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*J Neurophysiol* 85:89-97, 2001.

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# Selective Expression of Electrical Correlates of Differential Appetitive Classical Conditioning in a Feeding Network

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**Jones, Nick, György Kemenes, and Paul R. Benjamin.** Selective expression of electrical correlates of differential appetitive classical conditioning in a feeding network. *J Neurophysiol* 85: 89–97, 2001. Electrical correlates of differential appetitive classical conditioning were recorded in the neural network that underlies feeding in the snail *Lymnaea stagnalis*. In spaced training (15 trials over 3 days), the lips and the tentacle were used as CS+ (reinforced conditioned stimulus) or CS– (nonreinforced conditioned stimulus) sites for behavioral tactile conditioning. In one group of experimental animals, touch to the lips (the CS+ site) was followed by sucrose (the unconditioned stimulus, US), but touch to the tentacle (the CS– site) was not reinforced. In a second experimental group the CS+/CS– sites were reversed. Semi-intact lip-tentacle-CNS preparations were made from both experimental groups and a naive control group. Intracellular recordings were made from the B3 motor neuron of the feeding network, which allowed the monitoring of activity in the feeding central pattern generator (CPG) interneurons as well as early synaptic inputs evoked by the touch stimulus. Following successful behavioral conditioning, the touch stimulus evoked CPG-driven fictive feeding activity at the CS+ but not the CS– sites in both experimental groups. Naive snails/preparations showed no touch responses. A weak asymmetrical stimulus generalization of conditioned feeding was not retained at the electrophysiological level. An early excitatory postsynaptic potential (EPSP) response to touch was only enhanced following conditioning in the Lip CS+/tentacle CS– group but not in the Tentacle CS+/lip CS– group. The results show that the main features of differential appetitive classical conditioning can be recorded at the electrophysiological level, but some characteristics of the conditioned response are selectively expressed in the reduced preparation.

## INTRODUCTION

Invertebrates provide excellent model systems for studies on the cellular and molecular mechanisms of learning and memory (Krasne and Glanzman 1995) and have provided important information on the nature of changes resulting from associative conditioning. Gastropod mollusks demonstrate both nonassociative and associative learning (Byrne 1987; Carew and Sahley 1986). They also have relatively simple nervous systems and large neurons, which are accessible to intracellular analysis, and this has made them particularly valuable in the search for the cellular mechanisms of behavioral plasticity. One such model gastropod is the pond snail *Lymnaea stagnalis*. This animal previously was shown to demonstrate a variety of distinct types of classical and operant conditioning (Aude-sirk et al. 1982; Kemenes and Benjamin 1989a,b; Kojima et al.

1996; Lukowiak et al. 1996). Appetitive classical conditioning was one particular learning paradigm that was extensively studied in *Lymnaea* at both the behavioral and cellular level (Kemenes et al. 1997; Staras et al. 1998, 1999a; Whelan and McCrohan 1996). Following classical conditioning of the rhythmic feeding behavior using a lip-touch training paradigm, electrical correlates of nondifferential appetitive conditioning could be recorded in a semi-intact preparation. Changes occurred at several different sites in the feeding network, at the level of the central pattern generator (CPG) interneuronal network, the motor neurons, and the conditioned stimulus (CS) pathway (Staras et al. 1999a).

Kemenes and Benjamin (1989a) also showed that the touch learning paradigm in *Lymnaea* could be developed further to demonstrate a more complex form of appetitive conditioning, differential classical conditioning, a type of discriminative learning behavior. In differential conditioning, two CS sites, the lips and the tentacles, were used in the same animal. At one site (the CS+) touch was paired with sucrose, and at the other one (the CS–) touch was specifically unpaired. After 15 spaced trials discriminative learning was demonstrated. The aim of the present experiments was to determine whether neural correlates of this behavioral differential appetitive conditioning could be obtained. This was successful at the level of the sustained CPG-driven differentially conditioned fictive feeding pattern in both experimental groups. Another aspect of behavioral differential conditioning, weak asymmetrical stimulus generalization, was not retained in the reduced preparation. Moreover, a known electrophysiological consequence of appetitive conditioning, an increase of the amplitude of an early excitatory postsynaptic potential (EPSP) response to touch (Staras et al. 1999a) was only seen in the Lip CS+/tentacle CS– group but not in the Tentacle CS+/lip CS– group, indicating the selective expression of the neural correlates of behavioral differential conditioning.

## METHODS

### Animals

Wild-type specimens of adult *Lymnaea stagnalis* were obtained from animal suppliers (Blades Biological, Kent, UK). The snails 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.

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### Behavioral experiments

Before the experiment, animals were moved into three tanks in the laboratory. During the training procedure the animals were given a little lettuce at the end of each day's training so that they were in a semi-satiated state. All lettuce was removed from the home tanks 45 min before the first trial and the water was replaced daily.

**DIFFERENTIAL CONDITIONING TRAINING.** The simpler nondifferential appetitive conditioning paradigm uses lip touch as the CS followed by sucrose as the unconditioned stimulus (US) in a spaced training schedule (Kemenes and Benjamin 1989a; Staras et al. 1998, 1999a). Differential conditioning involved two sites on the body, the lips and tentacles, both of which could be used as CS sites (Kemenes and Benjamin 1989a). In a balanced design, both lips and tentacles act as reinforced conditioned stimulus (CS+) or nonreinforced conditioned stimulus (CS-) sites for touch. Thus touch at the CS+ site is followed by sucrose (the US) dissolved in water, but touch at the CS- site is only followed by water to equalize for the displacement caused by the addition of sucrose solution to the dish after touching the CS+ site (Fig. 1). As well as these two experimental groups, a third, naive group, was used where spontaneous feeding rates were measured and the lack of basic feeding responses to touch was ascertained.

