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Multiple-Phase Model of Memory Consolidation Confirmed by Behavioral and Pharmacological Analyses of Operant Conditioning in *Drosophila*

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XIA, S. Z., C. H. FENG AND A. K. GUO. *Multiple-phase model of memory consolidation confirmed by behavioral and pharmacological analyses of operant conditioning in Drosophila.* PHARMACOL BIOCHEM BEHAV **60**(4) 809–816, 1998.—Previous work on classical olfactory learning and memory in flies has suggested at least four distinct phases of memory consolidation. Similarly, our behavioral and pharmacological analyses also provided clear evidence for at least four pharmacologically distinct memory phases in flies after operant conditioning. Anesthesia-resistant memory (ARM) is present between about 20 and 120 min after training, and susceptible to disruption by the ATPase deactivating chemicals such as ouabain and ethacrynic acid (EA). Long-term memory (LTM) is activated at least 150 min after training, and can be disrupted by protein synthesis inhibitors such as cycloheximide (CXM). In addition, a very short-term memory (pre-STM) is demonstrated by feeding flies with potassium chloride (KCl), which has been shown to disrupt the short-term memory. These observations confirm our previous argument that memory formation in flies involves an intricate, multiple-phase pathway of consolidation. © 1998 Elsevier Science Inc.

Operant conditioning Memory formation Short-term memory Anesthesia-resistant memory
Long-term memory Potassium chloride Ethacrynic acid Ouabain Cycloheximide *Drosophila*

ONE common feature present in memory formation is that memory, existing as a short-living disruptable form immediately after training, is consolidated within a few hours into a long-lasting stable form. This consolidation processing is organized into phases, which are susceptible to disruption of a different group of amnestic agents, respectively. The multiple phases emerge at different times after training, and their duration and times of onset can vary with various tasks and species (3,10,22,23,30,34,36,39,41,52,54,56,57).

Drosophila can learn a variety of associative tasks (32,35,50), and their powerful genetics makes them a promising assay system for genes important for learning and memory (8,28,32). Learning behavior has been investigated in flies under operant conditioning, which involves visual-pattern avoidance conditioning of individual tethered flies at a flight simulator (11,12,55,57). In this novel learning task flies receive training and testing based on their visual recognition or discrimination (55). In analogy to the existing observations about memory consolidation [for reviews, see (10)], our previous experiments have suggested at least three distinct phases in memory formation after operant conditioning: 1) anesthesia-sensitive memory (ASM), which lasts about 20 min after training, and can be disrupted by cold-anesthesia; 2) ARM, which is cold-anesthesia resistant and protein synthesis insensitive; and 3) LTM, which is protein synthesis dependent and can be disrupted by CXM (57,58).

KCl, ouabain, EA, and CXM have been introduced as inhibitors of memory formation in various animals (13,22–24,33,43,52,53). Our previous work has suggested that KCl and CXM inhibit short-term memory (STM) and LTM, respectively, and ouabain disrupts memory later than 20 min after operant conditioning (57,58). However, we did not pro-

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duce the full time courses of memory retention after applying these disruptive drugs in most cases. Here we report results from studies of investigating 1) concentration effects of ouabain and EA; 2) the retention time courses within the first 180 min after training in flies fed with ouabain as well as EA and CXM, respectively; and 3) the existence of the pre-STM. The present observations support the multiple-phase model of memory consolidation proposed previously (57). In particular, the finding that memory reappears in EA-fed flies later than 150 min after training suggests that LTM may be independent of ARM.

METHOD

Flies

Drosophila of the wild-type strain "Berlin" were used throughout. Flies were grown at (24 ± 1)°C in a 14 L:10 D cycle with lights on at 0700 h and bred on standard corn meal/molasses food medium (29). Single male flies were prepared with a small hook of copper wire glued to their head and thorax, and experiments were carried out on them subsequently between 0800 and 2000 h the following day [for details, see (55,57,58)]. Each sample point included 8–10 pairs of flies, i.e., the paired measures from two flies in which one fly had the upright T and the other the inverted T associated with heat as a negative reinforcement.

