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Differential Neuroethological Effects of Aversive and Appetitive Reinforcing Stimuli on Associative Learning in *Lymnaea stagnalis*

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ABSTRACT—It is necessary to determine whether, in the same species and for the same behavior, aversive and appetitive conditioning yield different strengths and periods of either acquisition or retention. To this end, we first examined the effects of various chemo-sensory and physical stimuli on feeding and avoidance behavioral responses in the pond snail, *Lymnaea stagnalis*. Then, using these findings, we constructed classical-conditioning paradigms with aversive and appetitive stimuli. In the aversive conditioning paradigm, an appetitive stimulus (sucrose), which increased the feeding response, was paired with an aversive stimulus (KCl, quinidine sulfate or electric shock), which inhibited the feeding behavior. Upon presentation of KCl, the first type of aversive conditioning, which is generally called "taste-aversion learning with cessation of feeding response", was acquired quickly and persisted for up to a month. When using a noxious stimulus (quinidine sulfate or electric shock) inducing pain we additionally found the second type of aversive conditioning, in which the previously appetitive stimulus (sucrose) not only failed to increase the feeding response, but came to elicit an avoidance response. This second type conditioning took longer to acquire and persisted for a shorter period of time than the first type. On the other hand, the appetitive conditioning paradigm paired a neutral stimulus (vibratory) with an appetitive stimulus (sucrose). The strength and period for acquisition and retention of this appetitive learned response were very similar to those of the second type aversive conditioning but not to the first one. On the basis of these behavioral analyses, the neuronal mechanisms of the two types of aversive and appetitive conditioning were discussed.

INTRODUCTION

One important tool used by neurobiologists to study the mechanisms of learning and memory is the associative-learning paradigm (Dudai, 1990). Gastropod molluscs, such as *Aplysia*, *Pleurobranchaea*, *Hermissenda*, *Limax* and *Lymnaea* etc., which possess a relatively simple nervous system of large identifiable neurons at least partly mediating their behaviors, have been extensively chosen for investigations of learning and memory. Their behaviors are known to be easily analyzed and modified by associative-learning paradigms (Carew and Shahly, 1986; Mpitsos and Lukowiak, 1985; Willows, 1973). Moreover, some of these molluscs are capable of the higher-order associative learning (Cook and Carew, 1986; Kemenes and Benjamin, 1989a; Suzuki *et al.*, 1994) typically associated only with vertebrates.

In Pavlov's original classical-conditioning experiments, an initially ineffective conditioned stimulus (CS, i.e. tone of metronome), when paired with an effective unconditioned stimulus (UCS, i.e. food), eventually came to evoke the unconditioned response (UCR, i.e. mouth watering). In the above example, the UCS (food) was an appetitive or a rewarding reinforcer; however, an aversive or punishing stimulus could also have been used as an effective UCS. We thus know that it is possible to employ both aversive and appetitive classical-conditioning paradigms. Most conditioning studies in molluscs, however, have employed the aversive conditioning paradigm (Alkon, 1974; Carew *et al.*, 1981; Lukowiak and Sahley, 1981; Mpitsos and Collins, 1975; Sahley *et al.*, 1981), and so our limited understanding of how the molluscan nervous system mediates learning and memory has been primarily based on the results of aversive conditioning (Carew *et al.*, 1983; Granzman, 1995; Hawkins *et al.*, 1983; Ito *et al.*, 1994; Lukowiak and Colebrook, 1988; Sekiguchi *et al.*, 1991; Walters and Byrne, 1983). Whether similar changes occur in the nervous system when appetitive conditioning occurs has been largely unexplored in these molluscan model systems. It is important to determine whether in the same

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species and behavior there are differences between aversive and appetitive conditioning.

The pond snail, *Lymnaea stagnalis*, has been reported to undergo appetitive classical conditioning (Alexander *et al.*, 1982; Audesirk *et al.*, 1982; Kemenes and Benjamin, 1989a, b, 1994) and aversive operant conditioning (Lukowiak *et al.*, 1995). Thus, we reasoned that it might be possible to study the differences, if any, between aversive and appetitive classical conditioning of a single behavior in *Lymnaea*. We choose to study the feeding response of *Lymnaea*, and show here that there are significant differences in both the acquisition and retention of aversive vs. appetitive classical conditioning.

MATERIALS AND METHODS

Subjects

We used locally-reared pond snails, *Lymnaea stagnalis*, originally derived from the stocks of Vrije Universiteit in Amsterdam. They were fed with lettuce and turtle food (Tetra ReptoMin, TetraWerke, Germany), and were maintained on a 12: 12 light-dark cycle at 20°C. Prior to experimentation, pond snails (adults with 20 mm or longer shells) were removed from their home aquaria and placed in distilled water (DW) without access to food for 24 hr. All experiments were performed in the light period.

Aversive and appetitive chemical stimuli

An aversive response to a stimulus was defined as the withdrawal of the body into its shell, while an appetitive response was defined as an increase in the frequency of biting of a food substance. To determine which stimuli were aversive or appetitive, pond snails (N=20 in each group) were individually placed in a 200 ml beaker with 50 ml of DW and given a 10 min acclimatization period. We tested substances which taste sweet, salty, bitter, *umami* and sour to humans.

Kemenes and Benjamin have reported that a 100 mM sucrose solution (sweet taste to humans) evoked a reliable feeding response (appetitive stimulus) in 90% of pond snails tested (Kemenes and Benjamin, 1989). We thus applied 0.1, 1, 3, 5, 10 and 100 mM D-sucrose solutions to the lips of individual pond snails. 1 ml of each concentration was applied gently just in front of the lip of each animal for 5 sec. The following substances were tested in the same manner: NaCl (salty to humans, 0.1, 1, 10 and 100 mM); quinidine sulfate (bitter to humans, 0.1, 1, 3, 5 and 10 mM); Na L-glutamic acid (*umami* to humans, 0.1, 1, 10 and 100 mM); and 1% acetic acid (sour to humans). For each taste group we also employed control solutions which were applied in the same manner: DW; 10 mM D-cellobiose (a disaccharide which is not perceived as sweet by humans as a control for sucrose); KCl (0.1, 1, 10, 30, 50 and 100 mM); L-glutamic acid (10 mM); K L-glutamic acid (10 mM); Na acetic acid (10 mM); K acetic acid (10 mM). The number of bites in the 1 min period following the application of the substance was counted.

