In Vitro Appetitive Classical Conditioning of the Feeding Response in the Pond Snail Lymnaea stagnalis

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Kemenes, György, Kevin Staras, and Paul R. Benjamin. In vitro appetitive classical conditioning of the feeding response in the pond snail Lymnaea stagnalis. J. Neurophysiol. 78: 2351-2362, 1997. An in vitro preparation was developed that allowed electrophysiological analysis of appetitive conditioning of feeding in the model molluscan system, Lymnaea. The feeding motor program (fictive feeding) is well characterized at the cellular level and consists of identified central pattern generator (CPG) interneurons, motor neurons, and modulatory interneurons. Activation of a modulatory interneuron, the slow oscillator (SO), evokes the three-phase fictive feeding rhythm in the same semi-intact preparations where tactile stimuli can be applied to the lips. By pairing touch as a conditioned stimulus (CS) with stimulation of the SO as an unconditioned stimulus (US), we established an effective in vitro paradigm for appetitive conditioning. Before training, touch to the lips evoked only brief and weak activity in the feeding interneurons and motor neurons. After 6-10 conditioning trials, there was a significant enhancement in the fictive feeding response to CS alone. This was not seen in controls (CS only, US only, random CS and US) and in preparations where there was no initial brief response to touch before conditioning. Direct recordings from the protraction phase N1M interneurons during in vitro conditioning indicated that the enhancement of the fictive feeding is due to an increased activation of these CPG cells by mechanosensory inputs from the lips. We also found that the conditioned response was not due to a facilitated activation of modulatory neurons in the feeding network, such as the SO or the cerebral giant cells (CGCs), because the activity of these cells remained unchanged after conditioning.

INTRODUCTION

To date, some of the most important insights into the cellular mechanisms of learning, including recently described molecular modifications of gene expression, have come from work using aversive conditioning paradigms in gastropod model systems (Alkon 1984; Carew 1996; Gelperin 1983; Hawkins 1984). Appetitive learning, first described in higher animals (Pavlov 1927), is also present in gastropods and other invertebrates (reviewed in Carew and Sahley 1986). This type of learning is based on pairing a neutral conditioned stimulus (CS) with food as unconditioned stimulus (US). Unlike aversive learning, this important form of associative behavioral modification only has been analyzed in cellular detail in a few invertebrate organisms (Hammer 1993; Whelan and McCrohan 1996).

One of the gastropod models where appetitive learning already has been demonstrated clearly is the pond snail Lymnaea stagnalis (Audesirk et al. 1982; Kemenes and Benjamin 1989a; Kojima et al. 1996; Whelan and McCrohan 1996). Lymnaea can be classically conditioned by repeatedly pairing touch to the lip with food, and this appetitive learning shares important characteristics with conditioning in vertebrates (Kemenes and Benjamin 1989a,b, 1994). A cellular analysis of appetitive learning in Lymnaea seemed feasible because the neuronal activity underlying feeding, the unconditioned behavior, already was known in detail. This behavior is generated by a central pattern generator (CPG) system (Benjamin and Elliott 1989), similar to those also commonly found in higher organisms (reviewed in Jacklet 1989). The feeding CPG is connected synaptically to a variety of modulatory interneurons that can gate, initiate, and help maintain CPG-driven activity (Elliott and Benjamin 1985b; McCrohan 1984; Rose and Benjamin 1981a; Yeoman et al. 1994a,b) (Fig. 1). In the present analysis, we employed an in vitro conditioning paradigm using a tactile CS and activation of a modulatory interneuron, called the slow oscillator (SO), as the US. Touch to the lip is a neutral stimulus in the intact animal and in semi-intact preparations in the sense that unlike sucrose, it cannot evoke prolonged behavioral or fictive feeding. However, it can evoke a few fictive feeding cycles in intact animals (Kemenes and Benjamin 1994) and produce weak, brief activation of fictive feeding in the semi-intact preparation (Kemenes et al. 1986). It excites modulatory neurons and has access to the feeding CPG as well (Kemenes et al. 1986) (summarized in Fig. 1A). Current injection into the SO reliably drives a fictive feeding pattern in the same semi-intact preparation where touch can be applied to the lips, and this rhythmic response resembles unconditioned fictive feeding evoked by sucrose applied to lip receptors (Kemenes et al. 1986; Yeoman et al. 1995). Moreover, during strong sucrose-evoked activation of the feeding CPG, the SO is activated together with CPG interneurons and helps to maintain a fast, regular rhythm (Yeoman et al. 1995), indicating that this modulatory interneuron is part of the network that mediates the unconditioned feeding response to food. A major advantage of using the SO as the source of US is that its synaptic connections with other neurons of the Lymnaea feeding network (summarized in Fig. 1B) are well understood (Benjamin and Elliott 1989).

