PC_p in *Pleurobranchaea*, following food application to the lips in satiated individuals, was only seen when a more intact preparation was used (i.e. CNS and lips, plus all lip and buccal innervation). Experiments carried out on the lip-brain preparation (similar to the preparation used with *Lymnaea* in this Chapter) showed no difference in the inputs received by the PC_p in starved or satiated preparations (Kovac *et al.*, 1985). This difference was attributed to the buccal innervation not being present, i.e. information from gut mechanoreceptors, signalling gut distension, could not be received by the cerebral ganglia (Kovac *et al.*, 1985). This is in contrast to *Lymnaea* where the increase of IPSPs to CV1 in satiated animals was clearly seen in the lip-CNS preparation. This may indicate that the buccal mechanoreceptors are not involved in modifying CV1's activity following satiation, or at least that different neural pathways are involved in modulating feeding following satiation in *Lymnaea* and *Pleurobranchaea*.

Activity states of CV1 in relation to whole animal behaviour:

It is generally accepted that variations in behavioural responsiveness cannot be adequately explained by the effects of external stimuli alone. In 1989, Teyke *et al.*

hypothesised that variation of animal behaviour is partly controlled by the "central motivational state" of the animal. If this is the case, then the "central motivational state" (i.e. internal neural and hormonal changes, etc) may well be the basis of the different states of CV1 seen in the isolated CNS, i.e. *"Command-like"*, "disrupted" and "dissociated" state.

The activity of, and inputs received by, CV1 following satiation were very similar to the "disrupted" state described in the Results. It is therefore possible that the "disrupted" state was actually a result of these particular snails being fully satiated at the time of dissection. (Although the snails were kept sub-satiated throughout these experiments, it was impossible to stop a few individuals eating more than their fair share!). The *"Command-like"* state, in contrast, may have represented sub-satiated or starved conditions, where CV1 could generate spontaneous feeding cycles in the absence of food. The significance of the "dissociated" state is not clear. It is possible that this state may represent *Lymnaea* showing incompatible behaviour to feeding (e.g. withdrawal; Winlow *et al.*, 1992), or the short term "sucrose-unresponsive" state seen in the behaviour experiments. The mechanism by which the "dissociated" state occurs may involve changes of the strength of synaptic connectivity between CV1 and N1 interneurons. Another possible reason for the "sucrose-unresponsive" state is that the CV1 pathway, although obviously capable of initiating feeding following food application to the lips, is only one of several neural pathways which is involved in initiating feeding following food application to the lips. Therefore, it is possible that different pathways have varying effectiveness in initiating feeding under different conditions. This hypothesis is supported by behavioural experiments in this Chapter, which showed initiation of feeding by sucrose in the whole animal to be much more reliable (95%), than that in the semi-intact preparation (35%). This suggests that certain neural pathways may be missing in the lip-CNS preparation. In support of this, Kemenes *et al.* (1986) showed that initiation of feeding was more reliable when sucrose was applied to a more intact preparation (lips, tentacles, buccal

mass plus all cerebral and buccal neural innervation) compared to using a split lip-CNS preparation similar to the one used in this study. Kemenes *et al.* (1986) also demonstrated that the slow oscillator (SO) interneuron in the buccal ganglia and the cerebral giant cells (CGC) were excited by the application of sucrose to the lips, indicating that initiation and maintenance of feeding may involve several pathways in addition to CV1. The use of the lip-CNS preparation in this study, however, is still valid, as it is an excellent model for allowing the investigation of the role of the CV1 pathway and innervation from the lips, in the absence of buccal innervation effects.

Studies on *Limax* also showed that differences in behavioural responsiveness were related to the "central motivational state" of the animal. Delaney and Gelperin (1990c) showed that the spontaneous inputs received by CB_1 were important in determining the strength of feeding behaviour shown by the animal. If CB_1 received many spontaneous inputs prior to food application, then the excitation following food to the lips was much greater than in a preparation where there were few or no spontaneous inputs to CB_1 (Delaney and Gelperin, 1990c). The variability in the inputs received by CB_1 was proposed to be due to several different internal factors such as seasonal effects, satiation, or release of a hormonal factors which cause long lasting effects (Delaney and Gelperin, 1990c). Interestingly, behavioural studies on *Pleurobranchaea* suggested that feeding behaviour is less variable compared to *Lymnaea* and *Limax*, and motivation to feed could be "controlled precisely via nutritional history" (Davis, 1984). This may explain why different levels of spontaneous inputs to PC_p (equivalent to the different CV1 "states" seen in *Lymnaea*; this Chapter) have not been described.

In summary, the CV1 pathway appears to be at least partly responsible for initiation of feeding following food to the lips in *Lymnaea*, and this is mediated by a similar, but not

identical mechanism to that described for putative homologous interneurons in *Pleurobranchaea, Limax* and *Aplysia.* It is also proposed that CV1 has a similar role to its putative homologues in other gastropod molluscs in modulating the initiation of feeding, i.e. they all act as a locus where excitatory and inhibitory inputs from the external and internal environment (sensory and internal motivational state) converge, with the overall bias determining whether feeding is initiated or not. However, there are subtle differences in the nature of the subthreshold inputs received in each species and thus different behaviour patterns are displayed. These differences are thought to have resulted from divergent evolution of these species whilst adapting to, and living in, different environments.

CHAPTER 4 - RESULTS

THE EFFECT OF DIFFERENT SENSORY STIMULI ON THE FEEDING BEHAVIOUR AND NEURAL ACTIVITY OF *LYMNAEA*

THE EFFECT OF DIFFERENT SENSORY STIMULI ON FEEDING BEHAVIOUR AND NEURAL ACTIVITY OF *LYMNAEA*.

INTRODUCTION

There has been much research into taste transduction mechanisms of vertebrates (for review, see Roper, 1992), and it is now well accepted that vertebrates can discriminate between four different taste qualities: sweet (sugars), sour (acids), salty (salts) and bitter (e.g. quinine). Although there have been no equivalent taste studies on gastropod molluscs, it has been demonstrated that most gastropods can discriminate between different food types and have a selective diet (for review, see Croll, 1983). One example of this is *Aplysia californica* which has been shown to have well developed distance chemoreception for its "favourite" food, the red seaweed *Laurentia spp.* (Kupfermann and Carew, 1974).

Studies on the feeding behaviour of *Lymnaea* indicate that it has a rather "unselective diet". Examination of the crop contents of 40 specimens of *Lymnaea* revealed the presence of many different foods, including fragments of green vascular plants, strings of algae and blue-green cells, and occasionally, whole and fragmented animal matter (Bovbjerg, 1968). In contrast to other herbivorous gastropods, *Lymnaea* was also shown to have no ability to detect vegetable matter at a distance, even though the predominant part of their diet is vegetable. However, *Lymnaea* was shown to have strong distance chemoreception for animal matter, with individuals showing a preference for it over vegetable matter (Bovbjerg, 1968). A later study by Kemenes *et al.* (1986) showed that *Lymnaea* also exhibits biting activity in response to different sugars (maltose and sucrose), and that the presence of quinine (a bitter "taste") resulted in inhibition of feeding. These studies suggest that even though *Lymnaea* does not show as much food preference as other gastropods, it can discriminate some chemostimuli, resulting in different behavioural responses; i.e. differing strengths of feeding response, or

inhibition of feeding accompanied by withdrawal.

In Chapter 3, *Lymnæa's* response to 10^{-3} M sucrose was examined in detail, and it was shown that sucrose (or a sweet "taste") initiated feeding at least in part via the CV1 pathway, by changing the balance of inputs received by CV1. This Chapter investigates the effect of several different mechanosensory and chemosensory "taste" stimuli on the behaviour and corresponding neural activity of *Lymnæa*. The Results are presented in two sections, each including behavioural and electrophysiological data. The first section examines the effect of a feeding stimulant other than sucrose, fish food (part of *Lymnæa's* diet whilst kept in the laboratory). The second section investigates the effect of three different aversive stimuli: 1) a mechanical "prod" to the lips (mechanosensory); 2) quinine (bitter); and 3) citric acid (sour).

RESULTS A - FEEDING STIMULI

Fish food

Experiments were carried out to assess the ability of homogenised fish food (0.1 g Tetramin fish food in 10 ml distilled water) to initiate feeding in the whole animal and in the semi-intact preparation, as compared to 10^{-3} M sucrose.

For the behavioural experiments, 15 snails were randomly selected, and their feeding response (latency to first bite and number of bites in two minutes following both fish food and sucrose) was tested. The order of presentation of the two stimuli was randomised to minimise any possible habituation or sensitisation effects due to exposure to repeated feeding stimuli. The experiment was carried out between 1000-1200 hours on the same day to minimise environmental / circadian variation. These experiments were followed by electrophysiological tests on the semi-intact preparation.

Figure 4.1 shows the feeding response (latency to bite and number of bites in two
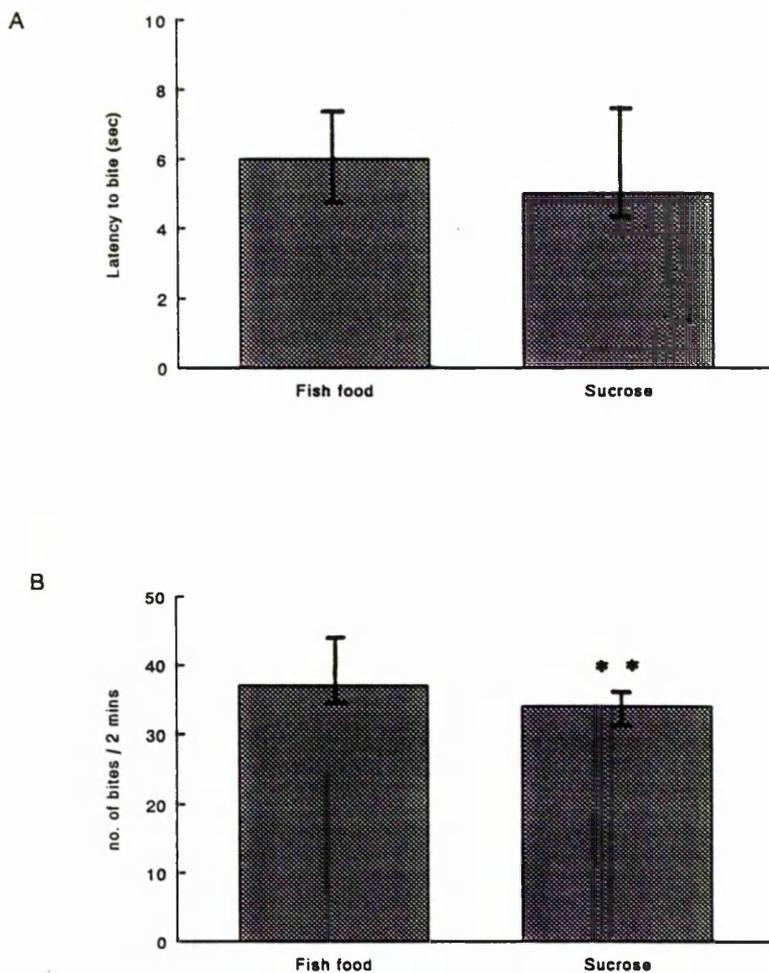
Figure 4.1 Comparison of feeding response to homogenised fish food and 10^3 M sucrose in the whole animal (n=15). Data are not assumed to be normal and are presented as medians and interquartile ranges. A) Latency to bite. There is no significant difference in latency to bite following application of fish food compared to sucrose (paired raw data; Wilcoxon ranked pairs test). B) The number of bites in two minutes. The no. of bites is significantly greater with fish food compared with sucrose (n=15) (paired raw data; Wilcoxon ranked pairs test). ** = p<0.001

minutes) to fish food and sucrose. Data are paired and are not assumed to be of a normal distribution so are presented as medians and interquartile ranges. There was no significant difference in latency to bite to either food substance (Wilcoxon ranked pairs test). However, there was an increase in the number of bites in two minutes in response to fish food as compared with sucrose ($p < 0.001$; Wilcoxon ranked pairs test). Examination of the difference in median number of bites to each substance, however, showed this difference (in real terms) to be small (in the region of only 4-5 more bites in two minutes in response to fish food as compared to sucrose). This indicates that, although both sucrose and fish food homogenate are reliable feeding stimuli (initiating feeding after approximately 5 seconds), fish food application appears to cause slightly faster feeding cycles than sucrose.

In 7/20 semi-intact preparations, application of fish food homogenate to the lip tissue in a previous quiescent preparation led to cessation or marked reduction of IPSPs, an increase in EPSPs, depolarisation of the membrane potential and rhythmic bursting of CV1. This activity in CV1 was accompanied by an initiation of feeding motor output, as seen by bursting activity in CV1 and in identified buccal motoneurons (Fig. 4.2A).

In 9/20 preparations application of fish food homogenate did not lead to initiation of feeding motor output but was still excitatory to CV1. This was seen as either a change in inputs received by CV1 (increase in EPSPs and decrease in IPSPs), or increased frequency and intensity of the ongoing feeding rhythm in CV1, or both (Fig. 4.2B).

In the remaining 4/20 preparations application of fish food homogenate resulted in no change in the activity of, or inputs to either CV1 or identified feeding motoneurons. This lack of response could be due to damage to the preparation during dissection or due to an "unresponsive state" (see Chapter 3).

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Figure 4.2 The effect of 0.1 ml fish food homogenate application to the lip tissue on the activity of CV1 and identified buccal motoneurons in the semi-intact preparation.

A) Initiation of feeding motor output. Fish food (FF) application to a quiescent preparation results in CV1 receiving less IPSPs and an increase in EPSPs, and the initiation of bursts of action potentials. This is accompanied by an initiation of feeding motor output, seen as rhythmic activity in the identified buccal motoneuron B4.

B) Increasing the frequency and intensity of feeding motor output (FMO). Application of fish food (FF) to a preparation showing ongoing feeding motor output results in an increased excitation of CV1. This is seen as increased EPSPs/reduced IPSPs to CV1, accompanied by an increase in the frequency and intensity of FMO i.e. the bursts of action potentials in both CV1 and the identified motoneuron B3.
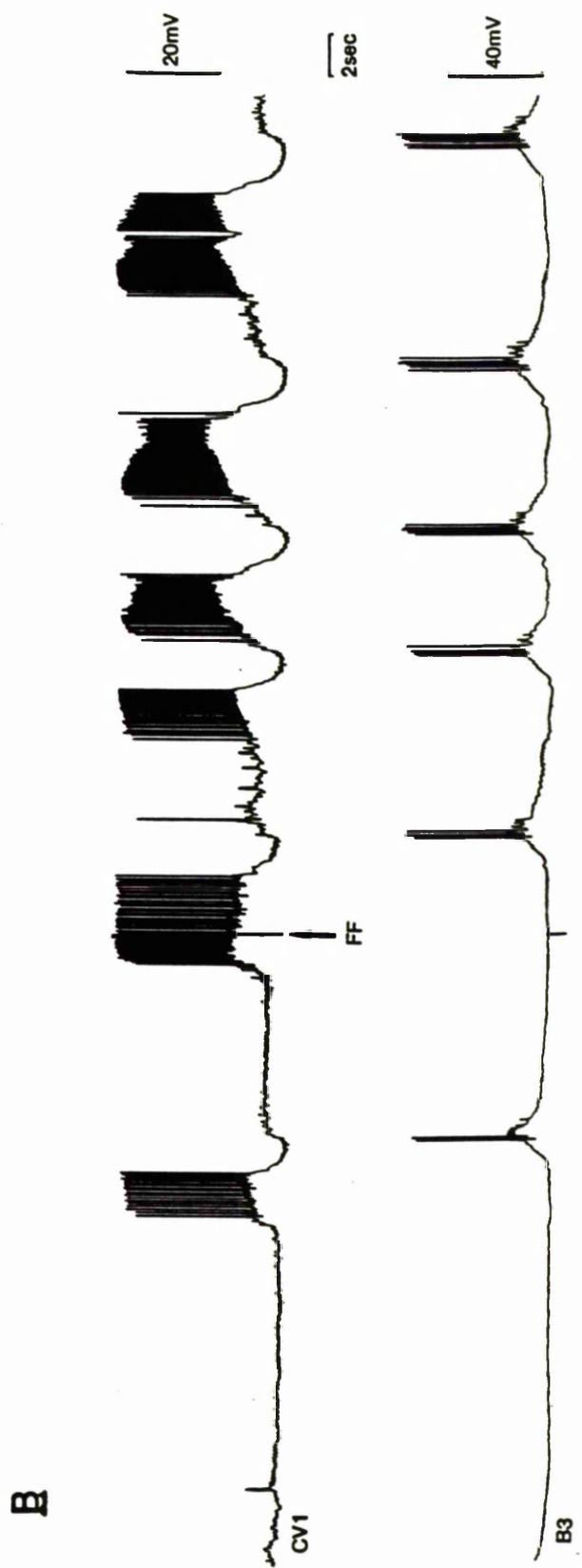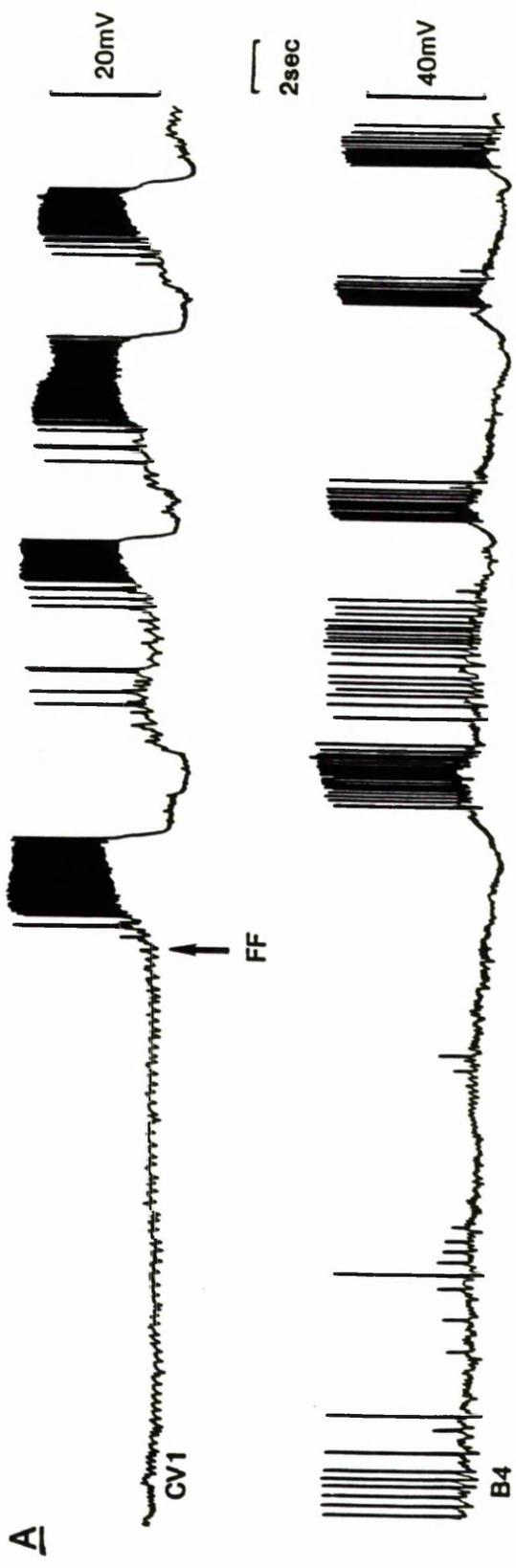
RESULTS B - AVERSIVE STIMULI

Withdrawal or escape responses are "protective responses" and take precedence over all other behaviours including feeding (Winlow *et al.*, 1992). The effect of several different types of aversive stimuli which caused withdrawal were examined using feeding and non-feeding snails.

Noxious mechanical stimulus

A noxious mechanical stimulus to the head was first used as it was the simplest method of inducing full withdrawal.

Non-feeding snails Ten specimens of *Lymnœa* were placed individually into a Petri dish containing a 5 mm depth of snail water. The snails were given five minutes to acclimatise to the environment and fully emerge from their shells. Then a sharp prod to the head was administered, using a blunt probe, with sufficient force to induce withdrawal but not to cause damage to the skin.

It was observed that in addition to withdrawal following the mechanical stimulus to the head, *Lymnœa* also showed a stereotyped "avoidance behaviour" pattern. This was characterised as:

i) immediate withdrawal with the snail remaining fully withdrawn for a short period (approx. 10 seconds); then,

ii) whilst still partially withdrawn in the shell, snails showed "head turning behaviour", i.e. the head-foot complex was turned away from the direction from which the prod was administered; followed finally by,

iii) active locomotion away from the site at which it received the prod. (NB: Timings are not given for each stage as the exact amount of force administered to each snail could not be

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assumed to be equal).

Feeding snails - Twenty specimens of *Lymnaea* were randomly divided into two groups: experimental (n=10) and control (n=10). Individual snails first received 0.1 ml 10^3 M sucrose to the lips to stimulate feeding. The number of bites in the first minute following sucrose application was recorded. After one minute of biting, the experimental group received a prod to the head sufficient to induce withdrawal whereas the control group received no mechanostimulus. Feeding movements were then recorded for a further minute. Results are shown in Fig. 4.3 and are presented as the median (and interquartile ranges) percentage reduction in the number of bites in one minute following a prod compared to the previous minute. The percentage reduction in number of bites in the experimental group was not significantly different from the control (Mann-Whitney U test; raw data). Therefore, the mechanostimulus did not cause a significant reduction in feeding. Observation of the snails' behaviour throughout the experiment explains this unexpected result. Experimental group snails all showed the stereotyped avoidance behaviour following the mechanostimulus, and, as reported by Winlow *et al.* (1992), ingestive feeding movements were never seen during full withdrawal. However, the withdrawal phase was only short-lived (only approx 4-5 seconds) compared to that seen in non-feeding snails, indicating that a state of food arousal reduces the withdrawal time. Therefore, the inhibition of feeding was short-lived as it only occurred during the withdrawal phase, and, upon head turning and locomotion, feeding movements were IMMEDIATELY seen to resume at a normal speed in all snails.

These results suggest that in the wild, if *Lymnaea* receives a noxious mechanical stimulus during grazing, its behaviour is not simply to withdraw and stop feeding, but to turn and locomote away from the area of the "danger" and resume feeding in the normal manner in a "safer" area.
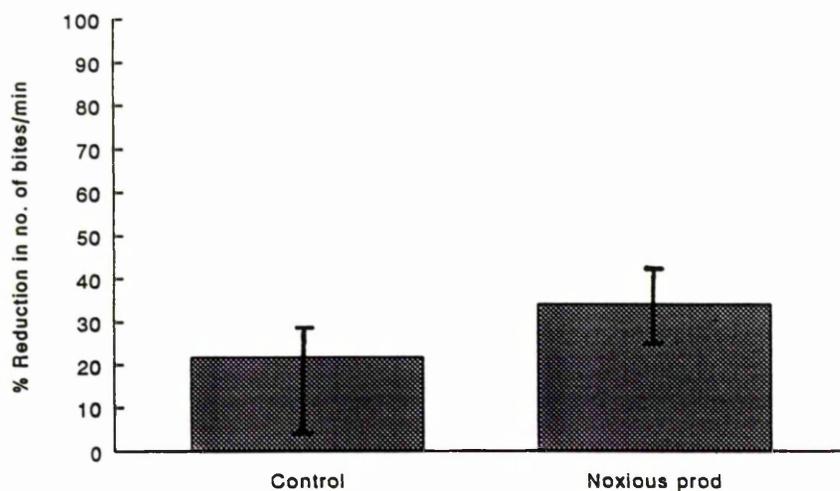
Figure 4.3 The percentage reduction of bites in one minute, caused by a noxious prod to the lips compared to no prod control after one minute of sucrose induced feeding (n=10 each group). Data are not assumed to be normally distributed, and therefore are presented as medians and interquartile ranges. There is no significant difference between the reduction of bites in one minute following a noxious prod and the no prod control (raw data; Mann-Whitney U test).

Quinine

Kemenes *et al.* (1986) reported that bathing snails in a 1% quinine sulphate solution induced strong withdrawal and inhibited feeding activity, both at the behavioural and the neural level. The effects of quinine were reassessed.

Non-feeding snails - Ten specimens of *Lymnaea* were randomly selected and their response to the application of both 0.1 ml of 0.026 M quinine chloride solution in snail water (equivalent to a 1% solution) and 0.1 ml snail water alone (i.e. mechanical stimulus only) to the lips, was investigated. NB: Quinine chloride was used as it had a higher solubility than quinine sulphate. The quinine chloride was applied by dripping it in a localised fashion close to the lips, rather than the full immersion of snails into the quinine solution (contrast Kemenes *et al.*, 1986). This was considered to be more environmentally relevant, as it could be the equivalent of *Lymnaea* coming into contact with a localised aversive stimulus. The order of application of the two substances was randomised to eliminate any accumulative application effects.

Following application of 0.026 M quinine chloride, none of the snails showed any biting movements, and all ten snails showed active whole-body withdrawal. This was in contrast to the slight tentacle and head withdrawal seen in response to the mechanical stimulus (i.e. snail water) only. A more detailed analysis of the group's behaviour showed that withdrawal in response to quinine chloride consisted of a stereotyped sequence of avoidance behaviour (Fig. 4.4), similar to that seen with a noxious mechanical prod. In this case, it was possible to measure the duration of each phase of the avoidance behaviour is given, as each snail received the same amount of quinine chloride.

i) within one second of application of 0.026 M quinine chloride all snails actively withdrew into their shells.

ii) this was followed by a whole body withdrawal or an inactive state, which lasted for 14.6

± 3.7 sec (mean ± standard deviation; n=10)

iii) after the inactive state, whilst still partially withdrawn, all snails showed head-turning behaviour, lasting for 14.3 ± 8.2 sec (n=10).

iv) Once the head was turned away from the quinine chloride, all snails actively moved away from that area.

Further experiments were then carried out to assess the effect of a more concentrated quinine chloride solution (i.e. a more aversive stimulus). A 0.13 M solution of quinine chloride was used as this represented a saturated solution.

A detailed analysis of the stereotyped avoidance behaviour was carried out on ten non-feeding snails. Application was in exactly the same manner as with 0.026 M quinine chloride (n=10) (Fig. 4.4).

i) there was active withdrawal into the shell within 1 second.

ii) the following whole body withdrawal lasted for 20.7 ±8.65 sec (mean ± SD; n=10). In addition, during this phase 50% of the snails showed regurgitation movements. Regurgitation was characterised by movement of the radula in the opposite direction to normal rasping and further out of the mouth (see demonstration of regurgitation section).

iii) head turning behaviour after withdrawal lasted for 14.5 ± 4.8 sec (n=10).

iv) once the head was turned away from the source of the quinine chloride, all snails showed active locomotion from this area.

The results from 0.026 M and 0.13 M quinine chloride cannot be directly compared, since the experiments were carried out on a different group of snails and on separate days. The behaviour seen with 0.13 M quinine chloride was similar to that with 0.026 M quinine chloride solution (Fig. 4.4). However, the response to 0.13 M quinine chloride was stronger,

Figure 4.4 Schematic diagram showing the mean duration of each behavioural phase of the stereotyped avoidance behaviour seen following application of 0.1 ml 0.026 M and 0.13 M quinine chloride to the lips of non-feeding snails (n=10, each group). Time courses given are means only (for details of standard deviations, see text).
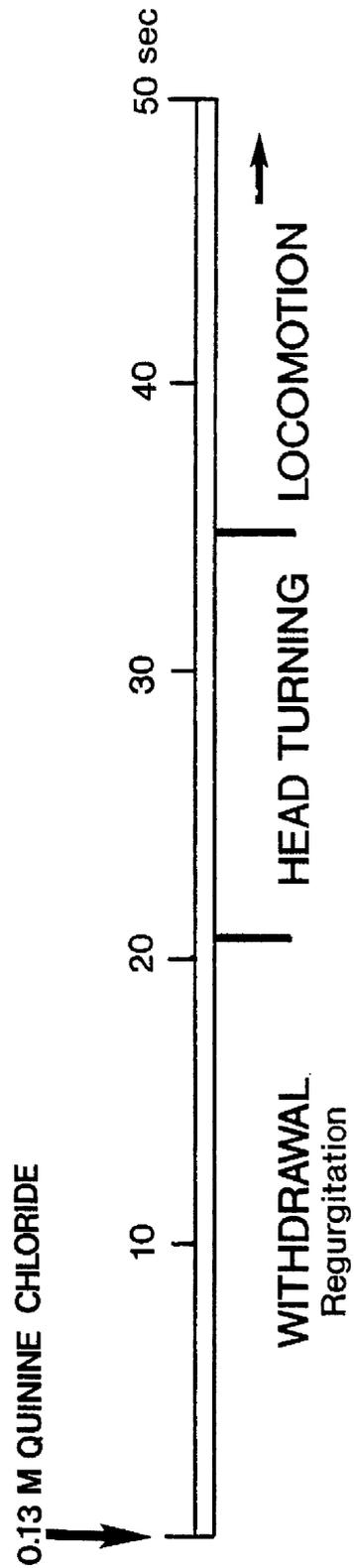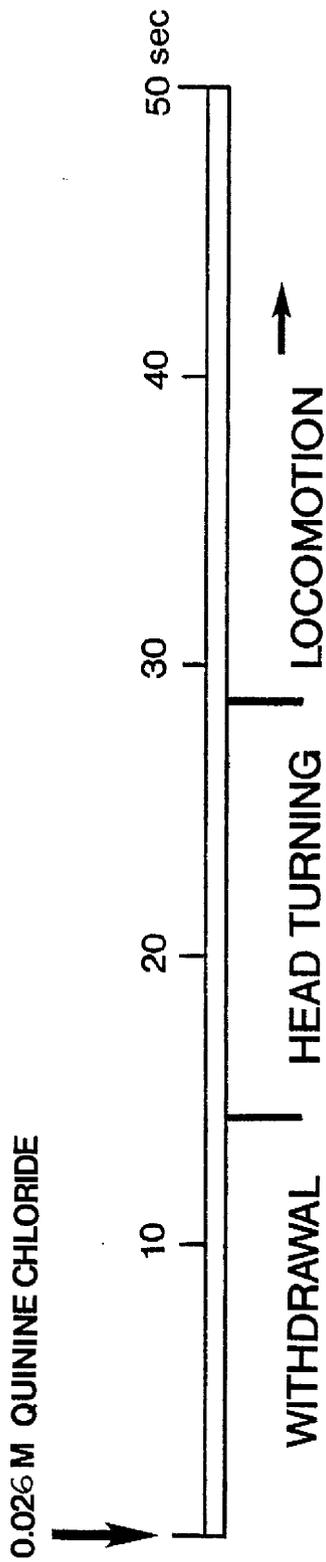
being characterised by an increase in mean duration of the withdrawal phase, and the presence of regurgitation behaviour in 50% of the snails. As the snails had not been feeding prior to application of quinine chloride, it is possible that the regurgitation response was a result of quinine chloride diffusing into the buccal cavity, mimicking ingestion of a noxious substance

Feeding snails. Twenty specimens of *Lymnæa* were randomly divided into two groups: experimental (n=10) and control (n=10). Individual snails first received 0.1 ml 10^3 M sucrose to the lips to stimulate feeding. The number of bites in the first minute after sucrose application was then recorded. After one minute of biting, the experimental group received 0.1 ml of 0.026 M or 0.13 M quinine chloride solution to the lips (the order of application of the two quinine concentrations was randomised to eliminate any accumulative application effects), and the control group received 0.1 ml of snail water. This method of application of quinine chloride was chosen as it could represent *Lymnæa* coming across a bitter plant (aversive feeding stimulus) whilst grazing. Feeding movements were then recorded for a further minute. (Regurgitation movements were recorded separately from typical biting activity).

Results are shown in Figure 4.5 and are presented as, A) the median ($\pm$ interquartile ranges) percentage reduction in the number of bites (ingestion) in 1 minute with the different concentrations of quinine chloride and snail water as compared to sucrose, and B) the number of regurgitations seen following the application of quinine chloride or snail water.

The reduction in the number of bites following 0.026 M quinine chloride was found not to be significantly different from the reduction in number of bites following application of snail water (Mann-Whitney U test; raw data). This suggests that 1% (or 0.026 M) quinine is not a powerful inhibitor of feeding, as Kemenes *et al.*, (1986) had previously indicated.

However, 0.026 M quinine chloride was obviously aversive, as there was a significant increase in regurgitation movements with quinine chloride compared to snail water (with which no regurgitation was seen) (p<0.001; Mann-Whitney U test). The stereotyped avoidance behaviour seen response to a noxious mechanostimulus in non-feeding snails was also seen following quinine chloride application, and, as with the mechanostimulation, inhibition of feeding only occurred during the withdrawal phase. Furthermore, the duration of the withdrawal phase was reduced compared to the non-feeding animal (5.7 ± 3.2 seconds feeding vs. 14.3 ±8.2 seconds non-feeding). This is again contributed to an aroused feeding state reducing the duration of withdrawal.

These results suggest that, in the wild, if *Lymnaea* ingests an aversive food stimulus during grazing, its behaviour is not simply to withdraw and stop feeding, as Kemenes *et al.*'s (1986) results might have suggested, but is to regurgitate the offending substance, turn and locomote from the area containing this food and resume feeding in the normal manner. This is very similar to the behaviour seen with noxious mechanostimulation, except for the additional regurgitation, which may be caused by the ingestion of the noxious chemosensory substance.

Application of 0.1 ml 0.13 M quinine chloride solution to the lips resulted in a significant reduction in number of bites, as compared to the snail water control (p<0.001; Mann-Whitney U test) (Fig. 4.5A). Observation of the snails' behaviour throughout the experiment showed that the reduction in feeding movements was not only due to a prolonged withdrawal phase compared to 0.026 M quinine chloride (8.2 ± 4.2 sec 0.13 M quinine chloride vs. 5.7± 3.2 sec 0.026 M quinine chloride, feeding snails), but also due to irregular biting activity in the head turning and locomotion phases. Application of 0.13 M quinine chloride also resulted in a significant increase in regurgitation movements as compared with snail water (p<0.001; Mann-Whitney U test; Fig. 4.5B).

Figure 4.5 The effect on the feeding response following of application of 0.1 ml of 0.026 M or 0.13 M (1% and 4%) quinine chloride and snail water to the lips of feeding snails. Data are not assumed to be of normal distribution and are presented as medians and interquartile ranges.

A) Percentage reduction in the number of bites in one minute caused by application of 0.026 M, 0.13 M quinine chloride and snail water to the lips, after one minute of sucrose-induced biting. There is no significant difference in the reduction of bites following 0.026 M quinine chloride compared to snail water control (raw data; Mann-Whitney U test). There is a significant reduction in number of bites following 0.13 M quinine chloride as compared to snail water control (** p= 0.01 level, raw data, Mann-Whitney U test). The increase in the reduction of bites with 0.13 M quinine chloride as compared to 0.026 M quinine chloride is significant (•• p= 0.08 level, raw data; Wilcoxon ranked pairs test, data are paired)

B) Number of regurgitations following application of 0.026 M, 0.13 M quinine chloride and snail water control. Application of both 0.026 M and 0.13 M quinine chloride cause a significantly greater number regurgitations compared to snail water (** p= 0.01 level, Mann-Whitney U test). There is no significant difference in the number of regurgitations following application of 0.026 M and 0.13 M quinine chloride (data are paired; Wilcoxon ranked pairs test).