Physical stimuli

The responses of pond snails to light, water flow, vibration and electric shock were also examined. Phototaxis (positive or negative) was determined by placing a pond snail in a 4×32 cm pool covered with an 8-step-grade shadowing sheet. Each grade covered a 4×4 cm space and was assigned a letter (A-H). The light intensity in zones A-H was 920, 680, 560, 440, 330, 250, 180 and 150 lx, respectively. Pond snails were either dark- or light- adapted before being placed in zone A or H. For dark adaptation, a pond snail was placed in zone H for 30 min with 5 lx of red light and a gate preventing its movement into the adjacent zone. At the end of this period, the white light was turned on and the gate removed. We observed the pond snail's location by zone at 5 min intervals for a 30 min period. We tested 25 pond snails in this manner. A similar procedure was employed for light adaptation, only in this case the pond snail was illuminated with 1000 lx for 30 min. Again 25 pond snails were tested. We also tested the activity of pond snails in different light intensities (1200, 550 and 5 lx). Their activity was measured as distance they moved in a 30 min period at each light intensity. We tested 24 pond snails for each light intensity.

To examine rheotaxis (taxis to water current stimulation) we employed a Y-pool (trunk length 9 cm, right and left branch length 15 cm, a 90° angle between right and left branch, a 4 cm width throughout, and water depth of 1.5 cm). Pond snails were placed at the end of the trunk, and water was perfused at 150 ml/min into either the left (N=10) or the right (N=10) arm. Their movements were observed for 30 min.

To examine their responses to vibration, single pond snails were first adapted for 10 min in 20 ml of DW in a 100 ml beaker. The beaker was then vibrated at 3 Hz with 2 cm amplitude for 1 min. The extent of withdrawal into their shells was observed (N=25). We also examined whether this response habituated by presenting a 15 sec vibration stimulus 10 times with a 6 min intertrial interval (ITI) (N=20).

An electric shock was administered to heads of single pond snails in 20 ml of DW in a 100 ml beaker. The shock duration was 30 msec (Carew *et al.*, 1983; Hawkins *et al.*, 1983; Walters and Byrne, 1983), and the current strength was varied at 10, 30, 50 and 100 μA (N=20 each).

Aversive classical-conditioning paradigm

We studied two types of aversive conditioning, herein called type 1 and 2. Type 1 is what is known in general as a taste-aversion learning with cessation of feeding response (Garcia and Koelling, 1966). In this conditioning procedure the CS comes not to induce eating after training, whereas before training it did. The definition of type 2 aversive conditioning is that the CS which initially induced eating before training no longer evokes the feeding response, but rather evokes a withdrawal reflex or an avoidance behavior. Thus type 2 conditioning is more aversive. Moreover, to equalize the strengths of stimuli, we made it a rule to employ the lowest possible concentrations of chemical substances or the weakest possible strengths of physical stimuli sufficient to evoke the feeding or withdrawal response in > 90% of pond snails used. These behavioral data are accumulated in the previous section, Aversive and appetitive chemical stimuli.

In a first series of experiments for building up the aversive conditioning, a 1 ml solution of 10 mM sucrose served as the CS and a 1 ml solution of 50 mM KCl served as the UCS. This conditioning paradigm is shown in Fig. 1. The CS initially induced a reliable increase in feeding response in > 90% of pond snails tested, whereas, the UCS resulted in withdrawal into the shell in > 90% of pond snails tested (see Results). The training apparatus consisted of a 100 ml beaker filled with 20 ml of DW, with an attached perfusion system. Throughout the experiment the beaker was constantly perfused with DW at 25 ml/min. Following a 10 min adaptation period, the CS and UCS were applied for 5 sec over the lips of the pond snails. The effective concentrations lasted for 15 sec in this perfusion system. The interstimulus interval (ISI) between the onset of the CS and that of the UCS was 20 sec. Pond snails were given 1 to 30 paired trials with a 10 min ITI between each paired presentation of the CS-UCS (1 trial: N=16; 5 trials: N=20; 20 trials: N=16; 30 trials: N=16). When pond snails were given 30 to 100 paired trials, a 30 min ITI was employed (30 trials: N=16; 100 trials: N=8). The reason for this change of ITI will be mentioned in Results. In all cases, the suitable ISIs and ITIs were determined after examination of some variations (see Results). A backward (UCS-CS) conditioning control (1 trial: N=16; 5 trials: N=40; 20 trials: N=16; 30 trials: N=16; 30 trials with long ITI: N=16), a CS alone (5 trials: N=20) and a naive (1 trial: N=16; 5 trials: N=20; 20 trials: N=16; 30 trials with long ITI: N=16; 30 trials: N=16) group were also employed. After the last ITI the feeding response (number of bites) evoked by the CS was counted using a blind procedure for 1.5 min. To determine the persistence of the learned behavior, the response to the CS alone was tested 10 min, 1 hr, 24

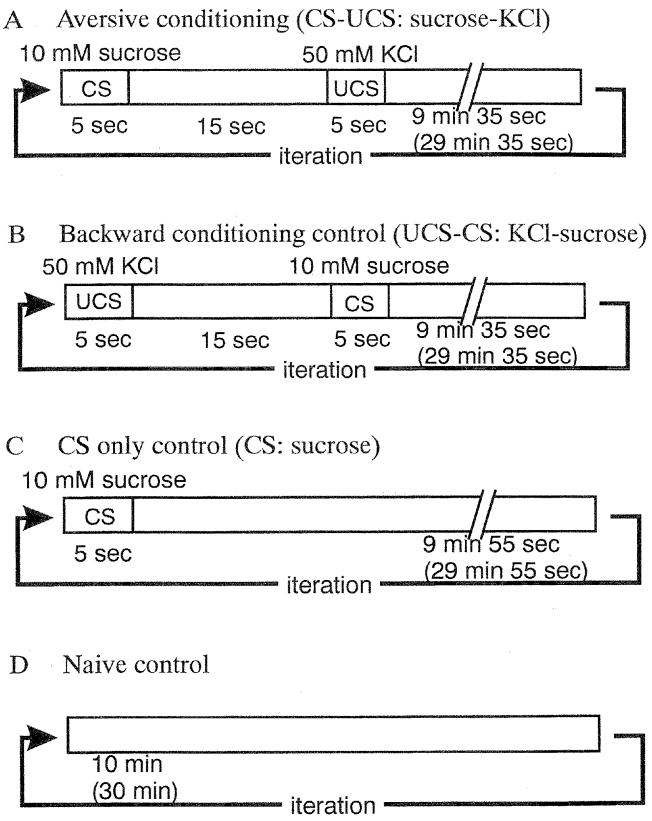

Fig. 1. Associative-conditioning paradigm with aversive stimulus in pond snails. In this case, the CS and the UCS were 1 ml solution of 10 mM sucrose and 1 ml solution of 50 mM KCl, respectively.

hr, and 7 and 30 days later.

