Neurophysiological Correlates of Unconditioned and Conditioned Feeding Behavior in the Pond Snail Lymnaea stagnalis

KEVIN STARAS, GYÖRGY KEMENES, AND PAUL R. BENJAMIN
Sussex Centre for Neuroscience, School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, United Kingdom

INTRODUCTION

In the quest to elucidate the cellular mechanisms of behavioral plasticity, two experimental approaches have been particularly successful. The first one is based on development of in vitro preparations where electrophysiological manipulation of neuronal pathways aims to mimic the behavioral conditioning paradigms that can produce learning in intact animals. This approach has been particularly useful in elucidating cellular and molecular mechanisms of a variety of nonassociative and associative types of learning in invertebrates (reviewed in Carew and Sahley 1986; Krasne and Glanzman 1995) and Hebbian-type synaptic plasticity in mammals (reviewed in Izquierdo and Medina 1995; Maren and Baudry 1995). With this approach, however, it is often difficult to find a causal link between the cellular mechanisms described and the behavior of the intact animal (see Morris 1994). The second experimental approach, also successfully used in a variety of invertebrate and vertebrate preparations (reviewed in Byrne 1987) is based on subjecting intact animals to behavioral conditioning and then looking for specific changes in the nervous system correlated with learning. Here, the direct links between cellular events and behavior of intact animals are easier to establish.

Ideally, both approaches should be used on the same system to help establish which cellular events are relevant to the behavior of intact animals. We already have reported that semi-intact preparations made from the pond snail Lymnaea stagnalis can be subjected to in vitro appetitive tactile conditioning, which results in the build-up of a conditioned fictive feeding response recorded on motoneurons and central pattern generator (CPG) interneurons of the feeding network (Kemenes et al. 1997). Here we use the alternative approach where behavioral training in intact Lymnaea was followed by electrophysiological analysis in semi-intact preparations to find a neurophysiological correlate of the conditioned feeding response. Lymnaea is a promising candidate for investigating the neuronal basis of appetitive learning because it can undergo reliable appetitive conditioning (Audesirk et al. 1982; Kemenes and Benjamin 1989a; Kojima et al. 1996) and the neuronal mechanisms underlying feeding are known in considerable detail. Feeding is generated by a CPG system (Benjamin and Elliott 1989), similar to that also commonly found in higher organisms (reviewed in Jacklet 1989). Importantly, rhythmic activity (fictive feeding) evoked by unconditioned or conditioned stimuli in the feeding CPG can be monitored in semi-intact preparations (Elphick et al. 1995; Kemenes et al. 1986) by intracellular recording of feeding motoneurons active in the three different phases of the feeding motor program (Rose and Benjamin 1979) (see Fig. 1).

So far, invertebrates have provided valuable models for the cellular analysis of aversive learning (Krasne and Glanzman 1995), but appetitive learning has only been analyzed at the cellular level in a few invertebrate models, the best known of which is the olfactory conditioning of the proboscis extension reflex in the bee Apis mellifera (Hammer 1993). In the present paper, we describe how neurophysiological traces of patterned unconditioned and conditioned feeding responses in intact Lymnaea survive into the semi-intact preparation and identify some of the neuronal changes associated with behavioral appetitive learning.

METHODS

Wild-type specimens of adult L. stagnalis, were obtained from animal suppliers (Blades Biological, Kent, UK). Animals were kept in groups in large holding tanks containing copper-free water at 18–20°C on a 12 h:12 h light:dark regime and fed lettuce three times a week. Before an experiment, animals were moved into
Behavioral experiments

Behavioral experiments were performed to first establish the level of unconditioned feeding responses of naive, intact animals to tactile and chemical stimuli. Later the animals were subjected to an appetitive classical conditioning procedure using touch to the lips as the conditioned stimulus (CS) and sucrose as the unconditioned stimulus (US).

Touch (CS) and sucrose (US) induced feeding responses in naive animals

Subjects (n = 9) were taken from the home tank and placed in test dishes containing 90 ml of copper-free water. During the transfer, the snails partially retracted into their shells from which they re-emerged once they were in the test dish (mean re-emergence time: 22.4 ± 3.5 (SE) s). Two minutes after emergence, each subject was presented with a lip touch, and any feeding movements were recorded for a further 2 min. In Lymnaea, feeding movements (rasps) consist of cycles of mouth opening and closing with accompanying extension and backward rotation of the radula of the buccal mass (Carriker 1946; Dawkins 1974). After testing, the animals were removed from the test dish, numbered with an indelible ink and replaced in the home tank environment. After a minimum of 2 h, the procedure was repeated, but this time the same animals were presented with sucrose, a strong unconditioned chemical feeding stimulus (Kemenes et al. 1986). Feeding movements were again recorded for a further 2 min after which the animals were rinsed in a clean-water tank to remove any residual sucrose and replaced in their home tanks where they were kept overnight before subsequent electrophysiological testing (see further text). The snails’ feeding behavior was observed using a mirror placed under the dish apparatus, which permitted a clear view of the snails mouthparts. These procedures are similar to those described by Kemenes and Benjamin (1989a,b; 1994; Kemenes et al. 1986; Tuersley and McCrohan 1987).