Here we report that pairing touch as a CS with SO stimulation as the US enabled us to establish an effective in vitro paradigm for appetitive conditioning in Lymnaea. We show that plastic changes in modulatory neurons alone do not account for the emergence of the conditioned fictive feeding response, but a facilitated activation of CPG interneurons by the tactile CS may underlie this in vitro appetitive learning.
Lymnaea (HEPES) buffered containing N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffered Lymnaea saline (Benjamin and Winlow 1981). The outer layers of the connective tissue sheath of the cerebral and buccal ganglia were removed with fine forceps and the ganglia treated with protease (SIGMA type XIV, Sigma Chemical, Poole, UK) to soften the inner layers of the sheath for microelectrode impalement. After treatment with protease, the preparations were washed for 10 min with saline using a fast perfusion system.

Electrophysiological recording techniques

Intracellular recordings were made from up to four neurons at a time. Electrodes were drawn from 2-mm capillary tubing (Clark Electromedical, Redding, UK) and filled with 4 M potassium acetate solution (Sigma Chemical), giving tip resistances of 30–80 MΩ. Signals were amplified with NEUROLOG NL102 amplifiers (Digitimer, Welwyn Garden City, UK) and fed to an oscilloscope (GOULD 1604, Gould Instrument Systems, Hainault, UK), a chart-recorder (GOULD TA240S) and a DAT recorder (BIOLOGIC DTR-1801, Biologic Science Instruments, Claix, France).

Selection and identification of neurons

The main goal of the experiments was to monitor neuronal changes occurring during in vitro conditioning of the feeding response. Therefore, all the neurons recorded were previously identified components of the feeding network (Fig. 1). These included the modulatory SO and cerebral giant cell (CGC) neurons, feeding motor neuronal (MNS, Fig. 1A) and the N1M projection-phase interneuron (Fig. 1B) of the CPG network. The SO cell always was impaled because its activation was used as the US. The synaptic connections of this cell with CPG neurons are summarized in Fig. 1B. These identified connections are crucial in the initiation of rhythmic fictive feeding, the unconditioned response (UR) by the SO. In addition to the SO, the activity of one or two feeding motor neurons (B1, B3, B4, B4CL, B6, or B7 cells) and the activity of one or both of the paired serotonergic modulatory interneurons (CGCs) were monitored (see Fig. 2).

By recording from at least one suitable motor neuron, we always could obtain information on activity in the whole CPG network.

**METHODS**

**Experimental animals**

Pond snails, L. stagnalis, obtained from animal suppliers (Blades Biological, Kent, UK), were kept in copper-free water in glass tanks on a 12 h:12 h light:dark regime and fed ad libitum on *L. stagnalis*, obtained from animal suppliers (Blades Biological, Kent, UK). By injecting depolarization stimuli (US) in the semi-intact preparation, we were able to subject the feeding network to classical conditioning trials in vitro.

**Semi-intact lip-tentacle–CNS preparation**

The semi-intact preparation consisted of the lips and tentacles separated into two halves by cutting down the midline and removing most of the foot. This was similar to the type of preparation first developed for in vitro studies of aversive learning in *Limax* (Chang and Gelperin 1980; Gelperin and Culligan 1984) and *Pleurobranchaea* (Kovac et al. 1985) and also previously successfully used to analyze chemical and tactile inputs to the feeding system in other mollusks, including *Helix* (Hernádi et al. 1984; Kemenes et al. 1985) and *Lymnaea* (Elphick et al. 1995; Kemenes et al. 1986; Yeoman et al. 1995). The split-lip preparation was used to facilitate the recording from modulatory neurons located in the cerebral ganglia (see below). The lip-tentacles were connected to the cerebral ganglia via the left and right median and superior lip nerves and the tentacle nerves. All other peripheral nerves were cut. The buccal ganglia were left connected to the cerebral ganglia via the paired cerebro-buccal connectives. The preparation was pinned down in a silicone elastomer (Sylgard)-lined dish containing N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffered *Lymnaea* saline (Benjamin and Winlow 1981).
Any one of the B1, B3, B4, B4CL, B6, or B7 neurons could be recorded for this purpose. Rhythmic activity of these and other buccal motor neurons was referred to as fictive feeding because it was similar to that produced rhythmic rasping in intact animals, when the buccal mass was present (Rose and Benjamin 1979). Patterned motor neuron activity is due to characteristic sequences of excitatory and inhibitory synaptic inputs from feeding CPG cells, so that by recording from selected types of motor neurons during fictive feeding, it was possible to monitor, indirectly, the activity of all three classes of feeding CPG cells. Of the motor neurons selected in the present experiments, the B1, B6, and B7 cells are excited during the N1 (protraction) phase but inhibited during N2 (rasp) and N3 (swallow); the B3 cell is inhibited during N1 but excited during N2 and N3 and the B4 and B4ACL cells are inhibited during N1 and the first phase of N2 but excited during the second phase of N2 and during N3.

