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Operant Conditioning of Escape Behavior in the Pond Snail, *Lymnaea stagnalis*

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ABSTRACT—Operant conditioning that the pond snail, *Lymnaea stagnalis*, suppressed its naturally occurring behavior of escape from a water tank was examined by using a negative reinforcement (i.e. an aversive stimulus) prepared outside the tank. During the training period, the number of escapes from a tank was strongly suppressed. One of behavioral factors for this suppression was confirmed as the elongation of latency to the first escape after training. The effects on the memory retention were examined in the massed and spaced training procedures. The latter procedure interposes a rest interval between three sets of 20-min training sessions, whereas the former has the same number of training sessions with no rest interval within 60 min. The memory retention by the massed training was observed within 20 min after training. By the spaced training, the learning acquisition was found to be stronger, which was observed as the slower latency to the first escape, than by the massed training, but the longer-lasting memory retention, which had been expected first, was not formed. These results suggest that once *Lymnaea* recognize the external environment is safe after training, they may extinguish their memory of the past situation quickly, resulting in no or very little difference in the memory retention by two different training procedures in this operant conditioning. Together with the facts that classical conditioning and its neuronal mechanisms in *Lymnaea* were previously clarified, the present findings may help to address not only the neuronal basis of operant conditioning but also the relation between classical and operant conditioning.

INTRODUCTION

Learning can be divided into 2 principal classes, nonassociative and associative learning, the latter of which includes 2 major forms: classical and operant conditioning (Dudai, 1989). Extensive attention has been paid to classical conditioning in gastropod molluscs which provide the advantage of a relatively simple central nervous system and the capacity to exhibit a wide array of learning phenomena (Ito *et al*., 1994; Sekiguchi *et al*., 1997; Kimura *et al*., 1998a, b, c). For example, in the pond snail, *Lymnaea stagnalis*, we studied classical conditioning by two different approaches, a conditioned taste-aversion learning and a visual-vestibular association learning (Kojima *et al*., 1996; Sakakibara *et al*., 1998), and found important insights in the cellular mechanisms of classical conditioning (Kojima *et al*., 1997), as well as the other groups grappled with it vigorously (Alexander *et al*., 1982; Audesirk *et al*., 1982; Kemenes and Benjamin, 1989a, b, 1994; Whelan and McCrohan, 1996; Kemenes *et al*., 1997).

In contrast, with the exception of works on head waving in *Aplysia californica* by Carew and coworkers (Carew and Sahley, 1986; Cook and Carew, 1986, 1989a, b, c; Cook *et al*., 1991; Kuenzi and Carew, 1991, 1994a, b; Fitzgerald *et al*., 1997) and on respiratory response in *Lymnaea stagnalis* by Lukowiak *et al*. (1996, 1998), very little is known about the cellular mechanisms of operant conditioning, particularly nothing for that using withdrawal response. Thus, we determined to examine whether gastropod molluscs are also capable of exhibiting operant conditioning with this well-studied response. The animal and the operant behavior examined in the present study were the pond snail *Lymnaea stagnalis* and its escape from a water tank. The escape from a water tank is a naturally occurring behavior that can be usually observed in laborato-

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ries. The motive force for this escape behavior originates in a locomotion to search food or mates. A negative reinforcement (i.e. an aversive stimulus), which always causes the withdrawal response in *Lymnaea* to avoid it, was prepared outside the tank, and thus the suppression of escapes was expected. After this examination is completed, we will address to analyze operant conditioning on a neuronal level to solve an important issue whether classical and operant conditioning represent fundamentally different forms of learning or whether they share at least aspects of a common underlying mechanism (Pearce, 1987).

The learning acquisition and memory retention of operant conditioning in *Lymnaea* was examined with massed and spaced training procedures. In psychology, spaced training, interposing a rest interval between the multiple training sessions, is known to produce stronger and longer-lasting memory than massed training, which has the same number of training sessions with no rest interval (Hintzman, 1974).

MATERIALS AND METHODS

Animals

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, *Lymnaea* (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.