Touch (CS) responses after appetitive conditioning

Initial tests were performed to establish that a conditioned response was attainable by pairing touch as the CS with sucrose as the US in the batch of snails involved in the present experiments. The hypothesis that the experimental animals were indeed being classically conditioned and not just undergoing nonassociative learning was tested using four different control groups. Because it would have been technically difficult to subject more than three groups of animals to training at the same time, two separate training experiments were performed to include all the controls. In each of these, a group of experimental animals were trained with two different control groups. The first group of experimental snails (n = 9) were trained with a US only and a handling control group (n = 8 in each), whereas the second experimental group (n = 8) was trained with a CS only (n = 8) and a random US + CS control group (n = 9).

Having established the level of conditioned response that can be built up in the experimental but not in any of the control groups in the batch of animals used in the present experiments, a third experiment then was performed to look at the neurophysiological correlates of the behaviorally conditioned response. In this experiment, we conditioned nine experimental animals, and these were compared both in vivo and in vitro with a random control group (n = 9), the most important control, which includes the use of all three stimuli (touch, food, and handling) that could have nonassociative effects on the feeding behavior.

The classical conditioning procedure in all three training experi-
ments was as follows: subjects from the experimental groups were removed from their home tank and placed in individual training dishes. Two minutes after transfer, each snail was given a touch to the lips (CS) followed immediately (interstimulus interval: <1 s) by presentation of sucrose (US) from a 10-ml syringe (0.01 M final concentration). After a further 2 min, the snails were transferred to a clean water rinsing tank. After 5 min rinsing, all snails were replaced in the home tank. In the CS only, US only, and handling control groups, the procedures were identical except that tactile stimulation or sucrose presentation or both were omitted. In random CS-US controls, the intervals between CS and US were varied between 0–30 min and the order (CS-US or US-CS) was randomized.

Both experimental and control animals received five trials a day with 60-min intertrial intervals. Training continued for 3 days so that a total of 15 trials were carried out. This spaced training protocol previously had been shown to produce successful chemical and tactile appetitive conditioning in *Lymnaea* (Alexander et al. 1984; Kemenes and Benjamin 1989a). In all control and experimental groups, the home tank water was replaced each evening and animals received a small amount of lettuce. After the 15th trial, animals were placed in clean water and left undisturbed for ≥2 h before testing them on the same day as the final training trials.

Testing was performed using a blind procedure, by an experimenter who had no knowledge of the training history of the group of animals being tested. During testing, an animal was removed from the tank and placed in a test dish containing 100 ml water, and all subsequent feeding events were recorded with the event recording software. After 2 min after emergence, the tactile stimulus was presented to the lips, and the feeding activity recorded for a further 2 min. The animal was removed, numbered using an indelible ink marked onto the shell, and replaced in the tank.

### Statistical analysis of behavioral data

The feeding responses to touch and sucrose were quantified by awarding a behavioral score to each animal. This was calculated by subtracting the rasp rate for the minute preceding a stimulus (touch or sucrose) from the rasp rate for the minute after the first rasp after the same stimulus. This scoring procedure is based on a method previously used by Kemenes and Benjamin (1994) in behavioral experiments with *Lymnaea*. In experiments with naive animals, the behavioral score data for the touch stimulus were compared with those for the sucrose stimulus in the same animals by nonparametric Wilcoxon matched-pairs signed-rank tests. In the conditioning experiments, the scores from the experimental and control groups were first subjected to a Kruskall Wallis *H* test (a nonparametric equivalent to a 1-way analysis of variance (ANOVA)). If this revealed a source of significant difference between groups, it was followed by nonparametric post hoc multiple comparisons (Dunn test) (Dunn 1964) between all the groups. In all experiments, as well as comparing the behavioral score data between the groups, we also compared the pre- and poststimulus (touch or sucrose) feeding rates within each group by Wilcoxon matched-pairs signed-rank tests.

### Electrophysiological experiments

These experiments had two main aims. The first one was to establish quantitatively if electrophysiological correlates of the unconditioned touch and sucrose responses in the whole animal could be measured in semi-intact preparations from the same animals. This was important because if the electrophysiological events underlying the unconditioned behavioral responses did not survive into the preparations, there would be little chance of finding any electrophysiological correlates of conditioning in the intact animal. The second and main aim of these experiments was to find out if the conditioned response established in the intact snails could be recorded electrophysiologically in the semi-intact preparations.