A

B
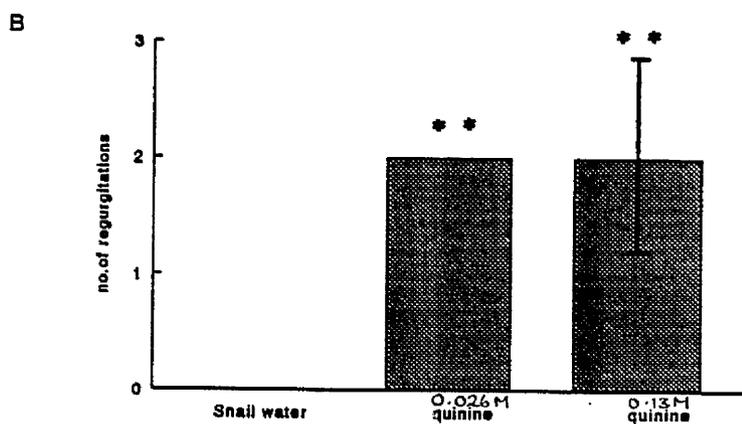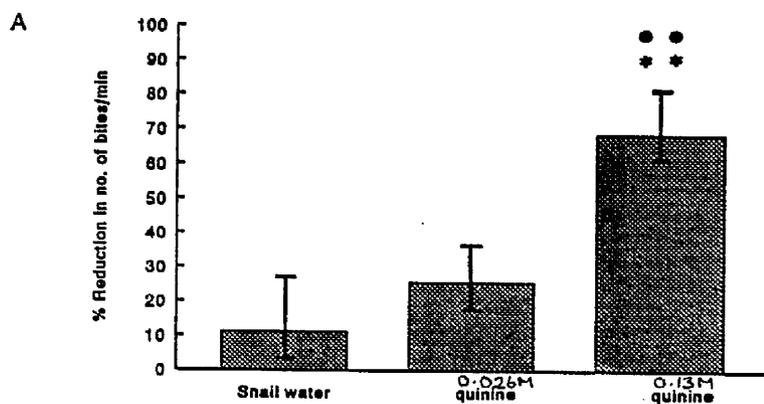
As the same snails were used for the 0.026 M and 0.13 M quinine chloride experiments, results could be directly compared as paired data. Statistical analysis showed that the reduction in feeding following 0.13 M quinine chloride was significantly greater than with 0.02 M quinine chloride solution (p=0.008; Wilcoxon ranked pairs test). However, there was no significant difference in the number of regurgitations between the two concentrations. OF QUININE

In summary, experiments on feeding and non-feeding snails showed that localised application of quinine chloride to the lips resulted in:

i) A stereotyped avoidance behaviour which could occur in the absence of ingestion of quinine chloride (i.e. in non-feeding snails). The withdrawal phase of this behaviour was concentration dependent, and biting movements were inhibited during this phase.

ii) If ingested, quinine chloride caused regurgitation behaviour and a reduction in biting activity. The number of regurgitations was not concentration dependent, which suggests that they represent a stereotyped short lived response to reject already ingested aversive-tasting material irrespective its strength. The reduction in biting following feeding, however, was concentration dependent; 0.026 M quinine chloride had little effect (except during the withdrawal phase), whereas the 0.13 M quinine chloride caused a significant reduction in feeding movements.

The cause of this reduction in feeding movements following application of 0.1 ml 0.13M quinine chloride should be considered. Application of 0.13 M quinine chloride to feeding snails resulted in exactly the same avoidance behaviour as with 0.026 M quinine chloride, i.e. turning from the area of quinine (bitter) taste and locomotion to a new area, presumably to avoid further ingestion of the aversive taste and to resume normal feeding. However, after withdrawal from 0.13 M quinine chloride, the feeding cycles seen were irregular, whereas with 0.026 M quinine chloride they were regular and uninterrupted. There

are two possible explanations for this difference. First the ingestion of the concentrated quinine caused *Lymnaea* to be more "tentative" in resuming normal feeding, a possible mechanism being that the prolonged withdrawal caused by the 0.13 M quinine chloride (compared to that with 0.026 M quinine chloride) greatly interrupted the feeding rhythm, making it less likely for the central pattern generator to resume regular feeding cycles after the withdrawal phase had finished. Alternatively, whilst *Lymnaea* was fully withdrawn, the 0.1 ml 0.13 M quinine chloride dispersed throughout the snail water, creating a mildly aversive-tasting environment. If this was the case, then the irregular feeding after the withdrawal phase could be the result of *Lymnaea* ingesting a mildly aversive taste every time it showed biting activity, aversive enough to disrupt feeding motor output from the central pattern generator, but not enough to cause withdrawal or regurgitation. This hypothesis was investigated in the following experiments.

Effect of quinine on food searching activity

The previous experiments using quinine chloride attempted to mimic *Lymnaea* coming into contact with a localised aversive stimulus by using a 0.1 ml drip of different concentrations of quinine chloride. However, how *Lymnaea* behaves in an environment containing aversive food stimuli only (specifically in relation to its spontaneous feeding activity or "food search" activity) was not studied. Kemenes *et al.* (1986) reported that *Lymnaea* showed strong withdrawal and inhibition of feeding when bathed in a 1% (0.026 M) quinine solution. However, this concentration of quinine was not relevant for this study, as my results showed that even 0.1 ml 0.026 M (1%) quinine chloride was enough to induce strong withdrawal and avoidance behaviour. The aim of the following experiment was to investigate whether a mildly aversive environment (which did not cause withdrawal) could inhibit spontaneously generated feeding activity, which is thought to represent "food search"

activity (Tuersley, 1986).

For this experiment a much weaker solution of quinine chloride was used. A 0.0014 M (equivalent to a 0.05%) solution in snail water was used as it was the strongest concentration in which *Lymnaea* could be placed without resulting in active withdrawal or obvious physical discomfort to the snail.

Six specimens of *Lymnaea* were starved for two days. Starved snails were used as they are known to show significantly greater food searching activity than satiated animals (Tuersley and McCrohan, 1987a). Searching activity (spontaneous feeding movements) of each snail was then scored for 1 hour using a BBC computer programmed as an event recorder (Azzopardi, 1986), in both snail water and 0.0014 M quinine chloride solution (30 minutes each). The order of exposure to the snail water and quinine chloride was randomised to eliminate any cumulative exposure/testing effects. Results are presented in two different ways: i) percentage reduction in searching activity (i.e. total number of bites in 30 minutes) in quinine chloride as compared to snail water. The percentage reduction was used so that the reduction in searching activity for each snail could be compared, i.e. any large differences in searching activity levels from snail to snail could be disregarded; and, ii) analysis of the bouting activity (grouping of biting activity) of snails in both snail water and quinine. Analysis of *Lymnaea*'s spontaneous biting activity in the absence of food by Tuersley (1986) showed that rasping behaviour was often organised into "bouts", i.e. series of frequent rasps followed by long intervals before the next bout of rasping.

The percentage reduction in searching activity for each snail is shown in Figure 4.6. Statistical analysis of the paired raw data (biting in each individual snail in snail water compared to 0.0014 M quinine chloride) showed the reduction in searching activity to be significant ($p < 0.05$; Wilcoxon ranked pairs test).
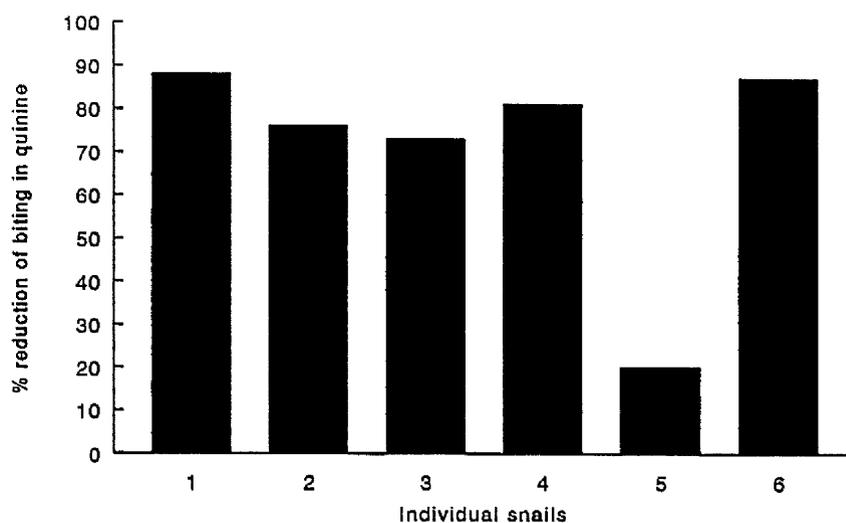
Figure 4.6 The percentage reduction of spontaneous biting by a 0.0014 M quinine chloride environment.
Percentage reduction in is calculated as:

No. of bites in quinine - No. of bites in snail water
No. of bites in snail water environment   X 100

Data are presented from six individual snails.

These results showed that 0.0014 M quinine chloride significantly reduced searching activity (spontaneous biting). However, it gave no indication of the distribution of biting over time (i.e. the bouts of biting). This becomes clear if the interbite interval is plotted against successive bites. Figure 4.7 shows representative biting data from one snail in snail water and quinine chloride. The troughs represent a series of bites in close succession (a "bout"; Tuersley, 1986), whilst the peaks represent bites separated by a long interbite interval (periods between bouts), in which *Lymnaea* usually showed locomotion behaviour. The area under the line is proportional to time.

The total number of bites in 30 minutes was less in quinine chloride than in snail water; the snails in snail water spent a large proportion of the time engaged in spontaneous searching activity consisting of prolonged bouts of frequent bites with few or no long interbite intervals (Fig. 4.7A). This is as expected from starved snails (Tuersley, 1986). However, the same snail in 0.0014 M quinine chloride showed modified searching activity. Although spontaneous biting was seen throughout the 30 minutes, the activity consisted of either single, isolated bites or very short bouts of biting (denoted by short troughs on Fig. 4.7B). This indicates that quinine chloride does not reduce spontaneously generated biting activity by completely inhibiting all feeding movements, but by reducing the duration of bouts of biting activity and increasing the duration of periods between bouts.

It has been previously demonstrated that *Lymnaea* shows increased searching activity when it is hungry (Tuersley, 1986). However, these results suggest that if *Lymnaea* ingests an aversive taste whilst searching it cuts short sampling in that area and moves to a new area to continue sampling. This hypothesis would explain why *Lymnaea*, whilst in the 0.0014 M quinine chloride environment, showed short periods of biting activity separated by relatively long interbite intervals in which there was usually locomotion, since it was attempting to find an area containing edible food.
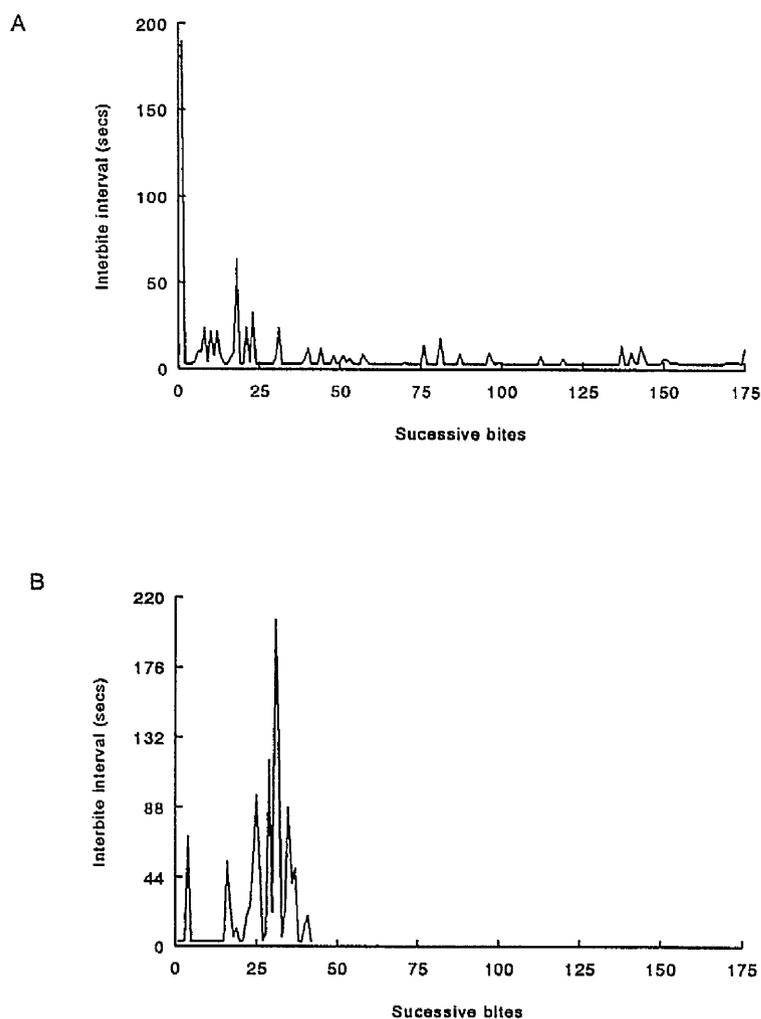
**Figure 4.7** Interbite interval plotted against successive bites in A) 0.014 M (0.05%) quinine environment and B) snail water environment. Graphs are plotted using representative data of biting activity from one snail. Peaks represent bout intervals, while troughs represent within bout intervals. The area under the line is equivalent to time. Biting was recorded over a 30 minute period in both environments.

Effect of aversive environment on the initiation of feeding.

The previous experiment suggested that the ingestion of a mildly aversive taste (0.0014 M quinine chloride) during food search activity, caused *Lymnaea* to cut short its sampling of that area and move to a new area in an attempt to find edible food. However, the previous experiment did not investigate the nature of the feeding response if the snail did actually come across a localised area of edible food, whilst in the aversive environment. The following experiment investigated the effect of a 0.0014 M quinine chloride environment on the initiation of feeding following a feeding stimulus to the lips.

Individual snails were placed into either a 0.0014 M quinine chloride solution or snail water and allowed 5 minutes to acclimatise to the environment. The order of the two environments was randomised to avoid any cumulative effects of exposure to testing. 0.1 ml of 10^{-3} M sucrose was then dripped near to the lips of the snail and the latency to first bite (seconds) and total number of bites in the first two minutes recorded.

The latency to bite and number of bites in both environments is shown in Figure 4.8. Data are not assumed to be normal and are therefore presented as medians and interquartile ranges. There was no significant difference in either latency or number of bites between the two environments (Wilcoxon ranked pairs test). This suggests that the initiation of feeding following application of feeding stimulus is not affected by an aversive environment, i.e. the response to feeding stimuli is unchanged.

From this and the previous experiment it is hypothesized that inhibition of feeding by quinine chloride occurs by inhibiting spontaneous feeding movements, rather than those in response to sensory cues.

In summary, if the results from all three behavioural quinine chloride experiments are considered (i.e. i) effect of localised high concentration of quinine chloride on stimulated
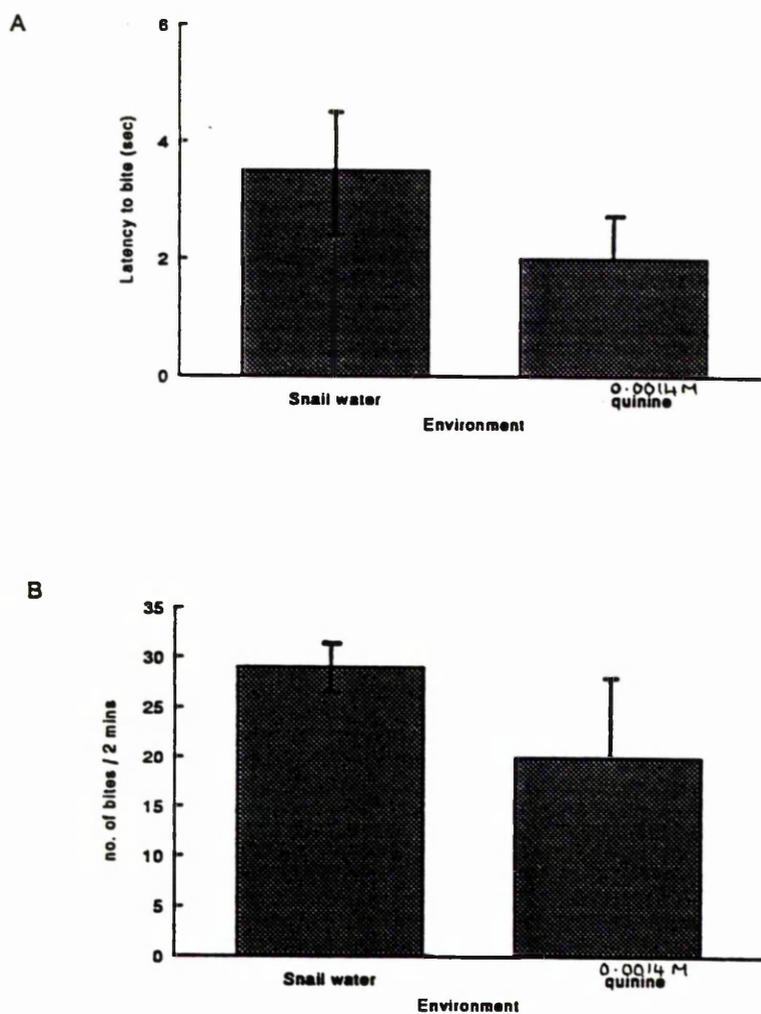
Figure 4.8 The feeding response to 0.1 ml 10^3 M sucrose to the lips in 0.0014 M (0.05%) quinine environment and control snail water environment. Data are presented as medians and interquartile ranges. There is no significant difference between the A) latency to bite or B) the number of bites in two minutes in either environment (Data are paired, Wilcoxon ranked pairs test).

feeding; ii) effect of low concentration quinine chloride on spontaneous feeding; and iii) effect of low concentration quinine chloride on stimulated feeding), it is proposed that quinine chloride acts in two different ways to inhibit feeding in *Lymnaea*.

i) In localised high concentrations quinine chloride causes a stereotyped avoidance behaviour, in which there is short-lived inhibition of all feeding movements, both food-stimulated and spontaneous biting.

ii) In widespread low concentrations quinine chloride reduces searching activity (spontaneous) by reducing bout length. Stimulated feeding, however, is not affected; snails show normal feeding responses to sucrose.

Citric Acid

The previous experiments demonstrated that quinine chloride did not totally inhibit feeding in the whole animal, except during the whole body withdrawal phase of the stereotyped avoidance behaviour, but instead encouraged the snail to move to a different area to search for food. This was similar to the results using a noxious mechanical stimulus.

The next series of experiments investigated the effect of a third type of aversive stimulus, acid (or sour taste), on *Lymnaea*'s feeding behaviour. Citric acid was chosen over other acids for two reasons: i) citric acid has been reported to inhibit feeding in *Helix* (P. Balaban, pers comm), and ii) it is naturally occurring in some plant material and so may be more relevant than some inorganic acids.

Non-feeding snails. Twenty specimens of *Lymnaea* were divided into two groups and the response to the application of 0.1 ml of 0.026 M citric acid solution in snail water recorded (the same molarity as the lower concentration of quinine chloride solution used in the previous experiment). This molarity was equivalent to a 0.54% solution of citric acid, pH=2.2 (lemon juice contains 5-8% citric acid; The Merck Index, 1968). Experiments were carried

out on non feeding snails in the same manner as the quinine experiments.

Following application of 0.026 M citric acid, snails showed very strong avoidance behaviour (Fig. 4.9) of a different nature to that seen with quinine chloride or a noxious mechanical stimulus:

i) within one second of application all snails actively withdrew into their shells.

ii) this was followed by a whole body withdrawal, which lasted for 44 ± 14 sec (mean $\pm$ S.D., n=10). During this time regurgitation movements and defensive expulsion of air from the pneumostome were seen in all snails.

iii) the withdrawal phase was followed by a writhing phase, in which there was much head turning and body writhing, with no coordinated locomotion. This phase lasted for 132 ± 57 sec.

iv) the head-foot was complex finally extended fully, followed by locomotion. The locomotion was away from the direction of citric acid application.

Feeding snails. Twenty specimens of *Lymnaea* were randomly divided into two groups, experimental (n=10) and control (n=10). Experiments were carried out in exactly the same manner as for quinine chloride, i.e. individual snails first received 0.1 ml 10^3 M sucrose to the lips to stimulate feeding, and the number of bites in the first minute after sucrose application was then recorded. After one minute of biting, the experimental group received 0.1 ml 0.026 M citric acid, and the control group received 0.1 ml of snail water. The number of bites in the following one minute was then recorded. Results are shown in Figure 4.10 and are presented as the percentage reduction in the number of bites in one minute with citric acid and snail water, compared to biting recorded in the previous minute following sucrose application, and the number of regurgitations seen following the application of citric acid or snail water.
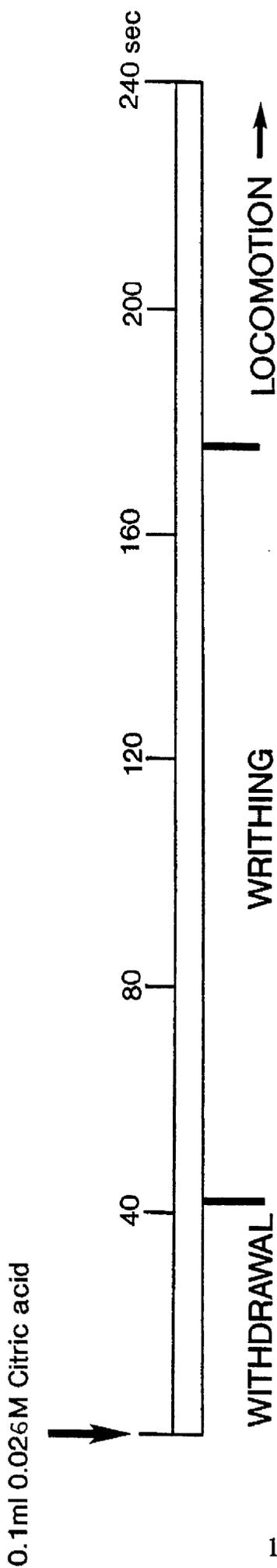
Figure 4.9 Schematic diagram to show the mean duration of the different behavioural phases in the stereotyped avoidance seen following application of 0.1 ml 0.026 M citric acid to the lips of non-feeding snail (n=10). Timings shown are mean duration only (for details of standard deviations see text).

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The reduction in the number of bites in one minute following 0.026 M citric acid was significantly greater than following application of snail water (p< 0.001; Mann-Whitney U test). In fact, following application of the acid, no biting movements were seen, since feeding was completely inhibited. Further observations after this one minute period showed that the inhibition of feeding was long lasting, with no snails showing any biting activity for a further five minutes after application of the citric acid. This is in contrast to results from both the noxious mechanostimulus and quinine chloride, which encouraged locomotion to, and feeding in, a new area. The number of regurgitations following citric acid was also significantly greater, as compared to application of snail water (p<0.001; Mann-Whitney U test); 8/10 snails showed regurgitation movements. Regurgitation movements were not seen in the other two but these movements probably occurred but were obscured whilst the snail was fully withdrawn within its shell.

In summary, application of citric acid not only resulted in strong avoidance behaviour, distinct from that seen with quinine chloride or a noxious mechanostimulus, but it also resulted in complete inhibition of feeding movements. It was noted that all snails showed normal locomotion and feeding responses 10 minutes after exposure to citric acid, indicating that the citric acid had no long term deleterious effects on behaviour.

Demonstration of regurgitation

In both the quinine chloride and citric acid experiments exaggerated backwards movements of the radula were assumed to be regurgitation. These movements of the radula were shown to be regurgitation movements by the following experiment.

Ten specimens of *Lymnaea* were placed into individual Petri dishes. Feeding was initiated by application of 0.1 ml 10^3 M sucrose to the lips, and once several feeding cycles had occurred, a 1mm x 15mm strip of lettuce was introduced to the mouth opening. Once the

Figure 4.10 The effect on the feeding response following of application of 0.1 ml of 0.02b M citric acid and snail water to the lips of feeding snails.

A) Percentage reduction in the number of bites in one minute caused by application of citric acid and snail water to the lips after one minute sucrose induced biting. There was a significant reduction in number of bites following citric acid application compared with snail water control, with no snails showing any biting (100% reduction) following application of citric acid (** $p= 0.001$ level, raw data, Mann-Whitney U test).

B) Number of regurgitations following application of citric acid and snail water control. Application of citric acid caused a significantly greater number regurgitations compared to snail water (** $p= 0.01$ level, Mann-Whitney U test).
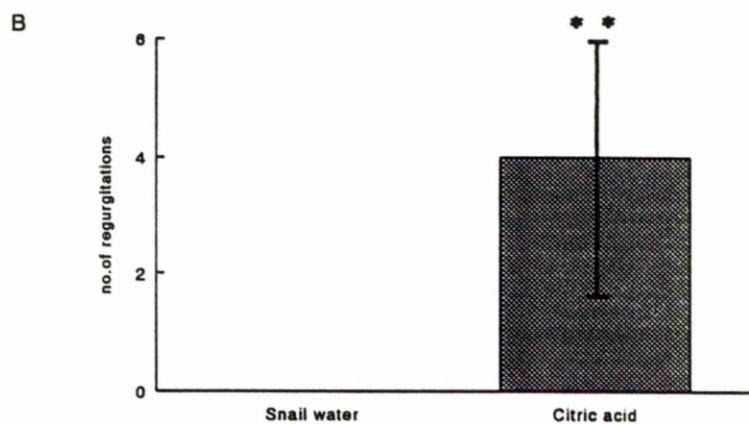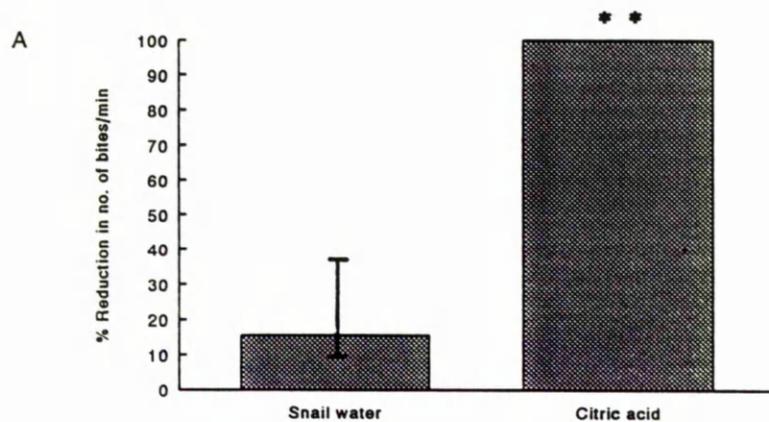
strip had been nearly completely ingested (usually after approximately four rasps), 0.1 ml of 0.026 M citric acid was applied to the lips. Citric acid was used as it was reliable at inducing the backwards radula movement assumed to be regurgitation. The number of regurgitation movements was then scored and the length of lettuce strip expelled measured.

In all 10 snails, citric acid application led to the expulsion of the lettuce strip. Therefore, the movements described in previous experiments did represent regurgitation. Egestion movements, although appearing to be exaggerated, were found to be less effective than ingestion, since each ingestive rasp was found to take in approximately 3 mm of the lettuce strip, whilst the average length of lettuce expelled by each regurgitation movement was calculated as 1.75 ± 1.15mm (n=10).

Electrophysiological experiments.

Electrophysiological experiments were carried out using a semi-intact preparation, to assess the neural pathways involved in the different responses to the three aversive stimuli, 1) noxious mechanostimulus, 2) quinine chloride and, 3) citric acid. As the behavioural experiments had shown that all three aversive stimuli caused stereotyped avoidance behaviour (withdrawal and locomotion), and also affected the feeding pattern in some way (i.e. caused regurgitation or inhibit feeding movements), recordings were made from neurons known to be involved in withdrawal and locomotion (the cerebral A cluster motoneurons; Benjamin *et al.*, 1985; Ferguson and Benjamin, 1991a,b; Winlow *et al.*, 1992), and feeding (CV1 and identified feeding buccal motoneurons).

Noxious mechanostimulus. In 11/11 semi-intact preparations a noxious mechanostimulus to the lip tissue led to a brief burst of summating EPSPs in cerebral A cluster neurons (lasting

1-2 seconds), sometimes depolarising them sufficiently to produce spiking (Fig. 4.11). This is in agreement with results reported by Ferguson and Benjamin (1991b). These results suggest that the cerebral A cluster cells are involved in mediating the stereotyped withdrawal response following a noxious prod to the lips, and that the neural pathways for withdrawal are intact in the lip-CNS preparation used, indicating that the lip nerves relay the mechanosensory information.

In 9/10 semi-intact preparations showing spontaneous feeding motor output, noxious touch to the lip tissue resulted in a short term disruption of the feeding rhythm (Fig. 4.12). Feeding motor output was monitored by recording from either or both of the identified buccal motoneurons B3 or B4, in which feeding cycles could be clearly identified by differing synaptic inputs and bursting activity. Disruption of feeding cycles was short-lived (approximately 4-5 seconds), and was characterised by a burst of spikes in B4, or IPSPs in B3 (Fig. 4.12). The time course of this disruption was comparable to the duration of withdrawal seen in the feeding whole animal following a noxious mechanostimulus.

In 5/5 feeding preparations, a noxious mechanostimulus led to a brief interruption of the feeding cycles of CV1 (Fig. 4.13). There was no corresponding change in the unitary inputs received by CV1, which may suggest that the interruption was indirect (i.e. due to feedback from changes in the CPG rhythm in the buccal ganglia).

Quinine chloride. In 10/10 semi-intact preparations application of 0.1 ml 0.026 M quinine chloride to the lip tissue resulted in a barrage of summating EPSPs to the cerebral A cells which depolarised the membrane sufficiently to produce prolonged firing lasting 5-10 seconds (Fig. 4.14A). The response to quinine chloride seen in the cerebral A cells was clearly distinct from the response seen with mechanical stimuli; it was stronger and more prolonged than the response to both a noxious prod and 0.1 ml saline to the lips in the same preparation (Fig.

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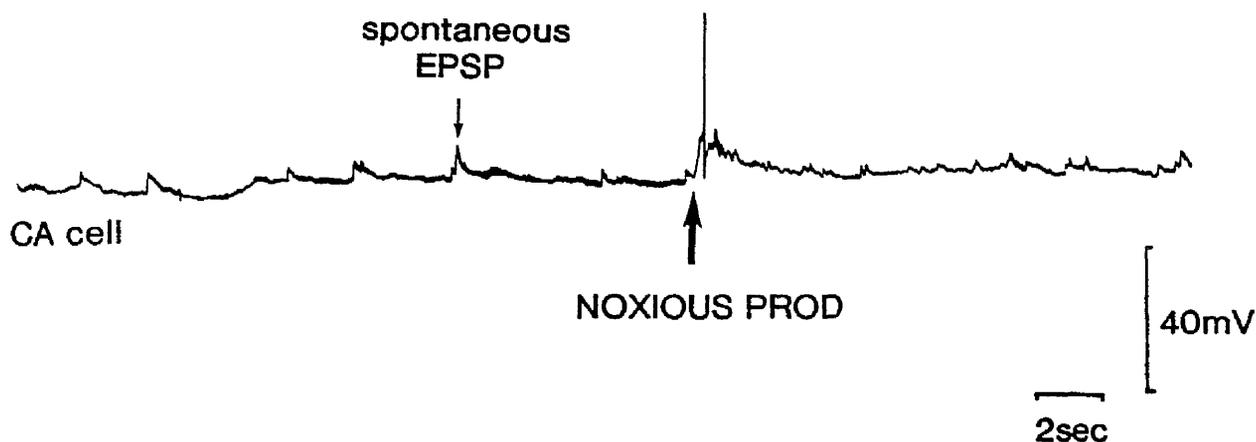
Figure 4.11 Effect of a noxious prod to the lips tissue of the semi-intact preparation on the activity and inputs of a cerebral cluster A motoneuron (CA cell). The CA cell receives spontaneous compound EPSP (labelled) in the absence of mechanostimulation of the lip tissue. A noxious prod to the lips results in the CA cell receiving a compound EPSP (lasting approx. 2 secs), sufficiently depolarising the CA cell to produce a single action potential.

Figure 4.12 Effect of a noxious prod to the lip tissue of a semi-intact preparation showing ongoing feeding motor output. The identified motoneurons B3 and B4 both show phasic bursting activity typical of FMO. A noxious prod to the lips results in a burst of action potentials in B4 and IPSPs to B3. This is accompanied by a short term disruption of the feeding rhythm, with one feeding cycle being prolonged by approx. 5 seconds.
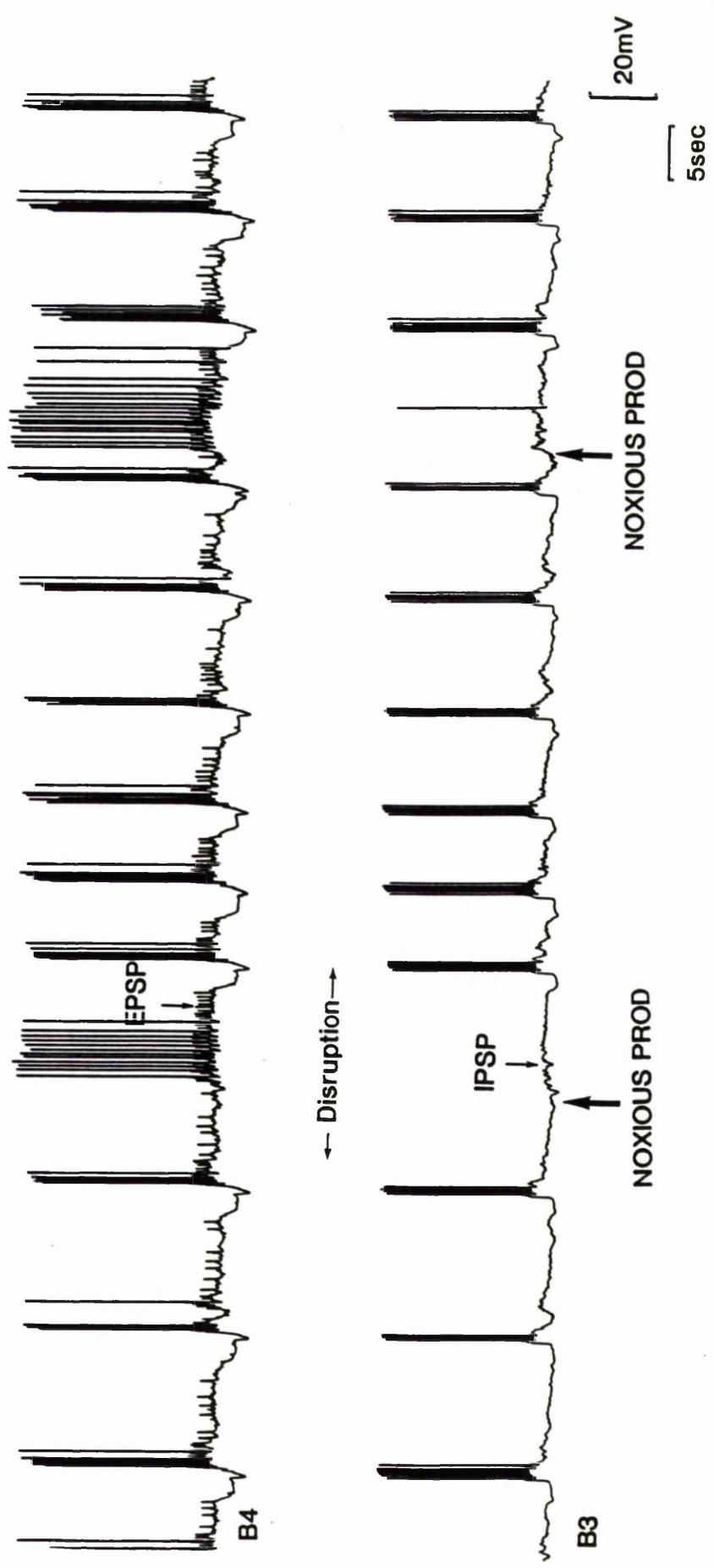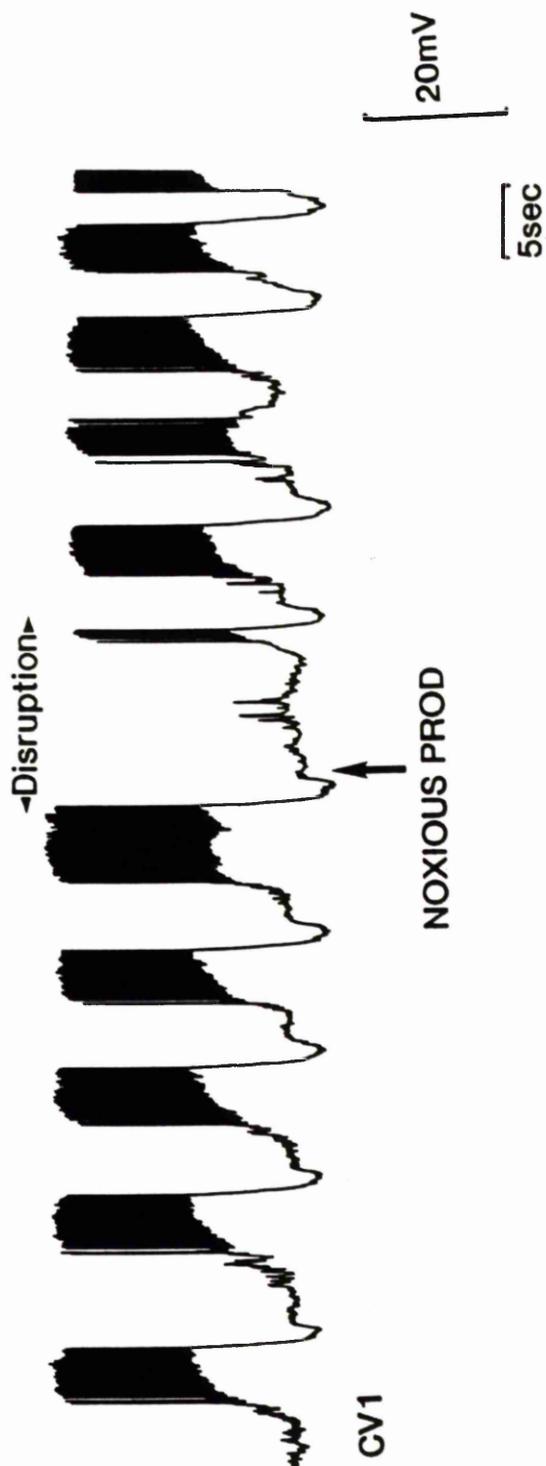
Figure 4.13 A noxious prod to the lips of a semi-intact preparation results in a short term disruption of CV1's feeding cycles. Since there are no immediate changes in inputs received by CV1, this disruption is probably due to feedback from the buccal CPG.