Conditioning paradigm

We chose an escape from a water tank as an operant behavior and prepared a negative reinforcement, which was a 100 mM KCl solution, outside the tank. We previously showed that a 100 mM KCl solution is an aversive stimulus, and hence evokes a whole-animal withdrawal response (Kojima *et al.*, 1996). A water tank used in this study was a lid of a 35 mm plastic petri dish (Nunc, Denmark), in which a single *Lymnaea* was placed with 3 mm depth of DW. For an adaptation, the petri-dish lids containing *Lymnaea* were set on a sheet of DW-soaked filter paper (240 mm ϕ) for 80 min (see Fig. 1). We called this period the pre-period. When *Lymnaea* tried to escape from the lids by sticking the heads out of the lids and touched the reinforcement with their lips (see Fig. 1), the experimenter helped them move back into the lids. This return into the lids was the same behavioral limitation on experimental animals in the case of the operant conditioning for other invertebrates, for example in head waving of *Aplysia* which was suspended in a water tank to keep its appropriate position for observation (Fitzgerald *et al.*, 1997). The number of their escapes was counted and summed up for each 20 min throughout the total experimental periods. Next, the lids containing *Lymnaea* were transferred on a sheet of 100 mM KCl-soaked filter paper for training. When *Lymnaea* escaped and touched the reinforcement with their lips, *Lymnaea* elicited the withdrawal response of their bodies into their shells. At that time the experimenter moved them back into the lids. For control, DW-soaked paper was used as a neutral reinforcement. Exposure time for the reinforcement in the massed training procedure was 60 min in a day; that in the spaced training procedure was 20 min in a day and was repeated for 3 days (Fig. 2). The determination for these training periods will be explained in the results. Then, the lids containing *Lymnaea* were transferred on a new sheet of DW-soaked filter paper for examination of memory retention. The retention period called the post-period was 60 min. We performed these experiments using a blind protocol: The experimenters for preparing the reinforcements and those for counting the escapes were independent; the reinforcements were not announced to the experimenter for counting the number of escapes. To examine whether *Lymnaea* have any fatigue during the training, we observed their voluntary activity just before and after the training period, that is, in the last 20 min of the pre-period and the first 20 min of the post-period. The tests performed in these 2 periods were called the pre- and post-tests. We put a single *Lymnaea* into a 4×32 cm pool, whose length was divided into eight equal parts (i.e. 4 cm). The depth of DW in this pool was 10 mm. The width and height of the pond were determined large enough so that *Lymnaea* moved freely in this pool. Voluntary activity was expressed as the number of parts passed by *Lymnaea* (arbitrary unit). The *Lymnaea* tested for the voluntary activity were different from those for the operant conditioning; the *Lymnaea* in the pre-test in the voluntary-activity tests were different from those in the post-test. In the pre- and post-tests, we also examined latencies to the first escape within 20 min. To measure these latencies correctly, we reset *Lymnaea* in the center of the lids at the starting time of the tests. The number of *Lymnaea* tested was 40 each.

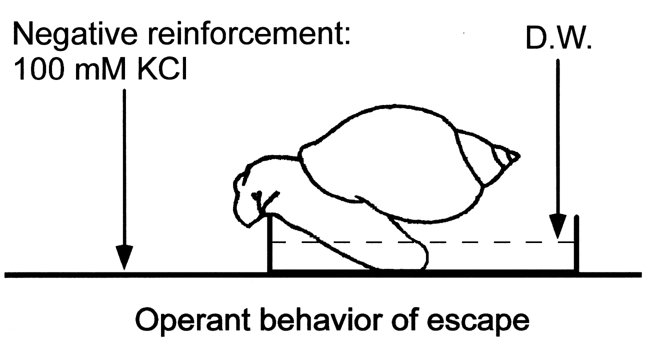

Fig. 1. Schematic presentation of training apparatus for operant conditioning in *Lymnaea*. A snail was set in a lid of 35 mm petri dish with DW. A 100 mM KCl solution soaked into a sheet of filter paper was employed as a negative reinforcement. For pre- and post-periods as well as for control, DW-soaked paper was used.

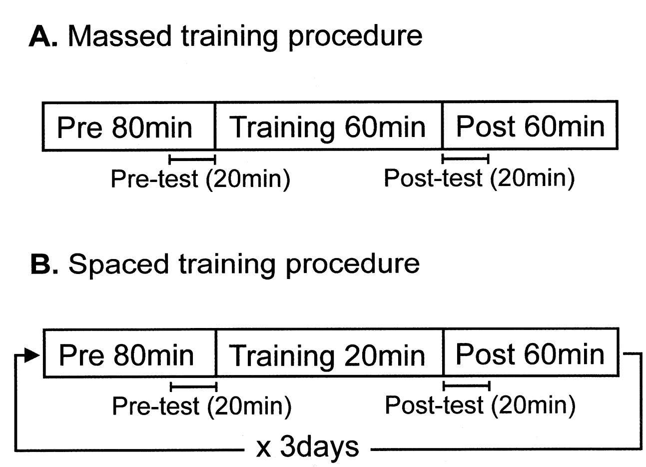

Fig. 2. Conditioning paradigm. (**A**) Massed training procedure performed within a day. (**B**) Spaced training procedure performed in 3 days. During the pre- and post-periods, the filter paper was soaked by DW. During the training periods, the filter paper was soaked by a 100 mM KCl solution for the conditioning group whereas by DW for the control group. Behavioral test during the last 20 min in the pre-period and that during the first 20 min in the post-period were called the pre- and post-tests, respectively.