The two groups of experimental animals were labeled Lip CS+/tentacle CS- or Tentacle CS+/lip CS-, depending on which site was positively reinforced (Fig. 1). To differentially condition the animals, subjects were taken from the home tank and individually placed in test dishes containing 90 ml of copper-free water. After 2 min in the test dish, the animals in the Lip CS+/tentacle CS- group were presented with a touch to the lips. As the animals were freely moving, the touch stimulus was presented using a hand-held probe with a tip made of a

thin wedge of soft, flexible plastic (see Staras et al. 1999a). The target zone on the lip structure was the median portion adjacent to the mouthparts including the leading edge of the lips as previously described in Staras et al. (1998). Within 1 s of the presentation of the tactile stimulus, sucrose (the US) was presented by injecting it into the water of the experimental dish from a 10-ml syringe so that the final concentration was 0.01 M. This was previously determined to be the most effective concentration for stimulating a high-frequency unconditioned feeding response (Kemenes et al. 1986). The pairing of touch with sucrose stimulus constituted one CS+ trial. After this, the animals were rinsed in a clean water tank to remove any residual sucrose before they were placed back into the home tank. Forty-five minutes after the CS+ trial, the animals were placed into test dishes again following the same protocol, and a tactile stimulus was applied to the tip of the left tentacle. This was followed within 1 s by water injected into the water of the experimental dish from a 10-ml syringe. This constituted one CS- trial. Again, the animals were then rinsed in a clean water tank before being placed back into the home tank. The interval between consecutive CS+ and CS- trials was 45 min. Five CS+ and 5 CS- trials were performed each day over a period of 3 days so that each animal received 15 trials of both CS+ and CS- in a spaced training regime. The second experimental group, the Tentacle CS+/lip CS- group, received the same number of trials over the same 3-day period, with the same intertrial intervals, but here, touch to the tip of the left tentacle (the CS+ site) was paired with sucrose (US), and touch to the lips (the CS- site) was only followed by addition of water to the training dish. A naive group of animals was used as a further control for both experimental groups. These had no experimental procedures performed on them, but were fed the same amount of lettuce and kept in an identical environment to the experimental animals during the trials. At the end of the 3-day training period, all three groups of animals were given a little lettuce in the home tanks and left undisturbed for 3 h before they were tested behaviorally.

**BEHAVIORAL TESTING OF THE DIFFERENTIALLY CONDITIONED FEEDING RESPONSE.** Testing was performed using a blind procedure, by a person who had no knowledge of the training history of the animals. During testing, animals were removed from the home tanks and placed in shallow test dishes containing 100 ml copper-free water. The test dishes were placed on a Perspex box with a mirror angled at 45° inside that allowed the animals to be observed while they were moving around on the bottom of the dish. In *Lymnaea*, feeding movements (rasps) consist of cycles of mouth opening and closing accompanied by extrusion of the toothed radula of the buccal mass. Each cycle of feeding was counted by visual observation using a hand-held counter.

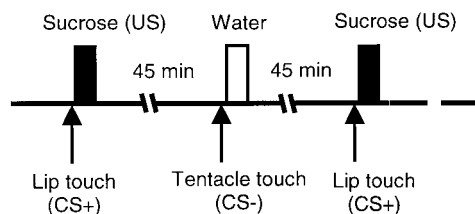
After emergence of the snail from its shell, any spontaneous feeding activity was recorded for 2 min, and then a touch stimulus was applied to the lips or the tip of the left tentacle. For a balanced test protocol, we alternated the order in which these two different test sites were used from one animal to another. After applying the touch stimulus to the test sites, feeding activity was recorded for a further 2 min before the animals were replaced into the home tanks. After all the animals had been tested on touch at one of the test sites, they were tested again after 90 min for a feeding response to touch to the other site. This testing regime ensured that approximately half of the animals in all three groups were first tested for a response to lip touch followed by a tentacle touch, and the remaining animals within the group were tested in the reverse order.

### Electrophysiological experiments

The main aim of these experiments was to record electrical changes in semi-intact preparations resulting from differential conditioning of the intact snails. Following training on three consecutive days snails were kept overnight in a sub-satiated state, and electrophysiological experiments were carried out on the following 2 days.

### Differential classical conditioning

#### Lip CS+/tentacle CS- Group



#### Tentacle CS+/lip CS- Group

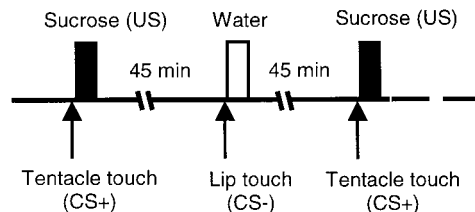


FIG. 1. Differential appetitive conditioning paradigms in *Lymnaea*. The top panel shows the training procedure used to differentially condition the Lip CS+/tentacle CS- group of experimental animals. One CS+ trial consisted of pairing lip touch, the reinforced conditioned stimulus (CS+) with sucrose, the unconditioned stimulus (US), and one CS- trial consisted of a tentacle touch, the nonreinforced conditioned stimulus (CS-) followed by addition of water to the training dish. The 2 different trials were separated by an interval of 45 min. The experimental animals received 5 CS+ and 5 CS- trials a day for 3 consecutive days. The training procedure used to differentially condition the Tentacle CS+/lip CS- experimental animals (bottom) was similar but with the CS+ and CS- sites reversed.

**GENERAL PROCEDURES.** Individual snails from experimental and control groups of animals were dissected under a light microscope in a silicone elastomer (Sylgard)-lined dish containing *N*-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid (HEPES)-buffered snail saline previously described by Benjamin and Winlow (1981). The preparations were then transferred to the electrophysiology chamber. Before recording, the outer ganglionic sheath surrounding the CNS was removed from around the buccal ganglia using a pair of fine forceps, and the inner ganglionic sheath was softened using a nonspecific protease (Sigma, XIV, Sigma Chemicals, Poole, UK) to aid intracellular recording. An experimenter who had no prior knowledge of the training history of the individual animals then performed the rest of the electrophysiological procedures.

**PREPARATIONS.** "Whole lip-tentacle-CNS" semi-intact preparations were developed that allowed the lips and tentacles to be stimulated by touch while simultaneously recording from neurons of the feeding system. The CNS was accessed by a dorsal body incision and all the peripheral nerves cut except for the paired superior and median lip nerves and the tentacle nerves. The buccal mass was excised, but a short length of esophagus was left attached to the brain to allow pinning out. The lips and the surrounding structures including the tentacles were arranged in the electrophysiological chamber facing upward so that the CS+ and CS- sites were accessible to tactile stimulation. The tactile stimulus was applied to either the lips or tentacle using an electromagnetic coil-driven mechanical probe that was designed to closely mimic the handheld probe used in the behavioral experiments (see Staras et al. 1999a). By pulling the intact CNS down below the peripheral head structures, the buccal ganglia could be stretched clear of the head structures over a raised Sylgard block so that the dorsal side was exposed for intracellular recording, as previously described by Staras et al. (1998).