### General procedures

Naive, random control or conditioned animals were dissected under a microscope in a silicon elastomer (Sylgard)-lined dish containing 2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES)-buffered snail saline (Benjamin and Winlow 1981). All the subsequent electrophysiological tests were performed by a second experimenter who was unaware of the behavioral history of the individual preparations. The preparation was transferred to a purpose-built Sylgard-coated electrophysiology chamber (volume 2–3 ml) containing saline and usually pinned dorsal-side up. The outer ganglionic sheath of the cerebral and buccal ganglia was removed using a pair of fine forceps. The second, inner sheath was softened using a nonspecific solid protease (Sigma, XIV, Sigma Chemical, Poole, UK).

#### Whole-lip CNS preparation

For these experiments, it was necessary to use a preparation in which the lip sensory-structures were left intact, and this led to the development of a whole-lip preparation (Fig. 1A). This proved to be more successful in getting reliable unconditioned responses (Staras et al. 1995) than the previously used ‘split lip’ preparations (Kemenes et al. 1986). The CNS was accessed by a dorsal body incision, and all peripheral nerves except for the median and superior lip nerves were severed. The buccal mass was excised, but a short length of oesophagus was left attached to the brain to allow pinning out. The posterior end of the foot was removed by a lateral incision and the preparation was arranged in the electrophysiology chamber so that the lip structures and mouth were accessible for stimulation. The buccal ganglia were stretched clear of the head structures over a raised block of Sylgard (to stabilize the preparation) and rotated 180° so that the dorsal side was exposed for intracellular recording (Fig. 1A).

#### Intracellular recording techniques

Glass microelectrodes (2 mm, Clark Electromedical, Redding, UK) were pulled on a Narashigi vertical electrode puller to a tip resistance of 10–50 MΩ when filled with 4 M potassium acetate solution. The electrode tips were dipped in a black etching ink (Rotring, part No. 595617) to improve visualization. The chamber containing the preparation was illuminated with a cold light source and viewed under a zoom microscope. A perfusion system driven by a peristaltic pump permitted a rapid exchange of saline within the chamber. Micromanipulators with attached headstage preamplifiers (Neurolog, Digitimer, Welwyn Garden City, UK) were arranged around the electrophysiology chamber, permitting up to four simultaneous intracellular recordings. Signals were fed into amplifiers (NL102G, Digitimer) incorporating a bridge-balance circuit for current injection and then outputted to a storage oscilloscope (GOULD 1604, Gould Instrument Systems, Hainault, UK) and a chart recorder (GOULD TA240S). All signals were recorded digitally using a DAT recorder (BIOLOGIC DTR-1801, Biologic Science Instruments, Claix, France).

#### Identification and selection of cell types

The main goal of the electrophysiological experiments was to monitor neuronal activity known to be underlying feeding responses in whole animals. This neuronal activity is called fictive feeding, and it is generated by a set of premotor CPG interneurons (Elliott and Benjamin 1985; Rose and Benjamin 1981b). These
neurons belong to three main types, N1, N2, and N3 (Fig. 1Bi), each active in one of the three behavioral phases of feeding, protraction (N1), rasp (N2), and swallow (N3). However, recording activity directly from these small interneurons in semi-intact preparations is technically difficult, and impalement with microelectrodes often triggers nonspecific excitation in them, making quantitative assessments of fictive feeding responses more difficult. An alternative and less invasive approach is to monitor CPG activity using identified buccal motoneurons that receive well-characterized synaptic inputs from the CPG during each phase of fictive feeding (Benjamin and Elliott 1989). An added advantage of this approach is that these large motoneurons could be located visually by size, position, and color so that they could be impaled with minimal disturbance to the rest of the system. In all experiments, the identified buccal motoneuron B3 was used as the main cell type to monitor fictive feeding because it is both large and has a very characteristic firing pattern (Fig. 1Bii). Previous work has established that the B3 cell is inhibited during N1 but is excited during both N2 and N3 (Benjamin and Rose 1979; Elliott and Benjamin 1985; Rose and Benjamin 1981a,b). This enabled us to obtain information on activity in all three classes of feeding CPG interneurons in response to tactile or chemical stimuli. In addition to B3, in all experiments, the B4 or B1 motoneuron or both also were recorded (Fig. 1A). The B4 cells are inhibited during N1 and the first phase of N2 but excited during the second phase of N2 and during N3, whereas the B1 cells are excited during N1 and silent in the N2 and N3 phases (Benjamin and Rose 1979; Elliott and Benjamin 1985; Rose and Benjamin 1981a,b).