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Figure 4.14 A) Application of 0.1 ml 0.026 M quinine chloride (QUININE) to the lips of a semi-intact preparation results in strong depolarisation of the cerebral A cluster motoneuron, and prolonged firing (lasting >10 secs). B+C) In contrast, application of a noxious prod or 0.1 ml snail saline (SALINE) to the same preparation, i.e. mechanostimulus only, results in immediate depolarisation of the CA cell, but only a brief burst of action potentials.

A

CA cell

QUININE

B

NOXIOUS PROD

C

SALINE

40mV
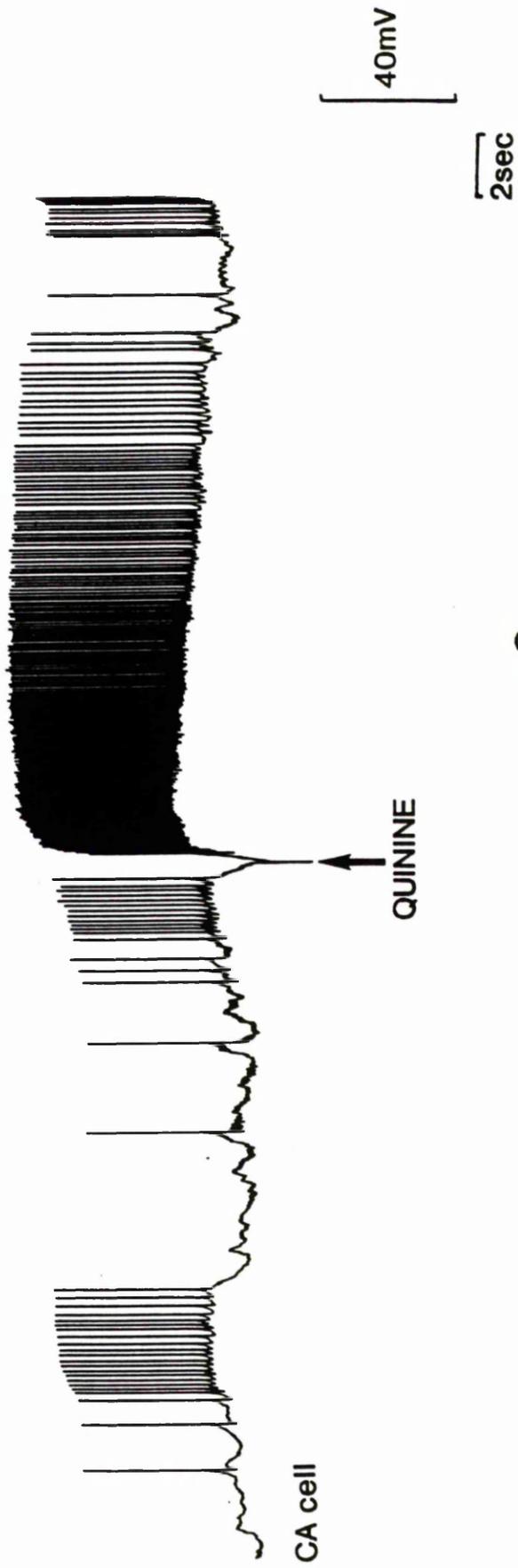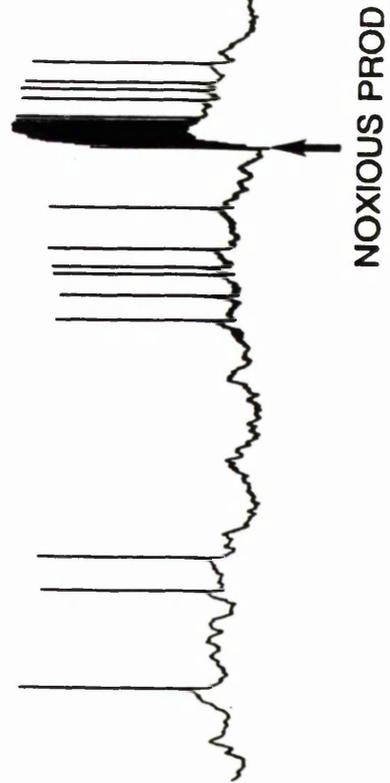
2sec
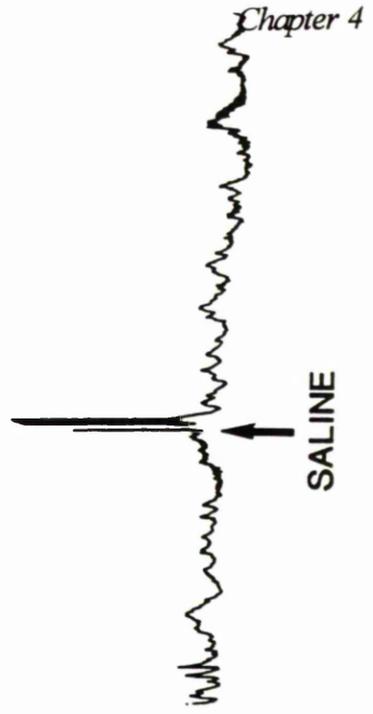
4.14B,C).

In 13/13 semi-intact preparations, application of 0.1 ml 0.026 M quinine chloride to the lips did not lead to any change in inputs to or activity of CV1 (Fig. 4.15). This suggests that inhibition of feeding movements by quinine chloride, which were observed in the intact snail, were not via the CV1 pathway. Application of quinine chloride to the lips also had little effect on the activity of the feeding motoneurons. In 8/12 preparations quinine chloride had no effect on the activity of buccal motoneurons either in preparations showing feeding motor output (Fig. 4.15) or quiescent preparations (Fig. 4.16A). In 3/12 preparations 0.026 M quinine chloride appeared to be *excitatory,* resulting in initiation of several feeding cycles (Fig. 4.16B). Only 1/13 preparations showed any inhibitory effect of quinine chloride, and this effect was very weak; spontaneous firing activity of B4 (not true feeding) was inhibited (Fig. 4.16C). There was no evidence of regurgitation activity (N1-N3 rhythm; Kyriakides, 1988) in any preparation.

Citric Acid. In 10/10 semi-intact preparations application of 0.1 ml 0.026 M citric acid resulted in a depolarisation of the membrane potential of the cerebral A cluster neurons and intense firing for prolonged period (> 20 seconds) (Fig. 4.17). Recordings, however, were often cut short owing to the microelectrode being displaced from the neuron, caused by movement in the lip nerves following the strong withdrawal response in the lip tissue of the semi-intact preparation. This withdrawal of the lip tissue was clearly seen with the naked eye and corresponded to activity in the cerebral A cluster neurons. The response to citric acid was very much stronger than that of an aversive mechanostimulus or 0.1 ml saline in the same preparation (Fig. 4.17). Although results from quinine chloride and citric acid application cannot be directly compared, citric acid appeared to have a stronger effect, producing a much more intense firing of the cerebral A cluster cells, and a much stronger withdrawal of the lip

Figure 4.15 Effect of application of 0.026 M quinine chloride (QUININE) to the lips of a semi-intact preparation showing feeding motor output. Quinine chloride application results in no change in the inputs to either the identified motoneuron B3 or CV1. The frequency of the feeding cycles is also unaffected. The three phases (N1, N2, N3) of the feeding cycle are indicated.
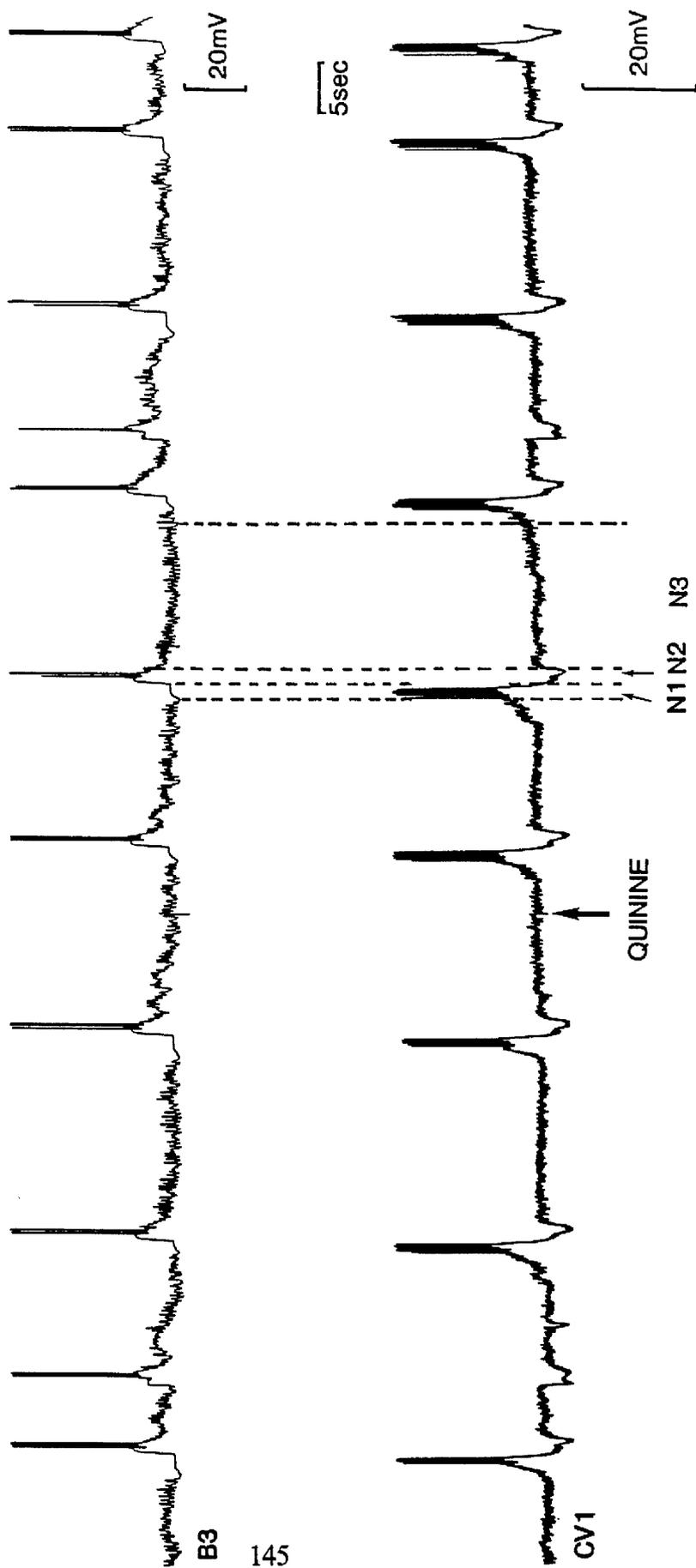
Figure 4.16 Effect of application of 0.026 M quinine chloride (QUININE) to the lips of a semi-intact preparation on the activity of the identified feeding motoneuron B4. A) B4 is quiescent. Application of quinine chloride has no effect on inputs to or activity of B4. B) B4 is initially quiescent. Application of quinine chloride appears to be excitatory, resulting in short-lived initiation of FMO, lasting 6 feeding cycles. C) B4 initially shows some spontaneous bursts of action potentials, (not true feeding). Application of quinine chloride results in B4 becoming quiescent.
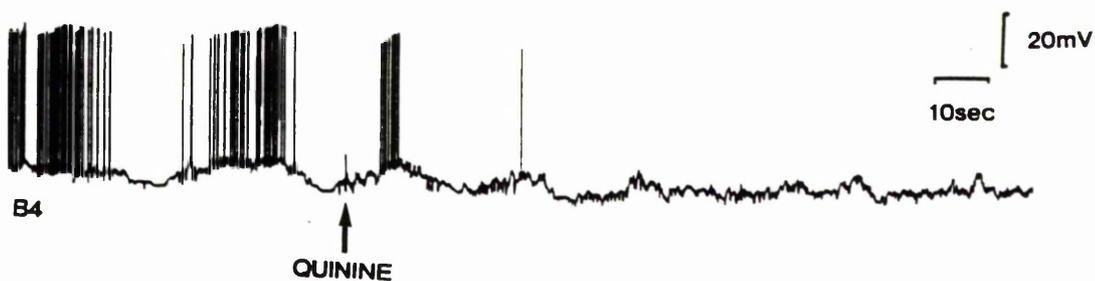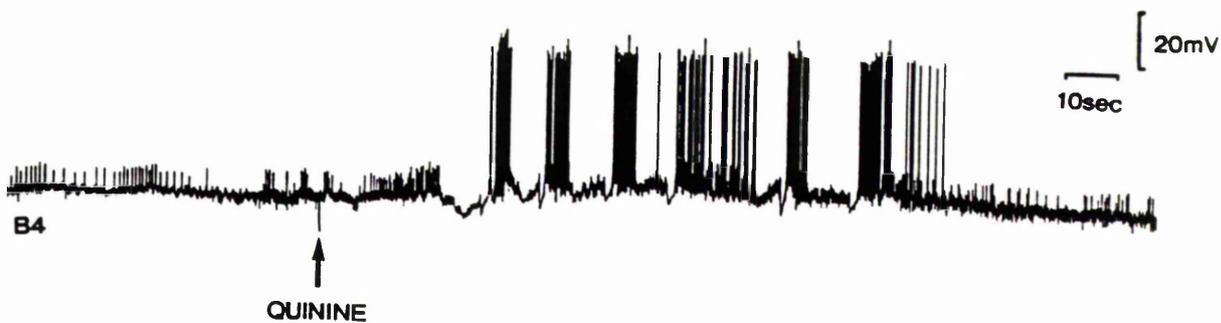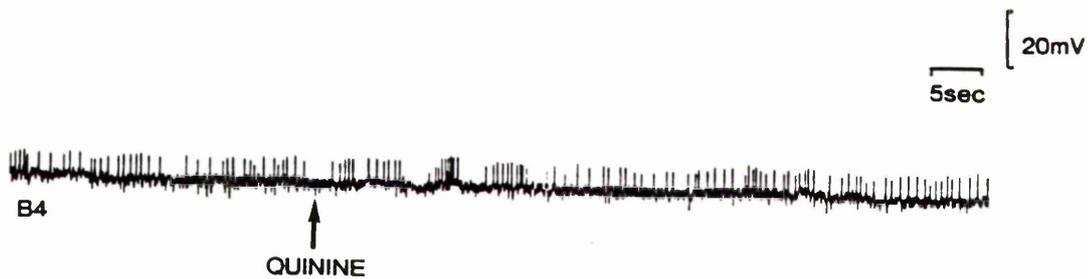
and tentacle tissue in the dish.

In 9/9 preparations, application of 0.026 M citric acid to the lips of the semi-intact preparation had no effect on the activity of buccal motoneurons (Fig. 4.18A, B), i.e. there was no evidence of inhibition of feeding or initiation of regurgitation (N1-N3 rhythm; Kyriakides, 1988) seen in the whole animal.

Recordings from CV1 were difficult following application of citric acid, owing to violent withdrawal of the lip tissue. This usually displaced the microelectrode and resulted in loss of the recording. However, some recordings were achieved (n=5). In 5/5 preparations application of citric acid resulted in no change in inputs to or activity of CV1 (Fig. 4.18B).

More intact preparation. The electrophysiology results obtained following application of citric acid (and quinine chloride) to the lips in the semi-intact preparation were not totally comparable with those from the whole animal. Although the neural correlates of withdrawal were clearly seen as strong activity of the cerebral A cluster neurons following application of citric acid or quinine chloride to the lip tissue, the inhibition of ingestive feeding movements and the increase in regurgitation movements seen in the whole animal were not reflected by the activity recorded from the feeding motoneurons. These results indicate that the semi-intact preparation used may not have been intact enough for citric acid or quinine chloride to affect feeding motor output. It is possible that aversive chemosensory stimuli only inhibit feeding once the stimulus has been ingested (taken into the buccal cavity), and that the semi-intact preparation used did not have the necessary sensory pathways intact. Likely candidates are the nerves originating from the buccal ganglia, which innervate the oesophagus, buccal cavity and lips (Kemenes *et al.*, 1986).

Some preliminary experiments were therefore carried out using a more intact preparation (modified from a preparation used by Kemenes *et al.*, 1986). The preparation

Figure 4.17 A) Application of 0.1 ml 0.02⌖ M citric acid to the lips of a semi-intact preparation results in strong depolarisation of the cerebral A cluster motoneuron, and very fast and prolonged firing. Cell is lost at end of recording. B, C) In contrast, application of a noxious prod and 0.1 ml snail saline (SALINE) to the same preparation, i.e. mechanostimulus only, results in immediate depolarisation of the CA cell (no delay), and much briefer bursts of action potentials.
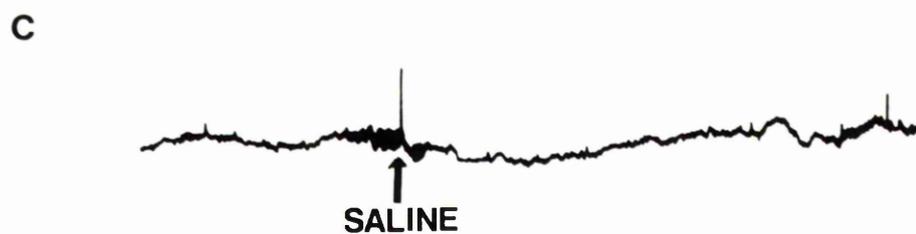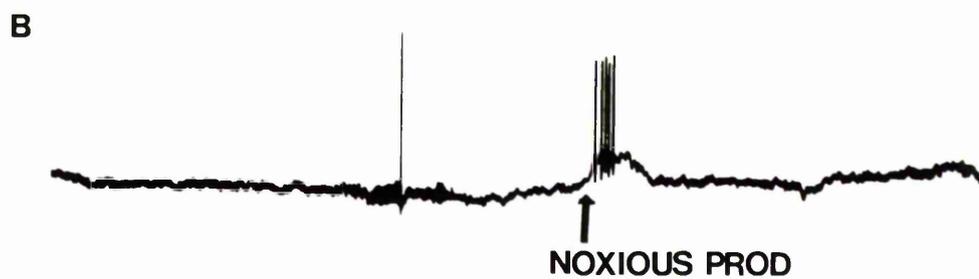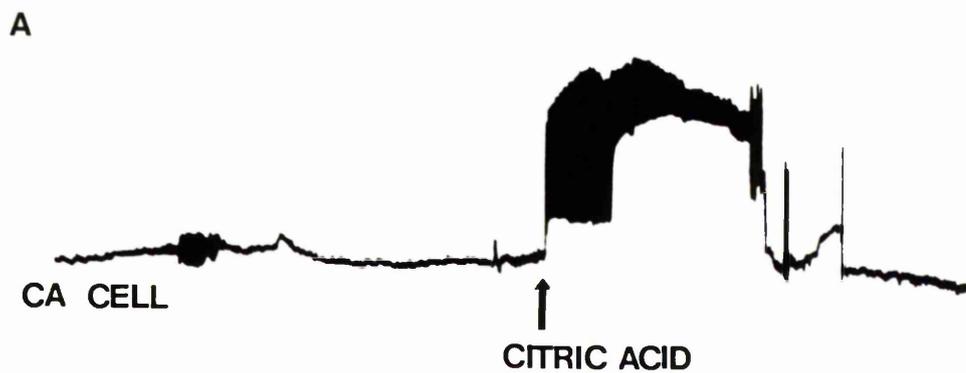
Figure 4.18 Effect of application of 0.026 M citric acid to the lips of a semi-intact preparation on the feeding system. A) B4 is quiescent. Application of citric acid does not result in any change in inputs to, or activity of B4. B) Preparation is showing FMO. Application of citric acid does not result in any change of inputs to CV1 or B4, or to the frequency of feeding.

A

B4

CITRIC ACID

2sec

B

B4

CV1

CITRIC ACID

20mV

5sec
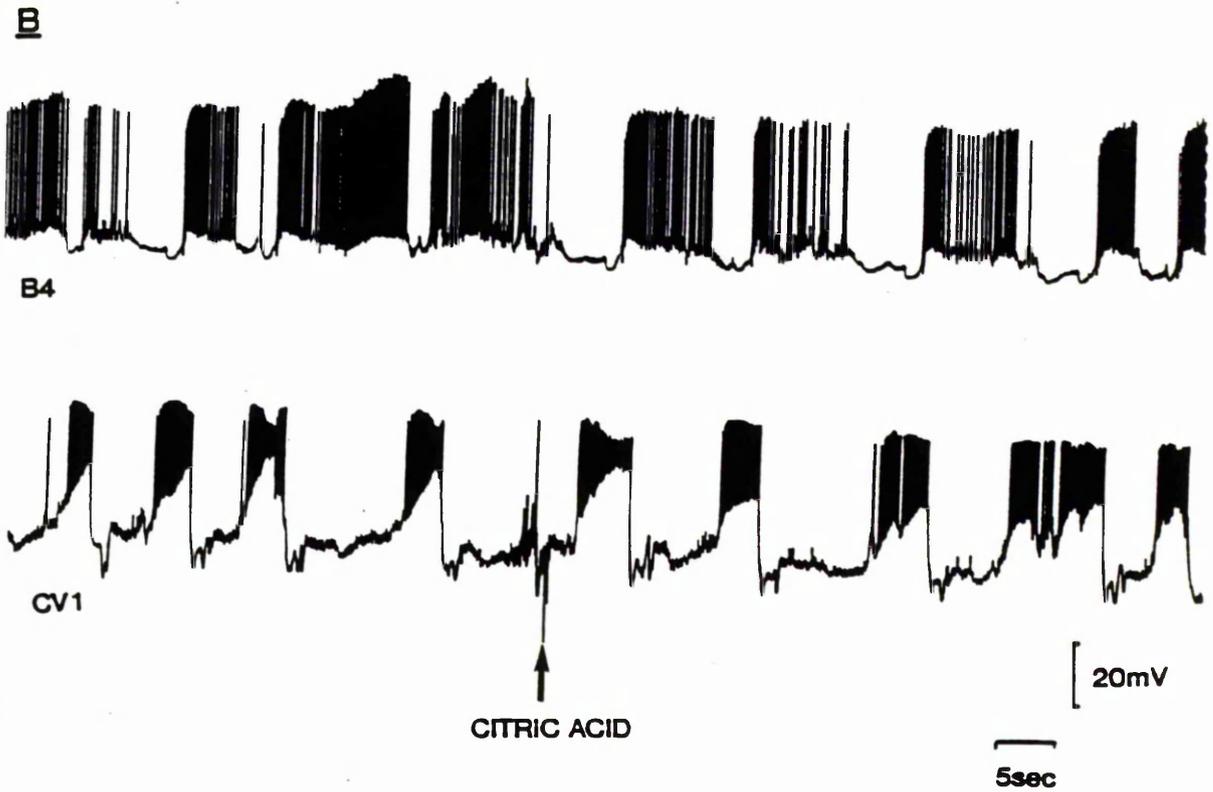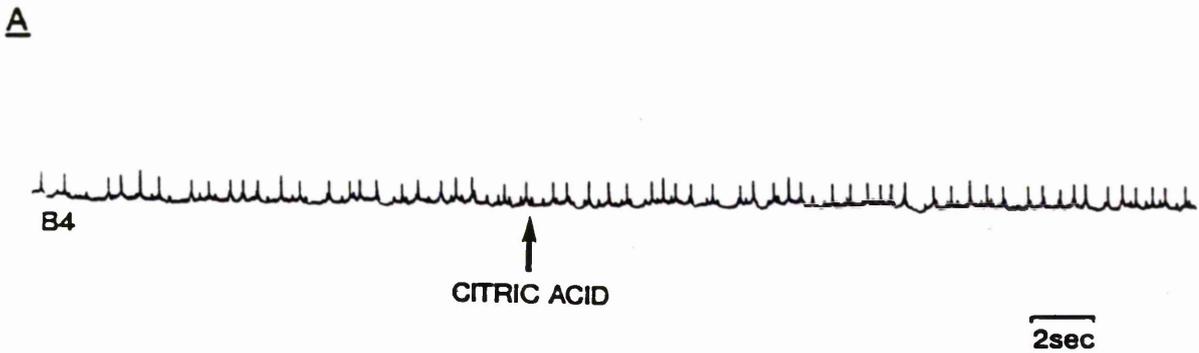
consisted of the anterior part of the snail being mostly intact, including the buccal mass, lips and tentacles. All innervation from the cerebral and buccal ganglia were left intact (including the paired superior and median lip nerves, and the paired laterobuccal, ventrobuccal and dorsobuccal nerves; Benjamin *et al.*, 1979). The saline bathing the outer mouth and tentacles was isolated from that bathing the exposed CNS using moulded pieces of blue-tak and sealing any gaps with vaseline (Fig. 4.19). Recordings were made from identified buccal motoneurons B3 and B4 before and after application of 0.1 ml 0.026 M citric acid to the lips of the semi-intact preparation. Citric acid was used as it reliably caused complete inhibition of feeding in the whole animal, and also induced regurgitation movements in feeding and non-feeding snails.

Recordings of buccal motoneuron activity following application of citric acid proved extremely difficult, owing to the microelectrode being displaced from the motoneurons by the strong movements of the anterior part of the snail and the buccal mass. However, short term recordings were achieved in two preparations. Figure 4.20 shows the activity of the buccal motoneuron B4. A noxious prod to the lips resulted in a burst of EPSPs, leading to firing lasting approximately 14 seconds. There was a delay of 2 seconds between the prod and activity in B4 (4.20A). Application of 0.1 ml 0.026 M citric acid to the lips in the same preparation, resulted in a prolonged increasing in spike frequency in B4 (>40 seconds) after a delay of 2 seconds (Fig. 4.20B). This tonic firing could reflect a mechanism of inhibition of feeding seen behaviourally following application of citric acid to the lips. However, there was no evidence of any regurgitation (N1-N3) rhythm as described by Kyriakides (1988).
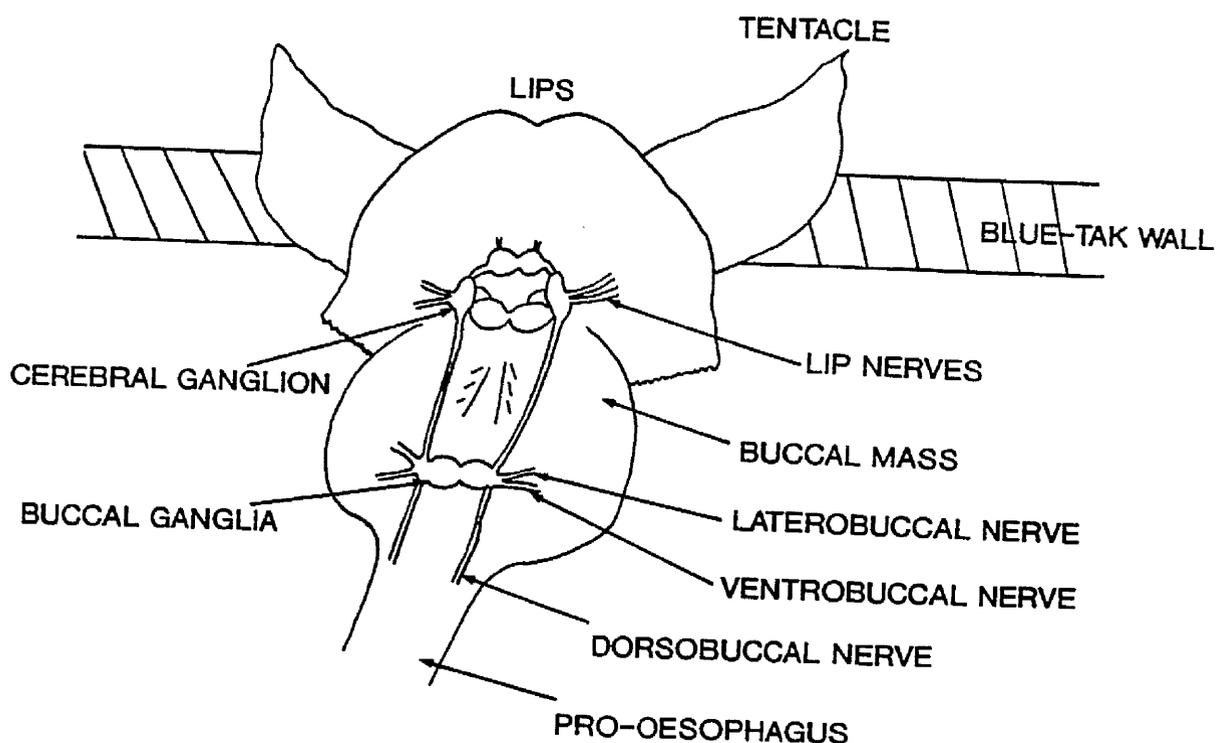
Figure 4.19 Schematic diagram of the more intact preparation modified from Kemenes *et al.* (1986). Most of the anterior part of the snail is left intact. The buccal mass, lips, tentacles and all innervation from the buccal and cerebral ganglia are left intact (the different nerves are labelled). The saline bathing the lips and tentacles was isolated from CNS, using a blue-tak wall and vaseline (labelled).

Figure 4.20 A) A noxious prod to the lips in the very intact preparation results in a short burst of EPSPs (lasting approx. 14 secs) in B4 which sufficiently depolarises the membrane potential to produce action potentials, after a delay of approx. 2 secs. B) Application of 0.026 M citric acid in the same preparation results (after a delay of approx. 2 secs) in a more intense and prolonged burst of action potentials in B4 lasting >40 secs. No evidence of regurgitation is seen. Both recordings are cut short due to electrode displacement following strong withdrawal of the preparation in the dish.
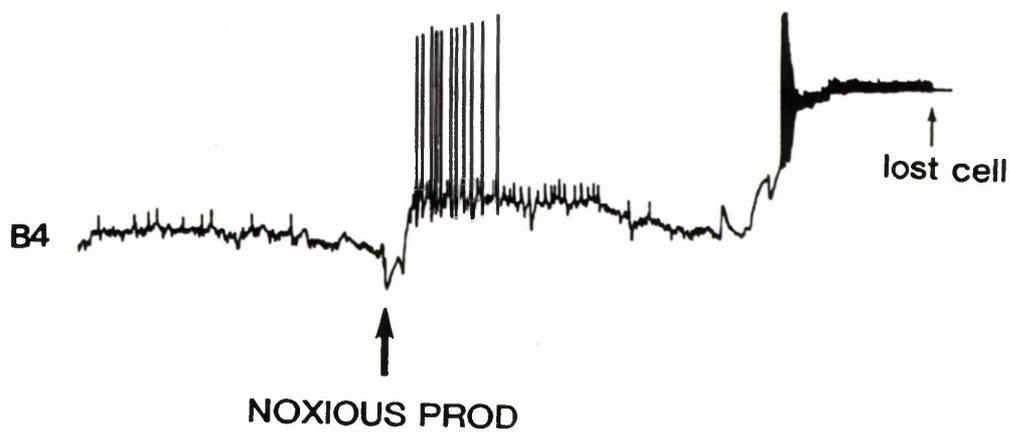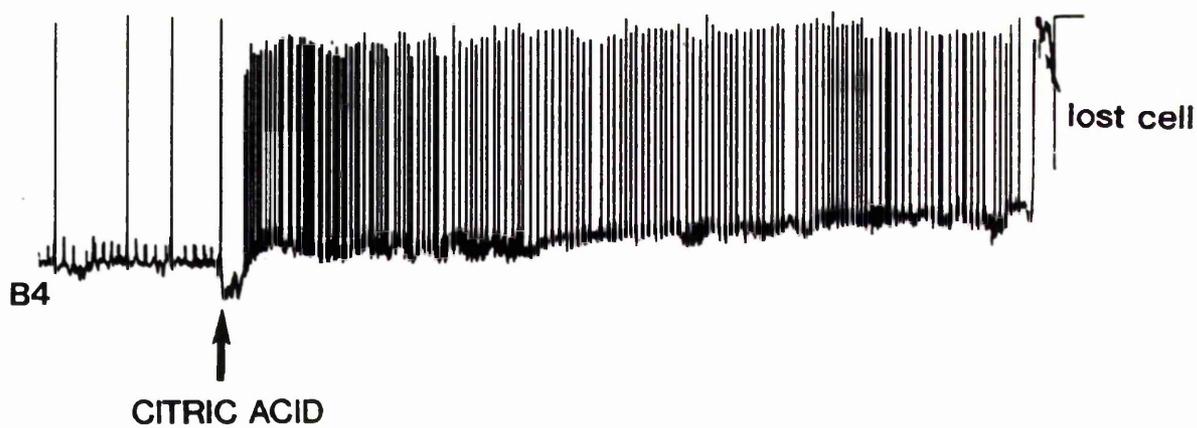
DISCUSSION

Feeding stimuli

The behavioural experiments in this Chapter showed that the only difference in *Lymnaea*'s feeding response to sucrose and fish food was that fish food caused slightly faster feeding than sucrose. One explanation for this faster feeding may be that fish food has a more complex structure than sucrose, and therefore stimulates more chemoreceptors, and thus excites the feeding system more than sucrose. Studies on *Pleurobranchaea* support this, since food stimuli containing a broad spectrum of components have been shown to excite more "units" in sensory nerves, at higher frequencies, than simpler food stimuli (Bicker *et al.*, 1982a). An additional factor which could have contributed to faster feeding to fish food, may be that fish food homogenate also excited mechanoreceptors (as well as chemoreceptors) due to it being a suspension of solid material. Tactile stimulation, in association with food, has been shown to be very important in *Aplysia* for initiating and maintaining feeding behaviour (Preston and Lee, 1973; Rosen *et al.*, 1982), and although no quantitative studies have been carried out, experiments indicated that *Limax* and *Lymnaea*'s feeding responses were prolonged when there was a tactile element to the food stimulus (Delaney and Gelperin, 1990c; Kemenes *et al.*, 1986).

