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Rapid taste-aversion learning by an isolated molluscan central nervous system

(neural plasticity/conditioning/slug/chemoreception)

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ABSTRACT **The isolated lips and nervous system of the terrestrial slug *Limax maximus* will produce some of the feeding behavior of the intact animal; i.e., they generate the rhythmic neural activity characteristic of ingestion in response to food extracts applied to the lips. This preparation will respond to a variety of food extracts that elicit feeding in the whole animal. This provides the opportunity for aversive conditioning experiments involving taste discrimination. Pairing lip chemostimulation by attractive food extracts with lip chemostimulation by using bitter plant secondary substances can cause the isolated brain to selectively suppress its neural response to one food extract while remaining responsive to another. Such isolated brains can learn after one or two trials and retain the learning for more than 8 hr.**

The terrestrial slug *Limax maximus* can show one-trial food avoidance learning lasting 3 weeks (1). A physiological analysis of the synaptic events causally related to this learning would be greatly aided if one could train the isolated brain to alter its food-related chemosensory input–motor output pathways in a manner analogous to the learning shown by the intact animal. Here we report that the isolated central nervous system shows a form of learning analogous to that displayed by the intact slug.

The most striking change in behavior shown by an intact slug when it learns to avoid a new food is an alteration in directed locomotion in response to food odor. The food stimuli we have conditioned slugs to avoid are inherently very attractive. Naive, hungry *Limax* rapidly orient to and approach food items such as potatoes, carrots, and mushrooms (2, 3). Slugs conditioned by toxicosis to avoid these food items typically will not orient to or approach the odor source (i.e., the food item) (1). Thus, learning alters the olfactory input–pedal locomotor output pathway. Although the olfactory input (4) and locomotor output (5, 6) have been partially characterized, a related pathway from lip chemoreceptors to feeding motoneurons is much more amenable to physiological analysis. Therefore we decided to condition the feeding pathway to produce experience-dependent alterations that would be analogous to refusal to feed in the intact animal.

A lip–brain–buccal ganglia preparation will reliably generate reproducible bouts of feeding motor program (FMP) in response to brief standardized chemostimuli applied to the lips (7, 8). The ease of stimulus control and the unambiguous nature of the neural output pattern led us to use this preparation to produce an *in vitro* analog of the *in vivo* learning.

Slugs were reared by using an enriched artificial diet (8). The entire central nervous system with the lip region was removed from a slug such that the three lip nerves (external, medial, and internal) retained their peripheral and central connections. The lip region was split into right and left halves and each half-lip was placed in a Plexiglas chamber with the lip nerves exiting the chamber via a narrow slit in its wall. The lip nerves were sealed into the slit with Vaseline. Solutions could then be perfused through the lip chamber to stimulate lip chemoreceptors. The perfusion system permits a train of measured chemostimuli to be administered via a continuous flow of saline† over the lips. The chemostimuli we used were extracts made from potatoes, carrots, mushrooms, and lab chow (rat pellets) by standardized techniques described elsewhere (8).

FMP was monitored by recording from four buccal ganglion nerve roots with suction electrodes. The amplified signals were either filmed directly from the oscilloscope or tape recorded for later analysis. A naive observer with no knowledge of the experiments was given the filmed records and a measuring algorithm that set minimal conditions of spike frequency and coordination for accepting a burst of activity as a feeding burst (which corresponds to a bite in the intact animal). For each train of feeding bursts, the cycle time, from onset of one burst to onset of the next burst, was measured. The instantaneous frequency of FMP was calculated as the reciprocal of cycle time and plotted as a function of time.

The most effective way to selectively depress the FMP response was to apply to the lips plant secondary substances, such as colchicine, nicotine, and tannic acid (which taste bitter to humans). The stimulus procedure during training was to apply a 30-sec positive chemostimulus (CS_1) such as potato, mushroom, or carrot extract, followed by a negative chemostimulus (CS) such as nicotine (1–2%),‡ colchicine (10–50 mM), or tannic acid (1%, wt/vol). The negative CS was typically applied 30 sec after the end of stimulation with the positive CS_1 while the FMP response to the positive CS_1 was still in progress. In some experiments the negative CS was applied after the response to the positive CS_1 ended, 5–7 min after application of CS_1. The negative CS was left in contact with the lips for 20 min. After an additional wait of at least 30 min, the positive CS_1 was applied again for 30 sec. If the brain emitted FMP in response to the positive CS_1, then another training trial was given by applying the negative CS a second time. Conditioning ended when the preparation gave a weak response or stopped responding to the positive CS_1 or after six training trials. During testing, a new positive chemostimulus (CS_2) was applied for 30 sec after a 30-min wait. Subsequently, positive CS_1 and CS_2 were delivered alternately at 30-min intervals until at least four sets of measurements were obtained.