In the semi-intact preparations, we tried to reproduce the tactile and chemical stimuli used in intact animals as closely as possible. The only difference was that instead of the hand-held probe that had to be used in the freely moving intact animals, we now could use an electromagnetic coil-driven mechanical probe (Fig. 1A) to deliver tactile stimuli of a consistent force-position, and color so that they could be impaled with minimal disturbance to the lips in the semi-intact preparations. However, the end of the probe consisted of the same thin wedge of soft, flexible plastic that was used in the behavioral experiments, and this hit the same target area on the lips in the preparations as in the intact animals. The feeding stimulus, sucrose solution at 0.01 M concentration, was the same as in the intact animals. It was delivered from the end of a thin plastic tube that was positioned near the lip chemoreceptors (Fig. 1A), and it was removed from the chamber within 2 min by rapid perfusion with fresh saline. We therefore were satisfied that the ways the tactile and chemical stimuli were applied in intact animals and preparations were sufficiently similar. Like the behavioral tests, the neurophysiological experiments on trained animals were performed blind so that the presentation of the tactile CS to a preparation was carried out without the identity of the subject (control or conditioned)

**FIG. 2.** Behaviorally recorded touch (CS) and sucrose (US) responses in naive, intact animals. Ai: example of the lack of a feeding response to tactile stimulation of the lips in a naive animal. Feeding movements (rasps) were recorded using event-recording software on a continuous time base (horizontal line). Each vertical line represents a single rasp. Aii: example of a typical feeding response to sucrose in a naive, intact animal. Presence of sucrose evokes a continuous series of rasps. Bi and Bii: statistical comparison of feeding responses in whole animals to touch and sucrose. Data are presented as medians and interquartile ranges. Bi and Bii: absolute feeding rates before and after the presentation of stimuli. Bii: differential scores calculated from the pre- and poststimulus data. Bi: summary of the feeding responses to the touch stimulus (CS) in a group of naive animals (n = 9). Feeding rate after touch presentation is not significantly different from the pre-CS feeding rate. Bii: summary of the feeding responses to the sucrose stimulus (US) in the same group (n = 9). Feeding rate after sucrose presentation is significantly larger than the presucrose feeding rate. Bii: comparison of the change in feeding rate after touch (CS) and sucrose (US) presentation. Change (increase) was significantly greater on the application of US than on application of CS (for detailed statistical data, see Results).
being revealed. All electrophysiological experiments with previously conditioned and control animals were completed within ~16–24 h after the last trial, with a dissection schedule designed to ensure that the range of intervals between training and electrophysiological tests were the same for experimental and control groups. We assumed that there was no loss of memory between the behavioral and electrophysiological tests because previous experiments have shown that appetitive conditioning with 15 trials produces long-term memory and that experimental snails tested 24 h after the last training trials show conditioned responses that are not significantly different from the responses seen shortly after the end of training (Kemenes and Benjamin 1994).

Analysis of electrophysiological data

All the analysis was performed blind, with the person analyzing records from individual preparations being unaware of their status as experimental or control animals. The number of fictive feeding cycles (sequences of N1, N2, and N3 phase activity) occurring on the B3 (and when these also were recorded on the B1 and B4) motoneurons of the semi-intact preparations were counted for 1 min before the application of a touch (CS) or sucrose stimulus (US) to the lips and for 1 min after the first cycle after the stimulus. The difference between the numbers of post- and prestimulus cycles yielded a score that was used as a measure for unconditioned or conditioned fictive feeding.

In experiments with naive animals, the fictive feeding score data for the touch stimulus were compared with those for the sucrose stimulus by nonparametric Wilcoxon matched-pairs signed-rank tests. In the conditioning experiment followed by electrophysiological analysis, the fictive feeding scores from the experimental and control group were compared by a Mann-Whitney U test. For all electrophysiological experiments, we also compared the pre- and poststimulus (touch or sucrose) fictive feeding rates within each group by Wilcoxon matched-pairs signed-rank tests.

RESULTS

Tactile (CS) and sucrose (US) responses in naive animals

INTACT ANIMALS. Animals (n = 9) were transferred to the experimental chamber, and their feeding responses to touch and sucrose were tested. This generated behavioral data on the effect of the presentation of the CS and the US before preparations from the same animals were tested electrophysiologically. CS and US in these tests with naive animals were only applied in the CS followed by US order because application of sucrose (the US) often leads to longer-term