Drugs and Feeding Regimen

The drugs were KCl (75 mM; Beijing), ouabain (0.1, 0.5, or 1.0 mM; Sigma), EA (1, 2.5, or 5 mM; Sigma) and CXM (35 mM; Sigma). A detailed description of the feeding regimen has been given previously (57). In brief, the flies, with their head already glued to the thorax, were fed with KCl, ouabain, EA, or CXM in 5% sugar solution (w/v), or sugar solution alone (control) at (24–25)°C for about 12 h before training. Individual flies were placed singly in small transparent chambers with a filter paper on their bottom that had been soaked in one of the above solutions. Immediately after training flies were tested for learning acquisition, or fed the solution again and measured for memory retention at a specific later time.

Learning Apparatus

The flight simulator has been described earlier (55,57). Briefly, the simulator establishes normal negative feedback between a fly's yaw torque and angular velocity of a visual panorama surrounding the animal [coupling coefficient $K = -11°$ $(s10^{-10}\ Nm)^{-1}$; for details see (55)]. One single fly was fixed to a torque meter measuring its yaw torque, placed in the center of the vertical panorama illuminated from behind, and allowed to control angular velocity of the panorama with its own yaw torque in a negative feedback loop. The angular position of the panorama was detected, and stored continuously in a computer for the purpose of evaluation of the learning scores and for control of the reinforcer. The visual landmarks consisted of four equally sized, T-shaped black patterns; two of them (opposing quadrants) were inverted. Negative reinforcement was provided by a microscope lamp that was lit during training, but not during testing.

Conditioning Procedure

The first fly was always conditioned to avoid the upright T paired with heat. The conditioning procedure consisted of one pretraining session, one massed training session, one spaced training session, and one 3-min test session (57). The pretraining session comprised three consecutive 2-min test periods without heat, during which the fly learned how to stabilize the panorama so as to improve its learning score (29). At the same time it was tested for its spontaneous preference in respect of the two visual patterns. The massed training session was composed of three consecutive 6-min training cycles. Following this session, in a 10-min interval during which the torque meter with the fly was lifted out of the panorama, the fly was provided with the respective drug solution or sugar solution alone soaked in a small piece of tissue. Subsequently, the fly was lowered into the center of the panorama about 2 min before the spaced training session began. This session consisted of three training-test cycles and one 6-min training cycle as mentioned above. One training-test cycle comprised two 2-min training periods and one 2-min test period. In the test session the fly was tested for learning acquisition or memory retention without heat reinforcement. Before testing, the panorama was set to a random position.

Evaluation of Data

The whole sequence of pattern motion for each fly was digitally recorded on a computer. Performance indices (pattern preference index before, avoidance index during, and learning index after training) were calculated for a flight period as $PI = (t_1 - t_2)/(t_1 + t_2)$, with t_1 and t_2 indicating the time the fly spent fixating the no-heat- and heat-associated quadrants, respectively. The pattern preference index (PPI) was defined as the maximal absolute PI (max|PI|) of the three PIs during the pretraining session. The index is a measure of the fly's ability to stabilize the panorama (29), and reflects indirectly the fly's visual perception ability and visual discrimination (58). The learning index (LI) during one 3-min test session or the avoidance index (AI) during one 6-min training cycle was defined as the average of PIs of two flies from one paired measure to rule out any possible spontaneous pattern preference or asymmetry of the setup (7,40,51,57). LI is a measure for the pattern-specific avoidance behavior acquired from training; i.e., the fraction of the time for the subject to avoid the heat-associated pattern minus that for it to avoid the alternative pattern during a test session. AI is a measure of the pattern-specific avoidance behavior shown by the fly to avoid heat punishment during training. Here, only behavioral performance during the four training cycles (i.e., three in the massed training session and the one in the spaced session) was used to analyze its avoidance behavior.