Recording the CGCs as well as the motor neurons was important for the following reason. In semi-intact preparations, application of a food stimulus to the lips is known to cause a parallel increase in the tonic firing frequency of the CGCs and activation of the feeding CPG, both of which are required to drive the rhythmic activity of buccal motor neurons (Kemenes et al. 1986; Yeoman et al. 1995). Moreover, the CGCs are known to receive excitatory inputs after tactile stimuli to the lips (Kemenes et al. 1986). Increased activation of the CGCs by the CS after conditioning might be an important change occurring during learning, so it was necessary to record the CGCs at all stages of the experiment. The paired CGCs fire together 1:1 because of conjoint electrotonic (McCrohan and Benjamin 1980) and chemical coupling (Goldschmeding et al. 1981), so it was only necessary to record one cell to monitor the synchronized activity.

In some experiments, activity of the CPG was recorded directly. Here, the NIM interneuron was chosen because it is the most important projection phase interneuron and starts the whole pattern of activity in the CPG (Elliott and Benjamin 1985a; Elliott and Kemenes 1992; Kemenes and Elliott 1994). Importantly, monitoring its activity yields direct information about any plastic changes that might occur at the level of the CPG.

The motor neurons and the CGCs were identified by their position, size, and basic firing pattern as reported previously (Benjamin and Rose 1979; McCrohan and Benjamin 1980; Rose and Benjamin 1981a). In addition to these criteria, the identification of the SO was confirmed by testing its ability to initiate and maintain fictive feeding (Rose and Benjamin 1981b). The N1M neuron was identified by its position and characteristic firing pattern during SO-driven fictive feeding and by testing its excitatory synaptic connections with B1, B7, and SO and inhibitory connections with B4, B4CL, and B3 (Elliott and Benjamin 1985b; Elliott and Kemenes 1992; Rose and Benjamin 1981b).

### In vitro conditioning protocol

Semi-intact preparations show varying levels of spontaneous fictive feeding activity (Kemenes et al. 1986). Only quiescent preparations (showing less than an average of 1 cycle/min of spontaneous activity during the first 5 min of the experiment before the pretraining tests) were used for conditioning experiments as this allowed the occurrence of conditioned responses to be ascertained more easily by quantitative analysis. Naive semi-intact preparations (n = 39) were first tested for neuronal responses to touch to the lips, the prospective CS. This was the same light tactile stimulus, applied with a soft brush, that had been used in previous, successful behavioral conditioning experiments (Kemenes and Benjamin 1989a,b, 1994). The brush stimulus was a single stroke across the lip, moving laterally from the medio-anterior surface and avoiding the tentacles or eyes. This was the same area stimulated in previous behavioral experiments (Kemenes and Benjamin 1989a,b, 1994).

In most naive preparations (n = 25 of 39), the light tactile stimulus produced weak but consistent effects (1–3 cycles of fictive feeding) on the activity of feeding motor neurons and interneurons, but in none of them did this or stronger and/or more prolonged tactile stimuli evoke prolonged rhythmic activation of the feeding network. The touch stimulus used as the CS evoked weak local contractions in the lip area being stimulated but it was not sufficiently strong enough to evoke centrally mediated withdrawal reactions, such as contractions of the lip half contralateral to the stimulus site in the semi-intact preparations, or whole body withdrawal in intact animals (Kemenes and Benjamin 1989a).

The pretraining CS test was repeated up to three times on both lip halves at ~2-min intervals, allowing a preconditioning touch response to be established. In preparations where an initial touch response was found, the lip half on the side giving the highest initial response to touch was used consistently as the CS site. When there was no difference in the initial touch response on the two sides or no initial touch response was found on either lip halves, the left half of the lip was used as the CS site. In all preparations, between 1 and 2 min after the last pretraining touch stimulus, a depolarizing current was injected into the SO interneuron (the prospective unconditioned stimulus, US) to evoke a fictive feeding pattern in the CPG and obtain data on the level of the unconditioned response. The amount of depolarizing current sufficient to produce strong enough spike activity in the SO to excite the feeding CPG varies from preparation to preparation and mainly depends on the size of the SO, which is variable. In each preparation, we used the minimum amount of current (between 0.5 and 1 nA) that could evoke fictive feeding activity falling into the range established by earlier work (10–15 cycles/min) (Benjamin and Elliott 1989).

The amount of current necessary to achieve this was established in two tests with the SO, before the CS and US tests, which were performed to obtain pretraining touch and SO response data. Fictive feeding usually was monitored as CPG-generated synaptic inputs and consequent patterns of spike activity in the motor neurons (the unconditioned response), except in two experiments where a CPG neuron, the N1M, was recorded directly as well. Only preparations that responded consistently to all three pretraining SO activations were used in the experiments.

Of the 39 preparations of the starting batch, 18 were assigned randomly to an experimental group, and the remaining 21 preparations were divided up randomly between three control groups. The preparations assigned to the experimental group were subjected to a training regime (Fig. 3A) that involved pairing touch (the CS) with activation of the SO (the US). In this in vitro appetitive training paradigm, a 1-s touch to the lips was followed within 10 s by activation of the SO using steady depolarizing intracellular current injection. In turn, activation of the SO led to the completion of the first CPG-driven cycle within 3–10 s. Our previous behavioral data (Kemenes et al. 1990) have shown that the mean latency from touch of normal food to the first rasp is 18 ± 7 (SE) s. Independent work (Tuersley and McCrohan 1987) found the latency to have a median of 17.5 s (interquartile range: 10–27). The delay between touch and activation of fictive feeding by the SO in vitro and between touch and activation of behavioral feeding by sucrose in vivo sufficiently overlap for the in vitro paradigm to have a close resemblance to the in vivo conditioning procedure. Activity in the SO was maintained until five full cycles of fictive feeding were achieved and the range of durations needed to accomplish this was 20–30 s.