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Neurophysiologically recorded responses to touch (CS) and sucrose (US) stimuli in naive semi-intact whole-lip preparations. Ai: example of a response to tactile stimulation of the lips recorded in the identified feeding motoneuron B3. Touch to the lips induces a brief depolarization on the B3 cell followed by a long-lasting hyperpolarization, but no activity typical of a fictive feeding rhythm is present. Aii: example of a fictive feeding response resulting from the presentation of a sucrose stimulus to the lips. Sucrose evokes characteristic cyclical synaptic inputs in the B3 neuron that reflect activity in the feeding CPG. B3 neuron receives strong depolarizing waves in the N2 and N3 phases of the feeding rhythm (N2 and N3 CPG inputs) that are sufficient to trigger bursts of action potentials. B: statistical comparison of feeding responses in semi-intact preparations to touch and sucrose. These preparations were made from the same animals for which the behavioral results are shown in Fig. 2. Data are presented as medians and interquartile ranges. Bi: summary of the fictive feeding responses to the touch stimulus (CS) in preparations from naive animals (n = 9) showing fictive feeding rates before and after the stimulus presentation. Fictive feeding rate after CS presentation is not significantly different from the pre-CS fictive feeding rate. Bii: summary of fictive feeding responses to the sucrose stimulus (US) in the same preparations (n = 9). Fictive feeding rate after US presentation is significantly larger than the pre-US fictive feeding rate. Biii: comparison of the change in the fictive feeding rate after touch (CS) and sucrose (US) presentation. Change (increase) was significantly greater on the application of US than on application of CS (for detailed statistical data, see RESULTS).
behavioral and electrophysiological changes (prolonged feeding/fictive feeding) outlasting the stimulus by very variable amounts of time (∼1–45 min). This, especially in semi-intact preparations (see further) where there is a practical time limit on how long one can wait after the application of US, would have made counterbalancing the CS-US order very difficult.

Effect of the lip-touch CS on feeding behavior

In the majority (7/9) of intact, naive animals, the lip-touch CS did not evoke any feeding movements. This lack of a feeding response to touch is shown in Fig. 2Ai. For the whole population of snails, which included the two responding subjects, the feeding rate both before and after the CS was still very low (medians: 1.0 and 0.0 raps/min, interquartile ranges: 0.0–3.5 and 0.0–1.0, respectively, Fig. 2Bi). A pairwise comparison using the Wilcoxon matched-pairs signed-rank test revealed that the pre- and post-CS feeding rates were not significantly different (Z = −0.7, P = 0.46).

Effect of the sucrose US on feeding behavior. In contrast to touch, the sucrose US caused strong reliable activation of feeding movements in the same animals (compare Fig. 2A, i and ii). All nine animals in the group showed a large elevation in the frequency of feeding movements after the sucrose was presented to the test chamber (from a median 1.0 raps/min (interquartile range: 0.0–2.0) to 17.0 raps/min (interquartile range: 14.5–20.0); Wilcoxon matched-pairs signed-rank test: Z = −2.6, P < 0.01, Fig. 2Bi), and this heightened level of feeding activity persisted until the subjects were removed from the dish.

Feeding rate scores in the intact snails (Fig. 2Bi) were significantly higher after sucrose application (median: 16.0 raps/min, interquartile range: 14.5–17.5) compared with touch (median: 0.0 raps/min, interquartile range: −2.5–

![Graphs](http://jn.physiology.org/ Downloaded from http://jn.physiology.org/)

**Fig. 4.** Responses to the touch stimulus (CS) in appetitively conditioned and control intact animals after 15 training trials. Two separate experiments (A and B), each with an experimental (CS + US) and 2 different control groups, were carried out. Conditioned feeding response data are presented as medians and interquartile ranges. Ai and Bi: summary of the level of feeding responses to a touch stimulus to the lips in the control (Ai: handling, n = 8; US only, n = 8; Bi: CS only, n = 8; random, n = 9) and experimental groups (n = 9 in each) showing feeding rates before (Bef.) and after (Aft.) the stimulus presentation. Statistical analysis revealed that the feeding rates in the 2 experimental groups after the CS presentation were significantly greater than the pre-CS rates in the same groups. No significant differences between pre- and post-CS data were found in either of the 2 control groups in each separate experiment. Ai and Bi: comparison of the change in feeding rates after tactile presentation for the 3 groups in Ai and Bi, respectively. In both experiments, the change (increase) in feeding rate in the experimental groups was significantly greater than in any of their respective controls (for detailed statistical data, see RESULTS). **–**–,**–**, median level of unconditioned feeding response (UR) to sucrose.
0.5) (Wilcoxon matched-pairs signed-rank test: $Z = -2.7, P < 0.01$).

**SEMI-INTACT PREPARATIONS.** Having established the basic feeding responses in the whole animals, the same animals were dissected to make semi-intact whole lip-CNS preparations ($n = 9$). Specific identified feeding motoneurons (predominantly the B3 cells, see METHODS) were targeted for intracellular recording, and the presence of CS and US responses were assessed in a quantitative manner.

**EFFECT OF THE LIP-TOUCH CS ON FICTIVE FEEDING BEHAVIOR.** A typical touch CS response in a semi-intact preparation is shown in Fig. 3Ai (this is the same animal shown in the behavioral example in Fig. 2Ai). In all the preparations ($n = 9$), a characteristic synaptic response was present on the B3 neurons, suggesting that the access of sensory pathways to the central motor network was retained. This synaptic response consisted of a brief initial depolarization followed by a long-lasting hyperpolarizing wave. However, the touch CS had little influence on the frequency of fictive feeding cycles as shown by the identical pre- and post-CS median rates of 0.0 cycles/min (interquartile ranges: 0.0–1.5) (Wilcoxon matched-pairs signed-rank test: $Z = -0.53, P = 0.59$, Fig. 3Bi). This is consistent with the behavioral findings in suggesting that statistically the CS is neither a promoter nor an inhibitor of feeding.