In contrast to the behavioural experiments, electrophysiological experiments using the semi-intact preparation did not show any difference in response to fish food and sucrose. Both stimuli had very similar effects on the inputs to, and activity of, CV1 and the same probability of initiating feeding activity (Table 4.1).

McCrohan postulated that CV1 was responsible for initiating feeding, and not for modulating the frequency of feeding (McCrohan, 1984b). This hypothesis was based on the

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Table 4.1 Comparison of the % excitatory effect (electrophysiological results) of sucrose (Chapter 3) and fish food on CV1:

	Sucrose (n=26)	Fish Food (n=20)
Initiation of feeding	35 %	35 %
Excitatory to CV1 (no initiation)	42 %	45 %
No effect	23 %	20 %

observation that CV1 only had a limited range of firing rates in the isolated CNS, and thus had limited ability to modulate the feeding rhythm (McCrohan, 1984b). The lip-CNS preparation used in this Chapter was completely free of any buccal innervation, and thus was a good model for studying the initiation and control of feeding by the CV1 pathway in the absence of buccal-innervated pathways. Therefore, since there was no discernable difference in the excitation of CV1 by fish food and sucrose, this suggests that the purely "triggering" role hypothesized by McCrohan (1984b) is correct. One candidate pathway for modulating the frequency of feeding is via the SO interneuron located in the buccal ganglia. This is because the SO has been shown to modulate the feeding rhythm over a wide range of frequencies (Rose and Benjamin, 1981a), and its location suggests that it receives sensory inputs from the buccal mass and cavity (structures that were absent from the lip-CNS preparation used in this Chapter).

Aversive stimuli

The aversive stimuli experiments in this Chapter revealed an unexpected behavioural response to strong localised aversive stimuli (noxious prod and 0.1 ml 0.026 M quinine chloride) whilst feeding. *Lymnaea*'s behaviour was not to simply withdraw and stop feeding, but was to show short term withdrawal, then turn and locomote from the area, resuming

feeding as it went. This is in contrast to behaviour exhibited by other molluscs when they receive localised aversive stimuli. For example, *Tritonia's* feeding is inhibited during the "escape run" locomotion (behaviour seen following an escape swim that can be triggered by sodium chloride crystals to the lips; Audesirk and Audesirk, 1980a,b, and *Limax, Helix* and *Pleurobranchaea's* feeding is inhibited for several minutes following application of quinidine/quinine or an electric shock (Delaney and Gelperin, 1990c; Balaban *et al*, 1987; Balaban, 1993; Mpitsos and Collins, 1975).

This contrasting behaviour could be due to *Lymnaea's* "motivation to feed" being greater than other molluscs, resulting in even strong aversive stimuli, such as the noxious prod and quinine chloride, only interrupting ingestive feeding movements for a short period (long enough for *Lymnaea* to withdraw and turn away from the aversive stimulus) before feeding is resumed. This behaviour would be well suited to *Lymnaea's* grazing behaviour in its natural environment, as it would allow *Lymnaea* to avoid patches of inappropriate food sources without inhibiting feeding altogether.

There are other pieces of evidence in support of *Lymnaea's* motivation to feed being relatively stronger than other molluscs. Firstly, the avoidance response of *Lymnaea* following strong aversive stimuli is very similar to the reported behaviour of other gastropods in response to weak aversive stimuli, e.g. *Helix* actively moves away from weak/moderate aversive stimuli (the author did not qualify the nature of the aversive stimuli; Balaban, 1993); and *Pleurobranchaea* only shows brief withdrawal (and interruption of feeding) following weak tactile stimuli, after which feeding is resumed (Kovac and Davis, 1980b). This suggests that *Lymnaea* perceives "strong aversive stimuli" in the same way that other gastropod molluscs perceive "weak aversive stimuli". This is supported by the fact that *Lymnaea's* ingestive feeding movements could only be completely inhibited when the aversive stimulus was presumably extremely painful since it caused writhing for several minutes (i.e. with citric

acid). Therefore, it is possible that feeding is rarely completely inhibited for long periods in the wild, since it is unlikely that *Lymnaea* would often come across a food stimulus as aversive as citric acid in its natural environment.

Studies on *Pleurobranchaea* (Kovac and Davis, 1980b) and *Aplysia* (Teyke *et al.*, 1989) suggested that feeding and withdrawal are reciprocal and not hierarchical behaviours, since the withdrawal amplitude is reduced in feeding animals (although never blocked), and conversely, feeding is reduced in animals avoiding aversive stimuli. A similar situation is proposed for *Lymnaea*, since the withdrawal (in response to an aversive stimulus) was shown to be reduced in feeding animals compared to non-feeding animals, and ingestive feeding movements were inhibited during full withdrawal. However, it is proposed that *Lymnaea's* strong motivation to feed compared to other gastropods, is due to the reciprocal relationship being heavily biased towards feeding. This would explain why *Lymnaea* only showed very short term withdrawal following aversive stimuli that were considered to be strong and caused relatively long-lasting inhibition of feeding in other gastropods.

The electrophysiological results on the effects of aversive stimuli in the semi-intact preparation, as with the feeding stimuli, did not totally reflect the behaviour seen in the whole animal. These anomalies, however, were extremely interesting, as they revealed different neural mechanisms underlying the (apparently) same avoidance behaviour to both noxious mechanostimuli and chemostimuli. Whilst the neural correlates of withdrawal were seen in response to all noxious stimuli, the short-term inhibition of ingestive feeding which was seen behaviourally during withdrawal, was only seen following the noxious prod. It was not clear if the inhibition of feeding by mechanostimuli was via the CV1 pathway, but it could be concluded that the inhibition of feeding by noxious mechanostimuli was via information from the lip nerves. Conversely, the inhibition of feeding by chemostimuli must be via other nerves

which were not present in the semi-intact preparation, e.g. buccal innervation. On the same premise, the CV1 pathway cannot be involved in the inhibition of feeding by aversive chemostimuli, and this was supported by the observation that neither quinine or citric acid had an inhibitory effect on CV1's activity. In fact, application of quinine chloride to the lip-CNS preparation sometimes resulted in initiation of feeding - completely the opposite effect to the short term inhibition of feeding seen in the whole animal. (N.B. It should be noted that these results do not agree with a paper by Kemenes *et al.* (1986), in which quinine sulphate was reported to have a "dramatically opposite effect to sucrose" when applied to the lip tissue of a lip-tentacle preparation, i.e. it inhibited feeding cycles. The reason for this anomaly is not known, although it should be noted that what was described in the paper's text as strong inhibition of feeding, was supported by a figure showing only a temporary disruption of feeding cycles).

Similar studies on other gastropod molluscs have given conflicting results on the effects of aversive chemostimuli in the lip-CNS preparation. Several papers reported similar findings to the results found with *Lymnaea* in this Chapter. In *Helix*, quinine was shown to excite withdrawal neurons (Maksimova and Balaban, 1984), whilst having the same effect as feeding stimuli on cerebral cells thought to be involved in initiating feeding, i.e. quinine was excitatory, not inhibitory (Kemenes, 1992). Similarly, studies on *Limax* found quinidine to initiate feeding in the semi-intact preparation, whilst in the whole animal it was a strong inhibitor of feeding (Culligan and Gelperin, 1983). However, in a later paper on *Limax,* quinidine sulphate was reported to inhibit CB_1 (the putative homologue of CV1) in a similar manner to an electric shock to the foot which was known to cause withdrawal and inhibit feeding. The authors concluded, therefore, that suppression of feeding by a stimulus that evokes withdrawal occurs at both the cerebral and buccal level in *Limax* (Delaney and Gelperin, 1990c). Similarly, an earlier paper studying *Pleurobranchaea* reported that the emetic

stimulus ethanol had an opposite effect to food on the inhibitory interneurons presynaptic to PC$_p$ (the putative homologue of CV1) (London and Gillette, 1984), i.e. that aversive chemostimuli inhibited feeding via a cerebral pathway. Therefore, there is some conflict as to whether *Lymnaea* is typical of gastropod molluscs, with aversive chemostimuli not inhibiting feeding via the cerebral (CV1) pathway.

One definite conclusion that can be made, however, is that inhibition of feeding in *Lymnaea* following a aversive stimulus cannot be a result of central inhibition of the feeding network by the withdrawal network, a mechanism proposed to occur in *Pleurobranchaea* (Kovac and Davis, 1980b). This is because excitation of the withdrawal network was seen to occur in the semi-intact preparation whilst feeding motor output remained unaffected. Behavioural experiments also indicated that central inhibition of the feeding network did not occur, since regurgitation movements were seen whilst the snail was withdrawn, and regurgitation movements have been shown to originate from modified output from the feeding network (Tuersley and McCrohan, 1988; Kyriakides, 1988).

Regurgitation has been reported to be part of the behavioural repertoire of many species of gastropod molluscs, including *Pleurobranchaea* (Croll *et al.*, 1985a), *Aplysia* (Morton and Chiel, 1993a,b) and *Lymnaea* (Bovbjerg, 1968). There have been several studies into the neural mechanisms underlying this behaviour in *Aplysia* (Susswein and Byrne, 1988; Kirk, 1989; Morton and Chiel, 1993a,b) and *Pleurobranchaea* (McClellan, 1982a,b, 1983; Gillette and Gillette, 1983; Croll *et al.*, 1985a-c), and in both these species it was found that neurons in the buccal ganglia (not the cerebral ganglia) were responsible for controlling regurgitation behaviour (namely the B31/B32 neurons in *Aplysia*, Susswein and Byrne, 1988, and the VWC in *Pleurobranchaea*, McClellan, 1983; Croll *et al.*, 1985b). The putative homologues of CV1 (the CBI-2 in *Aplysia*, Rosen *et al.*, 1991, and PC$_p$ in *Pleurobranchaea*,

Gillette *et al.*, 1982b) therefore, were not involved in initiating regurgitation. This has been shown directly in several studies on *Pleurobranchaea*, since stimulation of PC_p does not result in regurgitation (Croll *et al.*, 1985c), and the PC_p shown not to be active during egestion behaviour (Gillette *et al.*, 1982b). Therefore, it was proposed by Croll *et al.* (1985c) that in *Pleurobranchaea*, the ingestion command system is situated in the brain (i.e the cerebral ganglia) and the egestion command system is in the buccal ganglia. The results in this Chapter indicate that a similar arrangement is found in *Lymnaea*, since there was no evidence of the N1-N3 buccal rhythm (proposed to drive regurgitation behaviour; Kyriakides, 1988) following application of citric acid to the lip-tentacle preparation. This indicates that the CV1 pathway, like the putative homologous PC_p pathway in *Pleurobranchaea*, cannot be responsible for initiating regurgitation, and that the buccal ganglia are responsible for initiating regurgitation. This is logical since the buccal nerves innervate the buccal cavity and oesophagus, areas where food or other substrates have first to be present before they can be expelled.

The preliminary experiments using the more intact preparation, where the buccal mass and innervation were left intact, were designed to test the above hypothesis. Unfortunately the recordings achieved were not of good enough quality to make concrete conclusions, due to the instability of the preparation. However, there was evidence that aversive chemostimuli do have an effect on the feeding system via buccal innervation; the buccal motoneurons were excited by application of citric acid to the lips, in contrast to the lip-CNS preparation where citric acid had no effect. This excitation seen may have represented the mechanism by which ingestive feeding is inhibited, and/or the initiation of regurgitation behaviour.

CHAPTER 5 - RESULTS

THE ULTRASTRUCTURE OF THE LIPS AND TENTACLES OF *LYMNAEA*

THE ULTRASTRUCTURE OF THE LIPS AND TENTACLES OF *LYMNAEA*

INTRODUCTION

Lymnaea, like other gastropods, has a poorly developed visual system, and is thought to rely on chemical reception to detect distant objects (Bicker *et al.*, 1982a,b; Croll, 1983; Emery, 1992). Chemoreception in gastropod molluscs has been shown to be important for many of their behaviours, including homing, predator avoidance, social, reproductive (involving pheromones), and feeding behaviour (Bovbjerg, 1968; Croll, 1983; Emery, 1992).

A light and electron microscope study carried out by Zylstra (1972) on *Lymnaea* showed that epidermal sensory perception takes place by means of primary receptor cells whose cell bodies are located below the epidermis and whose free nerve endings extend from them to the epidermal surface. He located "rich sensory regions" of the epidermis (characterised by presence of subepithelial nerve cells) on the tips and ventral surface of the tentacles, the ventral surface and edge of the lips, the front edge of the foot and the pneumostome and mantle edge. This arrangement is typical of gastropod molluscs, with chemoreceptors being commonly found to be associated with the tentacles, lips and osphradium in many species (Emery, 1992). Studying these areas in *Lymnaea*, Zylstra (1972) also described six different types of sensory free nerve ending in the epithelium of *Lymnaea*. This study, however, did not give a detailed account of which free nerve endings were concentrated in which areas, or show any scanning electron micrographs (SEM) of the general arrangement and outer form of these sensory structures.

This Chapter, therefore, describes a preliminary study investigating the arrangement and structures of candidate chemosensory structures possibly involved in mediating feeding behaviour. Since this thesis is predominantly a study of feeding behaviour, only areas thought to be involved in chemoreception affecting feeding were studied, i.e. the tentacles and lip

area. Since a recent study by Emery (1992) presented SEM and TEM micrographs of the sensory structures of *Lymnaea's* tentacles, the results presented in this Chapter concentrate on the previously undescribed lip area.

RESULTS

The lip area.

Figure 5.1A shows a low magnification SEM micrograph of the mouth opening of *Lymnaea*. The different areas studied at higher magnification are labelled. The lips are fully open, equivalent to the late protraction and rasp phase of feeding when the radula is pushed fully out of the mouth. The radula was removed before fixation and is shown separately in Figures 5.1B and 5.1C at two different magnifications showing the whole structure (Fig. 5.1B) and the individual "teeth" (Fig. 5.1C).

The lips. SEM demonstrated that the lips are composed of many densely packed large microvilli-like structures (Fig. 5.2). The individual microvilli-like structures which make up the lip are extremely large, approximately 65 x 4 μm (Fig. 5.2). At the edge of the lip there are rows of shorter microvilli of similar thickness, with dimensions of approximately 30 x 4 μm and 50 x 4 μm respectively (Fig. 5.2B). These shorter structures may represent either, 1) distinct populations of "microvilli", 2) partially grown "microvilli", or 3) "microvilli" which have had their tips broken off during fixation.

TEM was carried out on horizontal serial sections taken from different levels along the vertical axis of the "microvilli" (Fig. 5.3). Sections taken from the top of the "microvilli" clearly show them as discrete individual structures (Fig. 5.3A). The "microvilli" appear to be skeletal structures consisting of a dense honeycombed core, surrounded by a less dense material. There is no evidence of nuclei, mitochondria or vacuoles etc. (Fig. 5.3A).

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Figure 5.1

A) Scanning electron micrograph of the mouth and surrounding area of *Lymnaea*. The mouth is open and the radula is not present. The lips and oral area (O.A.) are indicated. Scale is indicated.

B) Scanning electron micrograph of the radula of *Lymnaea*. Low magnification shows the general shape and structure of the radula. Scale is indicated.

C) Scanning electron micrograph of the radula of *Lymnaea*. High magnification shows the structure of the individual "teeth", which are arranged in rows. Scale is indicated.

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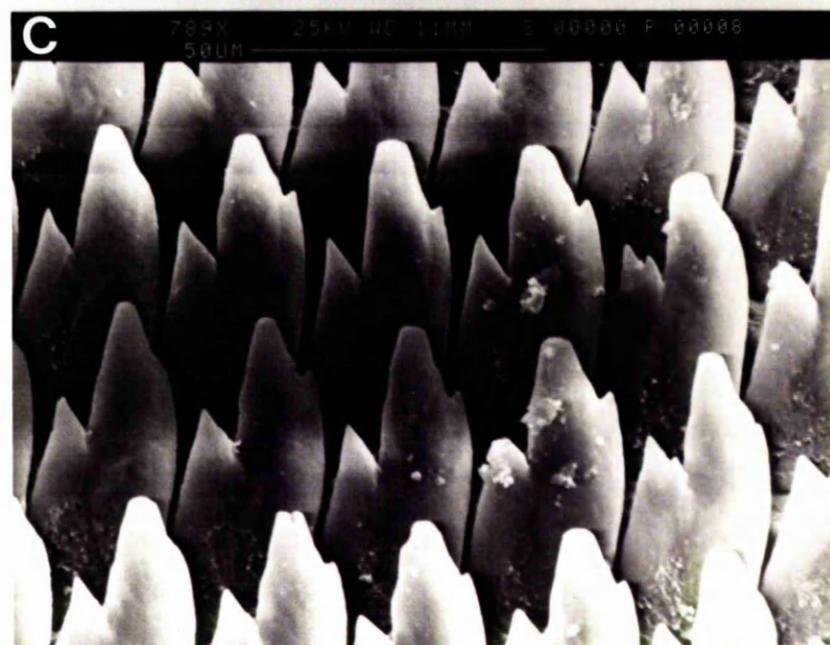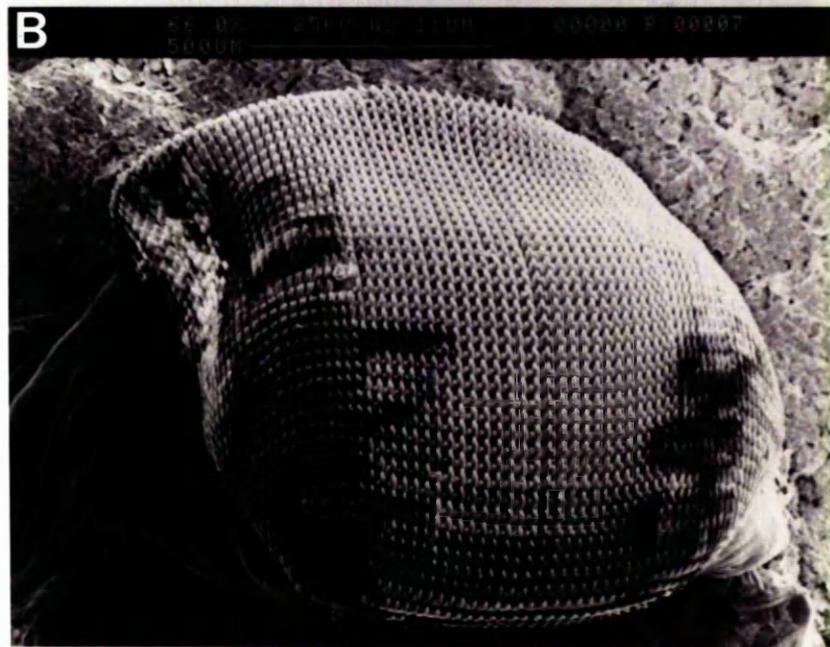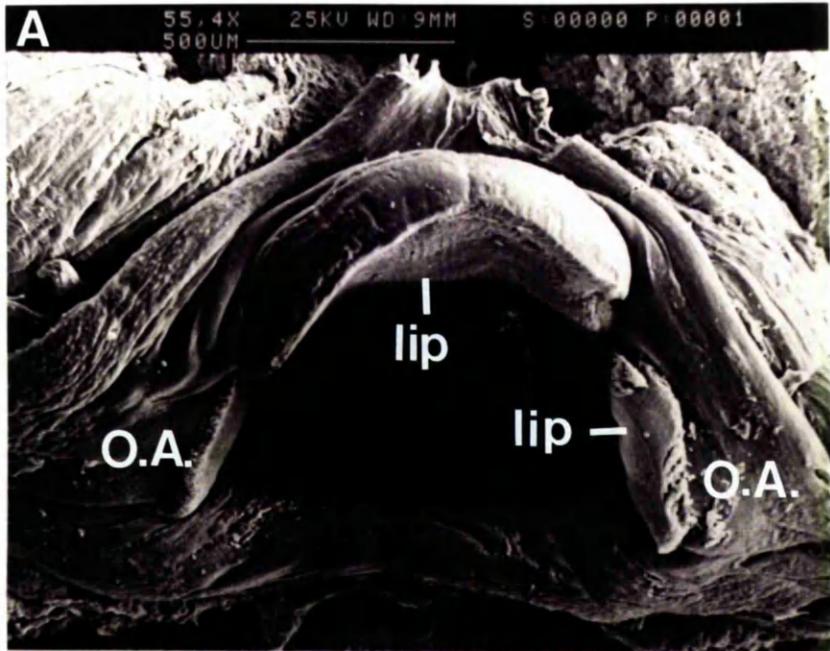
Figure 5.2

A) Scanning electron micrograph of the top lip showing it to be composed of densely packed large microvillous (mv) structures. Scale is indicated.

B) Scanning electron micrograph of one of the side lips. The individual "microvilli" (mv) are clearly seen. At the edge of the lip there are two shorter lengths of "microvilli" (labelled 1 and 2). Scale is indicated.
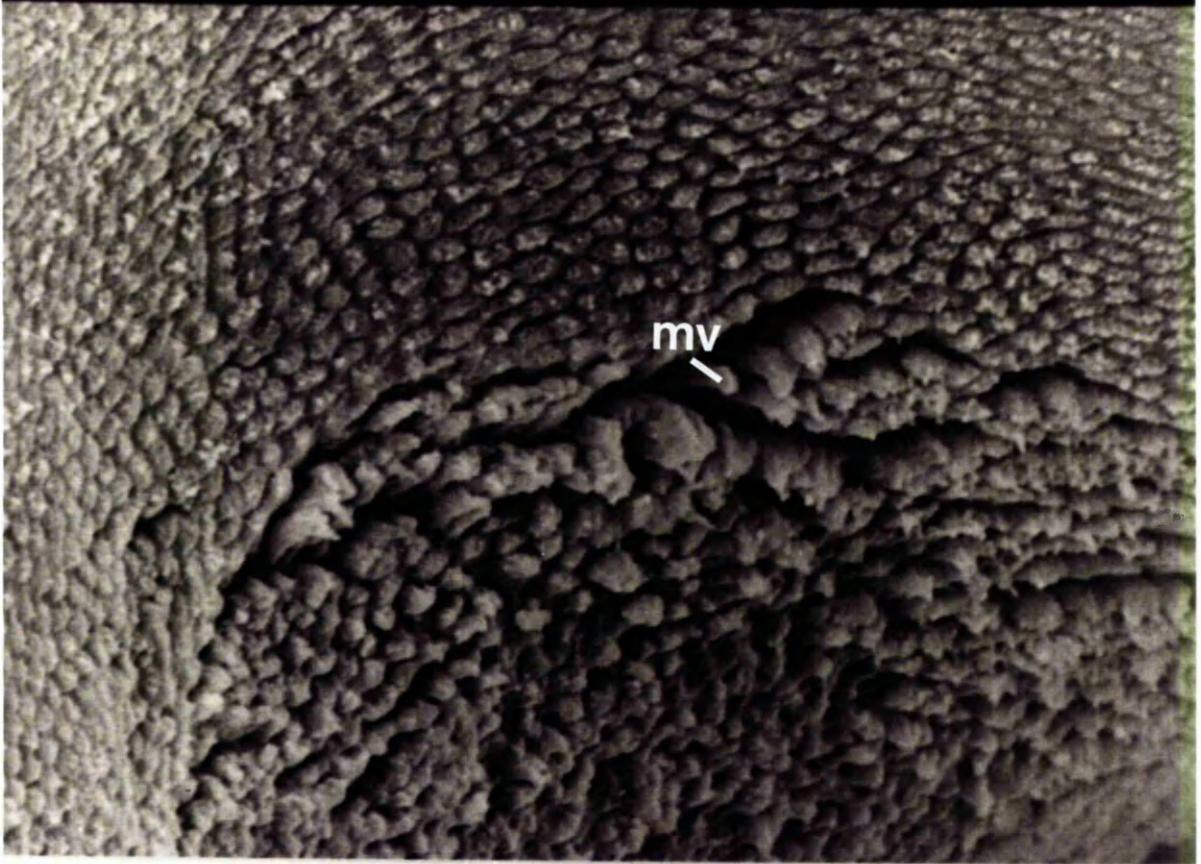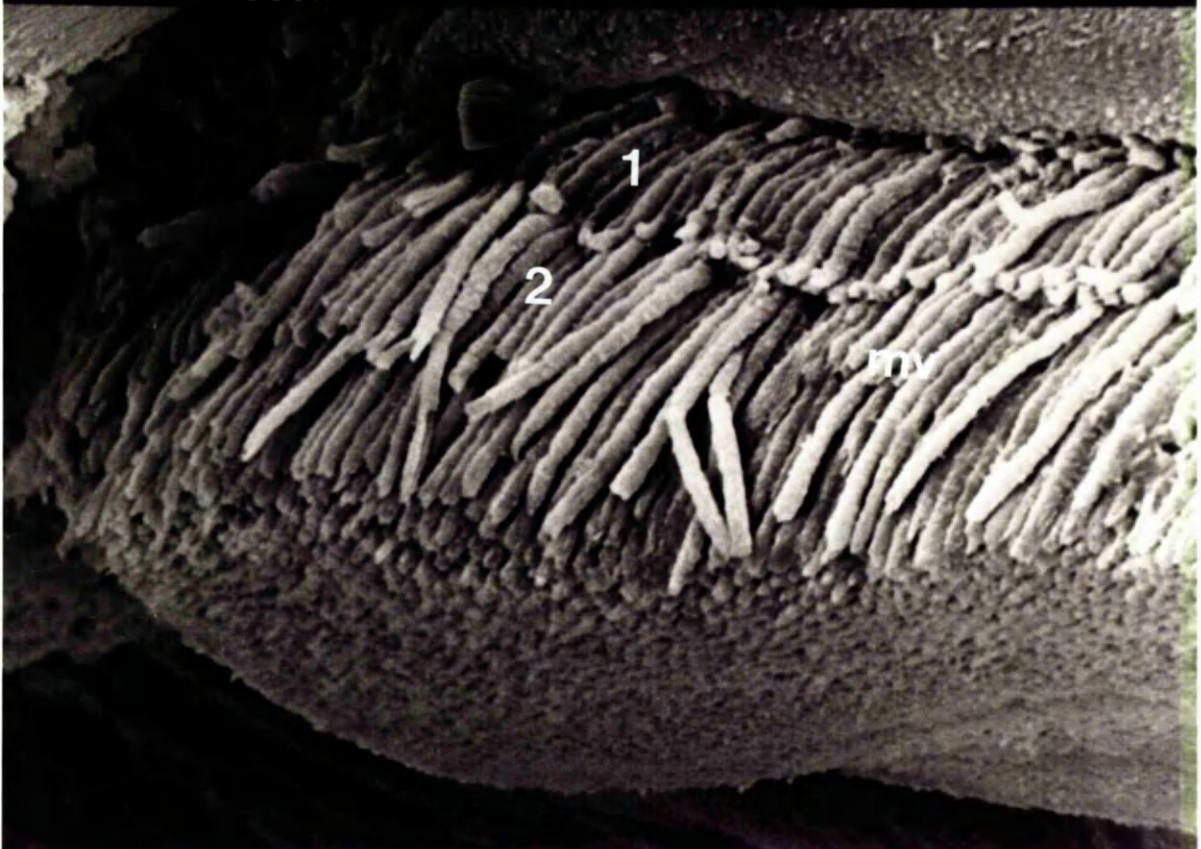
Figure 5.3

A) Transmission electron micrograph of a transverse section through the top of the large lip "microvilli". Each individual "microvillus" (mv) is clearly seen as a discreet structure. The "microvilli" are composed of a dense honeycombed core (darker material) surrounded by a less dense material. Scale bar = 5 μm.

B+C) Transmission electron micrograph of T. S. through the large lip "microvilli" (progressively deeper section to (A), i.e. closer to the epidermis). Individual microvillous structures appear to merge into an increasingly amorphous layer. Scale bars: B = 1 μm, C = 5 μm.

D) Transmission electron micrograph of T.S through the epidermal cells at the base of the large lip "microvilli". Cell membrane (cm), mitochondria (m) and rough endoplasmic reticulum (rer) are indicated. Scale bar = 1 μm.
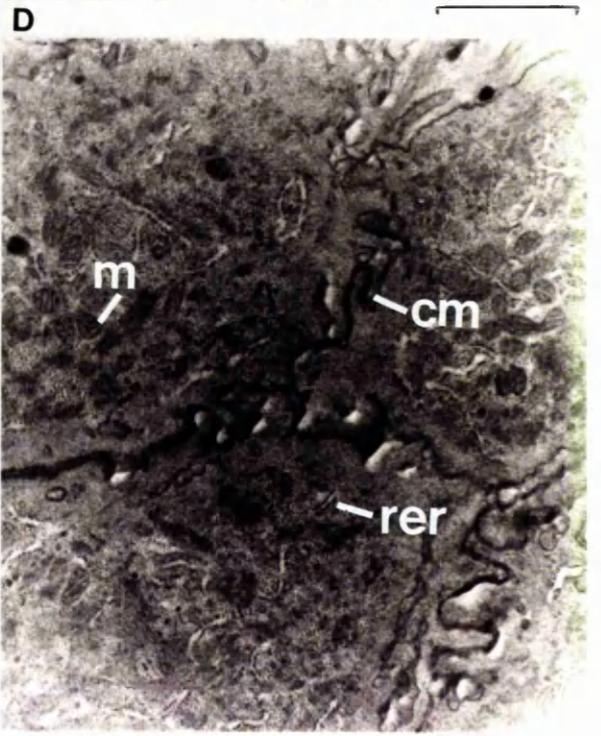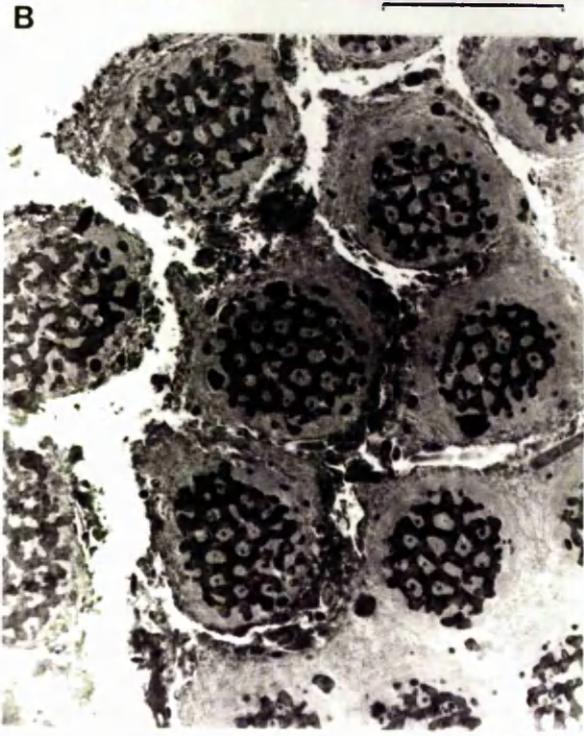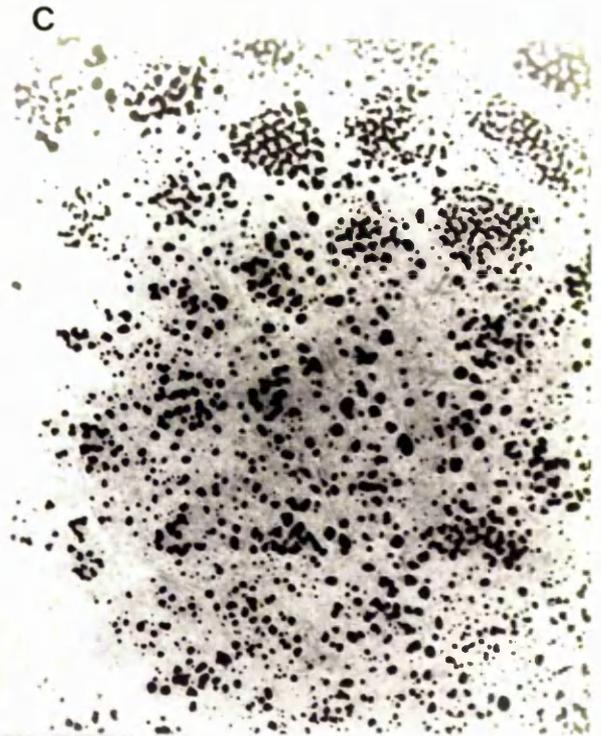
Sections from increasingly greater depths, show the "microvilli" structure to become increasingly amorphous nearer to the epidermis, with no evidence of mitochondria etc. (Figs. 5.3B,C). The epidermal cells at the base of the microvilli are in sharp contrast to the "microvilli", with mitochondria and other cell organelles clearly seen (Fig. 5.3D). Sections through the "microvilli" close to the epidermis, reveal the presence of occasional small clusters of cilia with the typical 9 + 2 arrangement of microtubular elements, surrounded by smaller microvilli (diameter 0.1 μm) (Fig. 5.4). This arrangement suggests that these cilia and microvilli originate from cells similar to the ciliated cells described in the oral area (see Fig. 5.5).