Statistical analysis

The data were evaluated for statistical significance ($p < 0.05$) with *t*-test.

RESULTS

Massed training effects

Lymnaea disliked the negative reinforcement (the paper soaked by a 100 mM KCl solution) by demonstrating the withdrawal response. Therefore, when *Lymnaea* were operantly conditioned by a massed training procedure, the number of escapes was very much suppressed during the training period ($p < 0.001$ vs. the pre-test). After the training, the number of escapes was still suppressed in the post-test ($p < 0.01$ vs. the pre-test, Fig. 3A). This suppression was not due to any fatigue of the conditioning group because Fig. 3B shows that no difference was observed in the voluntary activity between the pre- and post-tests. These findings, therefore, clearly show that *Lymnaea* are capable of acquisition and retention of operant conditioning. To find a behavioral factor for this suppression of escape, the latencies to the first escape were compared between the pre- and post-tests. The latency to the first escape was significantly slower in the post-test than that in the pre-test ($p < 0.01$, Fig. 3C).

We then compared the number of escapes of the conditioning group with that of the control one, particularly, to pay attention to the memory retention. *Lymnaea* adapted themselves to the training environment within 80 min of the pre-period because the number of their escapes was saturated at 60-80 min in Fig. 4A. In the training period, as described above, the number of escapes of the conditioning group was suppressed, whereas that of the control one was maintained at the maximal level. In the post-period, the number of escapes of the conditioning group was gradually returned to the same level as that of the control one. A significant difference ($p < 0.001$) between the number of escapes of the conditioning group and that of the control one remained in only the first 20 min of the post-period, i.e. in the period of the post-test, showing the memory retention of the operant conditioning in *Lymnaea*. This memory retention was not formed by the 20 min or 40 min training. That is, no differences were found in the period of the post-test between the numbers of escapes of the conditioning groups and those of the control ones by the 20 min or 40 min training. As for the 20 min training, the results will be shown in the section of spaced training (Figs. 5A and 6A). Therefore, we employed a 60 min training in the massed training procedure, because we could continue the experiments using this minimal training period to achieve our aims and compare the results with those in the spaced training procedure.

To reduce uncontrolled fluctuation of the behavior in *Lymnaea* and to show clearly the change in the number of escapes, the raw data in Fig. 4A were expressed as normalized response in Fig. 4B, C. In Fig. 4B, the data for the conditioning group were normalized by those for the control one. For assessment of this presentation in Fig. 4B, another normalization reported by Harrigan and Alkon (1985) was employed