**INTRACELLULAR RECORDING TECHNIQUES.** Glass microelectrodes, with tip resistances ranging from 10 to 60 M $\Omega$  when filled with 4 M potassium acetate solution, were used in the electrophysiological experiments. These were pulled on a Narashigi vertical electrode puller. The tips were dipped in black etching ink to improve visualization. The electrophysiology chamber in which the preparations were pinned was illuminated with a cold light source, and the preparations were viewed under a stereo microscope. To allow intracellular recording, micromanipulators with attached headstage preamplifiers (Neurolog, Digitimer, Welwyn Garden City, UK) were arranged around the electrophysiological chamber. 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).

**INTRACELLULAR RECORDING OF THE B3 MOTOR NEURON.** There were two main goals for the electrophysiological experiments. The first was to monitor fictive feeding activity in the semi-intact preparations, and the second was to record an early synaptic response to lip touch, previously reported on feeding neurons (Staras et al. 1999a,b). Both types of response were enhanced following training in earlier nondifferential tactile conditioning experiments (Staras et al. 1998, 1999a). The B3 motor neurons were used because food (sucrose) is known to elicit sequences of burst activity that are representative of fictive feeding activity throughout the feeding network. Similar patterns of neural activity were known to underlie feeding movements in the intact animal (Rose and Benjamin 1979). Feeding patterns are generated by a CPG consisting of three main types of interneurons (N1, N2, and N3) and at least three modulatory and command neurons (Benjamin and Elliott 1989). The B3 motor neuron receives inhibitory input in the N1, protraction phase and is excited in the subsequent N2 and N3, rasp and swallow phases (Rose and Benjamin 1981).

Therefore by intracellularly recording from the B3 motor neuron, it was possible to monitor indirectly the activity of all the components of the CPG. We impaled the B3 with two microelectrodes, one for recording the electrical signals and one for passing current into the cell. The current passing electrode was used to maintain the membrane potential of the cell at -65 mV, the average of previous measurements of B3 resting potential (Staras et al. 1999a). This current clamp allowed the early depolarizing synaptic response in B3 due to touch (input I of Staras et al. 1999a) to be recorded at a constant membrane potential. Changes in the amplitude of the early compound EPSP were recorded as a further monitor of the effect of differential conditioning.

**ELECTROPHYSIOLOGICAL TESTING OF THE DIFFERENTIALLY CONDITIONED FEEDING RESPONSE.** After the preparation had been set up by an experimenter who had no knowledge of its training history, spontaneous fictive feeding activity was recorded for 2 min, and then a touch stimulus was applied to one of the CS sites. This was repeated after a 2-min interval. After a further 2-min interval the other CS site was stimulated, and this was repeated again after another 2 min. Randomization of the testing regime was achieved by using preparations provided by a second experimenter that had been randomly selected from the three groups to be tested.

#### *Statistical analysis of behavioral and electrophysiological data*

The same types of statistical analysis were used for both the behavioral and electrophysiological experiments. All analysis was done blind with the person analyzing the records unaware of the group from which the snail/preparation had originated. First, the presence of a normal distribution of both prestimulus (spontaneous) and poststimulus (touch-evoked) rasp/fictive feeding rate data were established [Normal Probability Plot, SPSS (Norusus 1995)]. This justified the subsequent use of parametric analysis techniques to compare data (presented as means  $\pm$  SE) both within and between groups. A one-way ANOVA on the prestimulus data established that all three groups (Lip CS+/tentacle CS-; Tentacle CS+/lip CS- and Naive) were matched for both spontaneous rasping and fictive feeding rates before either a lip or a tentacle touch was applied [ $F(5,75) = 1.4$ ,  $P = 0.26$ , behavior;  $F(5,58) = 0.64$ ,  $P = 0.67$ , electrophysiology].

Responses to touch to the lips or the tentacle were quantified by awarding a difference score to each animal for each site. This was calculated by subtracting the number of rasps/fictive feeding cycles in the minute preceding the tactile stimulus from the number of rasps/fictive feeding cycles in the minute after the first rasp/fictive feeding cycle after the tactile stimulus. Poststimulus rasps/cycles were only counted if the first rasp/cycle occurred within 1 min after the stimulus; otherwise the poststimulus rate was regarded as zero. For the analysis of fictive feeding rates, the rates measured before and after the two stimuli to the same site were averaged. These are the same methods that were used by Staras et al. (1998, 1999a) to analyze behavioral and electrophysiological changes after simple appetitive classical conditioning. Paired *t*-tests were then performed on data *within* each group to determine whether touch to a site, either lip or tentacle, induced a significant change in the rasping/fictive feeding rate over prestimulus levels measured in the same preparations. Differences between responses to touch applied to the two different sites in the same group were also analyzed using paired *t*-tests. To make comparisons *between* different groups, first an ANOVA was performed separately for responses to touch to the lips and tentacle, respectively. Only when a source of significant difference was revealed was the data further subjected to post hoc analysis (Tukey's B test) to determine where the source of significant difference originated.

## RESULTS

*Behavioral analysis*

**LIP CS+/TENTACLE CS- GROUP.** In almost all of the Lip CS+/tentacle CS- animals (12 of 13), touch to the lips at test increased the feeding rate following training. The mean spontaneous feeding rate before touch was applied to the lips was  $1.3 \pm 0.8$  (SE) rasps/min. After tactile stimulation of the lips, the mean feeding rate rose to  $5.3 \pm 1.0$  rasps/min (Fig. 2Ai), and statistical analysis showed that there was a significant difference between the pre- and poststimulus data (paired *t*-test, *df* = 12, *t* = 6.1; *P* < 0.001).

In comparison, touch to the tip of the left tentacle in the same animals increased the feeding rate in only a small proportion of the animals (5 of 13), and the average increase was only small (Fig. 2Aii). The mean feeding rate before the tentacle was touched was  $0.8 \pm 0.5$  rasps/min, and in the 1-min test period after touch rose only marginally to  $1.0 \pm 0.4$  rasps/min. This increase in the feeding rate was not significant when the pre- and poststimulus data were compared (paired *t*-test, *df* = 12, *t* = 0.44; *P* = 0.7, Fig. 2Aii). A paired *t*-test comparing the differences between the pre- and posttouch data for both sites (lips:  $4.0 \pm 0.7$  rasps/min; tentacle:  $0.2 \pm 0.5$  rasps/min) also revealed a significant difference between the feeding responses to touch stimulus at the two sites (*df* = 12, *t* = 4.4; *P* < 0.001, Fig. 2Aiii) confirming that differential conditioning had been successful.