The first training trial was performed 2 min after the preconditioning CS and US tests, and subsequent trials were separated by 2-min intertrial intervals. In these experiments, 10 is the maximum number of pairings that can be achieved in any particular preparation without considerably increasing the risk of losing some of the neurons being recorded (unpublished observations), and this was the number of trials the majority of the experimental preparations.
Three different control groups were used to test for possible influences of nonassociative learning on the touch response after training. These were similar to the controls used in a previous, behavioral appetitive conditioning paradigm in Lymnaea (Kemenes and Benjamin 1989a,b, 1994): random CS and US (Fig. 3B, n = 6), touch (CS) alone (Fig. 3C, n = 8), and SO activation (US) alone (Fig. 3D, n = 7). The control preparations were subjected to the same pretraining CS and US tests as the experimental ones. Again, like the experimental ones, the majority of control preparations [random: 4 (6), CS alone: 5 (8), US alone: 5 (7)] were subjected to 10 trials but a minimum criterion of 6 trials again was used when this could not be achieved.

Before comparing the postraining responses, we performed a variety of statistical tests to confirm that the experimental and control groups were matched sufficiently in all their pretraining parameters. First, a nonparametric equivalent of a one-way analysis of variance (Kruskall-Wallis $H$ test) was used to confirm that the experimental and control preparations were matched according to the number of training trials (between 6 and 10) they received ($H = 0.84$, df = 3, $P = 0.85$). A Kruskall-Wallis $H$ test was also performed on the pretraining CS and US test data from all four groups of preparations (1 experimental, 3 controls); this confirmed that they were matched according to their mean pretraining responses to CS and US (Fig. 4, Kruskall-Wallis $H$ test for CS: $H = 1.59$, df = 3, $P = 0.66$; Kruskall-Wallis $H$ test for US: $H = 4.07$, df = 3, $P = 0.3$). Similarly to the experimental group (see RESULTS), in each control group, there was a number of preparations that did not give an initial weak response to touch [random: 2 (6), CS alone: 3 (8), US alone: 2 (7)]. A $\chi^2$ test therefore was performed that confirmed that the experimental and control groups were all matched for the proportion of preparations not showing an initial touch response ($\chi^2 = 0.26$, df = 3, $P = 0.95$). The results from the above set of analyses demonstrated that, despite the existence of differences between preparations in the responses to CS and US before training and also in the number of trials, all four groups were matched according to their pretraining parameters. We therefore were satisfied that the experimental design was sufficiently well balanced for statistically valid and meaningful postraining comparisons between the groups.

Statistical analysis of the fictive feeding responses to touch after conditioning

For the analysis of the touch-evoked fictive feeding data, nonparametric tests were used. This was necessary because preparations not showing an initial touch response as well as preparations showing weak initial touch responses were used in the experiments, and therefore no specific assumptions on the shape of the distribution of the data could be made. For quantitative analysis, we first calculated the differences between the post- and pretraining fictive feeding responses to touch by using pairs of data from each preparation from all four groups (1 experimental, 3 controls). This data was first subjected to a Kruskall-Wallis $H$ test (a nonparametric equivalent to a one-way analysis of variance). Next, data from the three control groups only were subjected to a Kruskall-Wallis $H$ test (a nonparametric equivalent to a one-way analysis of variance). Next, data from the three control groups only were subjected to a Kruskall-Wallis $H$ test, and this was followed by a comparison of the combined controls and the experimental group by a Mann-Whitney $U$ test.

Following the above comparisons between groups, Wilcoxon matched-pairs signed-rank tests were used for comparisons of pre- and postconditioning responses to touch within the same groups. The criteria for accepting the occurrence of a classically conditioned response were that the experimental group should show a difference between post- and pretraining levels of the response to touch that is significantly greater than differences found in controls, and this difference should be due to a significant increase of the response to touch in the experimental group compared with pretraining levels in the same group.
For the analysis of possible changes in the spiking frequency of the CGCs (a normally distributed variable), a parametric statistical test, the paired Student’s t-test was used. All statistical procedures were performed using SPSS version 6.1 (Norusis 1995).

RESULTS

In vitro conditioned fictive feeding responses

In the experimental group (n = 18), the mean difference (black bar in Fig. 4A) between the post- and pretraining touch responses was 4.2 ± 0.8 (SE) cycles/min. This was in contrast with much smaller differences (black bars in Fig. 4, B–D) in the control groups [random (n = 6): 0.5 ± 0.6, CS alone (n = 8): 1.3 ± 0.7, US alone (n = 7): 0.3 ± 0.5]. When these data were subjected to a Kruskall-Wallis H test, it revealed the existence of a source of significant difference between the four groups (H = 10.4, df = 3, P < 0.02). The same test, however, did not reveal any significant differences between the control groups (H = 0.62, df = 2, P = 0.73). A pairwise nonparametric test (Mann-Whitney U test) between the experimental and combined control data showed that the response to touch after training was significantly stronger in the experimental ones than in the controls (U = 78.5, P < 0.002).