Error bars in all figures indicated standard errors of the mean (SEMs). Samples (N) for experiments using LIs or AIs indicated the number of the paired measures from two flies; samples (n) for experiments using PPIs indicated the number of flies tested. Because PPIs as well as LIs and AIs as defined above distribute normally (57,58), statistical significances of the differences among two or more means of untransformed (raw) data were assessed with analysis of variances (ANOVA); if necessary, Tukey's honestly significant difference method (T-method) was used to assess unplanned pairwise comparisons between group means. Comparisons between the two means were also assessed with Student's t-test (47).

RESULTS

Inhibitory Effect of Ouabain on Memory Formation

In the experiments of Fig. 1, the flies were fed with 0.2 (open circles), 0.5 (closed circles), or 1.0 (open squares) mM

ouabain in 5% sugar solution, or sugar solution alone (control; closed squares) for 12 h before training, respectively; then in each case they were tested for learning acquisition immediately (0 min), or memory retention at 15 and 60 min after training. A two-way ANOVA, with feeding REGIMEN and TIME as main effects, indicated that the four feeding regimens produced different effects on learning indices, $F(3, 84) = 6.7$, $p < 0.001$, and regimen and time interacted, $F(6, 84) = 2.75$, $p < 0.05$. T-methods ($\alpha = 0.05$) from separate one-way ANOVAs confirmed that the three ouabain-feeding regimens exerted no effect on learning acquisition and 15-min memory retention, but significantly reduced 60 memory retention when compared with the control. In addition, the two regimens with 0.5 and 1.0 mM ouabain abolished memory at 60 min after training, $t(7) \leq 0.44$, $p > 0.7$ for ouabain+ vs. zero.

Inhibitory Effect of EA on Memory Formation

The inhibition of memory was also studied by feeding flies with 1.0 (open circles), 2.5 (closed circles), or 5.0 (open squares) mM EA in 5% sugar solution. The conditioned performance of the EA-fed flies paralleled that of the ouabain-fed flies in most respects, and thus, very similar results were obtained (Fig. 2). All the EA-fed flies produced the same learning acquisition and 15-min memory retention as the control flies, $t(14) \leq 1.37$, $p > 0.2$ for all comparisons. However, EA abolished 60-min memory retention in both the 2.5 and 5.0 mM groups, $t(7) \leq 1.5$, $p > 0.15$ for EA vs. zero.

Disruptive Retention Course Following Ouabain- and EA-Feeding Regimens

Populations of flies were tested for learning acquisition (0 min), and 10, 30, 60, 120, 150, or 180 min memory retention (Fig. 3), which had been fed with 5% sugar solution laced with 0.5 mM ouabain (closed circles) or 2.5 mM EA (open circles), or sugar solution alone (closed squares) for about 12 h before training. These experiments were all carried out under the same general conditions, and the only difference was the respective feeding procedures concerned. A two-way ANOVA, with feeding regimen and time as main effects, indicated that 1) the three feeding regimens produced significantly different

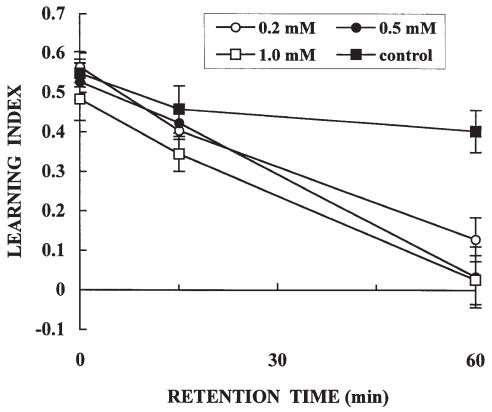

FIG. 1. Memory inhibition by ouabain. Different groups of flies were tested for learning acquisition or memory retention at 15 or 60 min after training, which were fed 0.2 (open circles), 0.5 (closed circles), or 1.0 mM (open squares) ouabain or sugar solution alone (control; closed squares) for at least 12 h before training. $N = 8$ for each group.