Abbreviations: FMP, feeding motor program; CS, chemostimulus.

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† The saline had the following composition in mM: Na^+, 55.4; K^+, 4.2; Ca^{2+}, 7.0; Mg^{2+}, 4.6; Cl^-, 80.1; $H_2PO_4^-$, 0.2; HCO_3^-, 2.5; and glucose, 5.0, at pH 7.6.

‡ One percent nicotine is 1 ml of a stock solution containing 44% nicotine base and 60% nicotine sulfate (wt/vol) added to 99 ml of slug saline.

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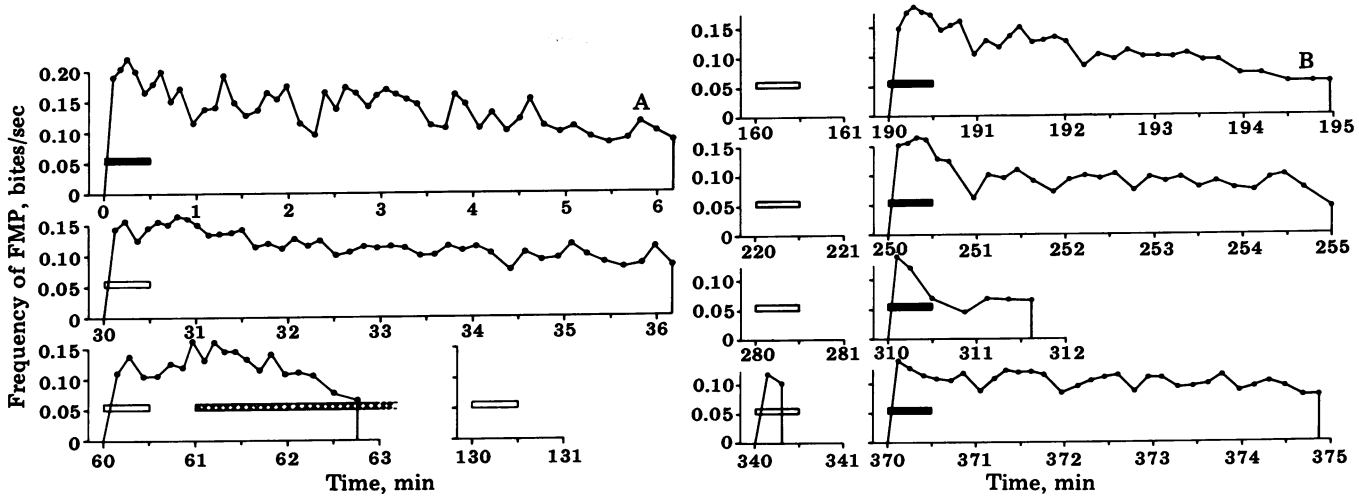

FIG. 1. (*A*) Responses of an isolated lip–brain–buccal ganglion preparation to standard potato, mushroom, and colchicine extracts applied to the lips *in vitro*. A single application of 50 mM colchicine to the lips for 20 min suppresses the brain's response to mushroom extract. (*B*) Continuation of testing of preparation shown in *A* to show that the suppressed response is selective for mushrooms. ■, Potato extract; □, mushroom extract; ▣, colchicine.

Selective suppression of the brain's response to positive CS_1 relative to CS_2 signaled selective taste-aversion learning and was shown to various degrees by different preparations. The responses were rated 4+ to 1+ based on three aspects of the FMP response: (*i*) ratio of the number of FMP cycles (equivalent to bites) elicited by positive CS_2 to that elicited by CS_1, (*ii*) number of tests with positive CS_1 that elicited no response, and (*iii*) number of training trials used to establish the differential response to positive CS_2 compared to CS_1.[§] Responses were scored ± if during testing the brain responded to both positive CS_1 and CS_2 or stopped responding to both positive CS_1 and CS_2. A third possible outcome during testing would be that a response to positive CS_1 returned or remained while an initial response to positive CS_2 decreased. This "antilearning" response pattern is designated by a minus sign.