A quantitative analysis of pre- and posttraining data within the same groups showed that in the experimental preparations (n = 18) the rate of fictive feeding in response to touch was significantly greater after training (5.5 ± 1.1 cycles/min) than before training (1.3 ± 0.7 cycles/min, Wilcoxon matched-pairs signed-rank test; Z = −3.3, P < 0.001; Fig. 4A). Importantly, no significant differences in posttraining response levels were found between the subgroup of experimental preparations (n = 13) that received 10 training trials and the subgroup (n = 5) that received <10 (but ≥6) trials (Mann-Whitney U test: U = 30.5, P = 0.84). This confirmed that the six-trial–minimum criterion for inclusion into the experimental group was justifiable in present experiments.

An example of a preparation that showed an increased posttraining fictive feeding response is presented in Fig. 5. The touch stimulus evoked only a single cycle of fictive feeding activity before conditioning, seen here as a sequence of N1, N2, and N3 phase, feeding CPG generated synaptic inputs on the B4 retractor phase motor neuron (Fig. 5A). Stimulation of the SO, on the other hand, evoked strong and prolonged fictive feeding in B4 (Fig. 5B), seen here and in other figures as repeated sequences of N1, N2, N3-phase synaptic inputs on the SO cell and on the motor neurons being recorded. After repeated pairings (7) of touch with stimulation of SO (Fig. 5C), the motor neuronal response to touch (Fig. 5D) became stronger and more prolonged than before training (Fig. 5A), indicating a conditioned response. The number of fictive feeding cycles during the first minute after the first cycle after the CS was similar for the CS after training (7 in Fig. 5D) compared with US before (6 in Fig. 5B) or during (7 in Fig. 5C) training and much greater than to the CS before training (1 in Fig. 5A).

In contrast, no significant change in the intensity of the response evoked by touch was observed before and after training in each of the control groups. This is shown for random touch (CS) and SO (US) (Fig. 4B), touch (CS) alone (Fig. 4C), and SO stimulation (US) alone (Fig. 4D) and confirmed by statistical analysis (Wilcoxon matched-pairs signed-rank tests performed separately on preparations from each of the three control groups, random: n = 6, Z =
FIG. 5. Example of a successful in vitro conditioning experiment. A: before training, touch (the CS) applied to the lips caused a small increase in tonic firing in the modulatory neuron CGC and weak fictive feeding activity in the B4 retraction phase motor neuron, seen here as a single cycle of sequences of N1 phase (inhibition), N2 phase (inhibition), and N3 phase (excitation) activity in B4. CPG-generated inputs also were present in the SO, but no spiking activity occurred. B: injection of depolarizing current into the modulatory interneuron SO initiated and maintained a strong unconditioned fictive feeding rhythm. Both the SO and the B4 cell received typical excitatory and/or inhibitory inputs indicating activity in the CPG network, triggered by the SO. A typical sequence of N1, N2, and N3 phase inputs is shown for 1 of the fictive feeding cycles. C: a conditioning trial in the semi-intact preparation consisted of a touch stimulus (the CS) applied to the lip, followed by activation of the feeding rhythm by current injection into the SO (the US). Note that a single cycle of fictive feeding, evoked by the touch (also see A), occurs immediately before the series of SO-driven, CPG-evoked cycles of N1-, N2-, and N3-phase inputs on B4. D: after repeated (7) pairings of touch with stimulation of the SO, the touch alone was able to evoke a conditioned fictive feeding response with clearly recognizable sequences of N1-, N2-, and N3-phase inputs on both B4 and SO. This response is more prolonged than the response to touch before training shown in A (see RESULTS for statistical analysis). Note that the SO did not show an enhanced response to touch after conditioning (the tactile and CPG inputs both remained subthreshold) and was therefore not driving the facilitated touch response in the B4 motor neuron. Neither did the CGC show an enhanced spontaneous firing rate or an increased response to touch after conditioning (see RESULTS for statistical analysis).

In this section, we demonstrated that in vitro conditioning leads to an overall increase in touch-induced fictive feeding (Fig. 6A) and after conditioning (Fig. 6B), although the unconditioned response to the stimulation of the SO was strong both before (Fig. 6A) and after 10 trials (Fig. 6C).