**EFFECT OF THE SUCROSE US ON FICTIVE FEEDING BEHAVIOR.** Presentation of sucrose to the lips had a very different effect on the activity of B3 compared with touch. After an initial strong excitatory synaptic input (Fig. 3Aii), seen on all B3 neurons recorded, the sucrose stimulus also produced feeding CPG-driven fictive feeding patterns in eight of the nine semi-intact preparations tested (example in Fig. 3Bii). The pre- and post-US median fictive feeding rates (0.0 and 5.0 cycles/min, interquartile ranges: 0.0–1.0 and 4.0–5.5, respectively, Fig. 3Bii) were significantly different (Wilcoxon matched-pairs signed-rank test: $Z = -2.5, P < 0.01$). The absolute frequency was lower than that seen in the behavioral results from the whole animal (compare Figs. 2Bii and 3Bii), but a significant difference (Wilcoxon matched-pairs signed-rank test: $Z = -2.5, P < 0.01$) still occurred when the median change in feeding activity after sucrose US (4.0 cycles/min, interquartile range: 2.0–5.5) was compared with the touch CS (0.0 cycles/min, interquartile range: −0.5–0.0) in the same semi-intact preparations (Fig. 3Bii).

**Classical conditioning of the feeding response to a tactile lip stimulus**

**APPETITIVE LEARNING IN INTACT ANIMALS.** To establish the level of the classically conditioned response attainable in the batch of animals used in the present experiments, two experimental groups (CS + US, $n = 9$ and 8, respectively) were trained, each with two control groups [handling control ($n = 8$), US only ($n = 8$); and CS only ($n = 8$), random CS + US ($n = 9$), respectively].

A nonparametric one-way ANOVA performed separately on the behavioral score data from the first set of groups (Fig. 4Ai) and the second set of groups (Fig. 4Bi) revealed a source of significant difference in each set (Kruskall-Wallis $H$ tests: $H = 9.9$ and 9.5, respectively; $df = 2, P < 0.01$ for both sets). Multiple post hoc Dunn tests established that this did not arise from differences between controls (handling vs. US only, $Q = 0.42, P < 0.5$; CS only vs. random, $Q = 0.40, P > 0.5$). However, the same type of post hoc comparison of the experimental groups with each of their controls showed that the conditioned touch response in both experimental groups was significantly stronger than in any of their respective controls (Fig. 4Ai: CS + US vs. handling, $Q = 2.51, P < 0.05$; CS + US vs. US only, $Q = 2.94, P < 0.01$). Fig. 4Bi: CS + US vs. CS only, $Q = 2.43, P < 0.05$; CS + US vs. random, $Q = 2.91, P < 0.01$). This stronger response in the experimental groups was due to a significant increase of the response after conditioning (Fig. 4, Ai and Bi, Wilcoxon matched-pairs signed-rank tests:

![Fig. 5. Responses to the touch stimulus (CS) in control and appetitively conditioned intact snails after 15 training trials. These animals were subsequently made into semi-intact preparations and analyzed neurophysiologically (see Figs. 6 and 7). Conditioned feeding response data are presented as medians and interquartile ranges. Ai: summary of the level of feeding responses to the touch stimulus in the random control group ($n = 9$) showing feeding rates before and after the stimulus presentation. No significant differences between pre- and post-CS data were found. Aii: change in feeding rate after tactile presentation for the data shown in Ai. Bi: summary of the level of feeding responses to the touch stimulus in the conditioned group ($n = 9$) showing feeding rates before and after the stimulus presentation. Statistical analysis revealed that the feeding rate in the experimental group after the CS presentation was significantly greater than the pre-CS rate in the same group. Bii: change in feeding rate after tactile presentation is statistically larger than the change in the random control group shown in Aii (for detailed statistical data, see RESULTS). **p < 0.01, median level of unconditioned feeding response (UR) to sucrose.**
With the same analysis, no significant differences between pre- and post-CS feeding responses were found in any of the total of four control groups used in the two experiments. Finally, a pairwise comparison of the conditioned feeding response in the two experimental groups showed that these were not statistically different (medians: 10.0 and 9.0 cycles/min, interquartile ranges: 0.0–11.8 and 3.8–10.0, respectively, $U = 28.0$, $P = 0.44$).

NEUROPHYSIOLOGICAL EXPRESSION OF BEHAVIORAL APPETITIVE LEARNING IN REDUCED PREPARATIONS. Behaviorally trained and tested control and experimental animals were subjected to in vitro intracellular recording to search for neurophysiological correlates of learning.