**TENTACLE CS+/LIP CS- GROUP.** The results with this group of animals were more complex than with the Lip CS+/tentacle CS- group. As expected, touch to the CS+ site after training increased feeding rates (in 15 of 17 animals) over spontaneous levels. The mean feeding rate before touch was  $1.2 \pm 0.4$  rasps/min; this increased to  $5.3 \pm 0.9$  rasps/min after touch, and this was statistically significant (paired *t*-test, *df* = 16, *t* = 2.6; *P* < 0.001), indicating successful conditioning of the CS+ tentacle site. However, increases in feeding rate were also observed at the CS- lip site (Fig. 2Bii). This occurred in a smaller proportion of animals (10 of 17, ~60% for the lips, compared with 90% for the tentacles), and the increase in mean feeding rate was smaller (less than twice,  $2.2 \pm 0.8$  rasps/min before;  $4.2 \pm 1.0$  rasps/min after, for lips compared with more than 3 times for the tentacle), but it was still significant (paired *t*-test, *df* = 16, *t* = 2.6; *P* < 0.02). This indicated that the effects of food reinforcement on the tentacle were being transferred to the alternative lip site, despite its CS- status. This suggests that stimulus generalization was occurring, but only in one direction, from tentacle to lips (Fig. 2Bii) and not from lips to tentacle (see Fig. 2Aii). Touching the CS+ tentacle site after training still produced significantly greater conditioned feeding responses than the CS- lip site in the same animals (Fig. 2Biii; paired *t*-test, *df* = 16, *t* = 2.1; *P* < 0.05) showing that there was still a distinction between the effectiveness of the two sites in eliciting conditioned feeding and that generalization was only partial.

**NAIVE GROUP.** The naive control group provided baseline data for comparison with the two experimental groups. It also showed that there were no significant differences in the before and after touch scores at either the lips (Fig. 2Ci) or tentacles (Fig. 2Cii), and it follows from this that simply touching either

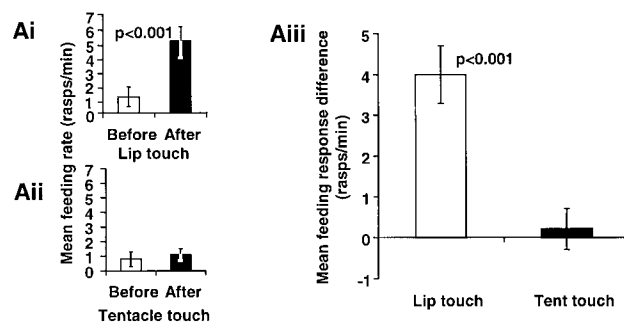
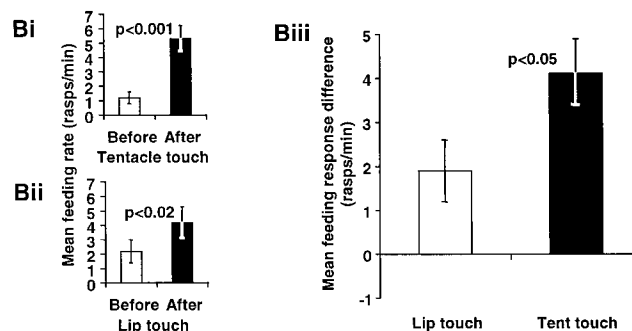
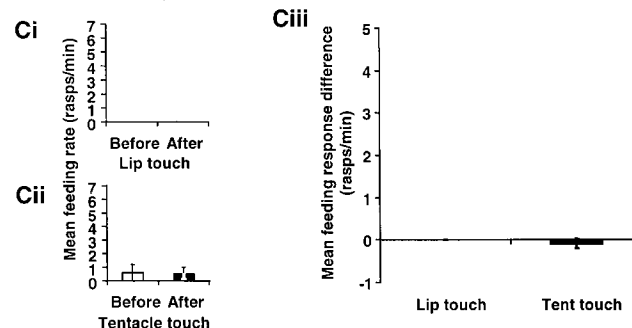
**A Lip CS+/tentacle CS- Group****B Tentacle CS+/lip CS- Group****C Naive Group**

FIG. 2. Behavioral analysis of differential classical conditioning in *Lymnaea*. **A:** pre- and posttouch feeding rate and feeding response data for the Lip CS+/tentacle CS- experimental group. The feeding responses here and in **B** and **C** were calculated as the mean difference between the post- and pretouch feeding rates. **Ai:** mean feeding rates ( $\pm$ SE) before and after touch to the lips (CS+), showing a significant increase after touch. **Aii:** mean feeding rates ( $\pm$ SE) before and after touch to the tentacle (CS-), showing no change after touch. **Aiii:** mean feeding responses ( $\pm$ SE) to touch to the 2 sites. The response to lip touch is significantly greater than to tentacle touch, showing that differential conditioning was successful. **B:** pre- and posttouch feeding rate and feeding response data for the Tentacle CS+/lip CS- experimental group. **Bi:** mean feeding rates ( $\pm$ SE) before and after touch to the tentacle (CS+), showing a significant increase after touch. **Bii:** mean feeding rates ( $\pm$ SE) before and after touch to the lips (CS-), also showing a significant increase after touch and indicating stimulus generalization from tentacle to lip. **Biii:** mean feeding responses ( $\pm$ SE) to touch to the 2 sites, calculated as the difference between mean feeding rates before and after touch. The response to tentacle touch is significantly greater than to lip touch, showing that despite the stimulus generalization, differential conditioning was successful. **C:** pre- and posttouch feeding rate and feeding response data for the Naive group. **Ci** and **Cii:** mean feeding rates ( $\pm$ SE) before and after touch to the lips and tentacles, respectively, showing no change after touch. **Ciii:** the lack of feeding responses to touch at the 2 sites.

of these sites does not induce any feeding responses (Fig. 2Ciii).