[§] The ratio of the number of feeding cycles elicited by positive CS_2 to the number of feeding cycles elicited by CS_1, the number of CS_1 tests with no FMP response, and the number of training trials needed to suppress the response to CS_1 are, respectively, as follows: 4+, 8–10, 3–6, and 1 or 2; 3+, 3–7, 0–2, and 2 or 3; 2+, 2, 0, and 3 or 4; 1+, ratio significant by the Mann–Whitney *U* test ($P < 0.05$), 0, and 4–6.

Brains can learn to selectively suppress their FMP output to positive CS_1 while maintaining their response to positive CS_2 (Table 1). This result was obtained in 75% of the last series (Series II) of experiments (12 out of 16). Some brains showed no decrease in response to positive CS_1 and CS_2 or stopped responding to both positive CS_1 and CS_2. This result was observed in 25% of the final series of experiments (4 out of 16). The third possible outcome, or antilearning response pattern, was never observed. Table 1 contains a summary of these results and of results from earlier experiments (series I) in which lower concentrations of the negative CS and shorter times of exposure to the negative CS were used. The testing procedure was the same for all experiments.

Fig. 1 shows data from a single preparation in the 4+ learning category. The first two bouts of FMP established that this preparation gave a clear response to both potato and mushroom extracts (Fig. 1*A*). After a single 20-min application of 50 mM colchicine to the lips paired with stimulation by mushroom extract, the response to mushroom extract was suppressed while the response to potato extract remained (Fig. 1*B*). Fig. 2 gives responses from another 4+ preparation to show that the response to potato could be suppressed while the response to mushroom extract remained unchanged. Here 1% nicotine

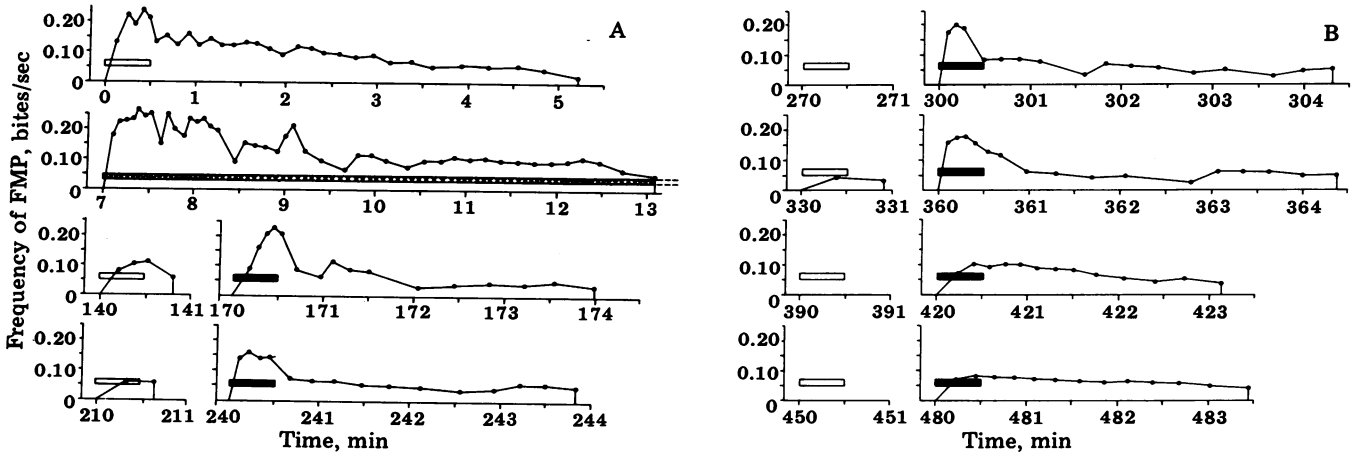

FIG. 2. (*A*) Pairing of lip chemostimulation by standard potato extract and 1% nicotine causes a suppression of response to potato while response to mushroom is maintained. (*B*) Continued testing of the same preparation demonstrates that the learning is clearly evident 6 hr after testing started. ■, Mushroom extract; □, potato extract; □, nicotine.