The oral area. SEM of the epidermis surrounding the lips (i.e. the underside of the head that is fully extended over the food substrate when the snail is actively feeding), shows it to be covered with a "carpet" of microvilli of approximate diameter 0.1 μm (Figs. 5.5 + 5.6). A "carpet" of microvilli is typical of pulmonate sensory epidermis (Emery, 1992). TEM of oblique sections taken from the oral area (Fig. 5.5B) show that the "carpet" of short microvilli arises from both non ciliated and ciliated epithelial cells. A coat of fine fibrillar material which extends beyond the tips of the microvilli is also seen (Fig. 5.5B). In addition to the microvilli "carpet" two types of candidate sensory structures were found using SEM. Figure 5.5A shows a SEM micrograph of the most common type: regularly spaced dense tufts of long cilia, approximately 10-15 μm long. TEM of longitudinal and oblique sections taken from this part of the oral area show that the 9 + 2 cilia and their basal feet have a parallel orientation (Fig. 5.5B). This suggests that these groups of cilia probably originate from non-sensory supporting epidermal cilia cells; Zylstra (1972) showed sensory free nerve endings to have a random orientation of cilia which was in contrast to the parallel orientation of cilia in non-sensory epithelial cells. However, it is possible that these ciliated cells are sensory,

Figure 5.4 Transmission electron micrograph of T.S through a clump of cilia (c) and small microvilli (mv), located in between the large lip "microvilli" close to the epidermis. Cilia show the 9+2 arrangement of microtubular elements. Scale bar = 0.2 μm.
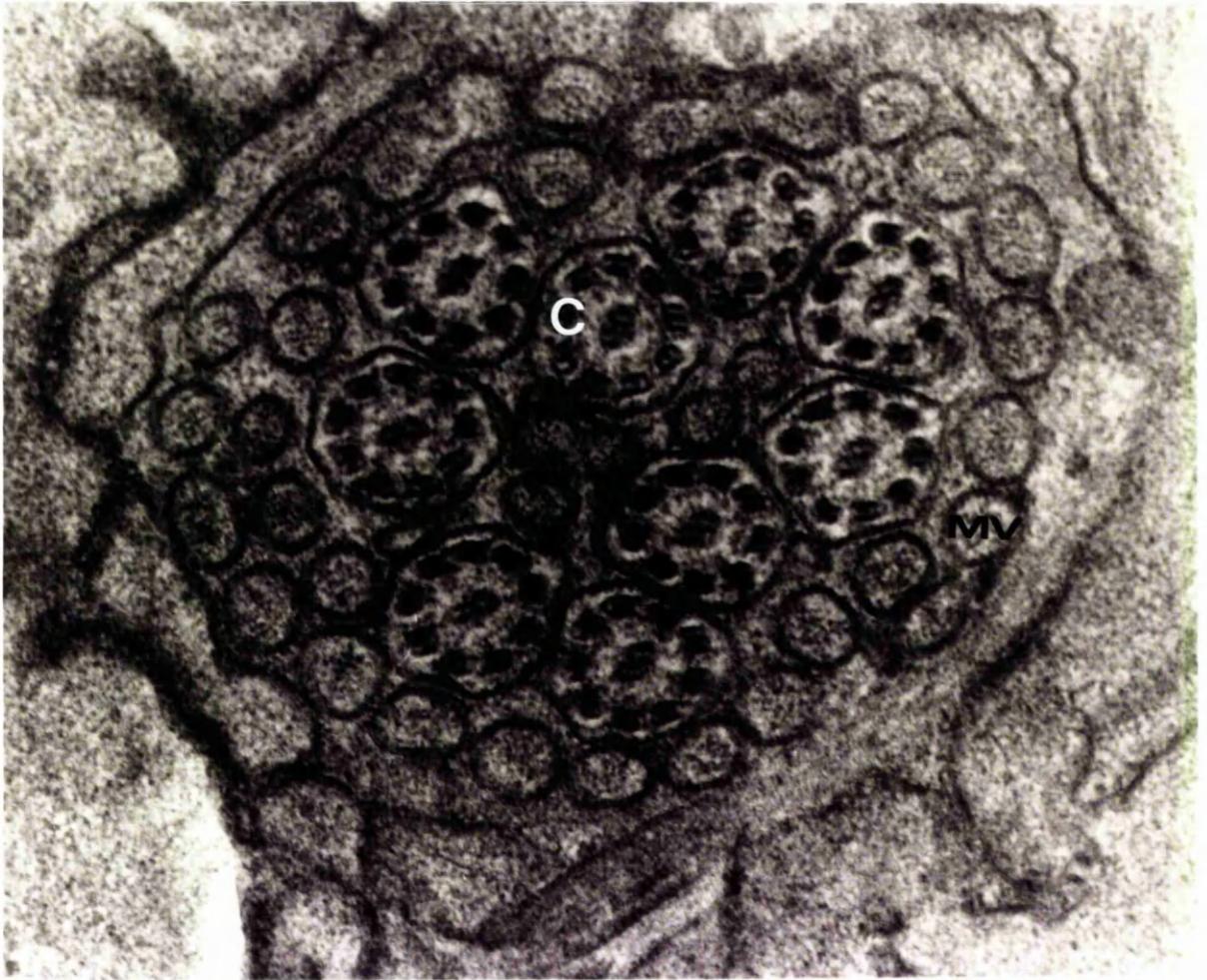
Figure 5.5

A) Scanning electron micrograph of the oral area showing dense tufts of cilia. Scale bar = 10 μm.

B) Transmission electron micrograph of an oblique section through the epidermis of the oral area. Both ciliated and non ciliated cells are seen to have microvilli (MV) at their surface. Cilia (C) and a fine fibrillar substance (F) extending beyond the microvilli tips are indicated. Note the parallel orientation of the cilia and basal feet (bf). Mitochondria (m) are indicated. Scale bar = 1 μm.

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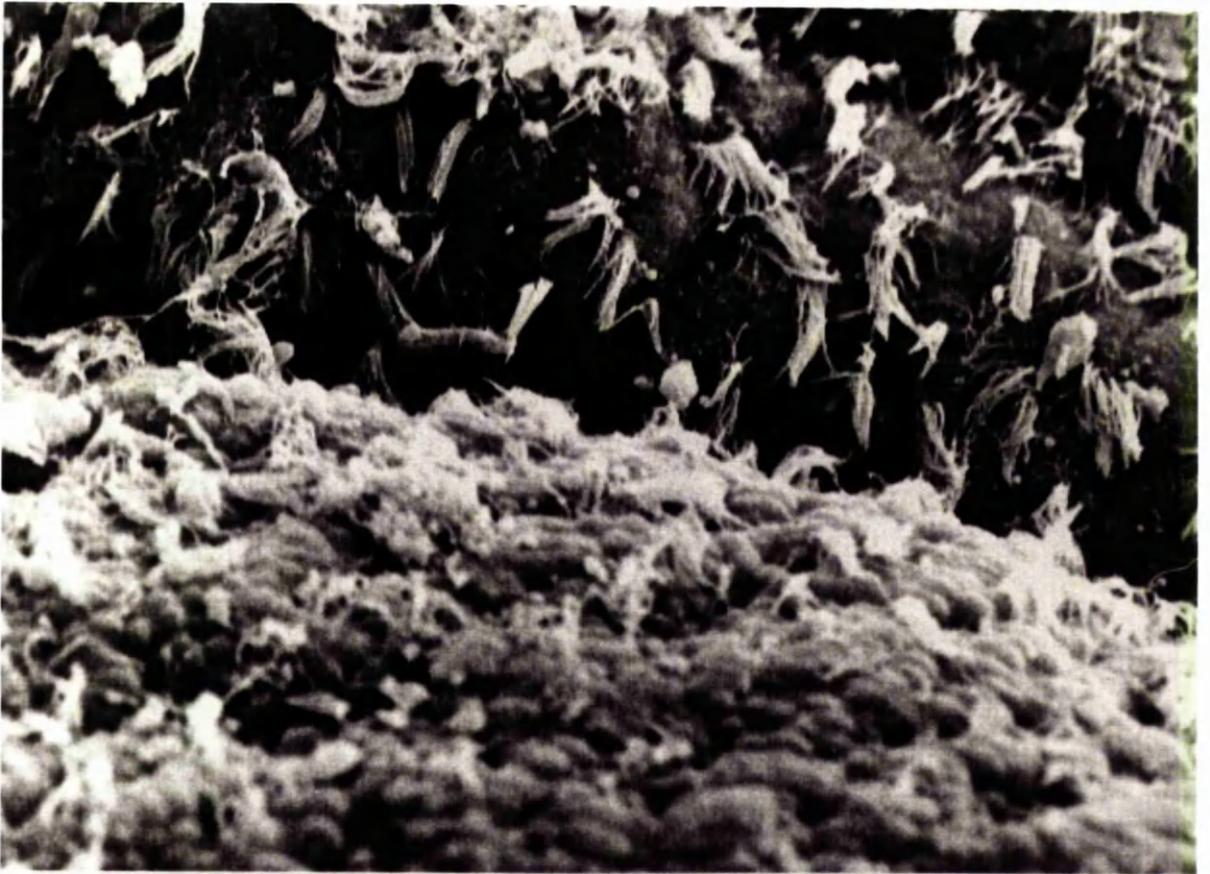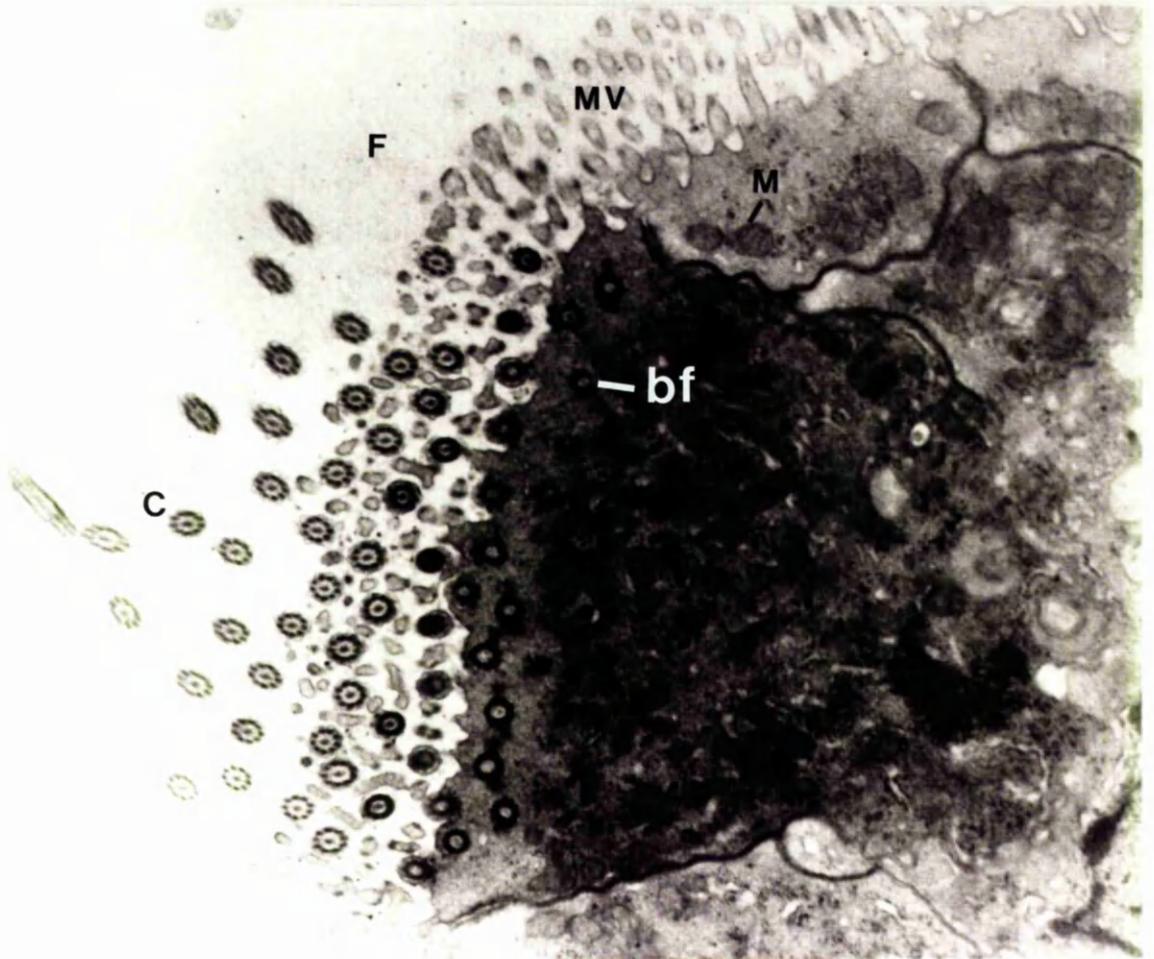
as free nerve endings Type 5 (described by Zylstra, 1972) were demonstrated to be predominantly found in the lip area, had between 10-40 cilia (length = 10 µm, 9+2 microtubular arrangement) at their surface, and sometimes could be confused with the non-sensory epidermal cilia cells (Zylstra, 1972). The second candidate sensory structure is shown in Figure 5.6. This structure was not as common as the cilial tufts, but in most cases the arrangement of a ring of 8-10 short (approximately 2 µm x 0.3 µm) microvilli/cilia surrounding a central two, was conserved. The microvilli/cilia are rigid and appear to arise from a "pit". This arrangement is more typical of sensory structures which could be involved in either mechano- or chemosensory signalling (Emery, 1992). TEM on longitudinal sections of the oral area did not locate these structures (presumably because their distribution was sparse), and so it is not clear whether they consist of microvilli or cilia. However, they are likely to be cilia, as similar structures composed of cilia were described on the tentacle of *Lymnaea* (Emery, 1992), and their diameter of 0.3 µm is more typical of cilia in this study (see TEM micrographs, Figs. 5.5, 5.7).

The tentacle

SEM of the ventral surface of the tentacle (i.e. the sensory area) demonstrated the presence of 1) tufts of long (>10 µm) cilia, and 2) clusters of shorter cilia (approximately 2 µm long) in either short stiff clumps or in a divergent circular structure (similar to the presumed sensory structures seen in the oral area) (Fig. 5.7). Both types of cilia emerge from a dense microvillous "carpet" (diameter approximately 0.1µm), as described by Emery (1992).

Figure 5.6 Scanning electron micrograph of the oral area showing circular arrangement of short cilia surrounding a central two short cilia. Cilia appear to arise from "pits" (arrow). Epidermal surface is covered by a "carpet" of microvilli (mv), typical of pulmonate sensory epidermis. Scale is indicated.

L= SE1   EHT= 25.0 KV   WD= 8   mm   MAG= X 14.0 K PHOTO= 17
2.00µm ├────────────┤
Holly oral veil single receptor

mv
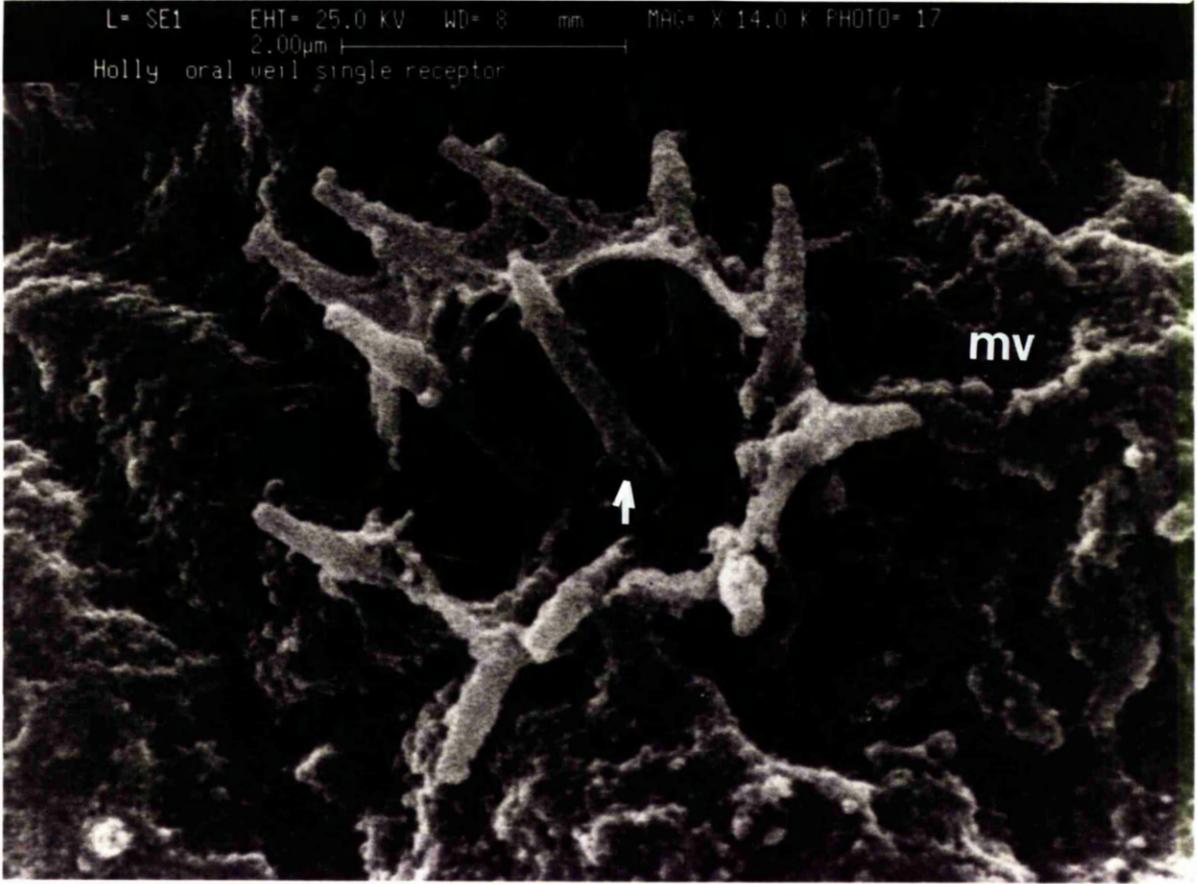
Figure 5.7 Scanning electron micrograph of the ventral surface of the tentacle showing tufts of long cilia. Clusters of shorter cilia in stiff clumps (A) and divergent circular structures (B) are also indicated. The epidermal surface is covered in a microvilli "carpet" (mv). Scale bar = 10 µm.
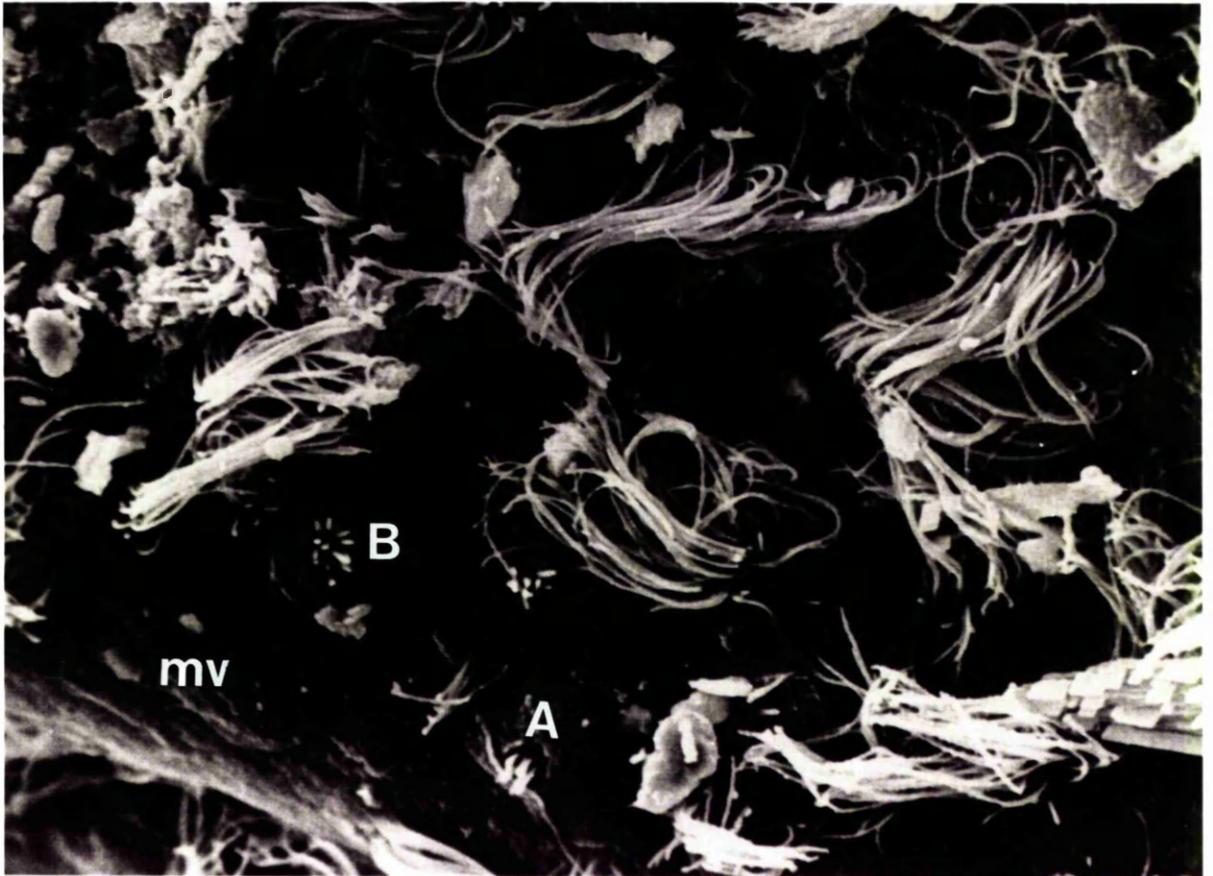
DISCUSSION

Similarities between the oral area and the tentacle.

The oral area and the ventral surface of the tentacles have very similar microvillous and cilia structures. This suggests that these areas serve a similar role, i.e. sensory transduction. In both areas, tufts of long cilia (>10 μm) and groups of rigid divergent cilia (<2 μm) were seen. The small groups of non divergent stiff cilia seen on the tentacles (this study and Emery, 1992) were not found in the oral area. This may be due to this structure being specific to the tentacles, but as the study was only preliminary, it may also be due to this structure being missed on the oral area. The ciliated structures, both on the tentacles and oral area, were shown to extend above a "carpet" of densely packed short microvilli. This "carpet" of microvilli (or microvilli border) is a feature of sensory epithelia of both terrestrial and aquatic pulmonates. In terrestrial snails and slugs (Stylommatophora), microvilli borders have been described in the olfactory area of the tentacular knob (Lane, 1963), and also in sensory region of the lips (Benedeczsky, 1977). The microvilli borders of terrestrial pulmonates differ from those of the aquatic pulmonates, in that the sensory cilia/microvilli do not extend above microvilli border, but are located in a spongy amorphous layer below the microvilli border; this is thought to be an adaptation to avoid drying out of the delicate membrane of the sensory structures (Emery, 1992). Therefore the microvilli border described in the oral area of *Lymnaea* is more typical of aquatic pulmonates, and is similar to the microvilli border described by Emery (1992) for *Lymnaea*'s tentacles, and also the olfactory organ of the freshwater snail *Helisoma trivolvis* (Emery, 1992). The fine fibrillar material which extended above the microvilli on the oral area of *Lymnaea*, was not described for the tentacles of *Lymnaea* (Emery, 1992). However, a similar material was described on the olfactory organ of *Helisoma*, but its function was unclear (Emery, 1992).

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It is not known whether either of the two types of cilial structures extending from the microvilli border in the oral area are sensory. However, it is likely that at least some of the cilia structures described are sensory, since cilia have been found to be the main chemoreceptive structures of sensory epithelia of molluscs; with ciliated sensory cells being described in the limpet (Hackney *et al.*, 1983), *Aplysia* (Emery and Audesirk, 1978), *Pleurobranchaea* (Davis and Matera, 1982; Matera and Davis, 1982), and many other gastropod species (for review see Emery, 1992). The study by Emery (1992) on *Lymnaea's* tentacles, demonstrated that tufts of longer cilia arose from the supporting epithelial cells, whilst the shorter stiff cilia arose from dendrites extending from clusters of olfactory neurons below the epidermis. The TEM of the oral area showed that the long cilia arising from the epidermal cells had parallel orientation, and this suggests that the long tufts of cilia may also (like the tentacular long cilia) originate from non-sensory supporting cells (Zylstra, 1972; Emery, 1992). However, the study is far from exhaustive and the TEM sections may have not included sections from different "sensory" tufts of long cilia. The TEM sections also did not locate the shorter "cilia", so it is unknown if these are sensory, or even definitely cilia. Before the exact role of these structures can be determined (i.e. whether they are mechano- or chemosensory structures) more detailed TEM studies on the oral area of *Lymnaea*, together with electrophysiological experiments, need to be carried out.

The lips

The structures described as the lips of *Lymnaea* were shown to be composed of densely packed large "microvilli". Considering the size of these structures (65 x 4 μm), it is surprising that they have not been described previously. The role of these large microvilli is not clear. One interesting observation is that the densely packed "microvilli" making up the lips, are similar to the microvillous "carpet" lining the sensory epithelium of *Lymnaea's*

tentacles and oral area, except that they are much larger. It is be possible, therefore, that *Lymnæa* has evolved a much larger microvillous "carpet" lining the mouth, to provide a rough surface for the radula to rub against (i.e. to act as a brush, directing the intake of food). This would aid the ingestion of slippery foods such as algae, the main food for *Lymnæa*. The "microvilli", however, may also be mechano- or chemosensory, with the sensory processing occurring at the epithelial cells at the base of the large structures. A study by Zylstra (1972) showed an abundance of sensory receptor cell bodies in "the region lining the mouth". This region described by Zylstra almost certainly corresponds to the "lips" described in this study, since the large lip microvilli line the inside of the mouth opening (although it does seem strange Zylstra did not report the presence of the large microvilli, which are difficult to miss!). Another possibility is that the clusters of cilia and smaller microvilli found in between the large lip microvilli are the main sensory structures in this area. This would suggest that, although the "microvilli" border is enlarged in the lip area to provide a mechanical "brush", sensory reception still occurs in this area by chemostimuli diffusing down to the sensory structures (cilia and microvilli) at the base of the microvilli. If this is the case, the lip area of *Lymnæa* would have evolved in a very similar way to that of the sensory epithelia of terrestrial pulmonates, i.e. the sensory structures have become embedded in the spongy, or amorphous, part of a microvillous "carpet". This process would, however, be an example of convergent evolution, since the reasons for the modification of the microvilli border are different in each case; *Lymnæa* needs the larger microvilli to provide extra friction to aid ingestion of food, and the terrestrial pulmonates need them to protect the delicate sensory receptors from drying out.

CHAPTER 6 - RESULTS

THE EFFECT OF ASSOCIATIVE CONDITIONING ON THE FEEDING SYSTEM OF *LYMNAEA*

THE EFFECT OF ASSOCIATIVE CONDITIONING ON THE FEEDING SYSTEM OF

LYMNAEA.

INTRODUCTION

There has been much research into the associative conditioning of gastropod molluscs, and a significant proportion of this work has involved modifying the feeding response, using both positive and aversive conditioning (see General Introduction). Neuroethological studies on *Pleurobranchaea* demonstrated that the PCNs (probable homologues of the CV1 population) were involved in modifying the feeding response following not only satiation, but also aversive conditioning to a particular food (Davis *et al.*, 1983). This suggested that the PC_ps (the putative homologue of $CV1_a$, Chapter 3 - this thesis) provided a locus at which the "decision" to feed was made, and that several different types of previous experience could modify the PC_p's ability to initiate feeding following application of food to the lips.

This Chapter investigates *Lymnaea's* ability to learn using a variety of different conditioning paradigms, and also further assesses the role of CV1 by investigating whether, like the PC_ps in *Pleurobranchaea*, it is involved in the modification of the feeding response following conditioning. The Results are divided into two main sections covering positive and aversive conditioning.

RESULTS A - POSITIVE CONDITIONING.

In 1982, Audesirk *et al.* demonstrated that *Lymnaea* showed positive conditioning (reward learning) when a neutral novel chemostimulus was paired with a feeding stimulus. In this section, this learning paradigm was exploited to see if the resulting behavioural changes (i.e. feeding in response to a neutral feeding stimulus), were correlated with changes

in neural activity in CV1 in the semi-intact preparation.

Positive conditioning paradigm. This was modified from Audesirk *et al.* (1982). Eighteen specimens of *Lymnaea* were divided into two groups: experimental positive conditioning (n=9) and random presentation control (n=9). Snails selected were from the same delivery batch and of medium size (approx. 1.5-2.0 g). This size was selected as learning is greatest in young snails (Audesirk *et al.*, 1982), and 1.5 g was found to be approaching the smallest size from which electrophysiological recordings could be made from CV1. Both groups of snails were starved for two days prior to the training to increase the motivation to learn (Audesirk *et al.*, 1982). On the first day of training the two groups were placed in separate tanks containing aerated snail water and a removable fine mesh lining. The mesh lining enabled the transfer of each group of snails to different tanks with the minimum of disturbance.

The conditioned stimulus (CS) was a 0.004% solution of amyl acetate ("pear drop" flavour) and the unconditioned stimulus (US) a 0.67% sucrose solution. The experimental group received 15 training trials (CS-US pairings) over a three day period: five trials a day with an inter-trial period of 90 minutes. At each training trial the experimental group was transferred (on the mesh) to a small tank containing 200 ml snail water. 50 ml of stock solution (0.024%) amyl acetate was then poured into the tank taking care not to pour the liquid directly on to the snails. Ten seconds later, 50 ml of sucrose stock solution (4%) was added in the same way and the snails left in this mixture for the next two minutes. The snails were then transferred to a rinse bin containing fresh snail water and then placed back in their original tank. The random control group received a snail water-US (sucrose) pairing each time the experimental group received a CS-US pairing (snail water was used as a substitute for the US to equalise mechanical disturbance), and then, at a random time in the inter-trial period,

received a CS (amyl acetate)-water pairing. The experimental group also received a water-water pairing at this random time, so that both groups received the same amount of handling throughout the experiment.

Behavioural testing was carried out blind, 24 hours and four days after the end of training. Results are presented as latency to bite (sec) and number of bites in two minutes following application of 0.004% amyl acetate to the lips. If no biting response was seen in two minutes individuals were awarded a latency score of 120 seconds.

The latency to bite and number of bites in response to 0.004% amyl acetate before (naive), 24 hours and four days after training are shown in Figure 6.1. The data were not assumed to be normally distributed and therefore were presented as the median and interquartile ranges. Prior to training (i.e. naive) there was no response to amyl acetate in either group (median latency = 120 sec, and median number of bites = 0.5). This was an important observation as it showed that *Lymnaea* does not to respond to amyl acetate before training. Twenty four hours after training the experimental group showed a significantly higher response to amyl acetate as a feeding stimulant compared to that of the random controls, with 100% of the experimental group responding. The decrease in latency to bite was significant $p < 0.025$ level, and increased number of bites significant $p < 0.001$ level (Mann-Whitney U test). Four days after training the learned response was fully retained; the biting response to amyl acetate in the experimental group (compared to random controls) was at the same level as at 24 hours (Fig. 6.1). This is in agreement with Audesirk *et al.* (1982) who found the memory to be retained for over two weeks.

Electrophysiological recordings.

Experiments were carried out two to four days after training, as the behavioural

Figure 6.1 The feeding response to 0.1 ml 0.004% amyl acetate before, and after positive conditioning (at one and four days).

A) Latency to bite. Prior to training (i.e. Naive) snails from both groups (n=18) show no response to amyl acetate as a feeding stimulus. This is denoted by the median latency to bite = 120 seconds (i.e. the arbitrary value awarded if no biting was seen in the 2 minutes after application of amyl acetate to the lips). At 1 day after training the experimental conditioned snails (experimental) show a significant decrease in latency to bite as compared to the random presentation control group (random contr) who show no response to amyl acetate ($p < 0.05$ level, Mann-Whitney U test, n=9 both groups). The significant decrease in latency to bite in the experimental group compared to the random controls is still at the same level four days after training ($p < 0.05$ level, Mann-Whitney U test, n=9 both groups).

B) Number of bites in two minutes. Prior to training (i.e. Naive) snails from both groups (n=18) show no biting in response to amyl acetate. The median is 0.5 (greater than zero) due to infrequent spontaneous biting. At 1 day after training the experimental conditioned snails show a significant increase in the number of bites in two minutes following amyl acetate as compared to the random presentation controls ($p < 0.001$ level, Mann-Whitney U test, n=9 both groups). This significant increase in biting in the experimental group is retained at the same level four days after training ($p < 0.001$ level, Mann-Whitney U test, n=9 both groups)

Data in both graphs are presented as medians and interquartile ranges. ** = $p < 0.05$ level, *** = $p < 0.001$ level, Mann-Whitney U test)

A

B

191
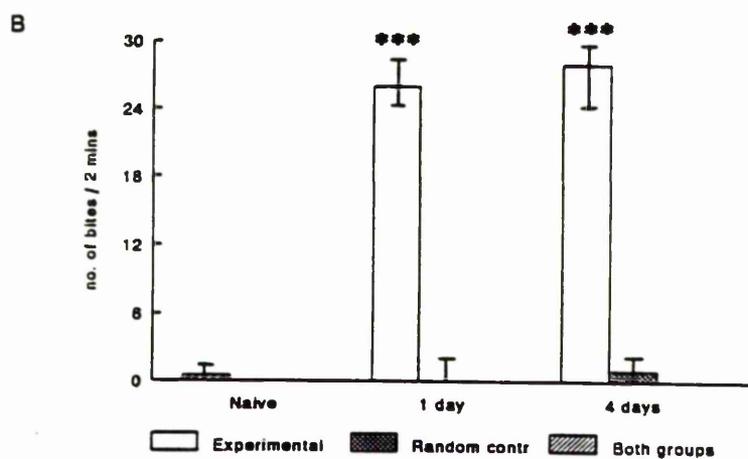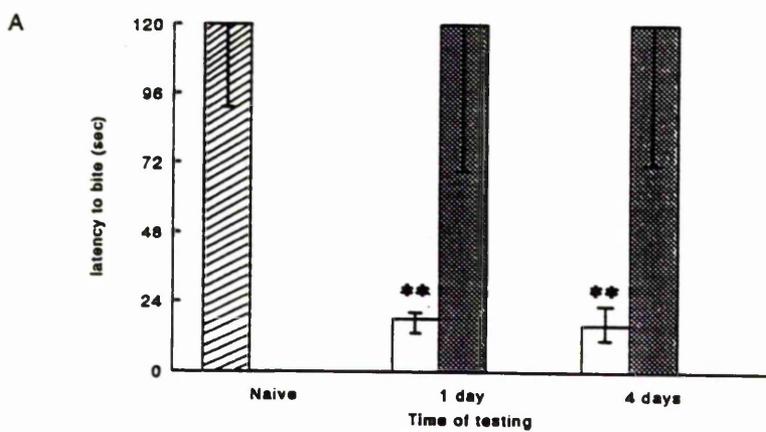
memory after positive conditioning had been shown to be retained fully over this time period. Recordings were made from CV1 and identified buccal motoneurons using semi-intact preparations taken from both experimental group (n=17) and random control group (n=16) snails from four further training experiments.

Experimental group In 5/17 semi-intact preparations, application of 0.004% amyl acetate to the lip tissue led to a depolarisation of CV1's membrane potential (by approximately 4 mV) and rhythmic bursting of CV1. This resulted in initiation of feeding motor output, as seen by rhythmic bursting activity in identified buccal motoneurons (Fig. 6.2A). The rhythmic bursting in CV1 was accompanied by an increase in EPSPs and temporary reduction in IPSPs. The IPSPs returned after approximately 15-20 seconds (Fig. 6.2A).

In 8/17 preparations, application of 0.004% amyl acetate did not lead to initiation of feeding motor output but still led to excitation in CV1. This was manifest as either: 1) a change of inputs received by CV1 (i.e. an increase in EPSPs and decrease in IPSPs; not shown); or 2) increased intensity (Fig. 6.2B) and/or frequency of ongoing feeding motor output.

Experiments using an isolated cerebral ganglia-lip preparation from experimental group snails (n=4) still showed amyl acetate to be excitatory, indicating that this "learnt" feeding response occurs in the cerebral ganglia and is not via the buccal ganglia (Fig. 6.3A). However this does not rule out the possibility that positive conditioning also results directly in changes in neural activity in buccal neurons.

In the remaining 4/17 preparations application of 0.004% amyl acetate resulted in no change in activity of, or inputs to, CV1 or the identified feeding motoneurons (Fig. 6.3B). This lack of response could be due to damage of the lip nerves during dissection, having too little of the lip tissue left attached to the brain, or to the feeding system being in an

Figure 6.2 The effect of 0.1 ml 0.004% amyl acetate application to the lip tissue on the activity in CV1 in a semi-intact preparation taken from an experimentally positively conditioned snail.

A) Initiation of feeding motor output. Amyl acetate application to a quiescent preparation results in CV1 receiving less IPSPs and an increase in EPSPs. CV1's membrane potential is depolarised by approximately 4 mV and bursts of action potentials are seen. This is accompanied by an initiation of feeding motor output as seen by rhythmic activity in the identified buccal motoneuron B4 and CV1. Phases of the feeding cycle (N1 and N2) are indicated. IPSPs are seen to return to CV1 approximately 12 seconds after application. NB: Spikes in B4 are attenuated.

B) Increasing the intensity of feeding motor output. Application of amyl acetate to a preparation showing ongoing feeding motor output results in an excitation of CV1 seen as increased frequency and duration of firing in CV1. This results in an intensification of feeding motor output as seen by stronger bursting activity of B3.