COMPARISONS BETWEEN LIP CS+/TENTACLE CS-, TENTACLE CS+/LIP CS-, AND NAIVE GROUPS. The inter-group analysis was useful in that it confirmed the basic discriminative phenomena revealed by the within group analysis. Thus the effect of lip touch (Fig. 2, Aiii–Ciii, open bars) was significantly greater in the Lip CS+/tentacle CS- group than in either the Tentacle CS+/lip CS- or the Naive group (Tukey's B test,  $P < 0.05$ ). Similarly, the effect of tentacle touch (Fig. 2, Aiii–Ciii, solid bars) was found to be significantly greater in the Tentacle CS+/lip CS- group than in either of the other two groups (Tukey's B test,  $P < 0.05$ ). However, the inter-group analysis was less successful in detecting the stimulus generalization revealed by the paired test in the Tentacle CS+/lip CS- group. This was indicated by the lack of significance when the difference score (Fig. 2Biii, open bar) to lip touch in the Tentacle CS+/lip CS- group was compared with baseline response to touch in the naive group (Fig. 2Ciii, open bar; Tukey's B test,  $P > 0.05$ ). Inter-group analyses are always less sensitive than their within group equivalents because of group differences, and this made it more difficult to confirm the fairly weak stimulus generalization.

### Electrophysiology

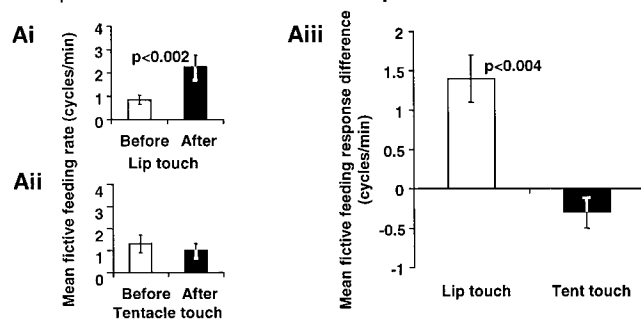
ANALYSIS OF TOUCH-EVOKED FICTIVE FEEDING. The same animals that had been behaviorally trained over 3 days were kept overnight in a sub-satiated state and semi-intact preparations made for electrophysiological recordings. The person performing the electrophysiological experiments on the snails was unaware of their behavioral scores, and the animals to be dissected were drawn randomly from the three groups (2 experimental, 1 naive).

LIP CS+/TENTACLE CS- GROUP. In 80% of the preparations (8 of 10), touch to the lips (CS+ site) increased the fictive feeding rate (Fig. 3Ai). The mean fictive feeding rate following touch increased to  $2.2 \pm 0.5$  cycles/min from  $0.85 \pm 0.2$  cycles/min before touch. When the data were analyzed, using a paired parametric test, the increase in mean fictive feeding rate was found to be significant ( $df = 9$ ,  $t = 4.2$ ;  $P < 0.002$ ; Fig. 3Ai). This increase in the fictive feeding rate is substantially lower than the conditioned feeding rates in response to touch in the intact animals, but similar differences between intact snails and semi-intact preparations following nondifferential appetitive conditioning were reported earlier by Staras et al. (1998, 1999a).

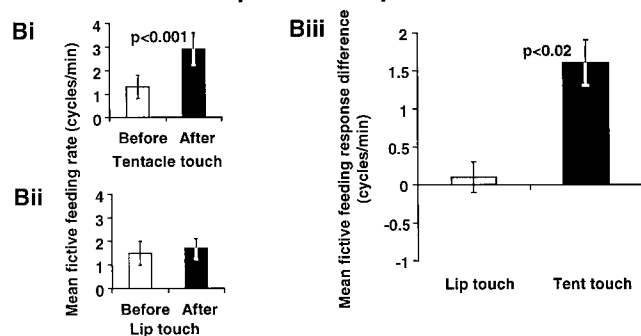
In contrast, touch to the tentacle (CS-) had little effect or tended to cause a decrease in the mean fictive feeding rate (4 of 10 snails). A decrease in mean fictive feeding rate (before,  $1.3 \pm 0.4$  cycles/min; after,  $1.0 \pm 0.3$  cycles/min) was potentially interesting because a CS- site might be predicted to reduce the response after training. However, statistically, this reduction in mean fictive feeding rate was not significant, and there was no significant difference in mean fictive feeding rate before or after testing (paired  $t$ -test,  $df = 9$ ,  $t = 1.3$ ;  $P = 0.2$ ; Fig. 3Aii).

Comparison of the data from lip and tentacle test sites showed that the differential conditioning that was apparent in the intact animals had survived into the semi-intact prepara-

## A Lip CS+/tentacle CS- Group



## B Tentacle CS+/lip CS- Group



## C Naive Group

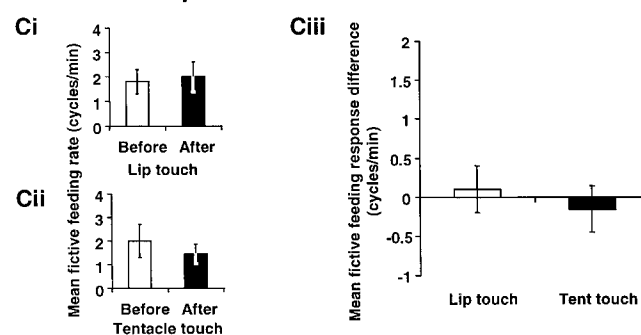


FIG. 3. Electrophysiological analysis of differential classical conditioning in *Lymnaea*. **A**: pre- and posttouch fictive feeding rate and fictive feeding response data for the Lip CS+/tentacle CS- experimental group. The fictive feeding responses here and in **B** and **C** were calculated as the mean difference between the post- and pretouch fictive feeding rates. **Ai**: mean fictive feeding rates ( $\pm$ SE) before and after touch to the lips (CS+), showing a significant increase after touch. **Aii**: mean fictive feeding rates ( $\pm$ SE) before and after touch to the tentacle (CS-), showing no change after touch. **Aiii**: mean fictive feeding responses ( $\pm$ SE) to touch to the 2 sites. The response to lip touch is significantly greater than to tentacle touch, showing that differential conditioning (see Fig. 2) survived into the semi-intact preparation. **B**: pre- and posttouch fictive feeding rate and fictive feeding response data for the Tentacle CS+/lip CS- experimental group. **Bi**: mean fictive feeding rates ( $\pm$ SE) before and after touch to the tentacle (CS+), showing a significant increase after touch. **Bii**: mean fictive feeding rates ( $\pm$ SE) before and after touch to the lips (CS-), showing no change after touch and indicating that the behavioral stimulus generalization from tentacle to lip (see Fig. 2Bii) did not survive into the semi-intact preparation. **Biii**: mean fictive feeding responses ( $\pm$ SE) to touch to the 2 sites, calculated as the difference between mean feeding rates before and after touch. The response to tentacle touch is significantly greater than to lip touch, showing that unlike stimulus generalization (see Fig. 2Bii), differential conditioning (see Fig. 2Biii) survived into the semi-intact preparation. **C**: pre- and posttouch fictive feeding rate and feeding response data for the Naive group. **Ci** and **Cii**: mean fictive feeding rates ( $\pm$ SE) before and after touch to the lips and tentacles, respectively, showing no change after touch. **Ciii**: the lack of fictive feeding responses to touch to the 2 sites.