Finally, we also tested to see if the initially spontaneously quiescent preparations involved in this experiment (see METHODS) showed an increase in spontaneous activity after the training procedure. After training, the mean frequency of spontaneous fictive feeding cycles in the 1-min period before touch in all 39 preparations was 1.2 ± 0.3 cycles/min, virtually the same as before training (1.3 ± 0.3). Paired comparisons (Wilcoxon matched-pair signed-rank test) within each group between pre- and postconditioning spontaneous activity did not reveal any significant changes (experimental: Z = 0.0, P = 1.00; CS: Z = −0.54, P = 0.59; US: Z = −1.34, P = 0.18; random: Z = −1.6, P = 0.10), so we were satisfied that all groups were subjected to both pre- and postraining testing with the CS against similarly low spontaneous backgrounds.

In this section, we demonstrated that in vitro conditioning by explicitly pairing touch (CS) with stimulation of SO (US) leads to an overall increase in touch-induced fictive feeding that is significantly greater than changes in control groups subjected to nonassociative protocols. The increase in the experimental group was due to the emergence of an in vitro-conditioned response to touch that was significantly greater than the pretrained touch response in the same group.

**Conditioning only occurs if there is an initial brief response to touch in the naive preparation**

Explicit pairing of lip touch with SO activation produced a statistically significant increase in the fictive feeding response to touch, indicating successful conditioning. However, the level of the response (5.5 ± 1.1 cycles/min) never reached that of the SO-stimulated unconditioned response
(9.1 ± 0.5, Wilcoxon matched-pairs signed-rank tests, n = 18, Z = -3.6, P < 0.01). A more detailed analysis of each preparation from the experimental group showed that this difference was due to 8 out of the 18 preparations showing virtually no conditioned response after training. In these eight experimental preparations, the mean posttraining fictive feeding response to touch was only 1.0 ± 0.4 cycle/min, with four preparations showing no response at all and four preparations showing only one to three cycles, respectively. This is in contrast with the other 10 experimental preparations where the mean posttraining touch response was 9.1 ± 0.8 cycles/min, with individual responses ranging between 5 and 13 cycles/min. A systematic examination of these two types of preparations indicated that a weak fictive feeding response to touch before training was an essential prerequisite for successful learning (Fig. 4). All 10 preparations that learned showed this type of pretraining response (mean, 2.9 ± 0.7 cycles/min), but 7 of the 8 preparations where conditioning was unsuccessful showed no pretraining touch response at all, and this difference was statistically significant (χ² = 14.3, df = 1, P < 0.01). When the paired comparisons between the level of conditioned and unconditioned response were restricted to the 11 preparations with clear (although weak) pretraining touch responses (including the preparation that gave no conditioned response despite showing an initial response to touch), there was now no difference in the mean conditioned response (8.5 ± 1.0 cycles/min) compared with the mean SO-driven unconditioned fictive feeding response (9.4 ± 0.9) in the same 11 preparations (Wilcoxon matched-pairs signed-rank tests, Z = -0.15, P = 0.87). Figure 5 is an example where touch in naive preparations produced an initial response, and this was followed by successful conditioning. Figure 7 is an example where no touch response occurred before training. This preparation had a normal SO-driven fictive feeding response (Fig. 7B) but showed no fictive feeding response to touch, monitored in the B4 motor neuron or the SO cell, before training (Fig. 7A). The touch remained ineffective (Fig. 7C) after 10 pairings of touch with SO-driven fictive feeding. In this and other similar preparations, despite the lack of an initial fictive feeding response to touch on buccal motor neurons and interneurons, the lip-CNS mechanosensory pathways and the interganglionic pathways appeared not to be damaged. In all seven preparations with no initial touch response, one or both CGCs were recorded, and, in each case, they both responded to touch and excitation to their synaptic follower motor neurons (not monitored here), indicating that the lack of a conditioned response was not due to a general decrease of the excitability of the feeding CPG network.

The data presented in this section indicated that a weak touch response before conditioning was necessary for successful conditioning to occur. When this was present, the conditioning resulted in the build-up of a conditioned response that reached the level of the unconditioned one.

**Changes in the firing rates of the serotoninergic modulatory CGCs do not contribute to in vitro conditioning**

Previous work on the modulatory role of a pair of giant serotonin-containing cerebral neurons, the CGCs, suggested...
FIG. 7. Conditioned feeding response did not occur in preparations that lacked an initial fictive feeding response to touch. A: before training, touch evoked no CPG-driven activity in the SO and motor neuron B4. It did, however, excite the paired CGCs. This showed that the lack of touch response on the buccal cells was not due to damage to the lip nerves, the main mechanosensory pathways between the lip and the CNS (see Fig. 2). The fact that the CGCs fired 1:1 and therefore were coupled showed that the cerebro-buccal connectives and the buccal commissure, which mediate ipsi- and contralateral mechanosensory effects in the buccal ganglia and the coupling between the CGCs, also were intact. Depolarization seen on B4 after touch here and in B are due to previously described synaptic connections between the CGCs and B4. In the same preparation, the modulatory interneuron SO could drive a regular fictive feeding pattern in the B4 motor neuron even though it was not responding to touch. B: after 10 pairings of touch with stimulation of SO, there was no increased fictive feeding response to touch alone when compared with A.