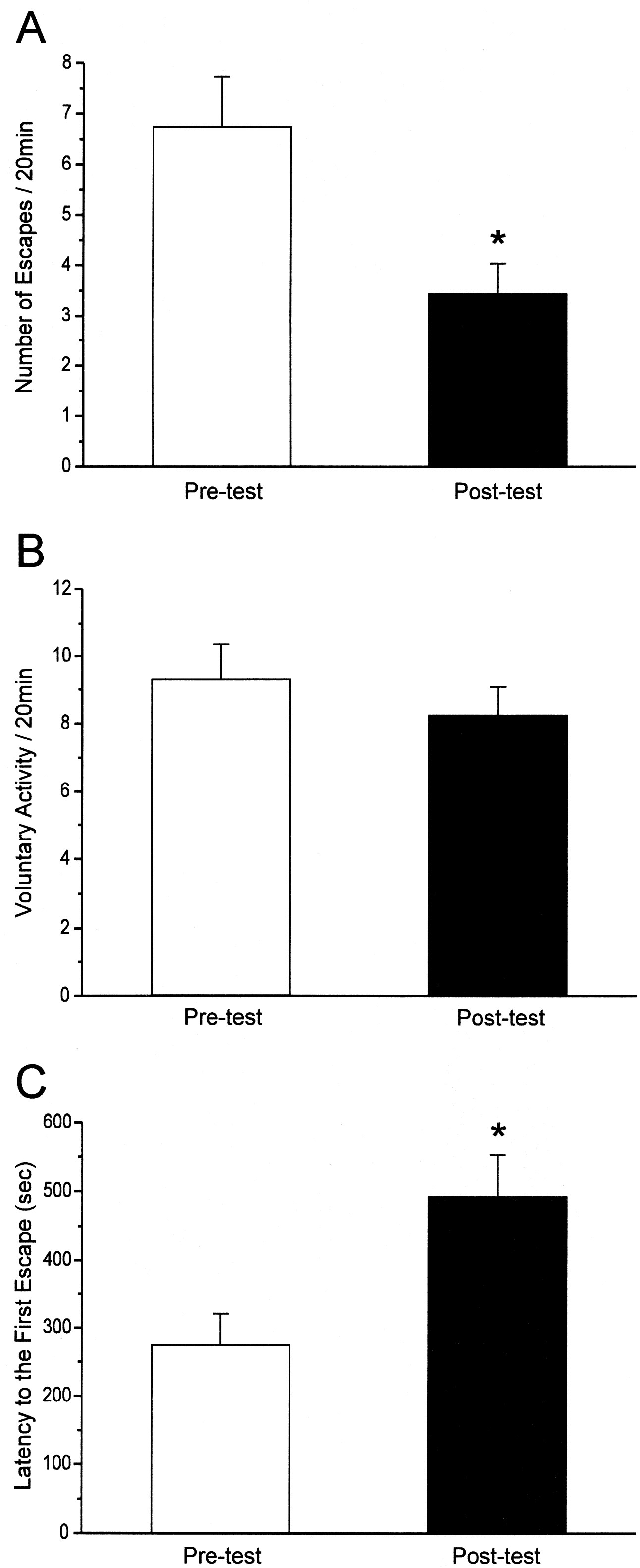

Fig. 3. Behavioral changes by massed training. Number of snails was 40 each. Data are shown as means ± SE. (**A**) Numbers of escapes of the conditioning group in the pre- and post-tests. (**B**) Voluntary activity of the conditioning group in the pre- and post-tests. No significant difference was observed. (**C**) Latencies to the first escape of the conditioning group in the pre- and post-tests. * indicates $p < 0.01$.