A

B4

N1 | N2

CV1

EPSP

AA

IPSP return

20mV

2sec

B

CV1

AA

20mV

2sec

B3
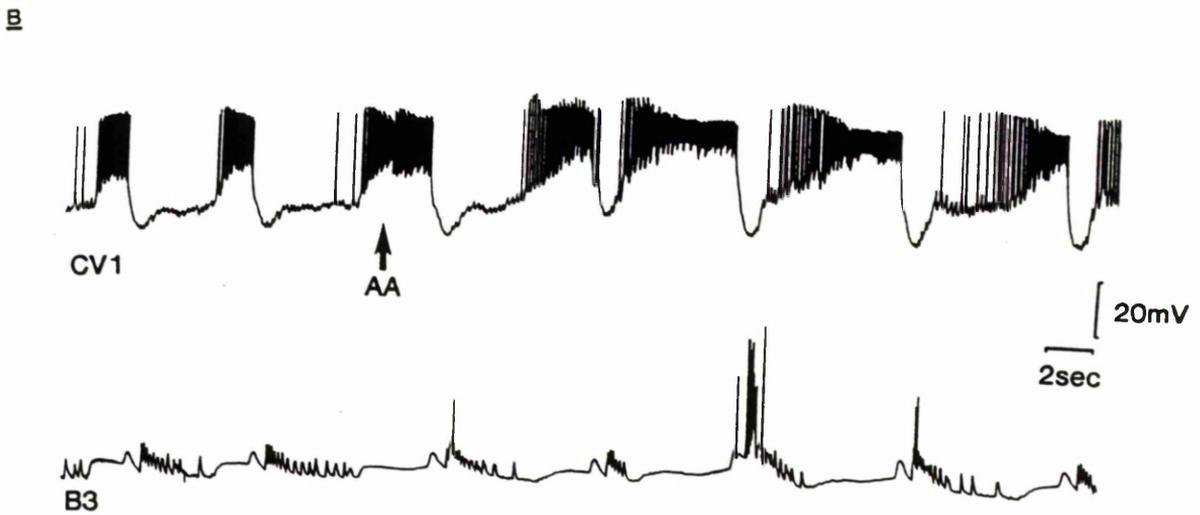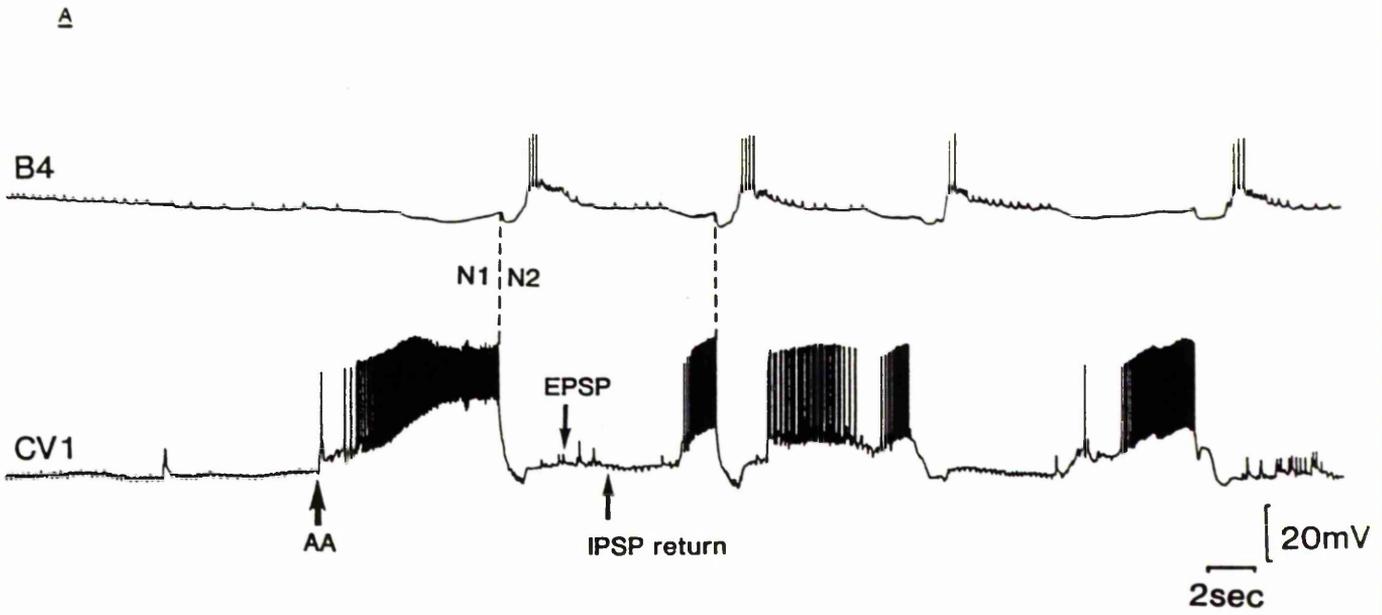
Figure 6.3 A) Effect of application of amyl acetate to the lip tissue of a cerebral ganglia-lip preparation taken from a positively conditioned snail. CV1 is initially quiescent and receives both IPSPs and EPSPs. Application of amyl acetate results in increased EPSPs to CV1 and rhythmic bursting activity. Note the absence of the deep N2 input after CV1's burst; this is because the cerebral ganglia do not receive inhibitory feedback from the buccal central pattern generator during the N2 phase of feeding.
B) Application of 0.1 ml 0.004% amyl acetate to lip tissue of a semi-intact preparation taken from positively conditioned snail results in no change in the inputs to, or activity of, CV1. This may be due to damage of the preparation during dissection or the feeding system being in an unresponsive state (see text and Chapter 3).
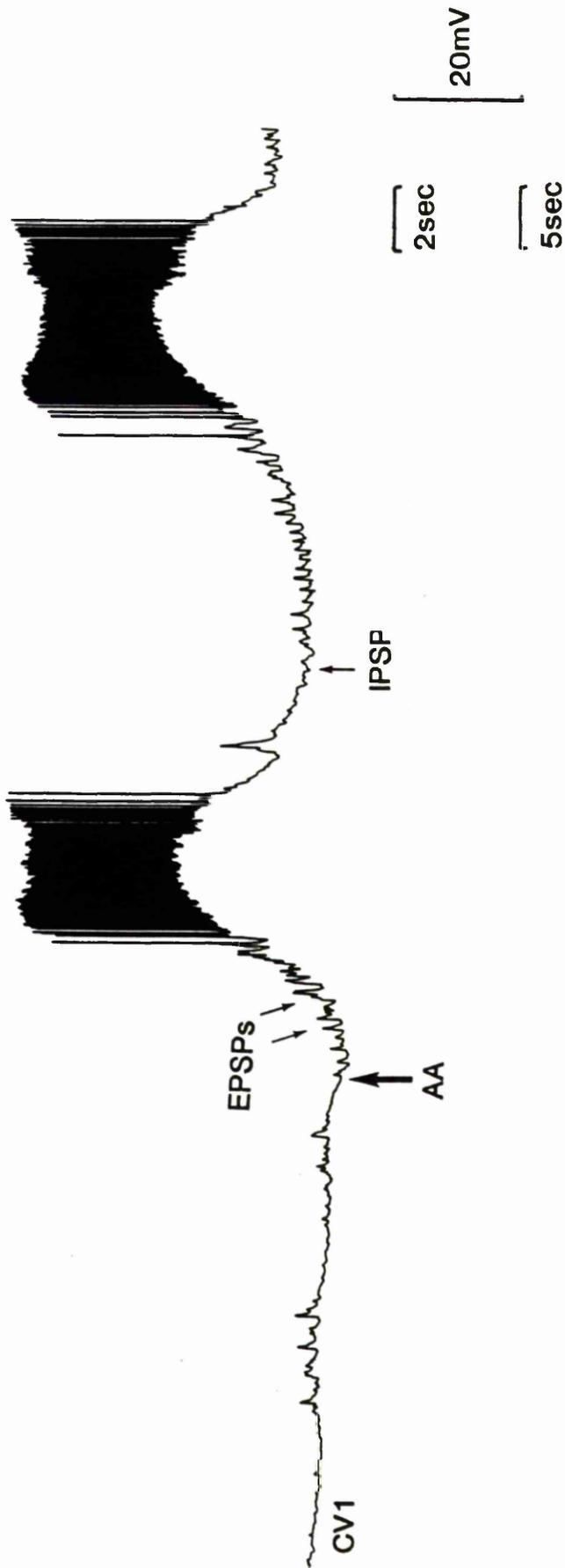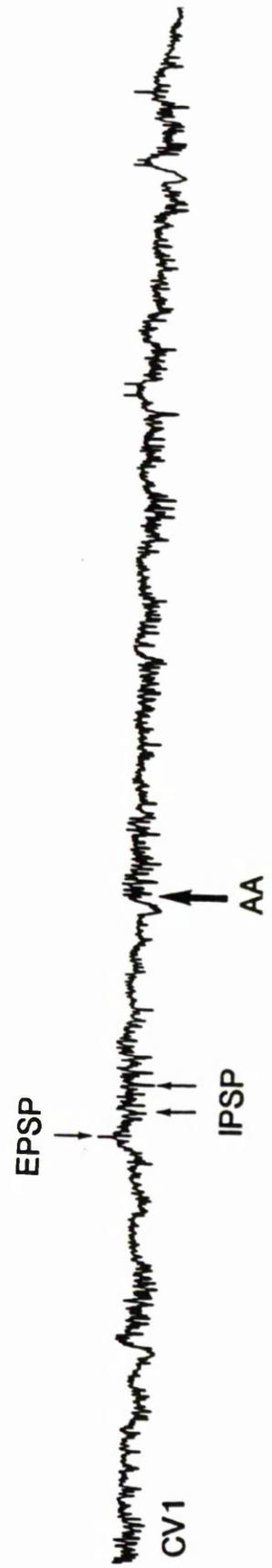
"unresponsive state" (Chapter 3).

Random control group - In 12/16 preparations, application of 0.004% amyl acetate to the lip tissue did not result in any change in activity in, or inputs to, CV1 (Fig. 6.4A). Application of amyl acetate did not cause any change in buccal motoneuron activity, which was in sharp contrast to the excitatory effect seen following application of 10^3 M sucrose to the same preparation, which led either to initiation or increased frequency of feeding motor output (Fig. 6.4B).

In 4/16 preparations, although application of amyl acetate did not lead to initiation of typical feeding motor output, it did appear to have an excitatory effect on CV1, causing increased EPSPs and intensification of ongoing bursting activity (Fig. 6.5). Therefore, the possibility that amyl acetate is weakly excitatory to CV1 (at least at the neural level) cannot be ruled out, and this questions the validity of amyl acetate being termed a "neutral feeding stimulus".

RESULTS B - AVERSIVE CONDITIONING

Although there have been several studies of positive conditioning in gastropod molluscs, the vast majority have used aversive conditioning of one kind of another. This is presumably due to the relatively faster speed of acquisition and greater strength of aversive conditioning as compared to non-aversive paradigms (Alexander *et al.*, 1982).

No previous reports were found on the aversive conditioning of *Lymnaea* to food, so the first aim of this section was to develop an aversive conditioning paradigm for *Lymnaea*, which involved conditioning the snail against a feeding stimulus. As stated earlier, electrophysiological studies showed that the PC_ps of *Pleurobranchaea* were involved in

Figure 6.4 The effect of application of 0.1 ml 0.004% amyl acetate to the lip tissue of a semi-intact preparation taken from a random presentation control animal.

A) Application of amyl acetate does not result in any change in inputs to, or activity of, CV1. (c.f. Figure 6.2A where amyl acetate to the lips of a positively conditioned snail results in an excitation and intensification of CV1's ongoing feeding motor output activity).

B) i) Application of amyl acetate does not result in initiation of feeding motor output, or any change in inputs to the identified motoneuron B4. ii) Application of 0.1 ml 10^{-3} M sucrose to the same preparation, however, results in initiation of feeding motor output, as seen by rhythmic bursting activity in B3. The three phases of feeding (N1, N2 and N3) are indicated.

A

CV1

AA

20mV

2sec

B i)

B4

AA

ii)

B3

SUCROSE

40mV

2sec

contd.

N1
N2
N3
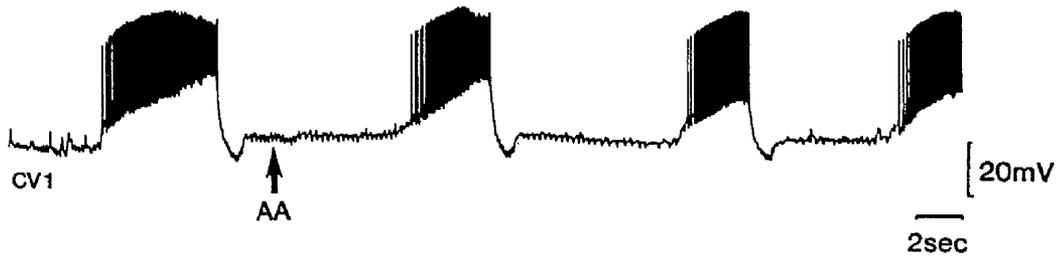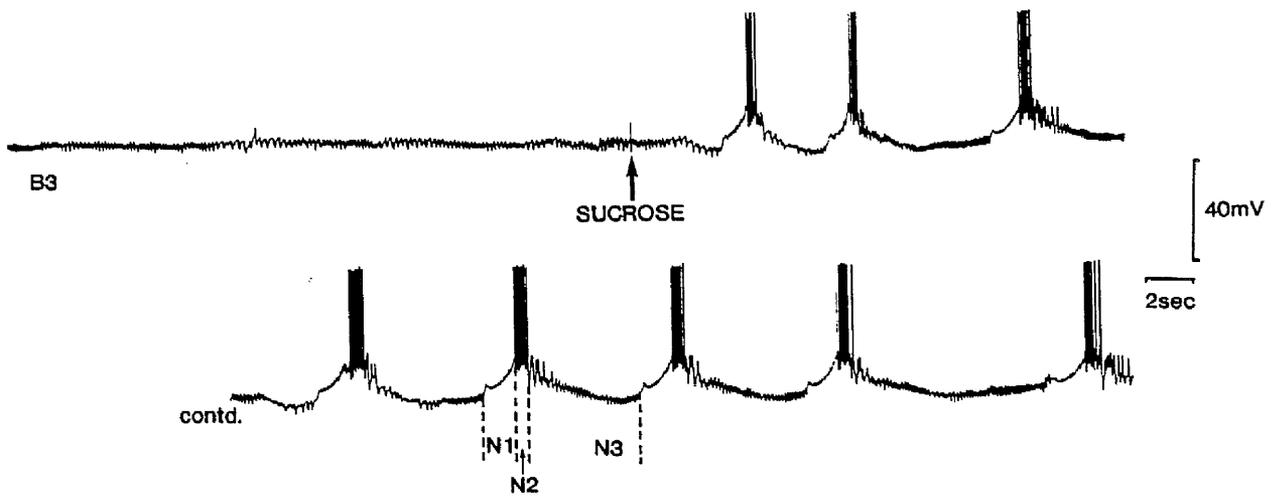
Figure 6.5 Application of amyl acetate results in a mild excitation of CV1 in a preparation taken from a random control group animal. This is seen as increased EPSPs and an intensification of bursts in CV1. There is little or no change in frequency of the feeding rhythm monitored from buccal motoneuron B4.

EPSP

AA

CV1

B4

20mV

2sec

201
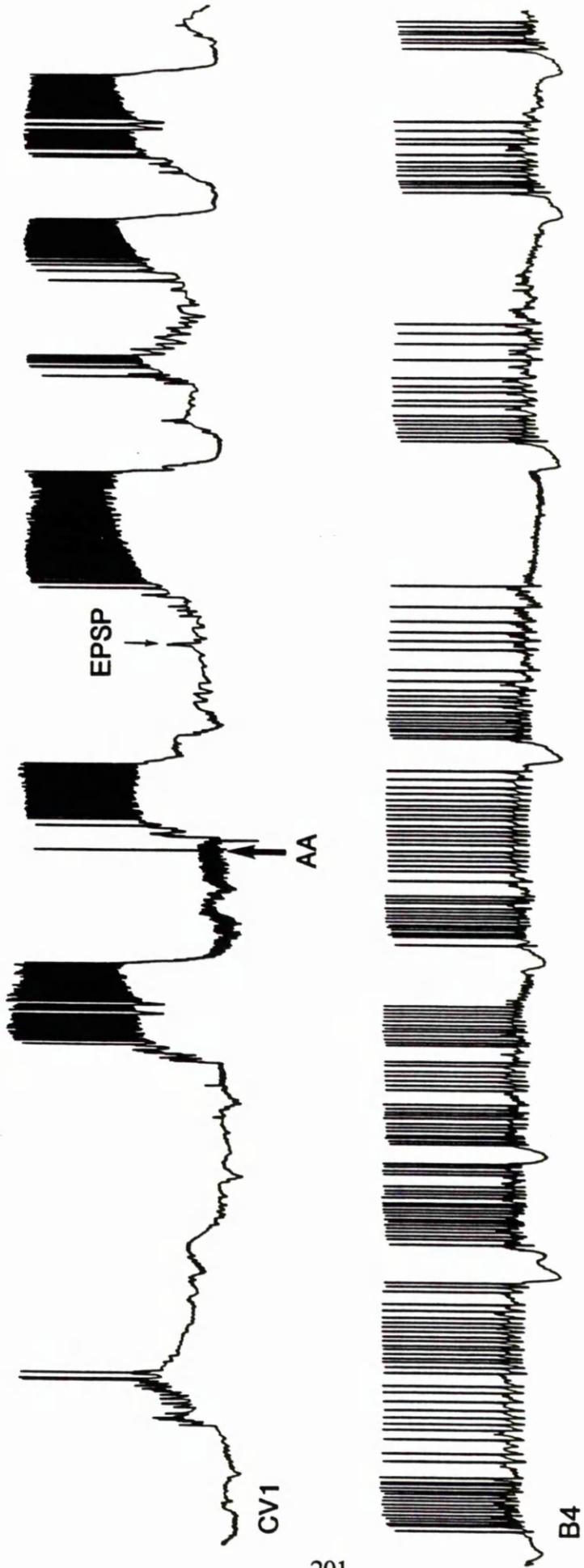
modifying feeding responses following aversive conditioning (Davis *et al.*, 1983), so the second aim of this section was to see if the presumed homologous interneuron in *Lymnaea* (CV1) was involved in modifying the feeding response following aversive conditioning.

Aversive conditioning paradigms

Experiment 1 - Sucrose / electric shock pairing.

The literature shows that the pairing of a strong electric shock with a food stimulus reliably led to rapid food aversion conditioning after only a few trials in several different species of gastropod molluscs (e.g. *Pleurobranchaea*; Mpitsos and Davis, 1973, Mpitsos and Collins, 1975, Davis *et al.*, 1980, Mpitsos and Cohen, 1986a-c, Mpitsos *et al.*, 1988; *Aplysia*, Carew *et al.*, 1981, Walters *et al.*, 1981; *Helix*, Maksimova and Balaban, 1984, Balaban *et al.*, 1987). An electric shock was therefore used as the aversive stimulus (US) in the first aversive conditioning paradigm used on *Lymnaea*. The CS was a 10^{-3} M solution of sucrose.

Eighteen specimens of *Lymnaea* were randomly divided into two groups: experimental (n=9) and random presentation control (n=9). Snails selected were of medium size (approx. 1.5-2.0 g), as this size of snail was found to be capable of associative learning in the positive conditioning experiments. The experimental group received 15 training trials (CS-US pairings) over a three day period: five trials a day with an inter-trial period of 90 minutes. This timing was expected to be amply sufficient, since: 1) several positive conditioning studies had shown this timing interval to be the optimum for associative learning in *Lymnaea* (Alexander *et al.*, 1982; Kemenes and Benjamin, 1989); and 2) it is generally accepted that aversive conditioning is more rapidly acquired than positive conditioning.

At each training trial, snails from the experimental group were removed from the home tank and placed individually into separate Petri dishes containing a 0.5 mm depth of snail water. The snails were allowed 5 minutes to acclimatise to the dish, and then 0.1 ml of 10^{3}

M sucrose (CS) was dripped close to the lips of the animal. One to two seconds after the presentation of the CS, the snail was given an electric shock (US), strong enough to cause immediate and full withdrawal into the shell followed by normal recovery. The electric shock was delivered into the snail water by placing electrodes (thin tungsten wire) either side of the animal. The shock was at 25 V (10 msec pulses at a rate of 50 per second) for 2 seconds. After each training trial, snails were immediately placed back into the home tank. The random control received a snail water-US pairing each time the experimental group received a CS-US pairing, and then at a random time in the inter-trial period received the CS (sucrose). The experimental group also received 0.1 ml snail water at this random time, so that both groups received the same amount of handling throughout the experiment. Behavioural testing was carried out blind, 1 hour and 24 hours after the last training trial. At each testing individual snails received 0.1 ml 10^3 M sucrose (CS) to the lips, and 1) strength of withdrawal 2) latency to bite, and 3) number of bites in two minutes, was noted.

The response to 10^3 sucrose solution is shown in Figure 6.6. Data were not assumed to be normal and were presented as medians and interquartile ranges. A full conditioned aversive response to sucrose (i.e active withdrawal) was not seen at either 1 hour or 24 hours after training. However, 1 hour after training the experimental group showed a significantly longer latency to bite ($p < 0.001$ level, Mann-Whitney U test) and a reduced number of bites in response to sucrose ($p < 0.005$ level, Mann-Whitney U test) as compared to the random control group. At 24 hours after training, however, there was no significant difference between the two groups in latency to bite or number of bites in two minutes following application of 0.1 ml 10^3 M sucrose to the lips (Mann-Whitney U test); i.e. the experimental snails showed no retention of the associative learning at 24 hours after training.

Although the paradigm used in Experiment 1 did lead to associative learning in the experimental animals, this learning was weak. Application of sucrose resulted only in a reduction in the feeding response, rather than causing active withdrawal. Also, retention of the learned association was short-lived; there was no retention at only 24 hours after training. This made it necessary to review the conditioning paradigm used.

First, the effectiveness of the US was questioned. The 25 V shock had been used as it was sufficient to produce complete withdrawal without any obvious long-term deleterious effects or death; therefore, in theory, it should have been a strong enough aversive stimulus. Pilot studies had shown that a delivery of a stronger shock was not possible as any greater voltage resulted in the death of a large a number of snails. However, the possibility that the short-lived, weak associative learning was due to *Lymnaea*s not strongly "recognising" an electric shock as an aversive stimulus (i.e. it was not environmentally relevant, as *Lymnaea* would never come across this type of stimuli in the wild) had to be investigated.

Experiment 2 - *Sucrose / mechanical stimulus pairing.*

Several studies on aversive conditioning in gastropod molluscs used noxious mechanical stimulation as an aversive stimulus (US) (Lukowiak and Sahley, 1981; Lukowiak, 1986; Balaban, 1993). Also, in Chapter 4 of this thesis, a mechanical "prod" to the head of *Lymnaea* was shown to induce withdrawal and inhibit ongoing feeding movements for the duration of the withdrawal. Taking this into account, a mechanical prod seemed to be a relevant aversive stimulus to use in conditioning *Lymnaea*.

Sixteen specimens of *Lymnaea* were divided into two groups: experimental (n=8) and random presentation control (n=8). Training was carried out in exactly the same manner to Experiment 1, except the US was a mechanical "prod" delivered by sharply poking the snail on the head with a blunt probe, with sufficient force to induce a strong withdrawal, but not

cause damage to the skin.

The response to 10^{-3} M sucrose is shown in Figure 6.7. As with Experiment 1, testing at both 1 and 24 hours showed no conditioned active withdrawal by the experimental group in response to sucrose. However, at one hour after training the experimental group showed a significant increase in latency to bite ($p<0.002$ level, Mann-Whitney U test) and decrease in number of bites in 2 minutes ($p<0.007$ level, Mann-Whitney U test) in response to sucrose, as compared to the random controls. This association, similar to Experiment 1, had been almost "forgotten" at 24 hours after training. There was no significant difference in the number of bites in two minutes between the two groups (Mann-Whitney U test) and the slight increase in latency to bite was only significant to the 0.1 level (Mann-Whitney U test).

Although the two experiments cannot be directly statistically compared as they were carried out using different groups of snails on separate days, these results suggest that there is little difference in 1) the strength of associative learning at 1 hour after training, or 2) the retention of the association, using either an electric shock or a mechanical prod as the US. Further experiments were therefore carried out, to investigate whether the strength of learning increased after changing other factors within the conditioning paradigm. A mechanical prod was kept as the US, as delivery posed less problems than administering an electric shock (e.g. no possibility of mechanical disturbance to the snail whilst positioning the electrodes).

Experiment 3 - *Fish food / mechanical prod pairing*

Studies on conditioning in several gastropod mollusc have shown that the strength of aversive conditioning is not only dependent on the US but also on the choice of CS. In *Pleurobranchaea* it was shown that individuals learnt much better when a CS of Budweiser

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Figure 6.6 The feeding response to 0.1 ml 10^3 M sucrose 1 and 24 hours after aversive conditioning to sucrose. The training paradigm consisted of fifteen training trials (CS-US pairings) over a period of three days; CS = 10^3 M sucrose and US = an electric shock (see text for details).

A) Latency to bite. One hour after training the experimentally conditioned group (experimental) show a significant increase in latency to bite compared to the random presentation control ($p < 0.001$, Mann-Whitney U test, n=9 each group). At 24 hours after training, however, there is no significant difference between the two groups, with the experimental group's median latency to bite returning to the random control levels.

B) Number of bites in two minutes. One hour after training the experimental group show a significant decrease in number of bites in two minutes as compared to the random presentation control ($p < 0.005$ level, Mann-Whitney U test, n=9 both groups). At 24 hours however, the number of bites shown by the experimentally conditioned group has returned to control levels with no significant difference between groups (Mann-Whitney U test).

Data for both graphs are presented as medians and interquartile ranges. *** = $p < 0.001$ level, ** = $p < 0.005$ level, Mann-Whitney U test.

A

B

Experimental   Random Contr
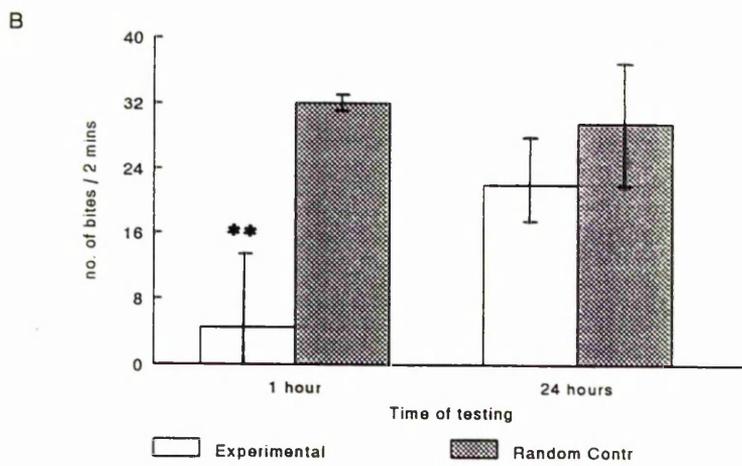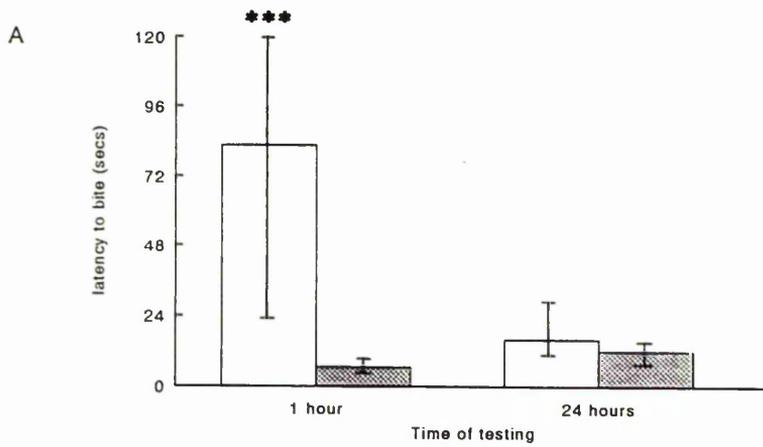
Figure 6.7 The feeding response to 0.1 ml 10^3 M sucrose 1 and 24 hours after aversive conditioning to sucrose. The training paradigm consisted of fifteen training trials (CS-US pairings) over a period of three days; CS = 10^3 M sucrose and US = a mechanical prod to the head (see text for details).

A) Latency to bite. One hour after training the experimentally conditioned group (experimental) show a significant increase in latency to bite compared to the random presentation control (p< 0.002 level, Mann-Whitney U test, n=8 each group). In fact, the median value for latency to bite of the experimentally conditioned group = 120 seconds (i.e. the arbitrary value awarded if no biting was seen in the 2 minutes after application of sucrose to the lips). At 24 hours after training, however, the median value for the latency to bite in the experimentally conditioned group is nearly back at control levels, with the difference in latency to bite between the two groups only just being significant (p<0.1 level, Mann-Whitney U test).

B) Number of bites in two minutes. One hour after training the experimental group show a significant decrease in number of bites in two minutes as compared to the random presentation control (p< 0.01 level, Mann-Whitney U test, n=8 both groups). At 24 hours however, the number of bites shown by the experimentally conditioned group has returned to control levels with there being no significant difference between groups (Mann-Whitney U test).

Data for both graphs are presented as medians and interquartile ranges. *** = p< 0.002 level, ** = p< 0.01 level, * = p< 0.1 level, Mann-Whitney U test.

A

B
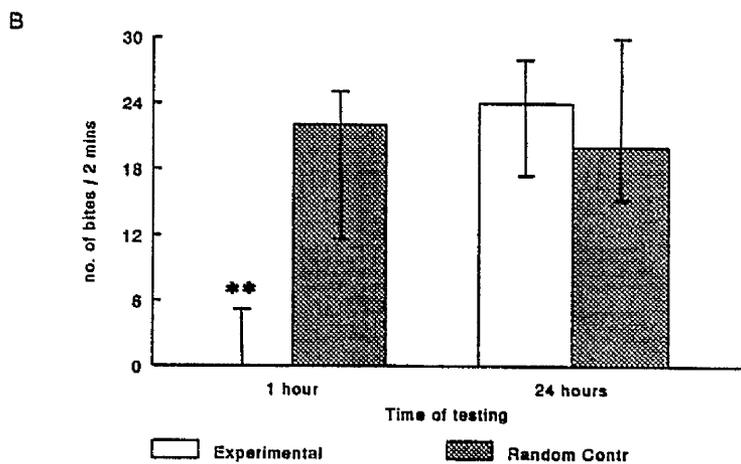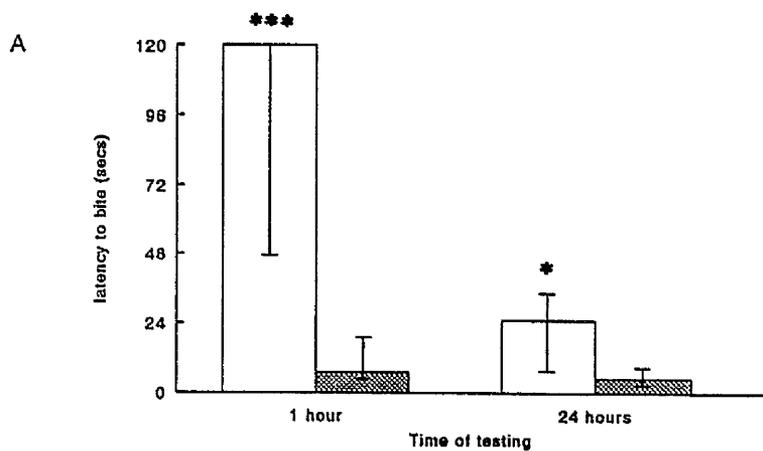
beer was paired with an electric shock, as compared to a CS of homogenated squid, even though both stimulated the same level of feeding before conditioning (Mpitsos and Cohen, 1986a).

Therefore, in this experiment, homogenised fish food (a known feeding stimulant to *Lymnaea*, see Chapter 4) was used as the CS, in place of a sucrose solution. Sixteen specimens of *Lymnaea* were divided into two groups; experimental (n=8) and random presentation control (n=8). Training was carried out in exactly the same way as Experiment 2, except that homogenised Tetramin fish food (0.1 g in 10 ml deionised water) was used in place of the 10^{-3} M sucrose solution.

The response to fish food homogenate 1 hour and 24 hours after training is shown in Figure 6.8. No conditioned withdrawal was seen in response to fish food. However results for latency to bite and number of bites in 2 minutes were very similar to those of the previous experiments. Associative learning was exhibited 1 hour after training as a reduction in feeding response to fish food in experimental animals as compared to controls (increase in latency to bite significant to the $p<0.02$ level and a decrease in number of bites in 2 minutes significant to the $p<0.01$ level, Mann-Whitney U test). There was also a total loss of this association at 24 hours after training (no significant difference between the experimental group and random control, Mann-Whitney U test).

Again results cannot be directly statistically compared to previous experiments, but they do suggest that the use of fish food homogenate as the CS does not result in any stronger learning compared to the use of sucrose. In fact learning appears to be weaker (i.e. significant only to the 0.02 and 0.01 level, compared to 0.001 level in previous experiments where the CS was sucrose). This is in agreement with studies carried out on *Pleurobranchaea* where it was shown that animals showed stronger discriminative aversive conditioning to food with

Figure 6.8 The feeding response to 0.1 ml fish food homogenate (0.1 g in 10 ml deionised water) 1 and 24 hours after aversive conditioning to fish food. The training paradigm consisted of fifteen training trials (CS-US pairings) over a period of three days; CS = fish food homogenate and US = a mechanical prod to the head (see text for details).

A) Latency to bite. One hour after training the experimentally conditioned group (experimental) show a significant increase in latency to bite compared to the random presentation control ($p < 0.02$ level, Mann-Whitney U test, n=8 each group). At 24 hours after training, however, there is no significant difference between the two groups, with the experimental group's median latency to bite returning to the random control levels.

B) Number of bites in two minutes. One hour after training the experimental group show a significant decrease in number of bites in two minutes as compared to the random presentation control ($p < 0.01$ level, Mann-Whitney U test, n=8 both groups). At 24 hours however, the number of bites shown by the experimentally conditioned group has returned to control levels with no significant difference between groups (Mann-Whitney U test).

Data for both graphs are presented as medians and interquartile ranges. ** = $p < 0.01$ level, * = $p < 0.02$ level, Mann-Whitney U test.

A
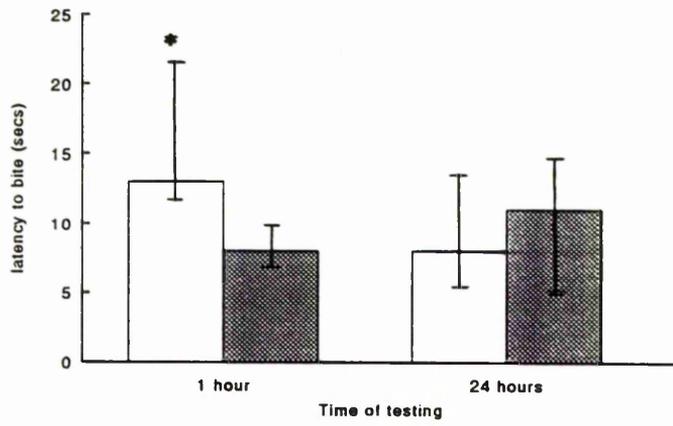
B
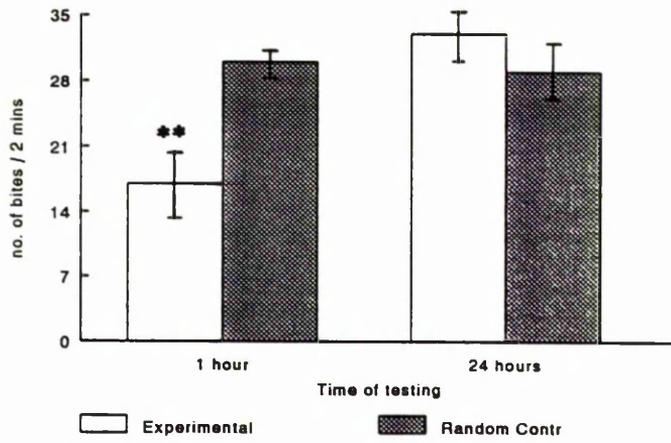
a simpler chemical make up than to more complex food (Mpitsos and Cohen, 1986a-c).

Experiment 4 - Effect of longer training period.