Two groups were used, a CS + US experimental group and a CS + US random control ($n = 9$ in each). The results of the behavioral conditioning procedure, which was identical to the method used in the previous experiment, are shown in Fig. 5. The absolute pre- and post-CS values are presented in Fig. 5A. Although the random control group (Fig. 5Ai) showed a marked, but nonsignificant decrease in rasp rate after the CS presentation (Wilcoxon matched-pairs signed-rank test: $Z = -0.77$, $P = 0.44$), the experimental group (Fig. 5Bi) showed a significant increase between the pre-CS and post-CS rates with the same test ($Z = 2.5$, $P < 0.01$). From these absolute values, median differences calculated by subtracting the pre-CS rate from the post-CS rate for each animal are shown in Fig. 5, Aii and Bii. The conditioned behavioral response was significantly higher in the experimental versus the control group (Mann-Whitney $U$-test: $U = 8.0$, $P < 0.004$). The magnitude of the conditioned response in the whole animals in this third experiment (median: 9.0 cycles/min, interquartile range: 5.5–12.5) was practically identical to those found in the previous two experiments (compare Figs. 4, Ai and Bi, with 5Bi and Figs. 4, Aii and Bii, with 5Bii). This confirmed that the subsequent electrophysiological experiments (see further) were performed on a group of animals that belonged to a batch that showed consistent classical conditioning of the feeding response.

All snails from the two groups of nine experimental and nine control behaviorally trained animals were tested in vitro. The animals were dissected into semi-intact whole-lip + CNS preparations, and CS-evoked fictive feeding cycles were measured electrophysiologically. An example of the occurrence of both a behaviorally conditioned response in the whole animal and its electrophysiological correlate in the semi-intact preparation is shown in Fig. 6A. Touch to the lips evoked feeding movements in the intact conditioned animal (Fig. 6Ai) and fictive feeding cycles in the semi-intact preparation made from the same animal (Fig. 6Aii). In contrast, neither feeding movements (Fig. 6Bi) nor fictive feeding (Fig. 6Bii) were evoked in the example shown for the random control animals. Also note that in this control preparation the initial synaptic inputs received by B3 after touch resembled those seen in naive animals (see Fig. 3Ai). However, the initial synaptic effect on B3 of the touch stimulus in conditioned animals resembled the initial synaptic
The statistical results showing the level of touch-evoked fictive feeding in reduced preparations made from the same animals that had been subjected to behavioral appetitive conditioning and control procedures (see Fig. 5) are shown in Fig. 7. The first two graphs give the absolute fictive feeding values recorded in the random control (n = 9, Fig. 7Ai) and experimental group (n = 9, Fig. 7Bi) for the minute before and after the presentation of the tactile CS. Although the random control group shows no change in the fictive feeding rate after the CS (Wilcoxon matched-pairs signed rank test: Z = -1.3, P = 0.18), the experimental group shows a significant increase between the pre- and post-CS rates using the same test (Z = 1.99, P < 0.05). Figure 7 also shows the summary data for this experiment in which the pre-CS fictive feeding rate is subtracted from the post-CS rate for the control (Fig. 7Aii) and the experimental groups (Fig. 7Bii). These data are significantly different in the experimental versus the control group (Mann-Whitney U-test: U = 18.5, P < 0.03). The results of this experiment indicate that a neurophysiological trace of the conditioned behavior, CS-evoked fictive feeding, established by the appetitive classical conditioning procedure in whole animals, survives into the semi-intact preparation and can be recorded as an electrophysiological correlate in identified neurons of the feeding system.

**DISCUSSION**

In this paper we demonstrated that neurophysiological correlates of patterned unconditioned and conditioned feeding responses could be found in semi-intact preparations in a molluscan model system *Lymnaea*. Unconditioned fictive feeding responses, evoked by chemical stimulation of peripheral chemoreceptors already have been reported in a number of molluscan preparations (Gelperin et al. 1978; Gillette et al. 1978; Horowitz and Senseman 1981; Nagahama and Takata 1990; Yoshida and Kobayashi 1992), including *Lymnaea* (Elphick et al. 1995; Kemenes et al. 1986; Yeoman et al. 1995). There have been two previous papers reporting conditioned fictive feeding in mollusks. These are an early study demonstrating tactile CS-evoked patterned activity recorded on a feeding-related nerve in preparations made from previously conditioned *Pleurobranchaea* (Mpirsos and Davis 1973) and a recent work by Whelan and McCrohan (1996), who reported conditioned fictive feeding responses evoked by a chemical CS (amyI acetate) in *Lymnaea*. However, our present work is the first study both demonstrating fictive feeding responses to a CS at the level of identified neurons in previously behaviorally conditioned snails and making direct quantitative comparisons between both unconditioned and conditioned feeding in intact animals and the cellular expression of these responses in semi-intact preparations made from the same animals.