A second series of experiments was performed using a 1 ml solution of 10 mM quinidine sulfate as UCS, and a 1 ml solution of 10 mM sucrose as CS. Our results as given in the previous section, Aversive and appetitive chemical stimuli, showed that the 10 mM quinidine sulfate was the lowest concentration to result in the withdrawal response in > 90% of pond snails. Pond snails, however, withdrew by a droplet of quinidine sulfate to the epidermis of their posterior feet, as well as to their lips. That is, we could conclude that quinidine sulfate has two stimulus pathways (bitter taste and pain) and that it is more noxious than KCl. The ITI, therefore, had to be increased from 10 to 30 min, even where ISI was of the same duration as in the sucrose-KCl conditioning. Pond snails were given 1 to 60 paired trials (1 trial: N=16; 5 trials: N=16; 20 trials: N=16; 30 trials: N=16; 60 trials: N=16). The maximum number of paired trials per day was determined as 10: for example, when the pond snails should have received 30 trials, the 10 paired trials per day were given to them for 3 days. A backward (UCS-CS) conditioning control (1 trial: N=16; 5 trials: N=16; 20 trials: N=16; 30 trials: N=16; 60 trials: N=16), a CS alone (5 trials: N=16) and a naive group (1 trial: N=16; 5 trials: N=16; 20 trials: N=16; 30 trials: N=16; 60 trials: N=16) were also employed. After the last ITI, the response to the CS alone was tested using a blind procedure for 1.5 min. The persistence of the learned response was tested 1 hr, 24 hr, and 3, 7, 14 and 30 days later.

A third series of experiments were performed using a 30 msec duration of 100 μA electric shock on the head as the UCS. This UCS was not a taste stimulus but the weakest shock sufficient to induce the withdrawal response in > 90% of pond snails (see Results). The CS was a 1 ml solution of 10 mM sucrose; the ISI was 20 sec; the ITI was 30 min. One to 70 paired trials were given to pond snails (N=16 each for 1 to 50 paired trials, and N=14 for 60 and for 70 trials), but the maximum number of paired trials per day was 10. A backward (UCS-CS) conditioning control (N=8 each for 1 to 50 paired trials, and N=6 for 60 and for 70 trials) and a naive group (N=8 each for 1 to 70 paired trials) were also employed. After the last ITI, the response to the CS alone was tested using a blind procedure for 1.5 min. The persistence of the learned response was tested 1 hr, 24 hr, and 3, 7, 14 and 30 days later.

Appetitive classical-conditioning paradigm

We used a vibratory stimulus as CS and paired it with a UCS of 10 mM and 100 mM solutions of sucrose, the latter of which perfectly initiated a feeding response. The same application method of sucrose and the same training apparatus as in the aversive conditioning experiments were employed.

The CS (vibration, 3 Hz and 2 cm amplitude for 15 sec: a neutral stimulus (see Results)) was followed immediately by the application of the UCS (1 ml solution of 10 mM or 100 mM sucrose). Pond snails received 1 to 60 paired trials (1 trial: N=20; 5 trials: N=20; 20 trials: N=20; 30 trials: N=20; 60 trials: N=16) with an ITI of 6 min. The maximum number of paired trials per day was 30. The ISI and ITI were determined in the same way as detailed in the aversive conditioning. A backward (UCS-CS) conditioning control (1 trial: N=20; 5 trials: N=20; 20 trials: N=20; 30 trials: N=20), a CS alone (30 trials: N=20) and a naive group (1 trial: N=20; 5 trials: N=20; 20 trials: N=20; 30 trials: N=16; 60 trials: N=16) served as controls. A blind procedure was used to determine if associative learning had occurred by counting the number of bites for 3 min following the presentation of the test CS. The persistence of appetitive learning was tested by the presentation of the CS alone 1 hr, 24 hr, and 3 and 7 days later.

Statistical analyses

The feeding responses (number of bites) were evaluated for statistical significance ($p<0.05$) with *t*-test. The responses to physical stimuli were evaluated with one-way ANOVA. Type 1 and type 2 aversive responses were classified by χ^2-test.

RESULTS

Responses to chemical stimuli

The feeding responses (number of bites/min) to the various chemical stimuli presented to the lips of the pond snails are shown in Fig. 2. The presentation of DW to the lips was taken as the control response. As can be seen in Fig. 2A, the feeding response increased in a sigmoid fashion with the increasing concentration of D-sucrose. Concentration of D-sucrose $\geqq$ 5 mM brought about a significant effect on feeding ($p<0.01$). We observed that a 10 mM sucrose stimulus induced the feeding response in > 90% of the pond snails, and that a 100 mM sucrose achieved this effect in all the pond snails. The presentation of 10 mM D-cellobiose (which does not taste sweet to humans) produced no significant increase in the feeding response as compared to the presentation of DW.

The feeding response evoked by NaCl was more complicated (Fig. 2B) than that of sucrose. NaCl at concentrations of 1 mM and lower had no effect on feeding, at 10 mM significantly increased feeding ($p<0.005$), and at 100 mM resulted in whole-animal withdrawal. 100 mM NaCl was therefore considered an aversive stimulus.