that their gating/enabling function depended on an increase in their level of tonic firing after food stimulation (Kemenes et al. 1990; Yeoman et al. 1994a,b). This raised the possibility that the changes in the responsiveness of the feeding circuitry to touch may, at least in part, result from an enhanced firing rate of the CGCs as a result of conditioning. However, we found no evidence that either the spontaneous CGC firing rate (Fig. 8A) or the CGCs’ response to touch (Fig. 8B) increased significantly after successful conditioning of the fictive feeding response. The mean spontaneous rate of CGC firing before training was 48.4 ± 4.5 spikes/min (data from 8 preparations, each showing a conditioned response after 6–10 trials). After conditioning, the mean spontaneous CGC firing rate was 52.4 ± 4.6 spikes/min, not significantly different from the mean preconditioning firing rate in the same preparations (Fig. 8A, paired t-test: \( t = 1.3, \text{df} = 7, P = 0.23 \)). This indicated that conditioning did not result in an increased spontaneous firing rate in the CGCs.

Typically, a touch to the lips evoked a brief acceleration of activity in the CGCs both before (Fig. 5A) and after training (Fig. 5D). However, there were no significant differences in the response to touch (paired t-test: \( t = 0.9, \text{df} = 7, P = 0.40 \)) before and after training (Fig. 8B), indicating that conditioning did not result in an increased responsiveness of the CGCs to tactile inputs either.

**SO interneuron does not mediate the facilitated touch response after conditioning**

One obvious mechanism for a previously ineffective touch stimulus to trigger CPG-driven fictive feeding would be if the SO neuron showed a facilitated response to touch after training. This, however, did not happen in any of the preparations that showed in vitro conditioning. The examples shown in Figs. 5 and 9 demonstrate a complete lack of spike activity in the SO neuron after the touch stimulus either before (Figs. 5A and 9A) or after conditioning (Figs. 5D and 9B). The SO receives synaptic inputs as feedback from the CPG interneurons (Fig. 1B), and these can be seen on the SO after successful conditioning of the feeding response (Figs. 5D and 9B). However, none of these CPG inputs led to any spike activity in the SO itself.

**Facilitated activation of the N1M central pattern generator interneuron may underlie the conditioned motor neuronal response**

Neither the CGCs nor the SO seemed to be involved in the facilitated response to touch after in vitro conditioning.
Given that the conditioned response (CR) involved the activation of the feeding CPG, this suggested that the CR might be due to direct effects on neurons of the CPG network. The most obvious targets were the N1M cells. These fire during the first, protraction phase of the feeding cycle (Elliott and Benjamin 1985a) (Fig. 1B) and are essential for activation of the CPG rhythm (Kemenes and Elliott 1994). In two in vitro conditioned semi-intact preparations, it was possible to record from the protraction phase N1M CPG interneuron and the SO long enough to compare changes in their activity taking place during conditioning. In the preparation shown in Fig. 9, touch to the lip before conditioning evoked a single cycle of activity in N1M, SO, and the B1 protraction-phase motor neuron (Fig. 9A). During training, this initial activation by touch was followed by a more prolonged activation triggered by injection of depolarizing current into the SO interneuron (Fig. 9A). After six conditioning trials, touch alone evoked the same short-latency prolonged activity in N1M that was evoked by the SO during training (Fig. 9B). As expected, there was no enhanced response to touch in the SO after conditioning (compare Fig. 9, A and B), so conditioning cannot be occurring via the previously described SO → N1M excitatory pathway (Fig. 1B). This again confirms that touch-induced SO activity is not necessary for a facilitated CPG and motor neuron response to occur (also see Fig. 5D).

**DISCUSSION**

This paper shows for the first time that it is possible to carry out in vitro appetitive conditioning of the molluscan feeding system. Remarkably, food stimulation of the feeding response, acting as the US in behavioral conditioning of the whole animal (Kemenes and Benjamin 1989a), can be replaced by stimulation of a single central interneuron, the SO, in the semi-intact in vitro preparation. It was shown previously that this neuron activates fictive feeding in the isolated or semi-intact preparation (Elliott and Benjamin 1985b; Kemenes et al. 1986; Rose and Benjamin 1981b). After 6–10 pairings of touch with activation of the SO, a conditioned fictive feeding rhythm could be evoked by touch alone on both the N1M and B1. Note that the SO was not contributing to the maintenance of the facilitated N1M response to touch after conditioning.
than one type of neuron, or may be caused mainly by a last-
change in a single neuron (such as the N1M), which could trigger rhythmic activity in other neurons of the feed-
ing network.

The similarity of the *Lymnaea* and bee findings indicates that at least in invertebrates stimulus of single identified central interneurons can be used as the US for in vitro condi-
tioning. In *Aplysia* aversive learning, direct activation of central facilitator interneurons, important in modulating syn-
aptic transmission between mechanosensory neurons of the CS pathway and motor neurons of the US pathway (Byrne 1987), produced the same presynaptic facilitation in the sensory neurons that an electric shock applied to the tail US site could produce (Hawkins and Schacher 1989; Hawkins et al. 1981; Mackey et al. 1989). This suggests that in both appetitive and aversive conditioning in invertebrates, intra-
cellular stimulation of identified central neurons can substi-
tute for a sensory US, although it is not yet known if during appetitive learning such neurons play a presynaptic facilitat-
ning role as well as evoke the unconditioned response.