tions, with increases in fictive feeding occurring with lip touch (CS+ site) but not with tentacle touch (CS- site). The difference in the response (Fig. 3Aiii) of the two sites was confirmed by statistical analysis (paired *t*-test, *df* = 9, *t* = 3.9; *P* < 0.004). An example of touch responses recorded electrophysiologically in a semi-intact preparation is shown in Fig. 4A. Touch to the lips (the CS+ site) induced sustained bursts of fictive feeding activity (details of the N1, N2, and N3 CPG synaptic inputs are shown in the *inset* of Fig. 4A) in the B3 motor neuron but nothing, apart from an early subthreshold EPSP, following tentacle touch (the CS- site).

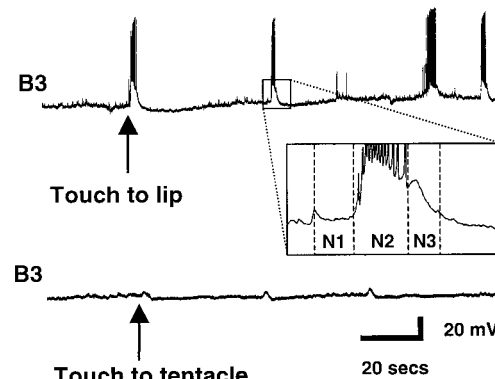
**TENTACLE CS+/LIP CS- GROUP.** Touch to the tentacle CS+ site increased fictive feeding rates in the semi-intact preparation (Fig. 3Bi). This occurred in 11 of the 12 preparations tested (mean fictive feeding rates before  $1.3 \pm 0.4$  cycles/min and after  $2.9 \pm 0.9$  cycles/min), and the increase was statistically significant (paired *t*-test, *df* = 11, *t* = 4.8; *P* < 0.001). This result was similar to the equivalent behavioral response seen in the whole animals. However, touch applied to the CS- site produced different results from those obtained in the same animals in the behavioral experiments. Instead of stimulus generalization and an enhanced response to touch on the lips, no increase in fictive feeding was observed in the semi-intact preparations. There was a small increase in mean fictive feeding rates (from  $1.5 \pm 0.5$  cycles/min to  $1.6 \pm 0.4$  cycles/min; Fig. 3Bii), but this was not statistically significant. Most of the preparations (7 of 12) did not respond at all, with the remainder showing one or two cycles of activity in the minute after touch. In the example shown in Fig. 4B, the early EPSP evoked a burst of spikes, but then only one burst of spikes was seen in the 1 min following touch in the B3 motor neuron. This contrasts with the tentacle CS+ site where a more sustained pattern of fictive feeding was recorded with five bursts of spikes following the early EPSP-evoked activity (Fig. 4B, *bottom trace*).

**NAIVE GROUP.** As predicted from the behavioral experiments, the naive group did not show fictive feeding responses (Fig. 3Ciii) at either the tentacle or lip test site, and this is illustrated in an example of the electrophysiological records shown in Fig. 4C.

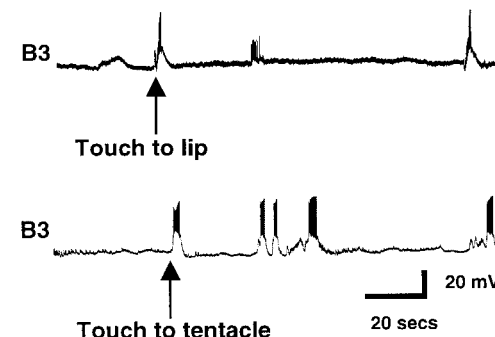
**COMPARISONS BETWEEN LIP CS+/TENTACLE CS-, TENTACLE CS+/LIP CS-, AND NAIVE GROUPS.** The statistical comparison of the electrophysiological data between groups confirmed what was expected from successful differential conditioning. The main significant differences were between the conditioning shown for lip touch in the Lip CS+/tentacle CS- group (Fig. 3Aiii, open bar) versus the other two groups. The effect of lip touch in this group was significantly greater (Tukey's B test, *P* < 0.05) than that of lip touch in the Tentacle CS+/lip CS- (Fig. 3Biii, open bar) and Naive groups (Fig. 3Ciii, open bar). For tentacle touch the response in the Tentacle CS+/lip CS- group was significantly greater (Fig. 3Bii, filled bar) than either the Lip CS+/tentacle CS- group (Fig. 3Aiii, filled bar) or the Naive group (Fig. 3Ciii, filled bar; Tukey's B test, *P* < 0.05).

We conclude that the effect of differential conditioning with either the lips or the tentacle as CS+ sites survives as a fictive feeding pattern in the semi-intact preparation but without the asymmetrical stimulus generalization seen in the behavioral experiments.

### A Lip CS+/tentacle CS- Animal



### B Tentacle CS+/lip CS- Animal



### C Naive Animal

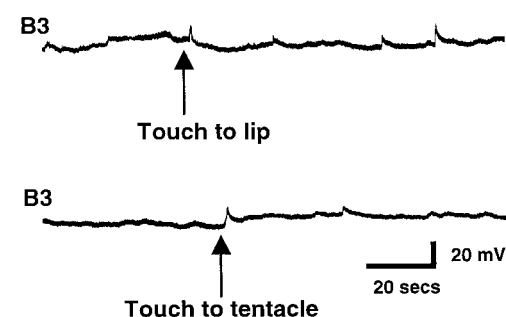


FIG. 4. Examples of electrophysiological traces from each of the 3 groups in response to touch to each tactile site recorded from the B3 motor neuron. A: Lip CS+/tentacle CS- group. *Top trace*: an example of a lip touch (CS+) induced increase of the fictive feeding rate. The *inset* shows a 10-s period of B3 bursting activity (in rectangle on B3 trace) on an expanded time (8 times) and voltage scale (4 times). Synaptic inputs (N1, N2, and N3) from the feeding central pattern generator (CPG) are indicated below the expanded trace. *Bottom trace*: a typical response to touch to the tentacle (CS-) after differential conditioning, where no cycles of activity are induced. B: Tentacle CS+/lip CS- group. *Top trace*: touch to the lips (CS-) producing a weak response in B3. *Bottom trace*: touch to the tentacle (CS+) inducing stronger fictive feeding than the lip touch shown in the *top trace*. C: naive group, both electrophysiological traces show that touch to either the lips or the tentacle in this group induces no cycles of activity in the feeding network's CPG as monitored on the B3 motor neuron.