A 1 mM KCl stimulus had no effect on the feeding response, while concentrations of KCl in excess of 10 mM KCl induced the withdrawal response and served as an

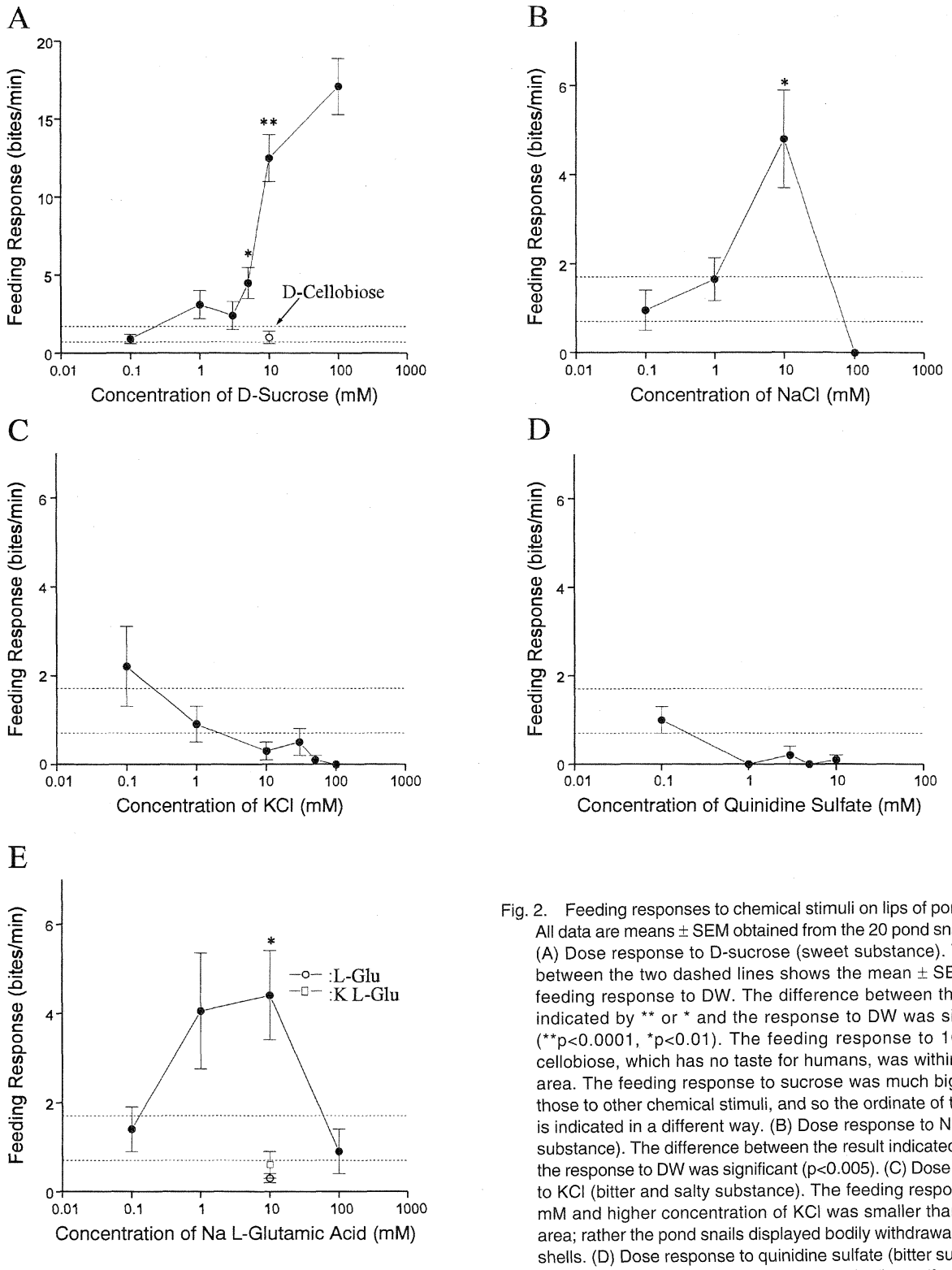

Fig. 2. Feeding responses to chemical stimuli on lips of pond snails. All data are means ± SEM obtained from the 20 pond snails used. (A) Dose response to D-sucrose (sweet substance). The area between the two dashed lines shows the mean ± SEM of the feeding response to DW. The difference between the results indicated by ** or * and the response to DW was significant (**$p<0.0001$, *$p<0.01$). The feeding response to 10 mM D-cellobiose, which has no taste for humans, was within the DW area. The feeding response to sucrose was much bigger than those to other chemical stimuli, and so the ordinate of this figure is indicated in a different way. (B) Dose response to NaCl (salty substance). The difference between the result indicated by * and the response to DW was significant ($p<0.005$). (C) Dose response to KCl (bitter and salty substance). The feeding response to 10 mM and higher concentration of KCl was smaller than the DW area; rather the pond snails displayed bodily withdrawal into their shells. (D) Dose response to quinidine sulfate (bitter substance). The 1 mM and higher concentration of quinidine sulfate induced withdrawal responses in pond snails. (E) Dose response to Na L-glutamic acid (*umami* substance). The difference between the result indicated by * and the response to DW was significant ($p<0.01$). Both L-glutamic acid and K L-glutamic acid induced withdrawal response in the pond snails due to the effects of potassium ion and low pH.

aversive stimulus (Fig. 2C). In particular, a 50 mM KCl stimulus evoked the withdrawal response in > 90% of the pond snails.

Quinidine sulfate at concentrations of 1 mM and higher also served as an aversive stimulus (Fig. 2D). The concentration of quinidine sulfate which induced withdrawal response in > 90% of the pond snails was 10 mM.

Na L-glutamaic acid significantly increased the feeding response at a concentration of 10 mM ($p<0.01$, Fig. 2E). L-glutamic acid (10 mM) and K L-glutamic acid (10 mM) both evoked the withdrawal response and thus inhibited the feeding response (Fig. 2E). It was possible that the withdrawal response was triggered by the potassium salt of K L-glutamic acid and the non-neutral pH of the L-glutamic acid (10 mM, pH 3.4). These effects were confirmed as follows: The feeding response was inhibited by 1% acetic acid (pH 3.0) and a 10 mM K acetic acid solution (pH 7.0), but not by a 10 mM Na acetic acid solution (pH 7.0) (data not shown). Moreover, a 10 mM KCl solution buffered with 10 mM HEPES-KOH (pH 7.0) evoked the withdrawal response, while 10 mM HEPES-KOH (pH 7.0) was a neutral substance. Basic solution (10 mM L-lysine, pH 9.1) also resulted in a suppression of feeding, while 10 mM L-glycine (pH 7.0) had no effect on feeding (data not shown).

Responses to physical stimuli

If pond snails were either positively or negatively phototaxic we would expect them to congregate at either end of the 8-step gradient. Although we found that they stayed at the ends for the longest period of time, they showed no particular preference between the light and dark ends (data not shown). Moreover, there were no differences in their levels of activity in the dim (5 lx), moderate (550 lx) or bright (1200 lx) light environments. Pond snails did, however, respond with a withdrawal reflex to an on-off stimulus consisting of 5 times repeated illumination of a 1 Hz light (1200 lx) (38 out of 50 pond snails).