Studies on *Helix* demonstrated that an increased number of training trials resulted in increased strength of aversive conditioning (Balaban, 1993). Since changing the US and CS appeared to have little or no effect on the strength of learning in *Lymnaea*, the possibility that the weak learning and lack of long term memory (Experiments 1-3) was due to insufficient training trials was investigated. Experiment 4 also investigated whether 1) testing 1 hour after training acted as an extinction trial (i.e. presentation of sucrose (CS) without reinforcing US), resulting in the loss of the CS-US association and therefore explaining the lack of memory 24 hours after training; and 2) the reduction in feeding responses following training was specific to the conditioned stimulus only.

Twenty four specimens of *Lymnaea* were randomly divided into three groups: experimental (extinction) (n=8), experimental (naive) (n=8) and random presentation control (n=8). Training was carried out in a similar manner to Experiment 2 with 10^3 M sucrose as the CS and a prod as the US. The only differences were that there were two experimental groups, and the training was prolonged. Twenty five training trials were given over five days: 5 trials per day, with an inter-trial period of 90 minutes. Testing was also carried out in a similar manner to previous experiments at 1 hour and 24 hours after training, except in addition to testing the response to the CS (sucrose), the response to a different feeding stimulant (fish food homogenate), was also tested. Since neither group had received fish food homogenate during training, the comparison of the response to sucrose and fish food would demonstrate whether the learned response (reduction of feeding following training) was specific (only occurred after application of the conditioned stimulus (sucrose) to the lips), or if it was generalised. At 1 hour after training, the response to sucrose and fish food was only

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tested in the experimental (extinction) and random control groups. At 24 hours, however, the feeding response was tested in all three groups.

The response to 10^{-3} M sucrose at 1 hour and 24 hours after training is shown in Figure 6.9. No conditioned withdrawal was seen at either 1 or 24 hours after training in response to sucrose. However, at 1 hour, the experimental (extinction) group showed a significant increase in the latency to bite (p<0.02 level, Mann-Whitney U test) and reduced number of bites in two minutes (p<0.05 level, Mann-Whitney U test) in response to sucrose, as compared to random controls.

As with previous experiments, the experimental (extinction) group showed no retention of learning at 24 hours after training; both latency to bite and number of bites in response to sucrose were at the same level as random controls (Mann-Whitney U test). Therefore, the level of learning did not appear to be any stronger than that seen with 15 training trial over three days. In fact learning appeared to marginally weaker, with differences in latency to bite and number of bites between the experimental and control group being only significant to the 0.02 and 0.05 levels respectively (Mann-Whitney U test). Further experiments, in which training was carried out over even longer periods (i.e. 6 and 7 days), indicated that there was no improvement in learning or memory even after many training trials (data not shown; prolonged training usually led to the death of too many individuals within groups to allow for statistical analysis).

At 24 hours after training, the experimental (naive) group also showed no associative learning. Latency to bite and number of bites values were at control levels (no significant difference, Mann-Whitney U test) (Fig. 6.9). This indicates that the loss of association at 24 hours after training seen in the experimental group in this and previous experiments was not due to testing at 1 hour after training acting as an extinction trial, but was time dependent.

Figure 6.9 The feeding response to 0.1 ml 10^3 M sucrose 1 and 24 hours after prolonged aversive conditioning training to sucrose. The training paradigm consisted of 25 training trials (CS-US pairings) over a period of five days; CS = 10^{-3} M sucrose and US = a mechanical prod to the head (see text for details).

A) Latency to bite. One hour after training the experimental (extinction) conditioned group (Exp. Extin) show a significant increase in latency to bite compared to the random presentation control ($p < 0.02$ level, Mann-Whitney U test, n=8 each group) NB: no data for the experimental (naive) group (Exp. Naive) are shown, as the feeding response of this group was not tested until 24 hours after training. At 24 hours after training, there is no significant difference between the latency to bite in all three groups, with both experimental groups (Exp. Extinc and Exp. Naive) having a median latency to bite similar to the random control value. This suggests that testing at 1 hour after training does not act as an extinction trial resulting in loss of the association at 24 hours (see text).

B) Number of bites in two minutes. One hour after training the experimental (extinction) group show a significant decrease in number of bites in two minutes as compared to the random presentation control ($p < 0.05$ level, Mann-Whitney U test, n=8 both groups). At 24 hours however, the number of bites shown by all three groups are not significantly different, with both experimental group (extinction and naive) showing biting at control levels (Mann-Whitney U test). This suggests that testing at 1 hour after training does not act as an extinction trial (see above).

Data for both graphs are presented as medians and interquartile ranges. ** = $p < 0.02$ level, * = $p < 0.05$ level, Mann-Whitney U test.
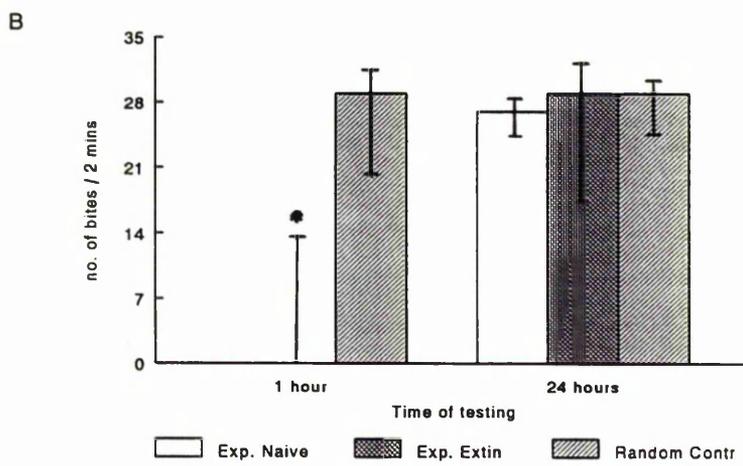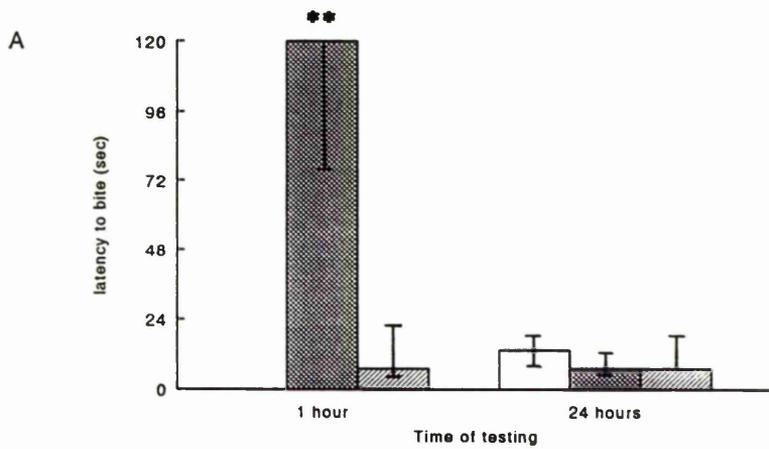
At 1 hour after training, application of 0.1 ml fish food homogenate to the experimental (extinction) group did not result in normal feeding at control levels as might have been expected. The latency to bite was significantly increased ($p<0.02$ Mann-Whitney U test) and number of bites in two minutes significantly decreased ($p<0.05$, Mann-Whitney U test) compared to the random controls. Since the feeding response to fish food and sucrose were tested in the same snails (the experimental extinction group) the response to the two stimuli could be directly compared. There was found to be no significant difference between the response to fish food and sucrose (data were paired, Wilcoxon ranked pairs test) (Fig. 6.10).

Therefore, the reduction in feeding responses seen in the experimental (extinction) group, was not specific to the conditioning stimulus (i.e. it was generalised). However, this generalised reduction in the feeding response was still a definite result of associative learning. This is certain, since reduced feeding responses were only ever seen in the experimental group, and not in the random controls which had received as many aversive stimuli. Therefore non-associative processes such as general depression of feeding as a result of repeated aversive stimuli, could not have been responsible.

Another interesting observation seen after training in all the aversive conditioning experiments was that, in addition to the reduced feeding responses at one hour after training, experimental snails appeared to be in a generally unresponsive or "still" state (Tuersley, 1986), similar to the spontaneously occurring sucrose-unresponsive state described in Chapter 3. This state was not seen in the random control animals. The unresponsive state was demonstrated by an increase in the time to emerge from the shell following handling (removal from home tank to Petri dish) (Fig. 6.11). At 1 hour after training the experimental (extinction) snails took significantly longer to emerge from their shells as compared to the
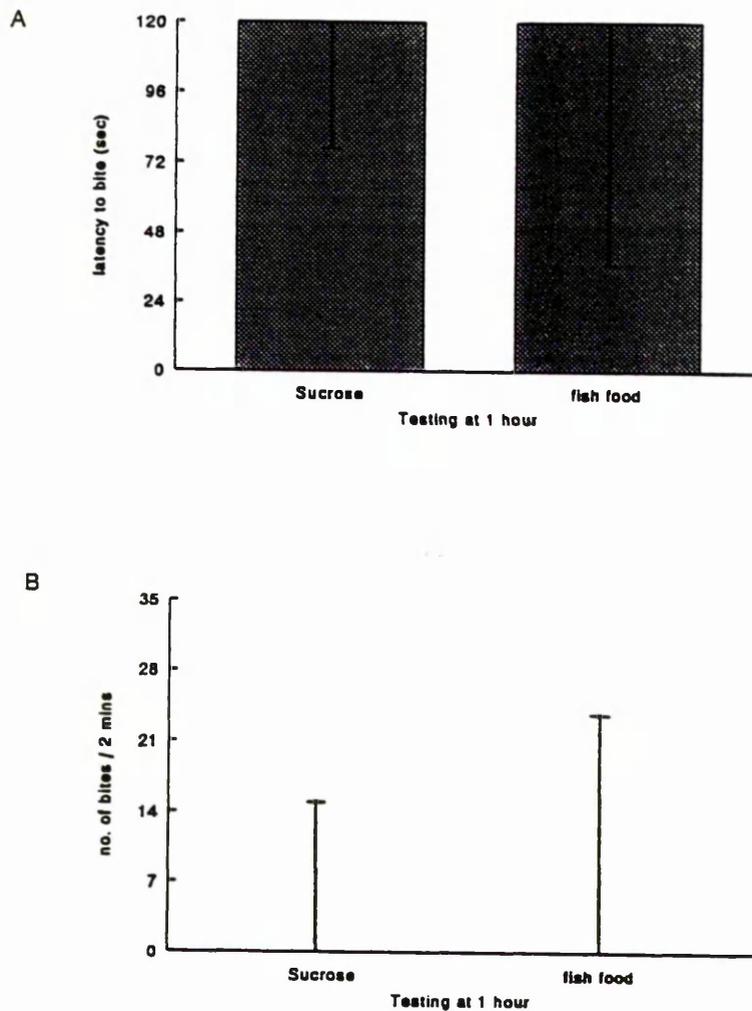
Figure 6.10 Comparison of the feeding response (at 1 hour after aversive conditioning training to sucrose) in the experimental (extinction) group (Fig. 6.9), to both 10^{-3} M sucrose and homogenised fish food. Data are paired, but are presented graphically as medians and interquartile ranges.

A) The latency to bite in response to sucrose and fish food homogenate is not significantly different (Wilcoxon ranked pairs test). The median latency to bite for both feeding stimuli = 120 sec (the arbitrary value given when there is no biting seen in two minutes).

B) The number of bites in two minutes in response to sucrose and fish food homogenate is also not significantly different (Wilcoxon ranked pairs test). The median number of bites for both stimuli = 0.
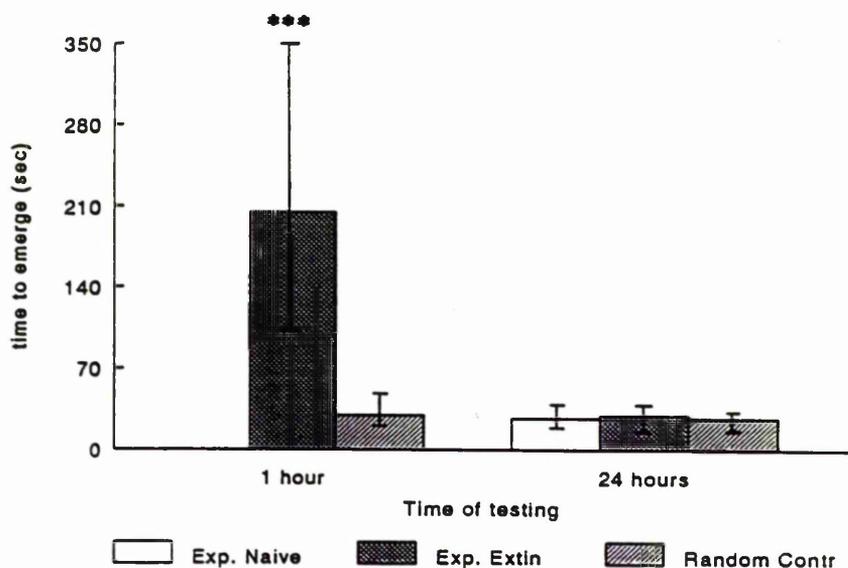
Figure 6.11 The time to emerge from the shell (a measure of the responsiveness of the snail) at 1 and 24 hours after prolonged aversive conditioning training to sucrose. The training paradigm consisted of twenty-five training trials (CS-US pairings) over a period of five days; CS = 10^3 M sucrose and US = a mechanical prod to the head (see text for details).

One hour after training the experimental (extinction) conditioned group (Exp. Extin) show a significant increase in time to emerge from the shell compared to the random presentation control ($p < 0.001$ level, Mann-Whitney U test, n=8 each group) NB: no data for the experimental (naive) group (Exp. Naive) are shown, as the response of this group was not tested until 24 hours after training. At 24 hours after training, there is no significant difference between the time to emerge from the shell in all three groups, with both experimental groups (Exp. Extinc and Exp. Naive) having a median time to emerge similar to the random control value. Increased time to emerge from the shell (i.e. reduced responsiveness of the snail) correlates well with the reduction in the feeding response (increased latency to bite, decreased number of bites in 2 minutes) seen in the same snails (Fig. 6.9). Data are presented as medians and interquartile ranges. *** = $p < 0.001$ level, Mann-Whitney U test.

random controls (p<0.001, Mann-Whitney U test). However, at 24 hours, there was no significant difference in time to emerge between the two groups. The experimental (naive) group also showed no significant increase in time to emerge at 24 hours, as compared to the controls (Mann-Whitney U test; Fig. 6.11). This suggests that the unresponsive state was directly correlated with associative learning and that the time course of this state was short lived, on a similar time scale to that of the reduced feeding responses.

From these observations it is hypothesised that the lack of response to any food stimuli seen after aversive conditioning using sucrose as the CS occurred because *Lymnaea's* conditioned response was not specifically to avoid sucrose by actively withdrawing when it came into contact with it (c.f. *Pleurobranchaea,* Mpitsos and Davis, 1973, and *Helix,* Balaban *et al,* 1987), but was to avoid all potential aversive food stimuli by going into a generally unresponsive state.

Experiment 5 - More stringent controls

To understand more fully the nature of *Lymnaea's* generalised conditioned response (i.e. a decrease in responsiveness) following aversive conditioning, experiments were carried out using several controls. This allowed the effect of the individual components of the conditioning paradigm to be assessed. Of particular interest was which of the components of the conditioning stimulus (i.e. the mechanical stimulus of the solution being dropped and the chemostimulus (sucrose)) were being associated with the aversive US to produce the generalised conditioned response.

Forty five specimens of *Lymnaea* were randomly divided into 5 groups (n=9 each group): 1) experimental aversive conditioned (CS-US pairing), 2) snail water-US pairing, 3) US only, 4) CS only and 5) random unpaired. The CS used was a 10^3 M solution of sucrose, and the US a mechanical prod to the head. The experimental group received 25 training trials

over a 5 day period: 5 trials a day with an inter-trial period of 90 minutes.

Table 6.1 - Aversive conditioning with stringent controls

Table to show the conditioning trial paradigms used on the different groups in Experiment 5. To equalise handling throughout training, each group received 0.1 ml snail water at the random time in the inter-trial period at which the unpaired control received the CS.

GROUP	TRAINING TRIAL	RANDOM TIME IN INTER-TRIAL PERIOD
EXP COND	Sucrose-Prod	Snail water
SNAIL H_2O-US	H_2O-prod	Snail water
US ONLY	Prod	Snail water
CS ONLY	Sucrose	Snail water
UNPAIRED	H_2O-prod	Sucrose

Table 6.1 shows the conditioning-trial paradigms used for each of the different groups at one training trial. As with all previous experiments, handling between groups had to be equalised throughout training, so each group received 0.1 ml snail water at the same random time that the unpaired control received the CS.

Behavioural testing was carried out blind, 1 hour and 24 hours after the last training trial.

The latency to bite in response to sucrose is shown in Figure 6.12A. At 1 hour after training there was a significant difference in the latency to bite between groups ($p < 0.005$, Kruskal-Wallis test). A non-parametric "Tukey-type" multiple comparison post-hoc test (Zar, 1984), showed that this difference was due to increased latency to bite in the experimental

conditioned group compared to all controls except the snail water-prod group (Table 6.2).

Table 6.2 Table to show the level of statistical significance to which the experimental conditioned group showed increased latency to bite compared to the other groups. The significance level was found using a "Tukey-type" multiple comparison post-hoc test.

COMPARISON	SIGNIFICANCE LEVEL (ONE WAY)
Exp cond / snail H_2O-prod	No significant difference
Exp cond / prod only	$p < 0.01$
Exp cond / sugar only	$p < 0.001$
Exp cond / unpaired	$p < 0.01$

The snail water-prod group also had a high median value of latency to bite compared to other controls. However the post hoc test showed that this increase in latency to bite was only significant when compared with the sugar only group ($p < 0.05$). There was no significant difference when comparing any other combination of groups.

At 24 hours there was no retention of associative learning. The experimental conditioned and snail water-prod groups' latency to bite was at the same level as all control groups (no significant difference using the Kruskal-Wallis test).

Figure 6.12B shows the number of bites in two minutes in response to sucrose following training. At 1 hour after training there was a significant difference between groups in the number of bites ($p < 0.001$, Kruskal-Wallis test). The multiple comparison post hoc test showed that this significant difference was attributable to two separate factors: 1) a decrease in number of bites in the experimental conditioned group as compared to all other controls (except the snail water-prod group), and 2) an increase in the number of bites in the sucrose only (CS only) group as compared to all other groups (Table 6.3).

Figure 6.12 The feeding response of the experimental conditioned and control groups (see Table 6.1 for details) to 0.1 ml 10^3 M sucrose 1 and 24 hours after aversive conditioning to sucrose. The training paradigm consisted of twenty five training trials (CS-US pairings) over a period of five days; CS = 10^3 M sucrose and US = a mechanical prod to the head (see text for details).

A) Latency to bite. One hour after training there is a significant difference in the latency to bite between groups ($p < 0.005$ level, Kruskal-Wallis test, n=9 each group). A "Tukey type" multiple comparison post-hoc test shows this difference to be due to the experimental conditioning group having a significantly longer latency to bite as compared to all other groups (except the snail water-prod group), and the snail water prod also having a significantly greater latency to bite than the sucrose only group (see Table 6.2 for list of significance levels). At 24 hours after training, however, there is no significant difference in the latency to bite between group (Kruskal-Wallis test). Data are presented as medians and interquartile ranges. Symbols for significance levels using the multiple comparison post hoc test: 1) In comparison with the experimental conditioned group ** = $p < 0.05$ level, *** = $p < 0.01$ level, **** = $p < 0.001$ level; 2) In comparison with the snail water-prod group ▲▲ = 0.05 level.

B) Number of bites in two minutes. One hour after training there is a significant difference in the number of bites in two minutes between groups ($p < 0.001$ level, Kruskal-Wallis test, n=9 each group). A "Tukey type" multiple comparison post-hoc test shows this difference to be due to the experimental conditioning group having a significantly smaller number of bites compared to all other groups (except the snail water-prod group), and the sucrose only group having a significantly greater number of bites compared to all other groups (see Table 6.3 for list of significance levels). At 24 hours after training, however, there is no significant difference in the number of bites in two minutes between groups (Kruskal-Wallis test). Data are presented as medians and interquartile ranges. Symbols for significance levels using the multiple comparison post hoc test: 1) In comparison to the experimental conditioned group * = $p < 0.1$ level, ** = $p < 0.05$ level, **** = $p < 0.001$ level; 2) In comparison with the sucrose only group o = $p < 0.1$ level, oo = $p < 0.05$ level oooo = $p < 0.001$ level.

A

B

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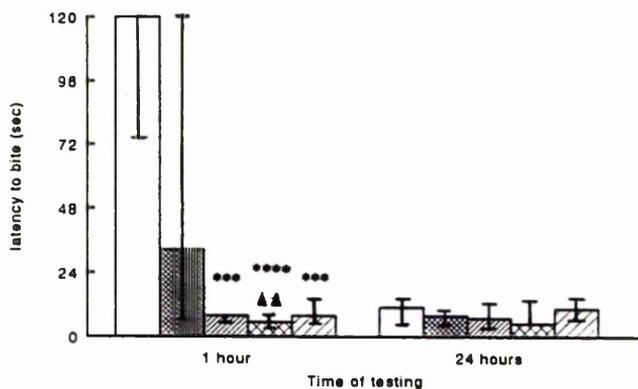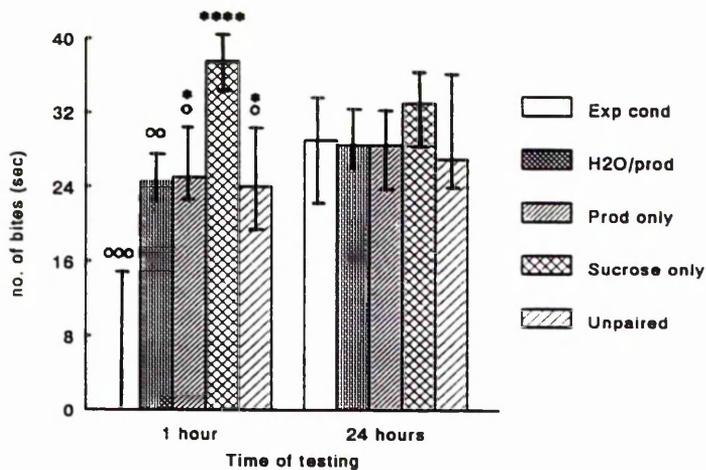
Table 6.3 Table to show the level of statistical significance to which 1) the experimental conditioned group showed reduced number of bites in two minutes as compared to the other groups, and 2) the sucrose only group showed an increase in the number of bites in two minutes compared to all other groups. The significance level was found using a "Tukey-type" multiple comparison post-hoc test. The data in brackets denote repeated data.

COMPARISON	SIGNIFICANCE LEVEL (ONE WAY TEST)
Exp cond / Snail water-prod	No significant difference
Exp cond / prod only	$p < 0.1$
Exp cond / sucrose only	$p < 0.001$
Exp cond / unpaired	$p < 0.1$
Sucrose only / s. water-prod	$p < 0.05$
Sucrose only / prod only	$p < 0.1$
Sucrose only / unpaired	$p < 0.1$
(Sucrose only / Exp cond)	$(p < 0.001)$

There were no other significant differences when comparing any other combinations of groups.

At 24 hours after training, there was no difference between groups in number of bites in response to sucrose (Kruskal-Wallis test), indicating that no associative learning or non-associative processes had been retained.

Figure 6.13 shows the level of responsiveness following training, expressed as time to emerge from the shell following handling. At 1 hour after training there was a significant difference in time to emerge between groups ($p < 0.001$, Kruskal-Wallis test). The multiple comparison post-hoc test showed that this difference was attributable to three separate factors:

1) An increased time to emerge in the experimental group as compared to all control groups except the snail water-prod group, 2) an increased time to emerge between snail water-prod group as compared to all other control groups (except for the unpaired group), and 3) a decreased time to emerge in the sucrose only group as compared to all groups (except the prod only group) (Table 6.4).

At 24 hours after training there was no retention of any associative learning or non-associative processes; there was no significant difference in time to emerge between any of the groups (Kruskal-Wallis test) (Fig. 6.13).

The differences in time to emerge from the shell between groups (i.e. the extent that each group was in an unresponsive or "still" state) was therefore closely correlated with the feeding response to sucrose (i.e. latency to bite and number of bites in two minutes). As expected, at 1 hour after training the experimental conditioned group showed a significant increase in time to emerge and a corresponding unresponsiveness to sucrose (increased latency to bite and decreased number of bites as compared to controls).

One interesting observation was that the snail water-prod group also showed some short-term associative learning, similar to, though to a lesser extent than the experimental group. There was significant increase in time to emerge compared to controls and corresponding increase in latency to bite in response to sucrose at 1 hour after training. This suggested that the snail water-prod group had weakly associated the mechanical disturbance of application of snail water with the aversive prod to the head. Curiously, the unpaired group, which had received just as many snail water-prod pairings as the snail water-prod group, did not show any associative learning. This could be attributed to the unpaired group's receiving a sugar solution (a feeding stimulant) at the random time within the inter-trial

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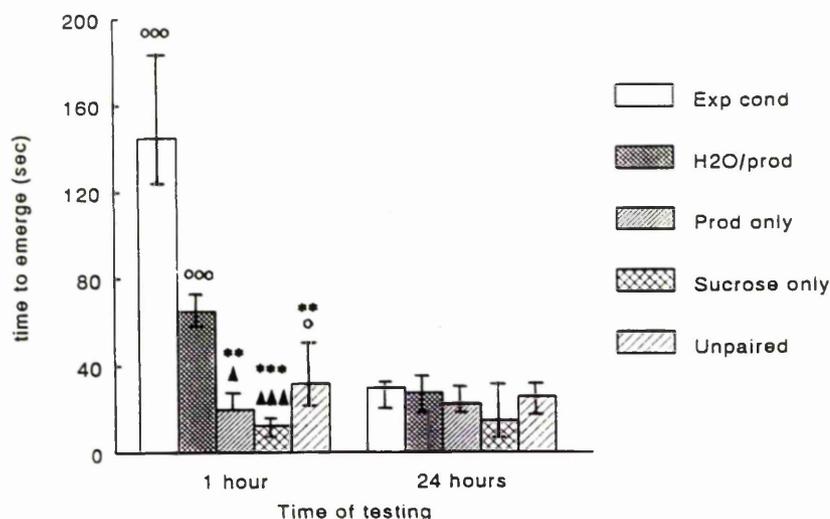
Figure 6.13 The time to emerge from the shell (i.e. a measure of responsiveness) of the experimental conditioned and control groups (see Table 6.1 for details of the different groups), at 1 and 24 hours after aversive conditioning to sucrose. The training paradigm consisted of twenty five training trials (CS-US pairings) over a period of five days; $CS = 10^3$ M sucrose and $US =$ a mechanical prod to the head (see text for details).

One hour after training there is a significant difference in the time to emerge from the shell between groups ($p < 0.001$ level, Kruskal-Wallis test, n=9 each group). A "Tukey type" multiple comparison post-hoc test shows this difference to be due to, 1) the experimental conditioning group taking significantly longer to emerge from the shell compared to all other groups (except the snail water-prod group), 2) the snail water-prod group taking a significantly longer time to emerge compared to all other control groups (except the random unpaired presentation group), and 3) the sucrose only group taking a significantly *shorter* time to emerge from the shell compared to all other groups (except the prod only group) (see Table 6.4 for list of significance levels). At 24 hours after training, however, there is no significant difference in the latency to bite between group (Kruskal-Wallis test). Data are presented as medians and interquartile ranges. The relative time it takes each group time to emerge from the shell following conditioning (i.e. the reduced/increased responsiveness of the snail) correlates well with the a relative strength in the feeding response (longer/shorter latency to bite, decreased/increased number of bites in 2 minutes) seen in the same snails in Figure 6.12. Symbols for significance levels using the multiple comparison post hoc test: 1) In comparison to the experimental conditioned group * = $p < 0.05$ level, ** = $p < 0.005$ level, *** = $p < 0.001$ level; 2) In comparison with the snail water-prod group ▲ = $p < 0.025$ level, ▲▲▲ = $p < 0.001$ level; and 3) In comparison to the sucrose only group ○ = $p < 0.1$ level, ○○○ = $p < 0.001$ level.

Table 6.4 Table to show the level of statistical significance to which 1) the experimental conditioned group and 2) the snail water-prod group showed greater unresponsiveness (or longer time to emerge from the shell) compared to the other groups, and 3) the sucrose only group showed an increased responsiveness (decrease in time to emerge from the shell) compared to all other groups. The significance level was found using a "Tukey-type" multiple comparison post-hoc test. The data in brackets denotes repeated data.

COMPARISON	SIGNIFICANCE LEVEL (ONE WAY TEST)
Exp cond / snail water-prod	No significant difference
Exp cond / prod only	$p < 0.005$
Exp cond / sugar only	$p < 0.001$
Exp cond / unpaired	$p < 0.05$
S. water-prod / prod only	$p < 0.025$
S. water-prod / sugar only	$p < 0.001$
S.water-prod / unpaired	No significant difference
(Sugar only / exp cond)	$(p < 0.001)$
(Sugar only / S.water-prod)	$(p < 0.001)$
Sugar only / prod only	No significant difference
Sugar only / unpaired	$(p < 0.1)$

period, in contrast to the snail water-prod group receiving only snail water. Therefore the "mechanical stimulus" received by the unpaired group in the absence of an aversive stimulus was sucrose, which possibly acted as a positive reinforcing stimulus, cancelling out any association between a mechanical stimulus and aversive prod. This hypothesis was backed up by the observation that 1 hour after training the sugar only group took significantly less time

to emerge from their shells compared to the other groups and showed a corresponding increased number of bites in response to sucrose as compared to other groups. This increased responsiveness in the sugar only group is likely to be simply a result of a short-term non-associative process such as facilitation (i.e. repeated exposure to sucrose resulted in a more active arousal state).

These results indicate that *Lymnaea* associates both the mechanical and chemostimulus components of the CS with the aversive US, resulting in the generalised unresponsive state. However, the mechanostimulus component is only weakly associated compared to the much stronger association with the chemostimulus component. Interestingly repeated exposure to the chemostimulus (sucrose) alone (i.e. not paired with the aversive US) resulted in the opposite effect to when it was paired, with the feeding response being increased (facilitated), rather than being suppressed by the snail going into the unresponsive state.

Experiment 6 - *"Context fear" learning?*

Previous research on *Aplysia* (Colwill *et al.*, 1988a,b) and *Helix* (Balaban, 1993; Balaban and Bravarenko, 1993) has shown them to be capable of context or environmental conditioning. The animals associated aversive conditioning with the environment in which they were trained. Therefore, when tested, experimentally conditioned animals gave stronger conditioned responses to the CS when in the training environment compared to a new, contrasting environment. Other researchers found that, following aversive conditioning, *Aplysia* showed evidence of a fear-like state in response to the CS. In addition to the expected conditioned response (withdrawal), individuals showed an increase in other defensive behaviours (e.g. inking) as well as reduced feeding responses (Carew *et al.*, 1981; Walters *et al.*, 1981).

Experiments 4 and 5 indicated that *Lymnaea's* conditioned response following aversive conditioning was not to actively avoid the CS by withdrawing, but to go into a short term (<24 hours) unresponsive state after training, so avoiding all possible aversive stimuli. This strategy of *Lymnaea* "burying its head in the sand" to avoid aversive stimuli, seemed impractical in the wild, as it could result in *Lymnaea's* being unnecessarily unresponsive for long periods even when it is in no danger. The following experiment investigated whether the unresponsive state seen after training was a result of an, as yet undescribed phenomenon, "context-fear" learning, in which an animal is unresponsive whilst in the training environment. This new concept of "context-fear" learning combined the ideas of context and fear conditioning, with the CS which triggers a fear-like response (unresponsiveness) being the environment in which an individual had received a CS-US (sucrose-prod) pairing. If this was the case, experimentally conditioned individuals, when tested would take longer to emerge from their shells (be less responsive) in the training environment, compared to a new previously unexperienced environment.

Twenty four specimens of *Lymnaea* were randomly divided into three groups (n=8 each group): 1) experimental conditioned, 2) snail water-prod paired, and 3) naive. The first two groups had both shown short-term associative learning in Experiment 5. The CS was 10^{-3} M sucrose and the US was a mechanical prod to the head. Training of the experimental conditioned and snail water-prod groups was carried out in Petri dishes, in exactly the same manner as Experiment 5. The naive control group received no training and no contact with either environment before testing. The time to emerge from the shell (i.e. the responsiveness) was recorded 1 hour after training, in both the training environment (Petri dishes), and the new environment (a gravel-lined crystallising dish). The order of testing in the two environments was randomised to reduce any possible extinction trial effects.

The time to emerge in the two environments (data from all three groups) is shown in Figure 6.14. Data were paired (each group tested in the two environments) and presented as medians and interquartile ranges. Both the experimental conditioned and the snail water-prod paired group showed a slight reduction in time to emerge from their shells in the new gravel environment as compared to the training environment (p< 0.1 level, Wilcoxon ranked pairs test). This reduction was, therefore, not great enough to account completely for the unresponsive state seen after training. The naive group showed no significant difference in time to emerge from shell in either environment, indicating that neither environment was preferred by untrained snails. It should be noted that the naive group's time to emerge cannot be directly compared to the other two groups, since this group had not received any handling prior to testing. This probably explains the relatively long time to emerge from the shell in both environments (the snails had not become accustomed to handling, and therefore showed non-associative depression of arousal following handling; Tuersley, 1986).

These results indicate that "context-fear" learning may be a small contributory factor to the unresponsive state seen in *Lymnaea* following aversive conditioning. However, *Lymnaea's* conditioned response following aversive conditioning does appear to mainly comprise a generalised unresponsive state lasting for less than 24 hours.

Electrophysiological recordings

Electrophysiological recordings were made from aversively conditioned animals to assess any specific effect the short-lived conditioned response (general unresponsive state) might have on the feeding system of *Lymnaea*.

Specimens of *Lymnaea* were trained following the training protocol of Experiment 4. As with all aversive conditioning experiments in this Chapter, snails were kept sub-satiated throughout the experiment. Electrophysiological experiments were carried out 1-3 hours after
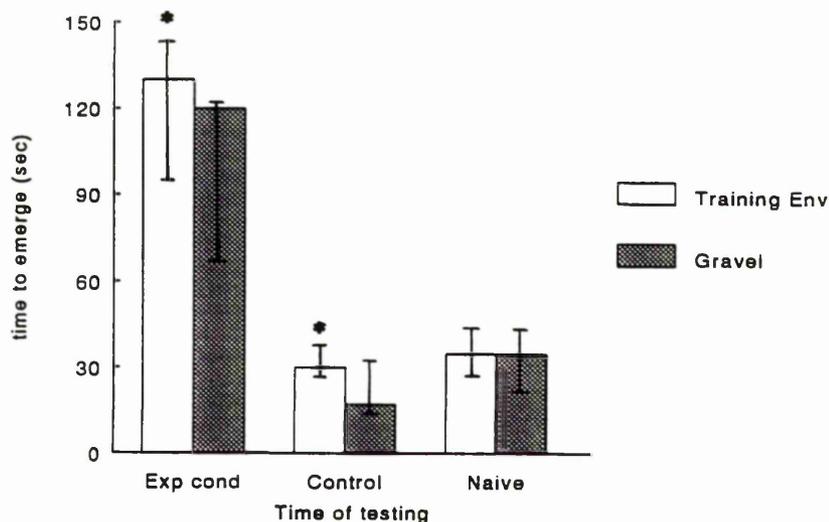
Figure 6.14 The time to emerge (i.e. the responsiveness) in the training environment (Petri dish) compared to a novel environment (gravel-lined crystallising dish), 1 hour after aversive conditioning to sucrose. Data are paired, but are presented graphically as medians and interquartile ranges. The time to emerge is significantly longer in the training environment compared to the novel gravel environment in both the experimental conditioned and the snail water-prod group ($p < 0.1$ level, Wilcoxon ranked pairs test, n=8 each group). There is no significant difference in the time to emerge in either environment in the naive group (Wilcoxon ranked pairs test).
* = $p < 0.1$ level, Wilcoxon ranked pairs test.

training, i.e. whilst in the unresponsive state. Recordings were made from CV1 using semi-intact preparations taken from both the experimentally conditioned group (n=7) and the random control group (n=6) (from four separate training experiments). Results were limited because of the difficulties of recording immediately (i.e. within a few hours) following training. However, some interesting findings were obtained.