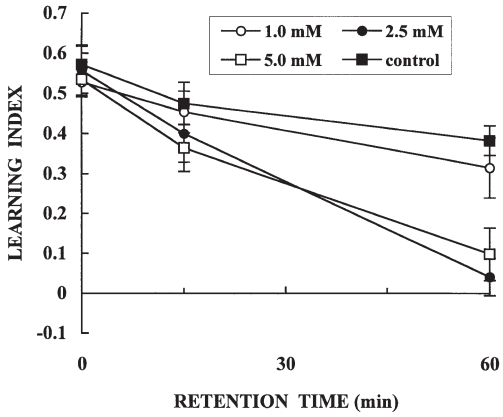

FIG. 2. Memory inhibition by EA. Populations of flies, fed with 5% sugar solution laced with 1.0 (open circles), 2.5 (closed circles), or 5.0 mM EA (open squares), or sugar solution alone (control; closed squares) for more than 12 h before training, were tested for learning acquisition or 15- and 60-min retention. $N = 8$ for each group.

effects on learning indices, $F(2, 168) = 31.3$, $p < 0.001$, and 2) procedure and time interacted, $F(12, 168) = 2.94$, $p < 0.01$.

Ouabain exerted no effect on learning acquisition, $t(16) = 0.23$, $p = 0.8$, and 10-min memory retention, $t(16) = 0.47$, $p = 0.65$, but significantly reduced 30-min memory retention, $t(16) = 4.1$, $p < 0.001$, when compared with the control. Then memory was abolished at 60 min, $t(8) = 1.12$, $p = 0.3$, for ouabain+ vs. zero) and no recovery of memory could be detected at least 180 min after training. Thus, ouabain may have no effect on STM, which is normally available within 20 min (58), but abolish memory formation later than 30 min after training.

EA exerted a similar effect on conditioned performance of flies within the first 120 min (i.e., leaving learning acquisition

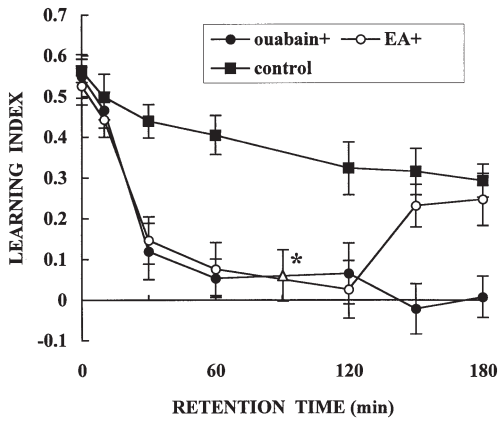

FIG. 3. Retention time course of memory in flies subjected to ouabain or EA feeding regimens. Populations of flies were fed with 0.5 mM ouabain (ouabain+; closed circles), or 2.5 mM EA (EA+; open circles) in 5% sugar solution or sugar solution alone (control; closed squares) for at least 12 h before training and then measured for learning acquisition or memory retention at 10, 30, 60, 120, 150, or 180 min after training. Another group of flies (open triangle with *; $n = 7$) was tested for 90-min retention without being fed with EA immediately after training. $n = 9$ for all other groups.

and STM undisturbed, but abolishing memory later than 30 min) after training. However, the EA-fed flies showed normal 150 and 180 min memory retention as the control, $t(16) \leqslant 1.1$; $p > 0.3$. Because LTM has been shown to be activated not later than 180 min (57), the observation indicates that it may be unaffected by this EA-feeding regimen.

The reappearance of memory in the EA-fed flies suggests that parallel processes may be involved in memory consolidation after operant conditioning. Otherwise, it will be necessary to explain why memory in the EA-fed flies reappeared later than 150 min after training. Due to the reappearance of memory, the action of EA cannot be attributed to effect on retrieval mechanisms. The second possibility is that the inhibitive effect of EA may diminish over time more quickly than that of ouabain, and 150 min after training may be beyond the acting time of EA. If so, it would be expected that the 90-min retention should be unaffected without introducing the drug immediately after training (one should keep in mind that the flies were usually fed the drug again immediately after training when tested for memory retention). The time interval of 90 min was obtained by subtracting 60 min (i.e., the duration of conditioning) from 150 min. However, their 90-min retention was near zero [open triangle in Fig. 3; $t(6) = 0.95$, $p = 0.35$] when the flies were not fed with EA immediately after training.