As regards rheotaxis, our experiments showed that pond snails moved randomly in a Y-character pool irrespective of the direction of DW: they have no rheotaxis (data not shown).

The vibratory stimulus resulted in weak withdrawal. That is, the pond snails retracted a portion of their bodies into the shell, leaving their lips and tentacles exposed. They appeared to habituate upon repeated presentation of this stimulus, no longer even exhibiting the incomplete withdrawal movements. We conclude that the vibratory stimulus could also serve as a neutral stimulus.

Electric shock of 100 μA administered to the heads of pond snails was sufficient to induce the withdrawal response in > 90% of the pond snails, but the shock with ≦ 50 μA was not.

Aversive classical conditioning

When the sucrose stimulus (1 ml of 10 mM sucrose) was paired 5 times with the KCl stimulus (1 ml of 50 mM KCl), significantly fewer feeding responses ($p<0.005$) were elicited by the sucrose stimulus than when the backward conditioning control (KCl then sucrose) was employed, or when sucrose was presented by itself, or when no stimulus (naive) was presented. These data are presented in Fig. 3A. We here used a 20 sec interval as the ISI because the effects of chemical stimuli did not last more than 15 sec in our perfusion system (see Materials and Methods). When the ISI was fixed at 20 sec, the ITI was varied at 5, 10, 20 and 30 min. We then found that a 10 min ITI paradigm could accomplish the optimal learning (data not shown). On the other hand, when the ITI was set at 10 min, the ISI was varied at -20, -5, 0, 5, 20, 30 and 60 sec. Consequently, the 20 sec ISI training could establish the optimal learning in the pond snails. Here we should note that ISI of -20 sec was also used in the backward conditioning control.

We tested the persistence of this taste-aversion conditioned response and found that feeding was significantly suppressed for at least 30 days ($p<0.01$, Fig. 3B). Thus pond snails are capable of aversive classical conditioning, which we have termed type 1 aversive conditioning (taste-aversion learning), and they can remember the association for longer than 1 month.

When the pond snails were trained by only one pairing of sucrose-KCl, they did not learn the taste aversion. Twenty paired trials induced the good conditioning ($N=16$, $p<0.0001$, vs. controls), but 30 pairings ($N=16$) did not in the case of 20 sec ISI and 10 min ITI. We supposed that the pond snails were desensitized to KCl or damaged by it, and so elongated the ITI to 30 min and determined the maximum number of pairings per day as 10 for the recovery. When the pond snails were paired with sucrose and KCl 10 times per day for 3 days (i.e. 30 paired trials) with 30 min ITI, they learned the taste aversion very successfully ($N=16$, $p<0.0001$, vs. controls).

To determine whether pond snails were capable of type 2 aversive conditioning in the sucrose-KCl procedure, we employed a similar training protocol (ISI=20 sec, ITI=30 min), but increased the number of paired trials to 100. Even with this increased training, the CS (sucrose) never elicited the withdrawal response ($N=8$).

We therefore employed 10 mM quinidine sulfate, which has two aversive-stimulus pathways to pond snails, with a long ITI (30 min) to evoke, if possible, type 2 conditioning. Five paired trials (Fig. 3C, D) and 20 paired trials (Fig. 3D) only elicited the type 1 taste-aversion learning. These learned behavior lasted for at least 30 days (Fig. 3D). In the 30 paired trials the CS elicited a withdrawal or head waving (i.e. type 2 aversive conditioning, Table 1) within 3 days following the end of training ($\chi^2=12.5$, $p<0.005$ vs. controls). After 3 days the CS only just resulted in a suppression of feeding (type 1 aversive conditioning), which continued to persist for at least 30 days (Table 1 and Fig. 3D). The increase of trial number to 60 did not elongate the persistence of type 2 aversion conditioning (as shown in a gray-line rectangle in Fig. 3D).

Seventy presentations of the sucrose-electric-shock-paired trial yielded type 2 aversive conditioning in the pond snails ($\chi^2=20$, $p<0.0001$, vs. controls), but 60 presentations did not. Surprisingly, this learned type 2 behavior was