As with aversive classical conditioning paradigms in *Aplysia* (for reviews see Byrne 1987; Carew and Sahley 1986), appetitive learning in *Lymnaea* was based on the amplification of a weak response (weak feeding motor output in response to touch) rather than on the formation of a completely new one. This was indicated by the observation that it was a prerequisite for successful in vitro conditioning that preparations should show a clear (albeit weak) fictive feeding response to touch before training. This is often called alpha-conditioning (Thompson and Berger 1984). Although this is an important difference from the original Pavlovian conditioning paradigm, it has the advantage that the existence of a physiologically measurable preconditioning re-
sponse allows the tracing of the pathways involved in the mediation of the CS in the naive preparation and the analysis of changes after conditioning. Importantly, the existence of a preconditioning response to CS seems to be a characteristic shared by both the present in vitro appetitive learning para-
digm and the in vivo appetitive learning paradigm involving feeding described previously (Kemenes and Benjamin 1989a). Both intact animals and semi-intact preparations where conditioning was successful showed a weak response to touch before training, and the rate of feeding response (real or fictive) to touch was in both cases between 1 and 3 rasps or cycles/min.

Our preliminary experiments showed that activation of fictive feeding by the SO seemed to be necessary for in vitro learning to occur (Kemenes, unpublished observations). This indicates that this form of appetitive learning in *Lym-
naea* is based on stimulus-response rather than stimulus-
stimulus conditioning. This also raises the interesting possi-
bility that the key event for in vitro learning to occur is the activation of the feeding CPG by the unconditioned stimulus and not the activation of the SO per se. This means that rhythmic activity in the feeding CPG alone may be adequate as a reinforcement, provided that it closely follows the acti-
vation of the tactile CS pathway. This has important similari-
ties to the long-established role of electrically induced brain activity as a positive reinforcement in both classical and operant learning in mammals (Malmo 1965; Olds 1962), which, in certain paradigms also can lead to unconditioned behavioral responses. For example, in the rabbit, cortical stimulation elicited movements of the face or neck and, when paired with a tone CS, produced learning comparable to that seen with peripheral unconditioned stimuli (Swain et al. 1992).

### Possible neuronal mechanisms underlying appetitive learning in *Lymnaea*

Most previous experiments on appetitive learning in mollusks were based purely on behavioral paradigms with intact animals (Audesirk et al. 1982; Croll and Chase 1980; Kemenes and Benjamin 1989a,b, 1994; Kojima et al. 1996; Pesciel et al. 1996; Sahley et al. 1990), and although they yielded very important data on the factors influencing the conditioned response, so far they have offered little direct insight into the neuronal mechanisms underlying this form of learning. However, in a few preparations, in vivo conditioning was followed by in vitro analysis in semi-intact preparations (Mpitsos and Davis 1973; Whelan and McCrohan 1996). Extracellular recordings made from feeding nerves of the buccal ganglia of *Pleurobranchaea* after repeated pairings of touch with food in intact animals, showed facilitated feeding CPG activity in response to electrical stimulation of the tentacle nerve carrying the CS pathway (Mpitsos and Davis 1973). These early experiments indicated that the main plastic change during tactile appetitive conditioning in mollusks may occur between the mechanosensory and CPG systems. More recent experiments also suggest that sensory to interneuronal pathways may be the sites of plasticity. For instance, in *Limax maximus*, where both aversive and appetitive conditioning of the feeding response were studied (Sahley et al. 1981, 1990), it was suggested that command-like cerebral-buccal interneurons may act as integrative cen-
ters involved in learning for inputs arising from mechanosen-
sory, taste, and odor stimuli (Delaney and Gelperin 1990; Sahley et al. 1992). A mechanism implicating cerebral-buc-
cal interneurons also is supported by recent work in *Lymnaea* demonstrating that the “CV1 neuron may be an important site of cellular plasticity in chemical appetitive conditioning” (Whelan and McCrohan 1996).

One obvious advantage of in vitro paradigms is the access to the same identified neurons before, during, and after condi-
tioning. By using this advantage in our in vitro learning paradigm, we were able to rule out some of the possible neuronal mechanisms that underlie appetitive learning in *Lymnaea* and select some of the likely mechanisms that might be involved. By analyzing changes in spike activity in the CGCs, we first showed that these serotonergic modula-
tory interneurons were unlikely to play an important role in the plastic changes occurring during in vitro conditioning in *Lymnaea*. We also eliminated the possibility that the SO interneuron might show a facilitated touch response after conditioning. This was important because both of these modu-atory cell types are known to have direct connections with the N1M type CPG interneurons (Elliott and Benjamin 1985a; Yeoman et al. 1996) and could have been responsible for the facilitated CPG and consequent motor neuron re-
sponse to touch after conditioning.

During appetitive learning, possible changes also could occur at synaptic connections between the CPG interneurons
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