A comparison of the in vivo and in vitro unconditioned feeding responses to touch and food was important because if the unconditioned behavioral responses do not survive the reduction to semi-intact preparations, there is very little prospect of finding any electrophysiological traces of the conditioned behavioral responses, which was the main objective of the present experiments. We found that both touch- and sucrose-evoked neurophysiological responses could be recorded in identified neurons of the feeding system. Tactile stimulation of the lips did not evoke prolonged feeding in intact animals or cause prolonged activation of fictive feeding in reduced preparations. On the other hand, sucrose caused reliable activation of both behavioral and fictive feeding patterns. However, the frequency of fictive feeding activity triggered by sucrose in the semi-intact preparations was consistently lower than the frequency of feeding in intact animals. One possible explanation for this is that the semi-intact preparations used in the present experiments did not have the buccal mass attached to the CNS. Previous work
on *Lymnaea* has demonstrated that chemoreceptors in the buccal cavity play an important role in full activation of fictive feeding (Kemenes et al. 1986). It also has been reported that key modulatory interneurons such as the serotoninergic cerebral giant cells (CGCs) show markedly different firing activity in intact animals and in reduced preparations (Yeoman et al. 1994), and this can also influence the rate of fictive feeding.

The most important finding of this work was that a neurophysiological expression of the conditioned feeding response was found in semi-intact preparations made from behaviorally trained animals. This was identified as patterned fictive feeding activity in motoneurons of the feeding system, triggered by the presentation of the lip touch CS, which evoked a significantly stronger response in experimental animals than in controls.

Like the unconditioned response, the conditioned fictive feeding response in semi-intact preparations was weaker than the behavioral responses recorded in intact animals. This may have been the consequence of either a decrease in the sensitivity to sensory stimuli or a reduced output from the CPG system in semi-intact preparations, probably caused by trauma resulting from the dissection procedure. Preventing this trauma would have been difficult, because anesthetization involving injection of MgCl$_2$ into animals is less feasible in *Lymnaea* than in large-bodied mollusks, such as *Aplysia*, used in previous learning studies. The effects of another widely used form of anesthetization, exposure to cold before dissection, are poorly understood in *Lymnaea* and therefore we did not risk using it either. A reduction in the magnitude of conditioned responses in semi-intact preparations derived from previously trained whole animals also has been reported in aversive conditioning in the slug *Limax maximus* (Gelperin and Culligan 1984). Despite this decrement in the in vitro response, in both the above *Limax* study and our present experiments, a clearly identifiable neurophysiological correlate of the conditioned behavior was found.

There are specific reported examples of the survival of complex, patterned conditioned nonfeeding type responses into semi-intact molluscan preparations, such as the facilitation of a fictive locomotory behavior triggered by an otherwise ineffective stimulus in the presence of an aversive CS in *Aplysia* (Carew et al. 1981). However, the neuronal circuitries underlying these responses have not been extensively characterized, and so the cellular mechanisms underlying these forms of behavioral plasticity are not clearly understood. In contrast, the circuitry responsible for the consummatory unconditioned feeding behavior in *Lymnaea* is well characterized (Benjamin and Elliott 1989), and appetitive conditioning to tactile stimuli has been demonstrated previously in both whole animals (Kemenes and Benjamin 1989a,b, 1994) and with an in vitro training paradigm (Kemenes et al. 1997). These earlier in vitro conditioning experiments demonstrated that conditioned fictive feeding responses, recorded on the same motoneurons used in the present study, can be built up in semi-intact preparations by repeatedly (6–10) pairing a touch CS to the lips with stimulation of the modulatory feeding interneuron slow oscillator as the US. The results from the above in vitro studies and from preliminary experiments with semi-intact preparations made from conditioned animals (Staras 1997) both indicate that a facilitated response to lip touch in identified feeding CPG interneurons is an important factor contributing to the expression of the conditioned fictive feeding response. Recently, putative mechanosensory cells of the tactile CS pathway also have been mapped both morphologically and electrophysiologically (Staras et al. 1995). The experiments reported in this paper bridge the gap between the purely behavioral analysis of learned responses in whole animals and studies of in vitro conditioned responses in reduced preparations and pave the way for a more detailed analysis of the cellular mechanisms of appetitive learning.

This work was supported by Biotechnology and Biological Sciences Research Council Grant GR/J33234 (UK).

Present address of K. Staras: Dept. of Physiology, Royal Free Hospital, School of Medicine, Rowland Hill St., London NW3 2PF, UK.

Address for reprint requests: K. Staras (c/o G. Kemenes), Sussex Center for Neuroscience, School of Biological Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, UK.

Received 24 September 1997; accepted in final form 20 February 1998.

REFERENCES