Table 1. Responses of isolated *Limax* central nervous system to taste-aversion training procedure

No. of exps.	−	±	1+ and 2+	3+ and 4+
Series I: 29	0	18	6	5
Series II: 16	0	4	7	5

sulfate was used as a negative CS. Note that nicotine by itself elicited FMP.

Standardized extracts of potatoes, carrots, mushrooms, and lab chow were used as positive CS_1 and CS_2 in various combinations. All of these stimuli were effective and reliable in eliciting FMP from the preparation. Because potato extract is the most effective among these four stimuli, we concentrated on attempts to train brains to stop responding to potato. Of 20 positive experiments with potato as positive CS_1, 12 had carrot as positive CS_2, 6 had mushroom as CS_2, and 2 had lab chow as CS_2. Of the three experiments that showed learning with potato as positive CS_2, mushroom, carrot, and lab chow each appeared as the positive CS_1 once. Bitter plant secondary chemicals were the most effective negative CSs tried. Nicotine sulfate (1–2%) and colchicine (10–50 mM) each yielded nine positive learning experiments. One 3+ learning result was obtained with 1% tannic acid. Weak learning (1+) was obtained on two occasions each with red pepper extract and with shock of the lips applied via spanning electrodes in the lip chambers.

The retention of the learning can be gauged from the fact that in the ten 3+ and 4+ experiments, the differential responsiveness of the preparation was still marked 6–8 hr after training ended but was not apparent after 18–20 hr. On two of these occasions, preparations that gave clear learning on one day were successfully retrained on the following day. In no case (eight experiments) did a brain that failed to learn on the first day show successful learning on the following day. Isolated lip–brain preparations survived for 3–4 days.

It is unlikely that the suppression of responses we observed was due to effects of the negative CS on lip chemoreceptors themselves. The fact that the preparation continued to respond to positive CS_2 argues against a destructive effect of negative CS on lip chemoreceptors, as does the demonstration of selective suppression with three different chemical species used as negative CS. A third observation also inconsistent with a destructive effect of negative CS on lip chemoreceptors is that, of the 22 brains scored as ± (Table 1), 16 (73%) continued responding to both positive CS_1 and CS_2 during testing in spite of repeated presentations of the negative CS.

The time delay between application of positive CS_1 and the negative CS varied from 0.5 to 9 min in these experiments. Two training strategies were used to determine this delay: we either ensured that the negative CS was applied during vigorous FMP triggered by the positive CS_1 (Fig. 1*A*) or we avoided this overlap of FMP and application of negative CS by waiting 7–9 min after application of positive CS_1 before applying the negative CS (Fig. 2*A*). Among the 10 best learners, the two training strategies were equally represented.

These experiments demonstrate that the isolated brain of *Limax* can show conditioning that is analogous to the food-aversion learning of the whole animal (1). The learning displayed by the isolated brain, like that of the intact animal, requires relatively few training trials for a robust effect. However, it is quite variable, with individuals showing responses ranging from one-trial learning to no learning. Improvements in experimental technique may well reduce this variability, as has already occurred for the whole-animal learning assay. The isolated brain differs from the intact animal in the retention of learning (6–8 hr in contrast to 7–21 days) and in the sensory input–motor output pathways involved. A different culture medium which can maintain *Limax* brains *in vitro* for 2 weeks or more (9) may allow longer lasting learning by isolated brains.

To investigate the behavioral implications of our *in vitro* "neural" learning, we are conditioning intact slugs in a similar task. Recent experiments show that intact animals can be conditioned reliably to selectively avoid highly palatable foods after one pairing of the food and a bitter substance (10).

Limax is one of several gastropod molluscs whose learning ability is being examined behaviorally and neurophysiologically. *Aplysia* can learn to associate shock and locomotion by using a nervous system well suited to cellular studies (11, 12). *Hermissenda* can learn to associate photic and vestibular inputs (13). A neural correlate of food avoidance conditioning by using shock as the aversive stimulus has been described in *Pleurobranchaea* (14, 15). This concerted effort combined with our ability to train isolated brains while recording cellular interactions gives real hope for progress in unraveling the synaptic fabric underlying associative learning.

Note Added in Proof. By using externally applied quinine as the aversive stimulus, we have found three robust and reliable forms of higher-order conditioning (second-order conditioning, Kamin blocking effect, and a transient unconditioned stimulus preexposure effect) in whole-animal studies with *Limax* (ref. 16 and unpublished data).

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