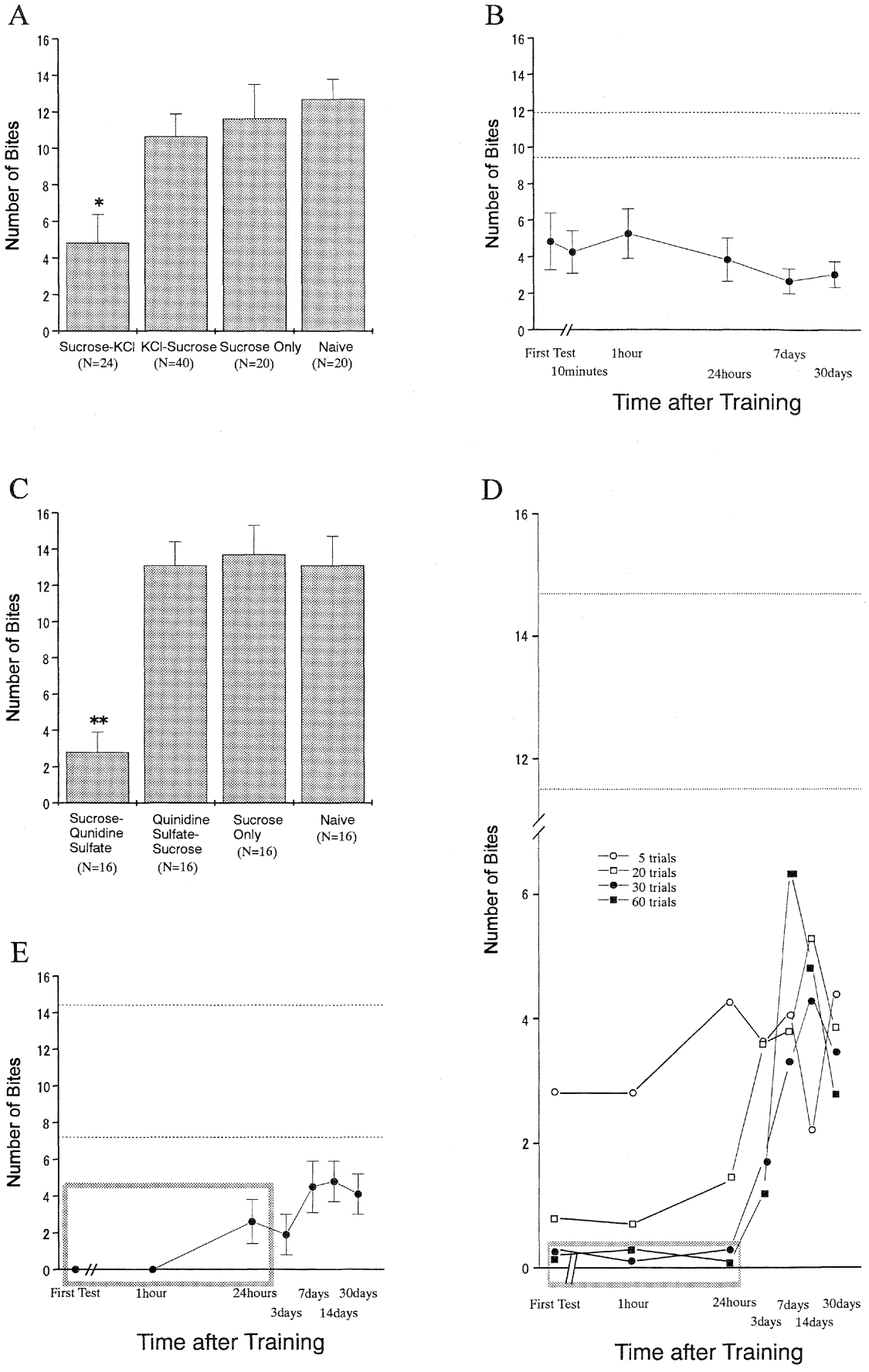

Table 1. Number of animals performing aversive or appetitive behavior in 30-paired-trial sucrose-quinidine sulfate aversive conditioning

Animals	Behavioral responses			
	Avoidance	None	Feeding	Conditioning type
Conditioned (N=20)				
first test	15	4	1	2
1 hr later	17	2	1	2
24 hr later	15	3	2	2
3 days later	5	8	7	1
7 days later	1	6	13	1
14 days later	0	3	15	1
30 days later	0	4	13	1
Controls				
UCS-CS (N=12)	1	0	11	–
CS alone (N=12)	2	2	8	–

Sucrose elicited a withdrawal or head waving within 3 days following the end of training (χ^2=12.5, p<0.005 vs. controls). Between 7 and 30 days after the training, 3 conditioned snails died.

extinguished within 3 days (as shown in a gray-line rectangle in Fig. 3E) as it was in the above sucrose-quinidine sulfate experiments. Therefore, these results in aversive classical conditioning suggest that the pond snails forget their withdrawal response to more aversive situations within 3 days, remembering only to close their mouths to the substances they liked before training.

Appetitive classical conditioning

When vibration (a neural stimulus) was used as a CS and paired with sucrose as a UCS, it came, after 30 paired trials, to evoke the feeding response. The backward conditioning, the presentation of the CS alone, and the naive group did not result in any significant increase in feeding, as the conditioned group did (p<0.01, Fig. 4A).

We found that it took at least 30 paired trials before the CS came to consistently evoke the increase in feeding (i.e. the unconditioned response, UCR). We here had to employ a 15 sec ISI and a 6 min ITI, because this ISI was the best interval, and because shorter and longer ITIs had failed to produce associative learning.

When we tested for the persistence of the learned response with vibratory stimulus used as the test CS, we found that associative learning was only maintained to 3 days (p<0.01, Fig. 4B). No learning was observed 7 days after the last training trial. Efforts to elongate the persistence of this

Fig. 3. Classical conditionings with aversive UCSs in pond snails. All data are means ± SEM except for (D). The ordinates in these figures are indicated as numbers of bites for 1.5 min. (A) Feeding response in sucrose-KCl conditioning. The CS and the UCS were 1 ml solution of 10 mM sucrose and 1 ml solution of 50mM KCl, respectively. The number of paired trials was 5. The CS used for the test suppressed the feeding response of the conditioned (sucrose-KCl) pond snails (namely, type 1 aversive conditioning was formed), but not that of the controls (KCl-sucrose, sucrose only and naive) (*p<0.005). The number of pond snails used is indicated as N. (B) Time dependence of retention of 5-paired-trial sucrose-KCl conditioning. Note that the time shown in the abscissa is to logarithmic scale. The area between the two dashed lines shows the mean ± SEM of the feeding response for the control (KCl-sucrose) pond snails in the first test (see A). The difference between the feeding responses of the conditioned pond snails and that of the control was maintained with at least p<0.01. (C) Feeding response in sucrose-quinidine sulfate conditioning. The CS and the UCS were 1 ml solution of 10 mM sucrose and 1 ml solution of 10mM quinidine sulfate, respectively. The number of paired trials was 5. The CS used for the test suppressed the feeding response of the conditioned (sucrose-quinidine sulfate) pond snails (namely, type 1 aversive conditioning was formed), but not that of the controls (quinidine sulfate-sucrose, sucrose only and naive) (**p<0.0001). (D) Time-dependent change in type of aversive memory in sucrose-quinidine sulfate conditioning. Note that the time shown in the abscissa is to logarithmic scale. The area between the two dashed lines shows the mean ± SEM of the feeding response for the control (naive) pond snails in the first test (see C). The conditioned pond snails with 5 or 20 paired trials retained type 1 aversive conditioning to 30 days (vs. the control, at least p<0.05). Responses of the 30-trial and 60-trial pond snails at the first test, after 1 hr and 24 hr later were withdrawal or avoidance; these behaviors were in agreement with the criterion of type 2 aversive conditioning. These type 2 aversive conditioning responses ended within 3 days (as indicated by the gray rectangle), and the aversive behavior changed from withdrawal or avoidance to simple cessation of biting (see Table 1). (E) Feeding response in sucrose-electric shock conditioning. Note that the time shown in the abscissa is to logarithmic scale. The area between the two dashed lines shows the mean ± SEM of the feeding response for the control (naive) pond snails in the first test. The CS and the UCS were 1 ml solution of 10 mM sucrose and 100 μA electric shock for 30 msec, respectively. The number of paired trials was 70. The CS used for the test completely abolished the feeding response of the conditioned (sucrose-electric shock) pond snails (N=14), but not that of the controls (electric shock-sucrose, sucrose only and naive). The responses of the conditioned pond snails at the first test, after 1 hr, and after 24 hr were withdrawal or avoidance; these behaviors were in agreement with the criterion of type 2 aversive conditioning. These type 2 aversive conditioning responses ended within 3 days (indicated by a gray rectangle), and the aversive behavior changed from withdrawal or avoidance to simple cessation of biting. Moreover, the number of feeding responses of conditioned pond snails on the 7th day was not significantly different from that in the control.