Disruptive Retention Course Following CXM-Feeding Regimen

The observation that EA left 150-min retention intact, suggests the following experiments to test whether memory formation at 150 min after training is protein synthesis dependent. The flies were fed with 35 mM CXM (open circles) in 5% sugar solution, or sugar solution alone (closed circles) for 12 h before training, and then tested for learning acquisition or memory retention at 60, 120, 150, or 180 min after training (Fig. 4). CXM left learning acquisition and memory within the first 120 min after training undisturbed, $t(16) \leqslant 0.53$, $p \geqslant 0.6$ for CXM+ vs. control, but abolished memory starting after 150 min, $t(8) \leqslant 1.14$, $p > 0.25$ for CXM+ vs. zero]. The result extends our previous argument that the protein synthesis-dependent LTM is activated later than 180 min after training (57).

Effect of KCl on Memory within the First 10 Min after Training

After fed with 75 mM KCl (closed circles), or 2.5 mM EA (open circles), or sugar solution alone (closed squares), respectively, different groups of flies were measured for learning acquisition or memory retention within the first 10 min after training (Fig. 5) with the test session shortened to 1 min. A two-way ANOVA, with feeding regimen and testing time as main effects, indicated that the three regimens produced different effects on learning indices, $F(2, 105) = 28.5$, $p < 0.001$. T-methods ($\alpha = 0.05$) from separate one-way ANOVAs indicated that 1) EA had no effects on learning indices at all tested times; and 2) KCl exerted no effect on learning indices at 0 and 1 min, but significantly diminished memory later than 3 min when compared with sugar feeding. In addition, learning index at 10 min was near zero, $t(7) = 0.36$, $p > 0.7$. Along with our previous observation that KCl feeding abolished memory starting from 5 min after training and no recovery of memory was detected 180 min later (58), KCl appeared to disrupt memory later than 3 min, but left the acquired heat-avoidance behavior undisturbed within the first 3 min after training.

Pattern Preference and Avoidance Behavior Unaffected by the Drugs Used

Pattern preference indices (PPIs) and avoidance indices (AIs) of the flies were shown in Fig. 6, which were fed with 0.5 mM ouabain (stripped columns), 2.5 mM EA (stippled columns), 35 mM CXM (crossed columns), 75 mM KCl (white columns) in 5% sugar solution, or sugar solution alone (gray ones) for 12 h before training. As for PPIs of flies (Fig. 6a), a one-way ANOVA with group as main effect revealed no significant between group effect, $F(4, 95) = 0.29$, $p > 0.2$. This may suggest that these drugs exert no effect on visual perception and visual discrimination of the flies so that they can stabilize the panorama as well as the control flies, producing nor-

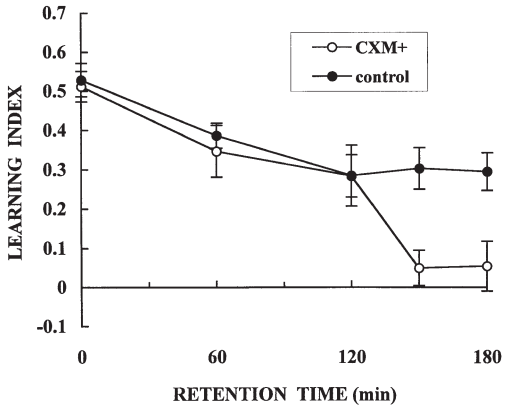

FIG. 4. Retention time course of memory in flies fed with CXM. The flies were tested for learning acquisition or memory retention at 60, 120, 150, or 180 min after training. These flies had been fed 5% sugar solution laced with 35 mM CXM (open circles), or sugar solution alone (control; closed circles) for at about 12 h before training. CXM has been shown to disrupt memory at 180 and 12 × 60 min after training (59). $n = 9$ for each group.