#### Analysis of the amplitude of the touch-evoked EPSP in motor neuron B3

Previous studies of Staras et al. (1999a,b) showed that the earliest response to lip touch on the B3 motor neuron was a compound EPSP. The maximum amplitude of the EPSP but

not the duration was enhanced following nondifferential appetitive conditioning (Staras et al. 1999a). A similar early EPSP also occurred in B3 following tentacle touch (e.g., Fig. 4C), so we examined whether the size of the responses from the two sites would change after differential conditioning.

Maximum EPSP amplitude (Fig. 5A) was measured with no knowledge of the origin of the preparation in the Lip CS+/tentacle CS- ( $n = 10$ ), Tentacle CS+/lip CS- ( $n = 11$ ), and Naive groups ( $n = 10$ ; Fig. 5B). The only significant difference was found within the Lip CS+/tentacle CS- group. As predicted by the successful differential conditioning of the behavioral response and its electrophysiological correlate, fictive feeding, the amplitude of the lip touch-induced EPSP was greater than the tentacle touch response (mean EPSP amplitude  $7.3 \pm 1.0$  mV,  $5.0 \pm 1.3$  mV, respectively; paired  $t$ -test,  $df = 9$ ,  $t = 2.3$ ;  $P < 0.05$ ; Fig. 5Bi). This is not due to any inherent difference in the amplitude of the responses from the two sites because there was no significant difference between the amplitude of EPSP responses to lip (mean EPSP amplitude  $5.3 \pm 1.5$  mV) and tentacle touch (mean EPSP amplitude  $3.9 \pm 1.1$  mV) in the naive group response (paired  $t$ -test,  $df = 9$ ,  $t = 1.0$ ;  $P < 0.3$ ; Fig. 5Biii). However, there was no significant difference between the amplitudes of EPSPs from lip (mean EPSP amplitude  $7.5 \pm 1.2$  mV) and tentacle touch (mean EPSP amplitude  $5.1 \pm 1.6$  mV) in the Tentacle CS+/lip CS- group (paired  $t$ -test,  $df = 10$ ,  $t = 1.5$ ;  $P = 0.15$ ; Fig. 5Bii) indicating no effect of differential conditioning on the EPSP response in this group.

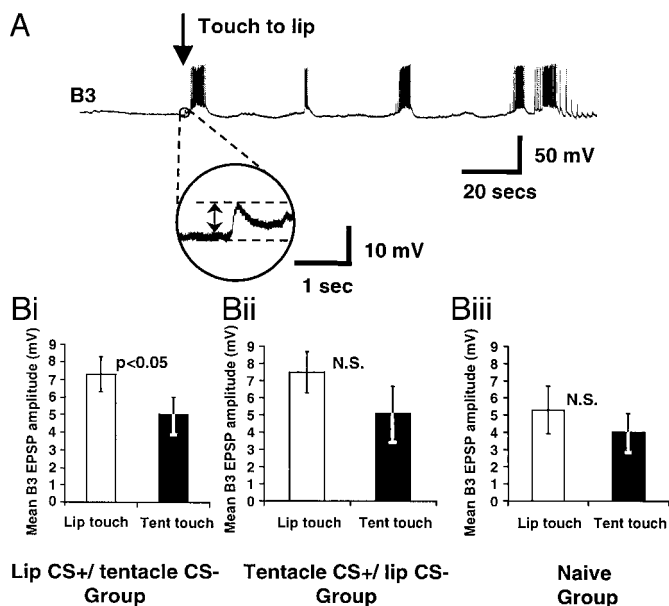


FIG. 5. Analysis of the early excitatory postsynaptic potential (EPSP) touch response in the B3 motor neuron. A: an example of an electrophysiological trace recorded from a B3 motor neuron in a Lip CS+/tentacle CS- animal where touch to the lips (CS+) also stimulated subsequent fictive feeding. A similar EPSP response can be evoked by touch to the tentacles (see Fig. 4). The early EPSP touch response is shown at higher gain with an indication of how the amplitude was measured. B: analysis of the amplitude of the early EPSP in each of the 3 groups. Bi: analysis of the amplitude of the early EPSP in response to lip touch and tentacle touch in Lip CS+/Tentacle CS- animals revealed a significant difference between the 2, where touch to the lips (CS+) produced a larger EPSP when compared with touch to the tentacle (CS-). Bii and Biii: in the Tentacle CS+/lips CS- and naive groups there was no significant difference (N.S.) between the 2 sites in relation to the amplitude of the early B3 EPSPs.

An ANOVA showed that the differences within the groups were greater than between the groups [ $F(5,56) = 1.15$ ,  $P = 0.67$ ] so no multiple unpaired comparisons could be justified between groups. Nevertheless, the within group analysis suggests that differential conditioning of the early EPSP was only possible in the Lip CS+/tentacle CS- group. This asymmetry of the effect of differential conditioning on EPSP amplitude was different from the result of the analysis of fictive feeding where differential conditioning occurred symmetrically in both experimental groups (Figs. 3 and 4).

## DISCUSSION

This paper has shown that neuronal correlates of differential appetitive classical conditioning can be recorded from semi-intact preparations of behaviorally conditioned *Lymnaea stagnalis*. This appears to be the first electrophysiological study performed on a single identified neuron in a preparation made from differentially conditioned whole animals. The main electrical correlate of the differential conditioning was found at the level of the CPG-driven fictive feeding pattern but only selectively at the level of the early EPSP response recorded on B3. Also the asymmetrical stimulus generalization seen behaviorally was not present as a correlated fictive feeding pattern in the semi-intact preparation.