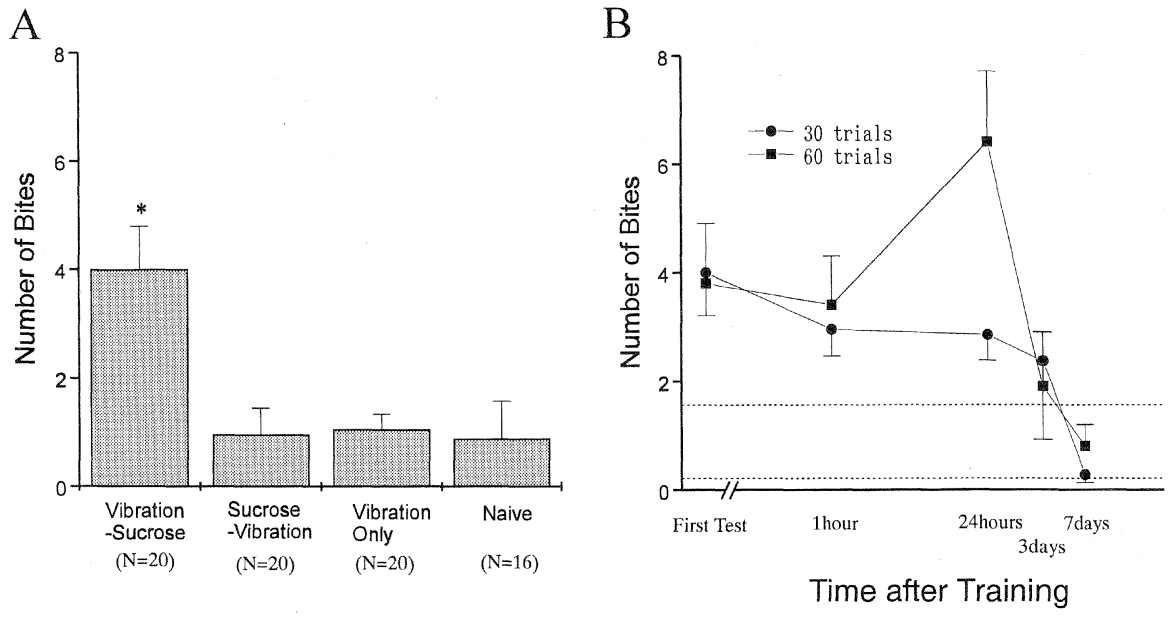

Fig. 4. Classical conditioning with appetitive UCS in pond snails. All data are means ± SEM. The ordinates in these figures are indicated as numbers of bites for 3 min. (A) The CS and the UCS are 15 sec vibration (3 Hz frequency and 2 cm amplitude) and 1 ml solution of 100 mM sucrose, respectively. The number of paired trials was 30. The test was performed to determine feeding responses to the vibration (CS) to the conditioned (vibration-sucrose) and the control (sucrose-vibration, vibration only and naive) pond snails. The difference between the results for the conditioned and the control pond snails was significant (*$p<0.01$). The number of pond snails used is indicated as N. (B) Time dependence of retention of vibration-sucrose conditioning. Note that the time shown in the abscissa is to logarithmic scale. The area between the two dashed lines shows the mean ± SEM of the feeding response for the control (naive) pond snails in the first test. The difference between the feeding responses of the conditioned pond snails with 30 or 60 paired trials and those of the control was maintained with at least $p<0.01$ within 3 days later.

appetitive response by increase of paired trials to 60 were in vain (Fig. 4B).

DISCUSSION

Using feeding behavior, i.e. the number of bites per unit time, and avoidance behavior as indices we found that pond snails respond quite differently to a number of chemical and physical stimuli. A number of substances were found which, when applied to the lips of pond snails, significantly increased the feeding response. Thus sweet (sucrose) as well as low concentrations of salty (NaCl) and *umami* (Na L-glutamic acid) substances resulted in increased feeding, and these were termed appetitive stimuli. On the other hand, bitter (quinidine sulfate) and sour (acetate) substances, as well as potassium salts and non-neutral pH substances, were found to suppress feeding behavior when applied to the lips, and were termed aversive stimuli. (Note: Needless to say, no one knows how these chemical substances taste to pond snails; we use human taste as a reference purely for the sake of convenience.)

In some cases the aversive stimuli not only inhibited the feeding response, but evoked either a withdrawal reflex where the pond snail pulled into its shell, or an avoidance behavior. Although pond snails possess photoreceptors they appeared to be neither positively nor negatively phototaxic. However, they did respond to a light on/off with a withdrawal response. Pond snails also possess statocysts, observable as white ball-like organs in the pedal ganglia (Syed and Winlow, 1991), but did respond with incomplete withdrawal movements to the vibratory stimulus used here. Pond snails exhibited neither positive nor negative rheotaxis. Finally, pond snails withdrew their bodies into the shells in response to 100 μA electric shock. The results obtained in this study thus allowed us to use these various stimuli as CSs and UCSs in aversive and appetitive conditioning experiments, and to have a benchmark for comparing the animal's capacity to form and maintain learned associations.

Previous reports have actually shown that pond snails have the capacity to acquire and retain learned associations (Alexander *et al.*, 1982; Audesirk *et al.*, 1982; Kemenes and Benjamin, 1989a, b, 1994; Lukowiak *et al.*, 1995). Our results confirm that pond snails are capable of associative learning and, further, that direct comparisons can be made between different types of aversive conditioning (type 1 and 2), as well as between aversive and appetitive conditioning. Since in all cases we have conditioned pond snails' feeding behavior, we are in a position to make direct comparisons between the acquisition and retention of the learned responses.

We defined type 1 aversive conditioning as characterized by a decrease in feeding response to a previously appetitive CS (generally, this is known as taste-aversion learning) and type 2 aversive conditioning as characterized not only by

suppression of the normally triggered feeding response, but also by a withdrawal or avoidance response to the CS. The UCSs used for the aversive conditioning were KCl, quinidine sulfate and electric shock. The association of CS with KCl elicited only type 1 aversive conditioning; that of CS with quinidine sulfate or electric shock provoked both type 1 and type 2 aversive conditioning.