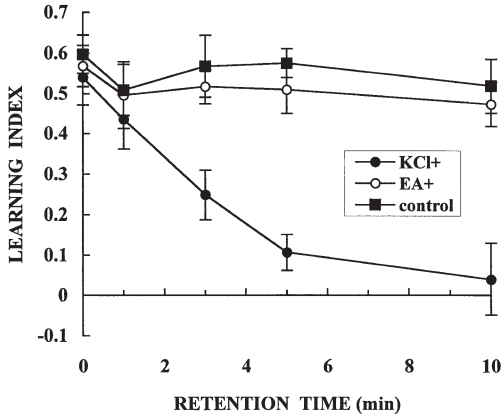

FIG. 5. Effects of KCl and EA on memory within the first 10 min after training. The flies were measured for learned avoidance behavior immediately (0 min), 1, 3, 5, or 10 min after training when fed with 75 mM (closed circles) or 2.5 mM EA (open circles) in 5% sugar solution, or sugar solution alone (control; closed squares) for about 12 h before training. $N = 9$ for each group.

mal pattern preferences. A two-way ANOVA, with feeding regimen and training CYCLE as main effects, indicated that 1) the five feeding regimens produced no different effect on AIs of flies (Fig. 6b) $F(4, 160) = 0.23$, $p > 0.2$, and 2) the four training cycles produced different effects on AIs, $F(3, 160) = 8.2$, $p < 0.001$. This indicates that these drugs exert no effect on flies' heat avoidance behavior and learning performance [referred to significant improvement in AIs with training, see (57)] so that flies performed normally during training.

DISCUSSION

Because flies were fed with the drugs for at least 12 h before training, these inhibitors should act during training and interfere with the brain in a variety of ways. To confirm an effect on memory, it is necessary to separate the behavioral deficits due to disruptive memory from those caused by nonspecific effects of inhibitors on the flies' visual perception or heat sensation or learning performance necessary for normal memory formation. All the disruptive drugs produced no effect on flies' spontaneous pattern preferences during pretraining, indicating that visual perception as well as discrimination, and the motor behavior of the flies were not disturbed (58). In addition, they exerted no effect on avoidance indices of flies during training. This may indicate that the drug-fed flies could normally avoid the heat-associated pattern, and associate it with heat, and improve their avoidance behavior based on their "experience" acquired or learned from the preceding training [for details, see (57,58)]. Finally, the drugs left learning acquisition intact measured immediately after training, suggesting that the drug-fed flies acquired the heat-pattern association presented during training. Taken together, all these inhibitors along with the feeding regimens do not interfere with subsequent neural function such as sensory perception and learning ability of flies.

Because no retention deficits were observed at 15 min for both ouabain and EA, and later than 150 min again for EA, and within the first 120 min for CXM after training, the inhibitive action of these drugs cannot be attributed, at least solely, to effects on retrieval mechanism. KCl has also been shown to interfere with memory formation, rather than memory retrieval (58). Therefore, the drugs should act by relatively specific biochemical mechanisms but not by rough disorganization of brain function, and the only reasonable explanation of their effects should be a specific disruption of some memory phase(s).

Ouabain and EA all left 10 and 15 min retention intact, but abolished memory starting after 30 min [i.e., at a time beyond the duration of STM, see (58)]. In addition, no recovery of memory was detected 90 min later when memory cannot be disrupted by CXM (57). Therefore, the memory phase inhibited by ouabain and EA may be ARM, which is normally available between 20 and 150 min after training and insensitive to cold anesthesia and protein synthesis (57,58). This interpretation confirms our previous argument that the ATPase deactivating chemicals such as ouabain interfere with ARM through the inhibition of the sodium–potassium interchange (58). The common action of ouabain and EA is their inhibition of Na^+/K^+ ATPase and thereby interference with the active transport of sodium and potassium across cell membrane (20,26). However, their inhibitive effect on memory in flies is different from that in chicks or rats trained with various tasks (3,19,21,23,24,43). In particular, ouabain, injected intracranially up to 5 min after training, induced amnesia at least 10 min after one-trial passive-avoidance training, corresponding to the appearance of middle-term memory (23,39).