### Discriminative learning and conditioned fictive feeding

The behavioral results showed that after differential conditioning the animals in the Lip CS+/tentacle CS- group were able to distinguish between touch to the lips and touch to the tentacle, with only touch to the lips increasing the feeding rate. This clearly showed the presence of discriminative learning. However, in the case of the Tentacle CS+/lip CS- group, the data were more complex with evidence for weak stimulus generalization as well as discriminative learning. Because the lip touch could evoke a generalized feeding response in the Tentacle CS+/lip CS- group, we have to assume that there is a site of convergence between the nonreinforced lip-brain and the reinforced tentacle-brain pathway, and it is likely to be upstream to the site(s) of plastic change(s) resulting from tentacle CS+ conditioning. On the other hand, the lack of a generalized response to tentacle touch in the Lip CS+/tentacle CS- group indicates that the nonreinforced tentacle-brain tactile pathway has no access to neurons that have undergone plastic changes after lip touch conditioning. This asymmetry could be due to differences between the innervation of the lips and tentacles, the former being innervated only by the lip nerves, whereas the latter is innervated by both the lip nerves and the tentacle nerve (Nakamura et al. 1999). Therefore lip conditioning may only affect neurons receiving tactile inputs from the lip, but tentacle conditioning may affect neurons receiving tactile inputs from both the lip and tentacle. Recently it was suggested that the expression of cellular correlates of nondiscriminative appetitive classical conditioning using the lips as the CS site in *Aplysia* is also specific to the stimulation of a particular lip-CNS pathway (the AT<sub>4</sub> nerve) but the effect of stimulating other peripheral nerves was not investigated (Lechner et al. 2000b). Behavioral work on appetitive classical conditioning in *Aplysia* (Lechner et al. 2000a) also showed that for the appetitive training to be successful, the US needs to

come into contact with both external (e.g., lips) and internal epithelia (e.g., foregut) of the animal and subsequently demonstrated that the esophageal nerve plays an important role in the mediation of the internal effect of the US during conditioning. These nondifferential conditioning experiments provided valuable insights into the organization of the US-mediating pathways, whereas our differential conditioning experiments provide new insights into the organization of the CS pathways that contribute to appetitive associative learning.

The robustness of the basic discriminative learning result was confirmed in the electrophysiological experiments. Here elevation of fictive feeding rates only occurred at the CS+ sites in both groups. However, the weak stimulus generalization seen in the behavioral data in the Tentacle CS+/lip CS− group did not survive as an electrophysiological response in the semi-intact preparation. Previous work showed that the neuronal correlates of both unconditioned and conditioned feeding were always weaker than the corresponding behavioral responses in intact animals (Staras et al. 1998) and as the behavioral stimulus generalization was weak both in the present and previous experiments (Kemenes and Benjamin 1989a), it appears that only the strongest aspects of the behavioral response survive.

#### *Asymmetrical differential conditioning of the early EPSP response*

Touch to the lips produces an early compound EPSP on the B3 motor neuron that precedes the onset of the rhythmic conditioned fictive feeding pattern. Previous work using the nondifferential lip touch conditioning paradigm showed that the amplitude of this EPSP was enhanced following reinforcement of lip touch with sugar in behavioral experiments, and this correlated with an increase in fictive feeding responses to touch (Staras et al. 1999a). If differential conditioning had been completely successful, then it would be predicted that touch to the CS+ sites in both the Lip CS+/tentacle CS− and Tentacle CS+/lip CS− experimental groups would produce larger EPSP responses than the corresponding CS− sites. In fact, only the lip CS+ stimulus in the Lip CS+/tentacle CS− group produced a significant enhancement in the amplitude of the EPSP response compared with the tentacle CS− stimulus, and there was no significant difference between the effect of touch at the two test sites in the Tentacle CS+/lip CS− experimental group, despite the occurrence of conditioned fictive feeding that normally follows the EPSP response.

Why should it be more difficult to use the tentacle as a CS+ site compared with the lips to condition this early EPSP response? One possibility is that the early EPSP response to tentacle touch is a correlate of an alternate behavior, withdrawal. Short latency EPSP responses to skin touch can be recorded in many neurons in the *Lymnaea* CNS, including motor neurons known to mediate whole body withdrawal responses (Ferguson and Benjamin 1991). It is generally known from vertebrate studies that stimuli that are a cue for an alternative behavior are more difficult to condition than completely novel or indifferent stimuli (Shettleworth 1973). Touch to the lips is more likely to be a part of the normal food stimulus (see Staras et al. 1999b) making touch responses evoked at this site more susceptible to conditioning by food reinforcers. The touch pathways from tentacles and lips both

produce a similar amplitude EPSP on the B3 motor neuron so the differential effect of conditioning suggests independent pathways mediating the early EPSP, one of which is more susceptible to appetitive conditioning than the other.

The fact that conditioned fictive feeding patterns can occur to tentacle touch in the Tentacle CS+/lip CS− group, despite a lack of enhancement of the early EPSP, was not entirely unexpected, as the two components could be dissociated in previous experiments on nondifferential conditioning by satiating the experimental animals between the behavioral and electrophysiological tests (Staras et al. 1999a).

#### *Discriminative learning in other model systems*

Discriminative learning has been previously demonstrated in other invertebrate systems, mainly mollusks. For example, differential conditioning of the defensive gill withdrawal reflex was shown in intact *Aplysia* (Carew et al. 1983), and more recently Hawkins et al. (1998) have shown that differential conditioning of the same reflex can be achieved in a reduced preparation. In vitro analogues of differential classical conditioning also have been used with great success in *Aplysia* (Hawkins et al. 1983; Murphy and Glanzman 1999; Walters and Byrne 1983), but so far no attempt has been made to analyze cellular traces of discriminative learning in preparations made from differentially conditioned whole animals. Discriminative learning was also reported in *Pleurobranchaea californica* (Mpitso and Cohan 1986a,b), and using muscle recording techniques it was shown that it survives dissection, and therefore features of whole-animal differential conditioning can persist into physiological preparations (Mpitso and Cohan 1986c). The above *Aplysia* and *Pleurobranchaea* examples used aversive paradigms, but appetitive differential classical conditioning also has been demonstrated in *Aplysia* (Colwill et al. 1997) and the terrestrial slug *Limax maximus* (Sahley et al. 1990). Appetitive differential classical conditioning was also investigated in an insect, the honeybee (Mauelshagen 1993), where a neuronal correlate of differential appetitive conditioning also was obtained after training had been performed in an isolated head preparation. The main advantage of using differential conditioning paradigms is that each animal/preparation can serve as its own control. This is particularly important in studies aiming to find cellular traces of behavioral classical conditioning where the variability of the data at both the behavioral and electrophysiological levels can considerably reduce the chances of finding such traces.

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