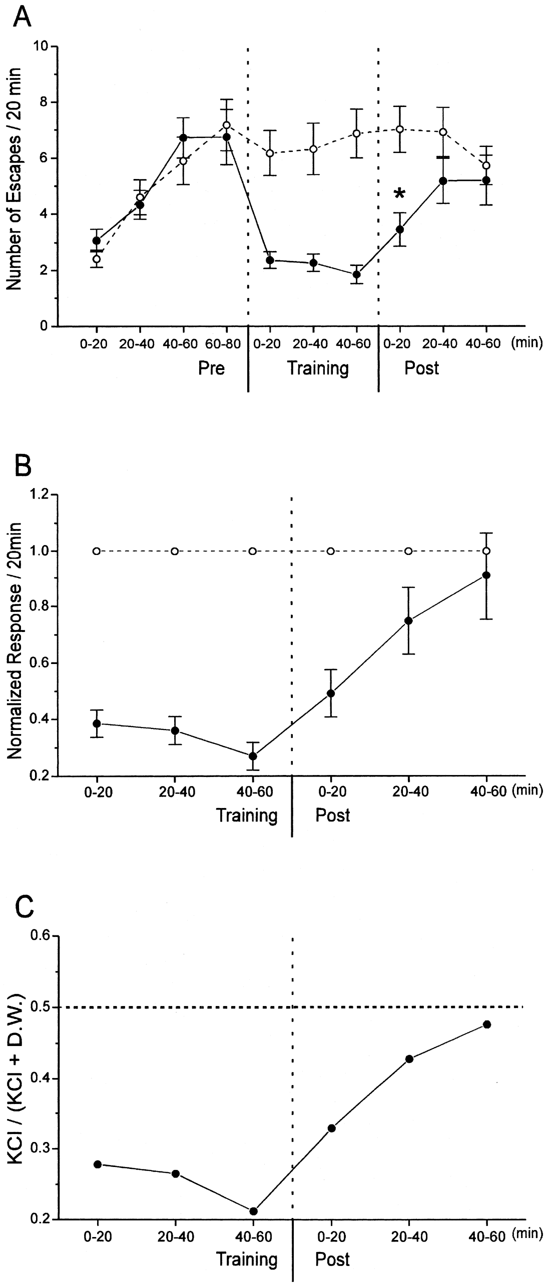

Fig. 4. Comparison of number of escapes of the conditioning group with that of the control group in the massed training procedure. Number of snails was 40 each. Data are shown as means ± SE. (**A**) Numbers of escapes in the pre-, training and post-periods. The solid line and closed circles indicate the conditioning group; the dashed line and open circles do the control group. All the animals could adapt themselves to the training apparatus within the pre-period (80 min). After the training, the number of escapes of the conditioning group was recovered to the same level as the control group in 60 min. * indicates $p < 0.001$. (**B**) Normalized data for the numbers of escapes. Special emphasis was given to the memory retention. The data for the conditioning group were normalized by those for the control one. (**C**) Another presentation for the normalized data for the numbers of escapes. We employed suppression ratio scores of the form A/(A + B), where A = response on any subsequent test, B = baseline response. In this study, A is the number of escapes of the conditioning group; B that of the control one.

and shown in Fig. 4C. Here, the raw data were converted to suppression ratio scores of the form A/(A + B), where A = response on any subsequent test, B = baseline response. In this case, A is the number of escapes of the conditioning group; B that of the control one. A score of 0.5 indicates that baseline and subsequent responses were equal; lower scores mean that test responses have decreased relative to baseline responses. Because the shapes of the response curves in Fig. 4B, C were very similar, the expression by normalized response like Fig. 4B was judged to be adequate. Accordingly, we employed the presentation of normalized response in other analyses. As for the latency to the first escape, no difference was found in the pre- and post-tests in the control group (data not shown).

Spaced training effects

In *Lymnaea* conditioned by a spaced training procedure, the number of escapes was also suppressed during the training period in any experimental day ($p < 0.001$ vs. the post-test). The changes in the numbers of escapes and the latencies to the first escape of the conditioning group in the pre- and the post-tests are shown in Fig. 5. Although the numbers of escapes of the conditioning group were not different between the pre- and post-tests at Day 1 (Fig. 5A), the latency to the first escape in the post-test was slower ($p < 0.05$, Fig. 5B). At Day 2, the number of escapes was suppressed in the post-test ($p < 0.005$, Fig. 5C); the latency to the first escape was slower in the post-test ($p < 0.001$, Fig. 5D). Interestingly, the number of escapes was recovered in the post-test at Day 3 (Fig. 5E), whereas the slow latency to the first escape was still maintained in the post-test ($p < 0.001$, Fig. 5F). Therefore, we concluded two points on the spaced effects on the operant conditioning: (1) The number of escapes was not suppressed (Fig. 5E). That is, the memory retention was not formed, at least not maintained longer than in the massed training procedure. (2) The elongation of latency to the first escape was significantly larger than that in the massed training procedure ($p < 0.05$, see Figs. 3C and 5F). That is, the learning acquisition was significantly stronger in the spaced training procedure. To confirm the conclusion of (1), we performed the spaced training one more day. Figure 5G, H shows that the number of escapes was not quite suppressed and that the latency to the first escape was slower. The spaced effects were not emerged at Day 4 (Fig. 5G). No fatigue was observed in the voluntary activity in the post-test at Day 4 (Fig. 5I).

Further analysis was performed to examine the spaced effects on the long-lasting memory in the operant conditioning. Figure 6A-D shows that the normalized response for the numbers of escapes at Day 1 to 4 in the training and post-periods. The summarized data are shown in Fig. 6E; the regression lines for these summarized data were obtained by a method of least squares in Fig. 6F. Compared with the memory retention in the massed training procedure, all the gradients of the lines but not that at Day 1 were fairly similar (Fig. 6F). Therefore, no effects by the spaced training procedure on the