As regards type 1 aversive conditioning, we found that it was acquired rapidly (5 pairings) and persisted for relatively long periods of time (at least one month). The increase of pairing (to 100) with stimulation only to the taste-sensory system using sucrose and KCl failed to form type 2 aversive conditioning. We here present a neuromodulation model for type 1 aversive conditioning in Fig. 5A.

The induction of type 2 aversive conditioning seems to require bodily pain (quinidine sulfate on the epidermis of posterior feet) rather than a bad taste stimulation (KCl or quinidine sulfate to the lips). As might have been expected, more trials were needed to acquire this learned type 2 response (30 vs. 5 trials), which did not persist for as long as the type 1 aversive conditioning (3 days vs. 1 month). On the other hand, electric shock also distributes pain, but not a bad taste, to pond snails. This may explain why more trials were needed to acquire the type 2 conditioning, as compared to the quinidine sulfate conditioning (70 vs. 30 trials). We can thus conclude that type 2 aversive conditioning requires more intense training for acquisition of the learned response, and does not persist as long as type 1 aversive conditioning.

However, our data further show that type 1 aversive conditioning is acquired and retained during the acquisition phase of type 2 conditioning (Fig. 3D and Table 1, and see Fig. 5B for a neuromodulation model of type 2 aversive conditioning). Even though type 2 conditioning with high repetitions of training did not persist for longer than 3 days, type 1 aversive conditioning continued to persist for at least one month (Fig. 3D). Whether or not this means that there is a sequential process at the neuronal level between type 2 and type 1 aversive conditioning remains to be determined, because the centrally located motoneurons involved in producing the withdrawal response of *Lymnaea* have been well studied (Inoue *et al.*, 1996; Ferguson and Benjamin, 1991a, b), but the data for the associated interneurons have not yet been accumulated. Our data from the type 1 aversive conditioning with KCl presentation also show that excessive training for type 1 conditioning does not necessarily provoke type 2 conditioning (Fig. 5A). In this regard, the advantages offered by the pond snail preparation are unmatched in any other preparation we know of. Although aversive learning in *Pleurobranchaea*, for example, was indicated by an active avoidance of food (Mpitsos *et al.*, 1978) much as in our type 2 learning, no change in avoidance behavior was reported.

We should add that type 1 response acquired by a 70-paired-trial electric-shock procedure did not last for more than 7 days (See Fig. 3E). This may be due to the low response in the control group, which had been tired of the 7-day training procedure.

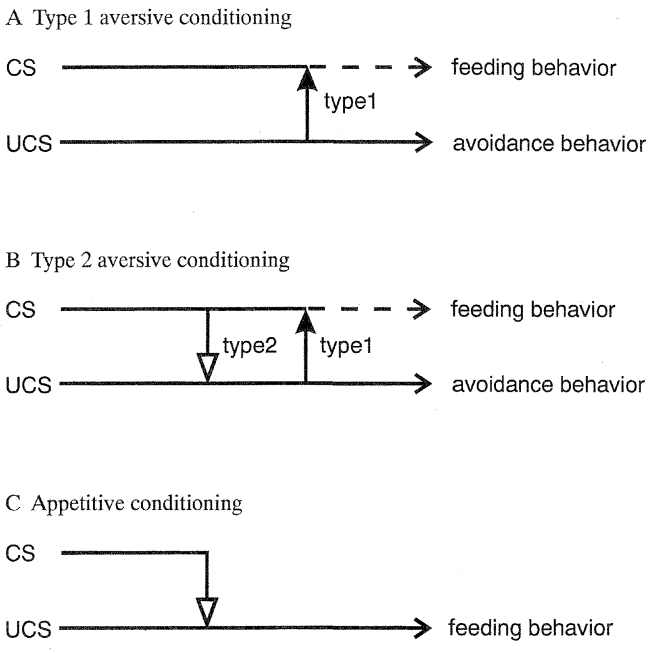

Fig. 5. Neuromodulation models for aversive and appetitive associative conditioning. The behavioral analyses performed in this study suggest such a working model for a neuronal study that a neuron in the CS pathway in both (B) type 2 aversive conditioning and (C) appetitive conditioning needs to evoke excitation of a neuron in the UCS pathway. Open arrows indicate excitatory projections and closed ones do inhibitory projections.

Recently, Kemenes and Benjamin showed the change of acquisition and retention of the appetitive conditioned feeding response after increasing the numbers of training trials in *Lymnaea* (Kemenes and Benjamin, 1994). However, we failed to elongate the retention of appetitive conditioning, even though an increase in trials (Fig. 4B). This inconsistency is still open to interpretation.

Because we have conditioned (aversively and appetitively) the same behavior we can begin to examine at the neuronal level why there are such differences in the acquisition and retention of these two types of classical conditioning. Previously, psychologists have suggested that these differences may be caused by differences in motivational systems. That is, two different motivational systems were postulated: one positive and one negative (Pearce, 1987). This is called as two-process theory. However, our data show that strengths and periods of acquisition and retention of type 2 aversive conditioning and those of appetitive conditioning were very similar. That is, both conditionings took many trials (30) to be acquired, and in both cases the learned behavior persisted for a short time (3 days). We will thus examine the neuronal mechanism of the type 2 aversive and appetitive conditioning by giving remarkable attention to this similarity which probably depends on such a system that a neuron in the CS pathway, which is not relevant to the unconditioned response at all before training, potentiates an excitatory input into that in the UCS pathway (Fig. 5B, C). We are now studying the changes in electrical responses in the regulatory

interneurons in the buccal and cerebral ganglia, which regulate the central pattern generator (CPG) for the feeding response in the buccal ganglia before and after the associative learning. Actually, our preliminary data suggest that the IPSP from the cerebral giant cell to the N1M interneuron in the CPG is prolonged in the taste-aversion conditioned pond snails (S. Kojima *et al.*, in preparation).

In conclusion, our behavioral analyses illustrated the differences between aversive and appetitive classical conditioning, clarified that the aversive conditioning consists of two types (type 1 and type 2), and showed that the acquisition and retention of appetitive conditioning is similar to type 2 aversive conditioning but not to type 1. These behavioral analyses, therefore, suggest that an excitatory projection from the CS to UCS pathway at the neuronal level changes similarly during the acquisition and retention in both type 2 aversive and appetitive conditioning.

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