Experimental conditioned group   In 7/7 semi-intact preparations there was no obvious change in the frequency of spontaneously occurring subthreshold inputs. This suggests that the reduction in feeding in the unresponsive state is not mediated centrally at the level of CV1 (c.f. satiation, Chapter 3). However, in 3/7 preparations, application of 0.1 ml 10^3 M sucrose resulted in a cessation of any ongoing feeding motor output, or activity of CV1 (Fig. 6.15A). This was the opposite to the response of CV1 following sucrose application to the lips in naive animals (Chapter 3). Figure 6.15B shows results obtained from one preparation, where application of sucrose resulted in an increase of very large amplitude IPSPs. CV1 was unusual in that it was completely quiescent. Prior to application of sucrose CV1 received both small amplitude IPSP and EPSPs. Upon application of sucrose, CV1 received groups of very large amplitude IPSP (Fig. 6.15B). These IPSPs had an amplitude of approximately 10 mV. Inputs of this amplitude had not been seen in any other experiments. Possible explanations for these very large amplitude IPSP are: 1) CV1's membrane potential was depolarised resulting in larger amplitude large IPSPs (usually 4 mV), or, 2) the very large IPSPs originate from another previously undescribed population of inhibitory interneurons presynaptic to CV1, which are activated following aversive conditioning.

In the remaining 4/7 preparations, following application of sucrose, there was no change in either the inputs received by, or activity of CV1 and identified buccal motoneurons (Fig. 6.15C).

Figure 6.15 The effect of 0.1 ml 10^{-3}M sucrose application to the lip tissue on the activity in CV1 and identified buccal motoneurons in a semi-intact preparation taken from a snail aversively conditioned to sucrose.

A) Prior to application of sucrose, CV1 received both EPSPs and IPSPs and was showing rhythmic bursts of action potentials. Upon application of sucrose there was a hyperpolarisation of the membrane potential and immediate cessation of all firing. This inhibition of all firing activity was long-lived with no firing seen for the subsequent duration of the recording (>2 minutes).

B) Prior to application of sucrose, CV1 shows no rhythmic bursting activity and receives both small amplitude IPSPs and EPSPs. Application of sucrose results in (after a delay of approx. 2 seconds) CV1 receiving groups of very large amplitude IPSPs (approx. 10mV). These input continued for over 2 minutes.

C) Application of sucrose results in no change in the activity of, or inputs to, CV1 or the identified feeding motoneuron B4.

A

CV1

SUCROSE

B

CV1

EPSP

IPSPs

IPSP$_L$

SUCROSE

IPSP$_L$

10mV

2sec

C

40mV

B4

2sec

20mV

CV1
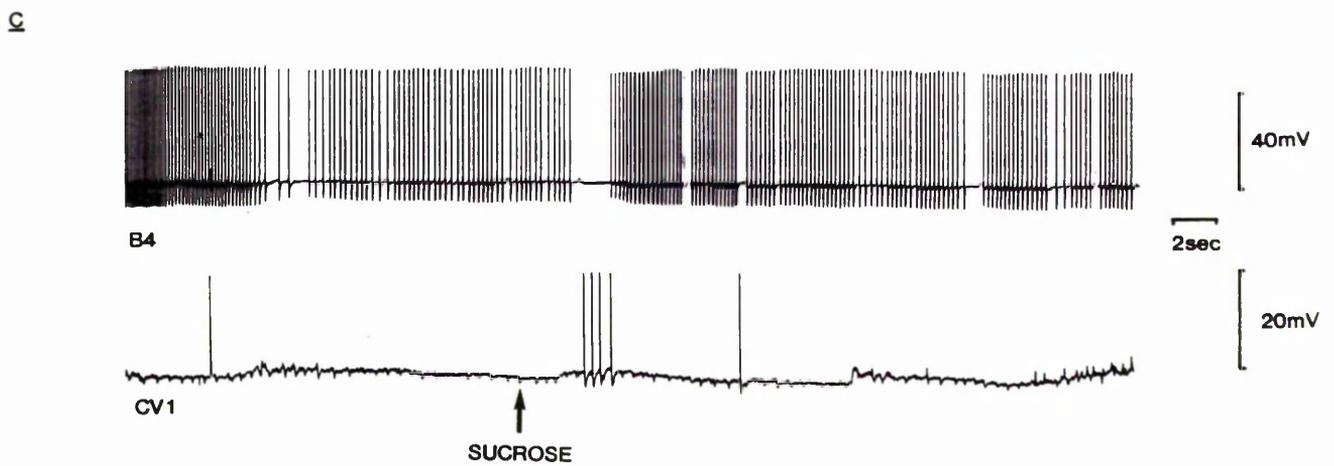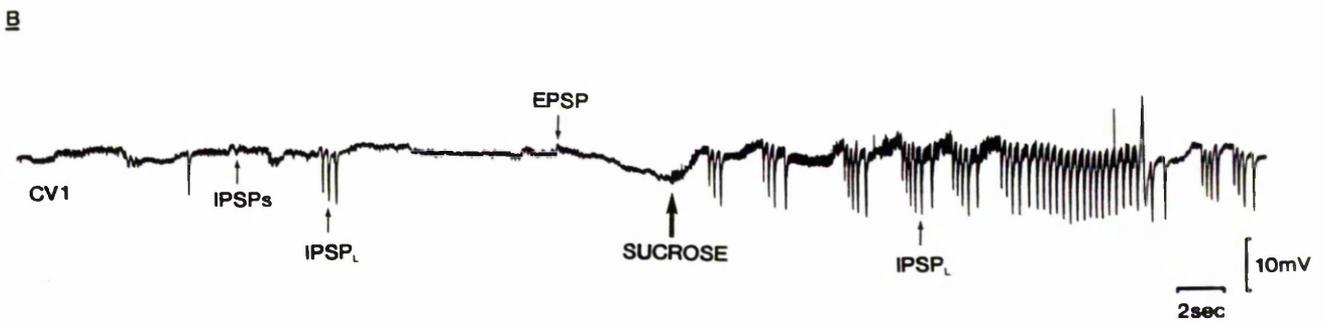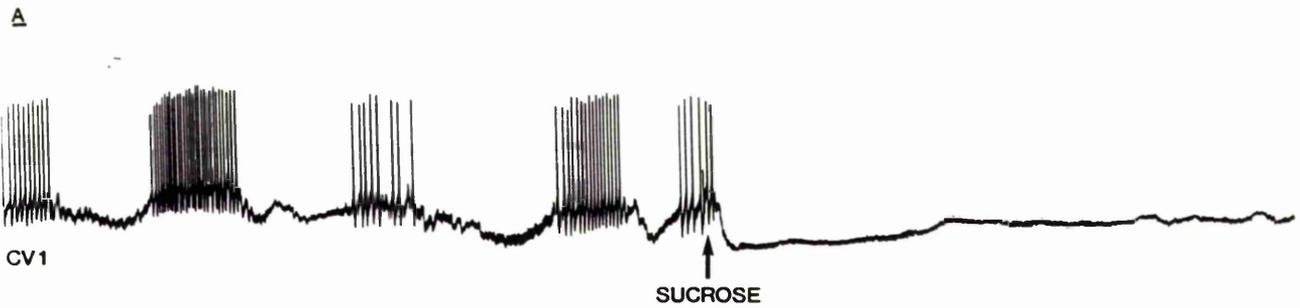
SUCROSE

Random control group In 6/6 preparations, CV1 received both types of IPSP, at similar frequencies to those received by the "trained" CV1s. In 5/6 preparations application of sucrose was, as expected, excitatory to CV1. This was seen either as 1) a depolarisation of the membrane potential and initiation of feeding motor output (Fig. 6.16), 2) an increase in EPSPs and decrease in IPSPs received by CV1, or 3) an increase in frequency or intensity of the feeding rhythm.

In the remaining 1/6 preparations application of sucrose did not result in any change in the activity of, or inputs to, CV1. This lack of response may have been due to damage during dissection.

Figure 6.16 Application of 0.1 ml 10⁻³M sucrose to the lip tissue of a random control snail results in initiation of a feeding rhythm, seen as bursting activity in CV1 and in the identified buccal motoneuron B4.
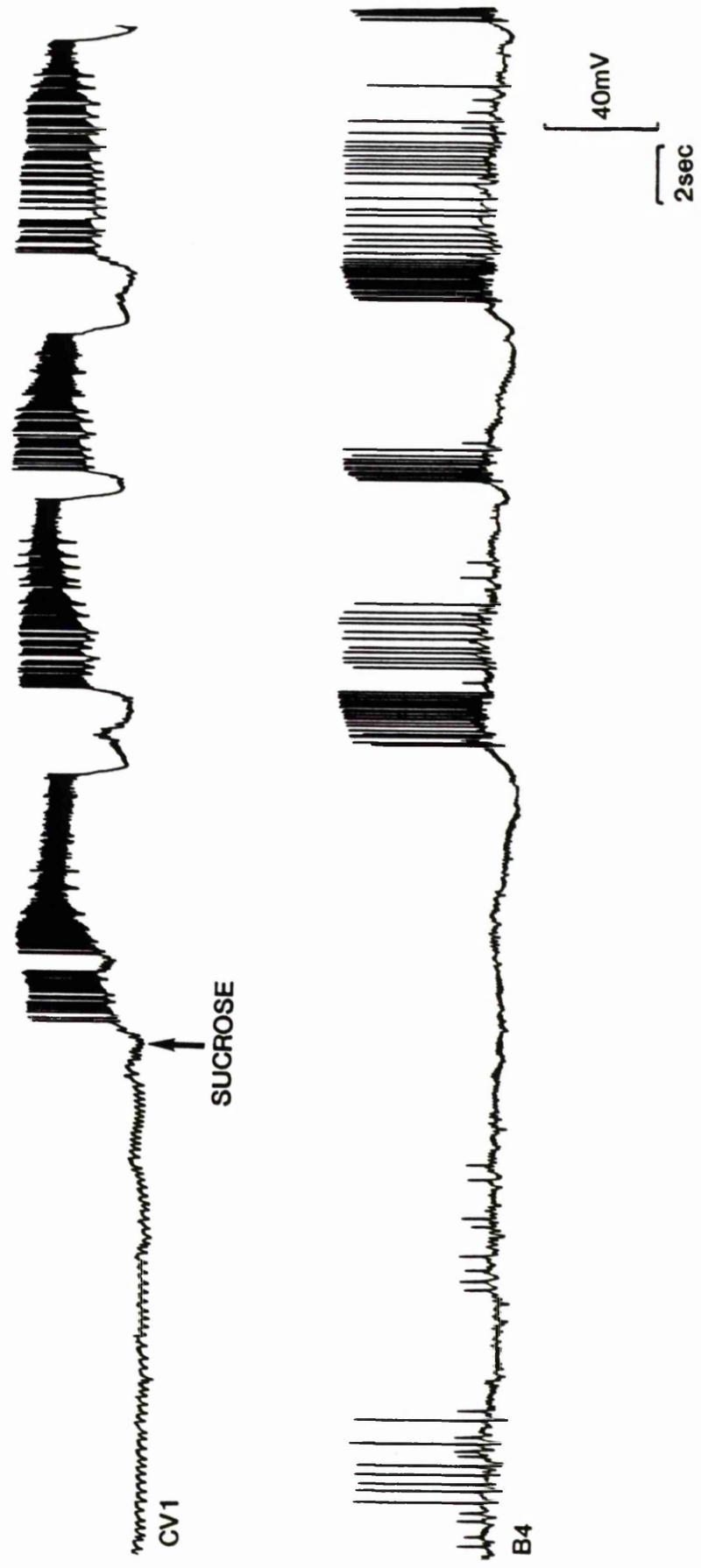
DISCUSSION

Positive conditioning

The appetitive conditioning paradigm developed by the Audesirks (Audesirk *et al.*, 1982, Alexander *et al.*, 1982, 1984) proved to be a good model for studying the neural correlates of learning. This was because, not only did it produce behavioural learning very reliably and rapidly, but a high proportion of trained snails also retained the learnt feeding response to amyl acetate in the semi-intact preparation.

Exploiting this, the results in this Chapter demonstrated that the learned feeding response to amyl acetate was at least partly mediated by the CV1 pathway; amyl acetate (after training) had a very similar excitatory effect to food stimuli on CV1. This can be shown by comparing the percentage excitatory effect of food stimuli (sucrose; Chapter 3, and fish food; Chapter 4) to amyl acetate in conditioned snails, on CV1 (Table 6.5).

Table 6.5 Percentage excitatory effect of amyl acetate on the feeding system of conditioned snails, compared to data obtained for the food stimuli, sucrose and fish food (Chapters 3 and 4). NB: The "Excitatory to CV1 (no initiation)" group represents preparations where there is an excitatory change in the inputs received by CV1, and/or a speeding and intensification of ongoing feeding motor output.

	Amyl acetate (n=17, conditioned snails)	Sucrose (n=26)	Fish Food (n=20)
Initiation of feeding	30 %	35 %	35 %
Excitatory to CV1 (no initiation)	47 %	42 %	45 %
No effect	23 %	23 %	20 %

In Chapter 3 it was proposed that CV1 acts as a locus where excitatory and inhibitory inputs converge, with the overall balance of inputs determining whether CV1 becomes active

and drives feeding motor output. Since amyl acetate caused similar inputs to be received by CV1 as those following food, the learning induced changes resulting in amyl acetate being excitatory to CV1 probably occurred presynaptic to CV1. One possible site for these changes is at the level of the neurons supplying the IPSPs and EPSPs to CV1. In this case, after training, amyl acetate would excite neurons supplying the EPSPs, and inhibit the neurons supplying the IPSPs. The exact mechanism by which this could occur is not known. It is also quite possible that other "learning-induced" changes may have occurred in different parts of the feeding network following positive conditioning, i.e. the buccal ganglia. This possibility will hopefully be investigated in the future.

Studies on the aversive conditioning of *Pleurobranchaea* showed that the PCN pathway (homologous to the CV1 pathway) was involved in modulating the feeding response following associative conditioning (Davis *et al.*, 1983; Davis, 1984; Kovac *et al.*, 1985, 1986; London and Gillette, 1986). It was demonstrated that the putative homologue to CV1 (PC_p), had a very similar role to CV1; in that it was not the actual site of the learning-induced changes, but only acted as a locus where modified synaptic inputs were received following application of food to the lips (Davis *et al.*, 1983; Davis, 1984; Kovac *et al.*, 1985, 1986; London and Gillette, 1986). Although these studies on *Pleurobranchaea* were on aversive conditioning, they, with the present study, do suggest that homologous cerebral-to-buccal interneurons in different species are involved in modulating feeding response following associative conditioning. In support of this, a recent behavioural study on both appetitive and aversive conditioning of *Limax* using odours, proposed that the putative homologous cerebral-to-buccal interneurons in *Limax* (the CBs; Delaney and Gelperin, 1990a-c) were involved in modulating the feeding response following associative conditioning (Sahley *et al.*, 1992).

One unexpected observation during the positive conditioning in this Chapter was that,

although behavioural feeding was never seen in response to amyl acetate in control snails, amyl acetate was sometimes mildly excitatory to CV1 in the semi-intact preparation. Studies on sensory receptors (thought to be food detectors) in the cerebral ganglion of *Tritonia*, showed amyl acetate to have an excitatory effect (Audesirk and Audesirk, 1980a,b), so it is quite possible that *Lymnaea*'s sensory receptors are also excited by amyl acetate in naive snails. This excitatory effect in the semi-intact preparation, however, was never great enough to initiate feeding motor output, and this was probably why a behavioural feeding response was never seen in the control groups. This result does not dispute whether learning occurred, but it does question whether it is valid to use the Audesirks' term "neutral feeding stimulus" when referring to amyl acetate, as it clearly has a mildly excitatory effect at the neural level. Interestingly, the "non-neutrality" of amyl acetate at the neural level is probably a key factor in allowing *Lymnaea* to learn to recognise amyl acetate as "food" so quickly. Studies on *Pleurobranchaea* showed that individuals learnt best when the conditioned stimulus (CS) had a small but finite chance of eliciting the same response as the unconditioned stimulus (US) (Mpitsos *et al.*, 1978); i.e. the association was made by the strengthening of existing neural pathways. Therefore, it is possible that the slight excitatory effect of amyl acetate in naive *Lymnaea* was somehow augmented by associative conditioning (by an as yet, unknown mechanism), to result in amyl acetate initiating feeding after training.

Aversive conditioning

In contrast to several other gastropod species (*Pleurobranchaea*; Mpitsos and Davis, 1973, Mpitsos and Cohen, 1986a-c; and *Helix*, Maksimova and Balaban, 1984, Balaban *et al.*, 1987), aversive conditioning paradigms carried out on *Lymnaea* did not result in long-term retention of a strong classically conditioned response (withdrawal in response to the CS), but led only to a short term (< 24 hours) generalised unresponsive state which did not need a CS

cue. This generalised unresponsive state was, however, a result of associative learning, since it was only ever seen in trained animals, i.e. the animals which had received the CS-US pairing. The short-term nature of the unresponsive state could have been a consequence of the learning being weak, and this is supported by the state also being "generalised" (i.e. not a specific response to the CS). Generalised responses are often seen at the start of the learning process before strong discrimination is made (Rachlin, 1970). Therefore, one possibility is that *Lymnaea* had not received sufficient training to make a true discrimination. This hypothesis, however, is unlikely since longer training paradigms were shown not to result in stronger learning, and strong associations were easily made by *Lymnaea* using the same trial spacing in the positive conditioning paradigms.

Early attempts by other workers to aversively condition *Lymnaea*, were also not very successful, with no, or only weak, learning seen even after many training trials (Buytendijk, 1921; Fishel, 1931, as quoted by Hyman, 1967). These findings were simply attributed to *Lymnaea* having poor learning capabilities. Since *Lymnaea* has recently been demonstrated to show strong positive conditioning (Audesirk *et al.*, 1982; Alexander *et al.*, 1982, 1984), this cannot be the case. Similarly, up until the early 1970s, *all* gastropod molluscs were considered to be poor learners (Willows, 1973). However, research in the last two decades also showed this to be untrue, with gastropods showing a range of learning capabilities, from "avoidance" learning right through to "social" learning (for review, see Suboski, 1992).

In 1978, Mpitsos *et al.* suggested that the paucity of learning seen by gastropods using the early conditioning paradigms, was due to worker's difficulty in assessing the kinds of stimuli that could be "meaningfully related to natural behaviour of these animals" i.e. be recognised as relevant by the animal. If this is the case, the poor aversive conditioning seen by *Lymnaea* in this Chapter could have been a result of the conditioned and unconditioned stimuli not being relevant. This, however, seems unlikely since; 1) the conditioned stimuli

used (sucrose or fish food) were both demonstrated to reliably stimulate feeding (Chapters 3 and 4), and so therefore should have been perceived by the animal as relevant feeding stimuli; and, 2) the unconditioned stimulus (electric shock or noxious mechanical prod to the head) induced a strong withdrawal in *Lymnaea*, and therefore should have been a relevant stimulus to the animal signalling "danger". Furthermore, electric shocks or noxious mechanical stimuli produced strong aversive conditioning in many other gastropod molluscs, including *Pleurobranchaea* (Mpitsos and Davis, 1973; Mpitsos and Collins, 1975; Davis *et al.*, 1980; Mpitsos and Cohen, 1986a-c; Mpitsos *et al.*, 1988), *Helix* (Maksimova and Balaban, 1984; Balaban *et al.*, 1987; Balaban, 1993), and *Aplysia* (Walters *et al*, 1981; Carew *et al.*, 1981, 1983; Colwill *et al.*, 1988a,b). This was attributed to "noxious stimuli always being relevant stimuli to the animal" (Alexander *et al.*, 1984).

Another important factor found to affect the speed and strength of learning is the nature of the CS and US. In 1990, Sahley stated that, although aversive conditioning to a certain food could occur using one of several different types of US (all of which were relevant since they were noxious to the animal), the resultant strength and retention of learning in *Limax* was dependent on the nature of the reinforcer (US), e.g. CO_2 gas-induced illness and bitter tastes (quinidine sulphate) resulted in quicker learning than a food lacking in an essential amino acids, but was less well retained. Balaban (1993) also showed the *strength* of the reinforcer to be important, with *Helix* only showing rapid aversive conditioning to food if the electric shock US was strong enough to elicit full body withdrawal and release of mucus. If a weaker reinforcement was used, i.e. only enough to induce tentacle/head withdrawal, learning still occurred but the speed of the elaboration of the conditioned response was much slower (Balaban, 1993). The two different US used in this Chapter (electric shock and noxious mechanostimulus) were both deemed to be very aversive, since they led to full body withdrawal and defensive forced expulsion of air from the

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pneumostome, and therefore should have been noxious enough to result in conditioning. However, neither US resulted in strong conditioning (withdrawal to the CS) following training.

Experiments on *Pleurobranchaea* also showed the type of CS to be important in the resultant strength of conditioning. One study found that animals learnt to avoid beer extracts better than squid, even though before training both feeding stimuli resulted in the same strength of feeding response (Mpitsos and Cohen, 1986a). There are two possibilities to why this might occur: 1) squid is close in composition to *Pleurobranchaea's* food sources in the wild, whilst beer is a novel food source derived from plant material (*Pleurobranchaea* is a carnivore). Therefore, it may be easier to condition *Pleurobranchaea* against beer, since it is more of a "foreign" substance, and thus, may be easier to associate with danger; or, 2) squid is a more complex food than beer, and therefore there are more individual components to associate with the US than beer. This could result in conditioning against squid being slower since the association would have to be more complex. The aversive conditioning paradigms in this Chapter also indicated that the nature of the CS could be important in determining the strength of *Lymnaea's* learning. The learning following aversive conditioning to sucrose (a relatively simple substance that *Lymnaea* would not come across in a pure form in the wild) was slightly stronger than that with fish food (a more complex food source which the snails are fed on whilst kept in the laboratory). However, despite the stronger learning to sucrose, the learning with both food stimuli was still very weak, generalised and short-lived.

One other possibility could be that sucrose and fish food tasted "too" attractive for *Lymnaea* to be easily conditioned against. This, however, is unlikely. In 1975, Gelperin suggested that *Limax* could not be trained to avoid attractive-tasting dog food containing metaldehyde (a molluscicide which caused obvious distress soon after ingestion; Gelperin, 1975), because the taste was too attractive. However, since different US such as quinine or

CO_2-induced illness could generate very strong and rapid aversive conditioning to attractive tastes such as carrot, potato or mushroom (Gelperin, 1975; Chang and Gelperin, 1980; Sahley *et al.*, 1981; Gelperin, 1986; Culligan and Gelperin, 1983; Sahley *et al.*, 1992), the lack of learning to avoid dog food is more likely to be simply be due to the US (illness caused by the metaldehyde) not being recognised as a noxious stimulus by *Limax*, since it would not usually come across this sort of illness in the wild.

Since the US and CS used in this Chapter are probably relevant and strong enough to result in conditioning, an alternative explanation has to be found for the weak generalised unresponsive state seen by *Lymnaea* following training. One possible explanation comes to light if *Lymnaea's* behaviour is considered. In contrast to other gastropod molluscs, *Lymnaea* is never seen to withdraw from food, instead it avoids feeding by either going into a generally unresponsive state (sucrose unresponsive state; Chapter 3), or by reducing spontaneous feeding movements (satiation; Tuersley, 1986; Chapter 3). Other gastropods such as *Pleurobranchaea*, *Limax* and *Helix*, however, can and do actively withdraw from food in certain situations, e.g. following satiation and aversive conditioning (*Pleurobranchaea*; Davis *et al.*, 1983). As mentioned in the previous section, learning has been proposed to be strongest when the CS has a small, but finite, chance of eliciting the same response as the US (Mpitsos *et al.*, 1978). This can be explained in terms of "behavioural hierarchy" and its related stimuli (Mpitsos and Collins, 1975; Mpitsos *et al.*, 1978). Learning is best when the US has a high probability of eliciting a behaviour (i.e. at the top of the behavioural hierarchy) and the CS has a small chance of eliciting the same response (i.e. at the lower end of the same behavioural hierarchy). Therefore, a possible explanation for a species like *Pleurobranchaea* showing such strong avoidance conditioning using the food-shock paradigm is that food on the lips (the CS), although usually resulting in feeding, can occasionally result in withdrawal

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(i.e. food is low in the hierarchy of stimuli that may induce withdrawal). Therefore, when the CS (food) and US (electric shock) are paired, strong learning occurs, since the CS and US are low and high in the withdrawal behavioural hierarchy respectively. Similarly, this behavioural hierarchy hypothesis offers an explanation as to why *Lymnaea* does not show the expected conditioned response (withdrawal) after conditioning. Since food to the lips never results in *Lymnaea* actively withdrawing, but only in feeding or being unresponsive, it is impossible for *Lymnaea* to make the association between the food CS and a noxious US withdrawal. This is because the two stimuli excite different behavioural networks (feeding and withdrawal), i.e. the CS and US responses are not in the same behavioural hierarchy. Therefore, it is possible that the "conditioned response" of *Lymnaea* (short term generalised unresponsive state) seen after aversive conditioning is adopted because *Lymnaea* pairs the food CS with the "not responding" component of feeding behaviour caused by the US, rather than to an active withdrawal component. In this case, the CS and US both have different effects on the same behavioural hierarchy (feeding), and the conditioned response of a "generalised unresponsive state" is adopted, since it is the only method that can be used by *Lymnaea* to avoid attractive tasting food.

Difference in positive and aversive conditioning capabilities in relation to *Lymnaea*'s habitat.

The results in this Chapter have shown that *Lymnaea* is unusual within the gastropod molluscs, and in fact, most of the animal kingdom, since it appears to show very strong appetitive conditioning but weak aversive conditioning. But why does this behaviour occur?

In 1970, Seligman put forward the principle that the ability of an animal to learn a particular association was dependent on the animal's "preparedness" to learn that paradigm, and this was governed by the animal's 1) sensory capabilities, 2) evolutionary history, and 3) ecological niche. *Lymnaea* has been shown to have the sensory capabilities to make both the

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aversive and positive associations, since control snails react appropriately to food and noxious stimuli (by feeding and withdrawing respectively), and amyl acetate is mildly excitatory at the neural level before conditioning. Therefore, since Part 1) of Seligman's principle is fulfilled, Parts 2) and 3) (evolutionary history and ecological niche) must be the basis of why *Lymnaea* is able to make strong positive and only weak aversive associations.

In 1984, Alexander *et al.* attributed the rapid one-trial positive conditioning in *Lymnaea* to its evolutionary history and ecological niche. *Lymnaea* has evolved to be a wide ranging omnivore, living in an environment where it comes into contact with many potential food sources (slow moving or stagnant water, freshwater ponds). Therefore, appetitive (positive) conditioning is very adaptive, as it allows *Lymnaea* to fully exploit its environment by learning to recognise novel food sources (Alexander *et al.*, 1984). This is in contrast to a carnivorous feeder with a limited diet such as *Pleurobranchaea*, which needs 100-140 training trials before appetitive learning is seen (Mpitsos and Davis, 1973). In support of this hypothesis, strong, rapid appetitive (positive) conditioning has also been seen in other wide-ranging omnivorous species, such as *Limax* (Sahley *et al.*, 1981, 1985, 1990, 1992). However, studies on *Limax* have also shown that it is adaptive to show rapid aversive conditioning, because as well as encountering many new potential food sources, *Limax* also encounters certain foods which are not appropriate (e.g. plants containing alkaloids) (Gelperin, 1975; Sahley *et al.*, 1981, 1992). In fact, studies on *Limax* showed that although appetitive conditioning was strong (learnt after less than 10 pairing trials), aversive conditioning to food was stronger, with *Limax* showing one-trial aversive conditioning (Sahley *et al.*, 1990).

Since *Lymnaea* is a wide-ranging omnivore like *Limax,* it is surprising that it too does not show strong classical aversive conditioning. One possible explanation for this is that *Lymnaea* is simply not "prepared" to make strong aversive associations because of the environment it has evolved in. There are two major factors why aversive conditioning may

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be adaptive for omnivores: i) to avoid predators, and ii) avoid inappropriate (harmful) foods. One hypothesis for poor aversive learning, therefore, could be that it is not adaptive for *Lymnaea* to make aversive associations because it lives in a relatively safe environment with very few predators. This, however, is not the case; in addition to *Lymnaea* being host to numerous commensals and parasites (including protozoa, ciliates, sporozoan parasites, trematodes and nematodes), it is also attacked by insects (e.g. the water beetle *Dytiscus marginalis*), fish, salamanders, frogs and toads (Hyman, 1967). This leads to a second hypothesis; despite these many predators, it is still not adaptive for *Lymnaea* to quickly learn to associate certain tastes with "danger". One possible reason for this is that "danger" may not always be associated with a particular food type, e.g. a carnivorous fish may be near to one patch of algae but not another patch of the same alga. Therefore, if *Lymnaea* made an association of attacks by fish with a particular algae, it would be inappropriate because it would stop *Lymnaea* from feeding from other perfectly safe areas of algae. Thus, it could be hypothesised that it is more adaptive for *Lymnaea*, if attacked, simply to withdraw, turn and move away from that area in search of a safer place to feed, i.e. show the stereotyped avoidance behaviour described in Chapter 4. If this were the case, then the "generalised unresponsive state" seen after aversive training in this Chapter may only represent behaviour seen in extreme cases where *Lymnaea* has been repeatedly attacked whilst feeding on a particular food. In this situation *Lymnaea* may respond by going into an unresponsive state for a few hours, by which time the predator hopefully will have moved on.

This hypothesis, although explaining how *Lymnaea* might deal with predators, does not take into account the necessity of avoiding the ingestion of harmful foods (such as plants containing alkaloids). One explanation could be that compared to the *Limax*'s terrestrial environment, there are very few inappropriate foods in the freshwater environment, resulting in *Lymnaea* having little need to evolve strong aversive conditioning like *Limax*. This view

is supported by the fact that plant alkaloids and other toxic secondary compounds, which are harmful if ingested, are mostly found in higher plants (i.e. herbaceous dicots) in the terrestrial environment (Salisbury and Ross, 1985). Therefore, if this is the case, the reason *Lymnaea* shows weak aversive conditioning is not because it has poor learning capabilities, but rather that it is not appropriate (or adaptive) in its environment to have evolved this type of learning. Another explanation for the poor learning seen in this Chapter, however, is that the paradigms used, although appropriate for studying predator avoidance, may not have been appropriate for studying avoidance of harmful food. This is because the US reinforcers used (electric shock and noxious prod) only had immediate and short term deleterious effects, and thus may not have been relevant to *Lymnaea*, since ingestion of harmful food would probably result in more long-term illness. Therefore, it is possible that *Lymnaea* is capable of showing strong aversive conditioning if the US reinforcer induces acute or chronic poisoning. Hopefully this possibility will be investigated in the future.

CHAPTER 7 -
CONCLUDING COMMENTS

CONCLUDING OBSERVATIONS AND COMMENTS

THE USE OF *LYMNAEA* AS A MODEL FOR NEUROETHOLOGICAL RESEARCH.

This thesis ends as it began: by supporting the use of invertebrate models for neuroethological research. In the General Introduction the advantage of using invertebrates was credited to the relatively simple behavioural repertoires and nervous systems of these animals. Although *Lymnaea* is considered to be reasonably "simple", I believe the work in this thesis has shown it to be complex enough to present some very interesting questions (and some possible answers) on how, and why, different behaviours are generated.

One key observation which has been made repeatedly throughout the thesis is that although *Lymnaea* has a similar feeding system to other gastropod molluscs, there are subtle differences. These differences have been proposed to be a consequence of the contrasting habitats in which the different species have evolved, which ultimately govern the animal's behaviour and its underlying neural mechanisms. *Lymnaea*, presumably because it evolved in an environment where there is an abundance of food, has become a grazing omnivore and spends a large proportion of its life feeding. A consequence of this, which has been repeatedly highlighted by this thesis, is that feeding is extremely important to *Lymnaea*. This has been illustrated by the fact that the neural system appears to be "geared" to feed. This means that factors which normally inhibit feeding in other species (e.g. satiation, aversive stimuli and aversive conditioning) have a much less pronounced effect on *Lymnaea's* feeding behaviour (Chapters 3, 4 and 6). In addition, *Lymnaea* shows spontaneous feeding movements in the absence of food (Tuersley, 1986; Chapter 3), a behaviour not shown by *Pleurobranchaea*, *Limax* or *Aplysia*, which further indicates that the feeding system is very active in *Lymnaea*. It is interesting to consider that the active state of *Lymnaea's* feeding system was probably a major factor in enabling workers to research and describe the feeding central pattern

generator in relatively greater detail (for review, see Benjamin and Elliott, 1989) than in other species. However, this "activeness" causes a problem when using *Lymnaea* as a model for studying mechanisms of inhibition of feeding, since inhibition rarely happens! Perhaps, therefore, *Lymnaea* is not a particularly appropriate model for this type of study. This, of course, does not make *Lymnaea* a redundant animal model, since differences between species are interesting in themselves, but it does highlight the importance of considering the relevance of using particular animal models before embarking upon neuroethological research.

THE USE OF THE SEMI-INTACT PREPARATION.

The results in this thesis highlighted a common problem within neuroethology: that electrophysiological results obtained from reduced semi-intact preparations do not always mirror behavioural observations (Culligan and Gelperin, 1983; Kemenes *et al.*, 1986): e.g. in Chapter 3, feeding was more reliably initiated by sucrose in the whole animal compared to the semi-intact preparation; in Chapter 4, citric acid reliably caused regurgitation and inhibition of ingestive feeding in the whole animal but not in the semi-intact preparation; in Chapter 6, positively conditioned snails showed a more reliable response to amyl acetate in the whole animal compared to the semi-intact preparation.

Whole animal behaviour is a result of many complex interactions between the external and internal environment. Therefore, for true behaviour to occur, the body has to be intact. This is a problem for neuroethologists, since recording neural activity requires the nervous system to be exposed, which of course will affect certain parameters, e.g. not all neural pathways are intact and neurohormonal influences are changed. An answer to this problem in the future may be to use chronic electrode implantation methods, which will allow recording of neural activity whilst the intact animal is behaving normally. This method is already being employed in molluscan neurobiology (Parsons *et al.* 1983; Fiore *et al.*, 1992;

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Balaban, 1993; Morton and Chiel, 1993a; M. Yeoman, pers. comm.). However, since the implanted electrodes have, as yet, only been used to stimulate or record extracellularly, this type of recording is still crude, giving no detailed information on the interactions of the neurons within neural networks. Consequently, with the chronic implantation method still being in its infancy, intracellular recordings from semi-intact preparations (despite their limitations) currently provide the best detailed information of how behaviour is generated, and hopefully should provide the ground work for the more sophisticated whole animal recordings of the future.

FINAL WORD......

If you were concerned that the life of the laboratory gastropod is not a happy one, then take comfort in the knowledge that gastropods sometimes *do* have a good time. Thanks to some rather bizarre research by Balaban (1993), it's now been shown that gastropods can feel "pleasurable sensations", and not only can they feel them, but they actively seek them out!

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Research showed that after chronically implanting stimulating electrodes (which could be operated by the snails themselves) into different "areas" of *Helix*'s brain; snails that had electrodes implanted in the sexual areas of the brain "self-stimulated" themselves significantly more times than sham-operated controls (Balaban, 1993; Balaban and Maksimova, 1993).

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