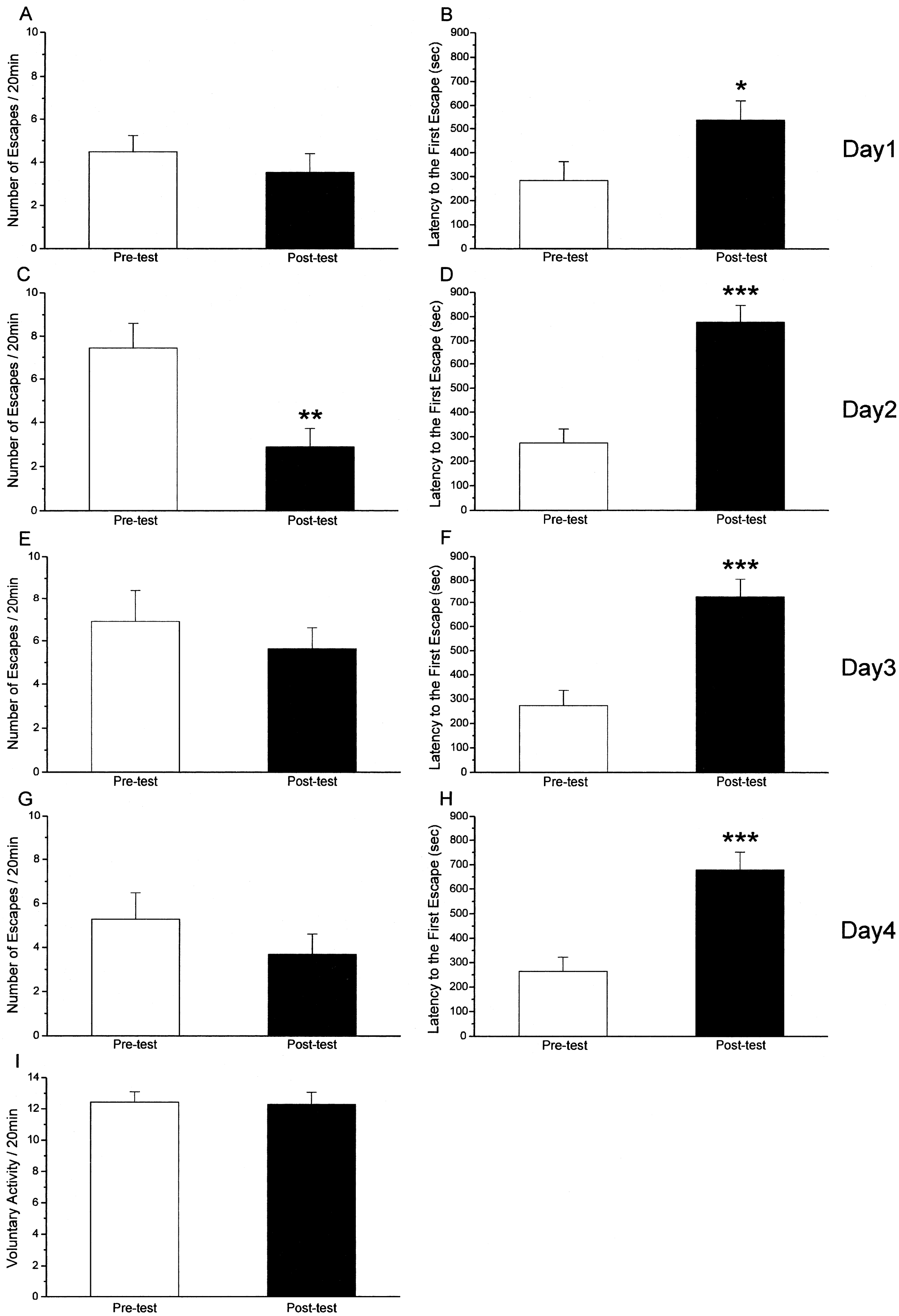

Fig. 5. Behavioral changes by spaced training. Number of snails was 40 each. Data are shown as means ± SE. (**A**, **C**, **E** and **G**) Numbers of escapes of the conditioning group in the pre- and post-tests. (**B**, **D**, **F** and **H**) Latencies to the first escape of the conditioning group in the pre- and post-tests. *, ** and *** indicate $p < 0.05$, $p < 0.005$ and $p < 0.001$, respectively. (**A** and **B**) The data were observed on Day 1; (**C** and **D**) Day 2; (**E** and **F**) Day 3; (**G** and **H**) Day 4. Because the data on Day 3 and Day 4 were similar, the spaced effects were not observed on Day 4. (**I**) Voluntary activity of the conditioning group in the pre-test at Day 1 and in the post-test at Day 4. No significant difference was observed.

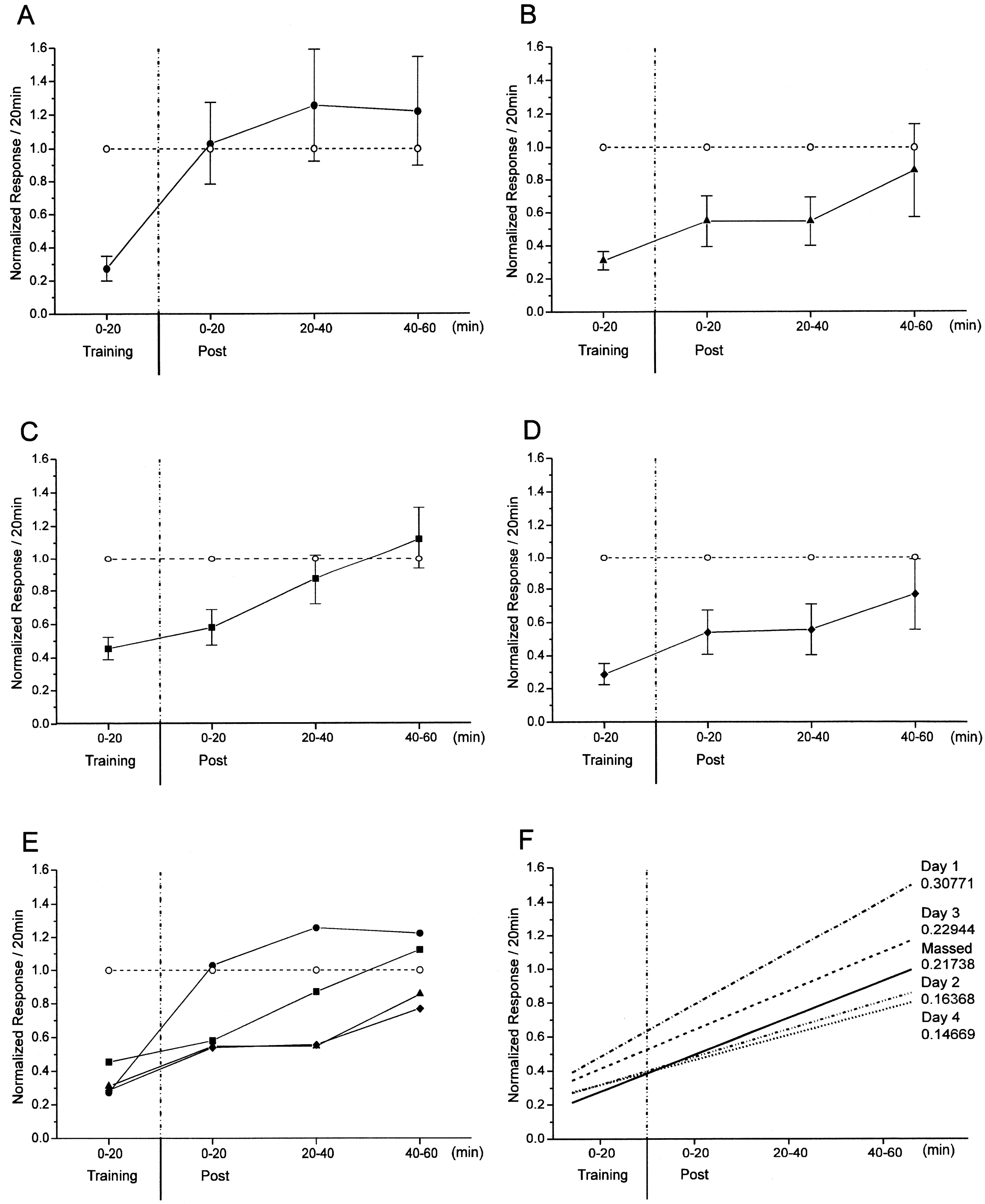

Fig. 6. Comparison of number of escapes of the conditioning group with that of the control group in the spaced training procedure for the memory retention. Number of snails was 40 each. Data are shown as means ± SE. (**A**-**E**) Normalized data for the numbers of escapes in the training and post-periods. The data for the conditioning group were normalized by those for the control one. The solid line and closed plots indicate the conditioning group; the dashed line and open circles do the control group. The data were observed on Day 1 (**A**); Day 2 (**B**); Day 3 (**C**); Day 4 (**D**); Summarized data (**E**). (**F**) Regression lines for the memory retention of the summarized data. These lines were obtained by a method of least squares. Values under a training name and a spaced training day are the gradients of the lines.

long-lasting memory retention were discovered beyond the massed training procedure.

DISCUSSION

We here reached the primary goal where we succeeded in conditioning *Lymnaea* by using the operantly escape behavior from a water tank and the negative reinforcement eliciting the withdrawal response outside the tank. During the training period, the number of escapes was strongly suppressed. One of behavioral factors for this suppression was confirmed as the elongation of latency to the first escape after training (Fig. 3). By the massed training procedure, the memory retention for this suppression was observed only in 20 min (Figs. 3 and 4). By the spaced training procedure, the learning acquisition was found to be stronger, which was observed as the slower latency to the first escape (Fig. 5F), than by the massed training one, but the longer-lasting memory retention, which had been expected first, was not formed (Figs. 5 and 6).

The reasons why we used two parameters, the number of escapes and the latency to the first escape, to estimate the memory retention should be described here. It could be easily expected that there was an inverse proportion between these two parameters: the number of escapes would be decreased as the latency to the first escape would be slower after the conditioning. Interestingly, Fig. 5 does not necessarily prove this relationship. In addition, Fig. 5 also suggests, but does not show clearly, that the latencies to the second or later escapes became faster in the post-test. The best way to represent these results for the memory retention may be to measure correctly the individual latency for all escapes. However, this experiment will spend too much time to obtain all the data in the present study. We, therefore, chose the two parameters, the number of escapes and the latency to the first escape, to well estimate the memory retention, even if the data could not give us enough information for the second or later escapes.

As described above, the memory retention in the massed training procedure and, if any, in the spaced training procedure was very short (Figs. 4 and 6). The reasons can be explained as follows: (1) *Lymnaea* tend to escape from the lids at the low frequency in the training period (Figs. 4 and 6), even though they well understand that the external environment is dangerous, causing the withdrawal response. This indicates the negative reinforcement employed in the present study was aversive but not noxious for *Lymnaea*. Assume that a very noxious reinforcement is employed, the retention may become longer, but the voluntary activity will be weakened. (2) Once *Lymnaea* recognize the external environment is safe in the post-period, they may extinguish their memory of the past situation quickly (Figs. 4 and 6). Therefore, the memory retention in the post-period is thought to be just hesitation in *Lymnaea* to go outside. This explanation is thought to be very reasonable because the results in Fig. 5 clearly showed that even though the latencies to the first escape were significantly slower in the post-tests, the numbers of escapes in the post-tests were not smaller than those in the pre-tests except at Day 2.

We had expected that the learning acquisition by the spaced training procedure was built by spending 3 days. However, Fig. 6F shows that the recovery from the suppressed response, which means the memory retention, at Day 2 was almost the same as that by the massed training procedure. The number of escapes at the post-test at Day 2 was shown to be suppressed (Fig. 5C). Therefore, the similar effects were acquired by the massed (60 min) training procedure and by the 2-day spaced (40 min) training procedure. The spaced training procedure may cause the training time to be reduced to two-thirds. The details for this issue will be studied in the future studies.

On basis of these present findings, we can progress the studies of neuronal mechanisms of the operant conditioning. However, these cellular analyses are not thought to be easy. Taking account of the result that the voluntary activity was not changed in the pre- and post-periods (Figs. 3C and 5I), the negative reinforcement did not weaken the *Lymnaea* mobility but made *Lymnaea* understand strictly to keep its position at a safe place. This suggests that the neuronal mechanisms may not be found in the pathways for the withdrawal response (Ferguson and Benjamin, 1991a, b; Syed and Winlow, 1991; Inoue *et al*., 1996). The interaction between the neural pathways for withdrawal response and those for locomotion (foot-muscle extension), which have not been identified so far, is probably important. In the case of head waving in *Aplysia*, it appears now that an analysis of the neuronal changes which mediate the operant conditioning was more difficult than first thought (Cook and Carew, 1989a, b, c).

We would like to add one comment on an opeantly appetitive conditioning. We also performed another experiment in a massed training procedure using a positive reinforcement which was a 100 mM sucrose solution. Sucrose is known to be an appetitive stimulus for *Lymnaea* (Kojima *et al*., 1996). Although the number of escapes during the training period became significantly larger ($N = 20$, $p < 0.01$), it was quickly returned to the control level in the post-test, that is, the memory retention was not observed (data not shown). In the both cases of aversive and appetitive paradigms, operant conditioning is characterized by a fact that the memory retention is very short. This important issue, which is completely different from classical conditioning (Kojima *et al*., 1996), may be due to a difference in a way of receiving a conditioned stimulus or a reinforcement. The experimental animals wait for a presentation of a conditioned stimulus in classical conditioning (i.e. a passive reception), whereas they try to receive a reinforcement by their naturally occurring behavior in operant conditioning (i.e. an active reception), even though the reinforcement is aversive (see Figs. 4 and 6). As a result, the operantly conditioned animals can quickly recognize that the reinforcement is removed and that they do not have to enhance or suppress their naturally occurring behavior. This point should also be considered in cellular mechanisms for operant conditioning.

In conclusion, the present study provides the operant conditioning in *Lymnaea stagnalis* by using a combination of a naturally-occurring escape behavior and a negative reinforcement eliciting a well-studied withdrawal response. Together with the fact that classical conditioning in *Lymnaea* was clarified, the findings in the present study may help to address not only the neuronal basis of operant conditioning but also the relation between classical and operant conditioning.

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