The fact that both ouabain and EA interfered with ARM in flies in the almost same way as they did for middle-term memory (MTM) in chicks and rats (3,23,39) suggests that MTM may be ARM in this learning paradigm. However, MTM and ARM are different memory phases, and the later has been considered to be a long-lasting memory form in olfactory learning in flies (10,52). In addition, single-gene mutant amnesiac has a diminishing effect on memory between 20 and 90 min, but no effect on memory later than 90 min after operant visual learning (Gong et al., in preparation). If the mutant interferes only with MTM in visual learning as in olfactory learning (52), MTM may be detected between 20 and 90 min after operant training. Nevertheless, we are still unable to test whether the ATPase deactivating chemicals interfere with MTM or ARM by observing the appearance of ARM with or without the drugs in cold anesthesia experiments. The reason is that no memory can be detected between 20 and 150 min after training in ouabain- or EA-fed flies (Fig. 4). Before we have the direct evidence to clarify the issue, we would like to argue that sodium pump activity may be involved in the formation of ARM, and the inhibition of sodium-potassium interchange is the important effect of the ATPase deactivating chemicals on memory in flies [also see (58)].

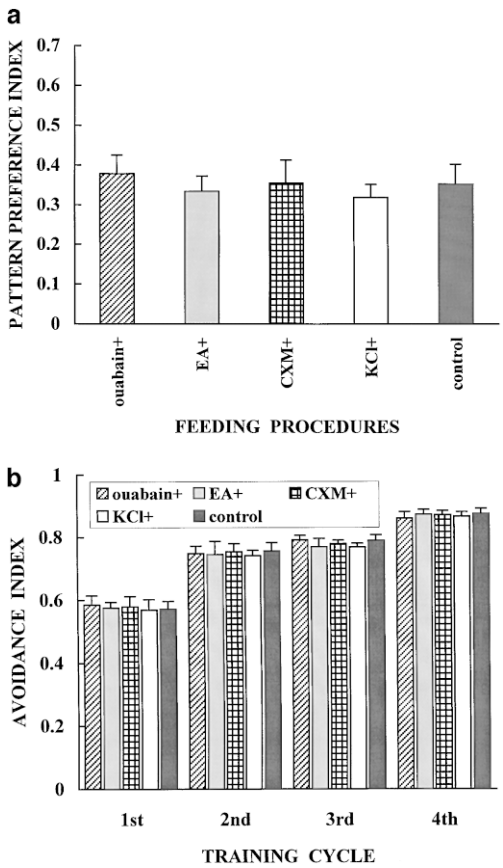

FIG. 6. Pattern preference indices (PPIs) and avoidance indices (AIs) of flies subjected to five different feeding regimens (for details see text). (a) PPIs of the flies. $n = 20$ flies for each group. (b) AIs of the flies during the four training cycles. $n = 9$ for each group.

However, ouabain and EA acted differently. Ouabain abolished memory later than 30 min, and no recovery of memory could be detected 150 min later when LTM is normally available (57). EA also interfered with memory between 15 and 150 min, but memory reappeared spontaneously later than 150 min after training. The reappearance of memory in EA-fed flies suggests memory consolidation may involve both sequential and parallel processes, and the formation process underlying LTM may be independent of that of ARM. EA is further assumed to disrupt ARM specifically, but leave STM and LTM undisturbed. The specific effect of EA on a memory phase is also found in some previous reports (3,19,43). However, the memory phase affected specifically by EA is STM in chicks when assayed in a visual discrimination paradigm or in rats assayed in an active-avoidance paradigm. It may be significant that EA was not fed but injected intracranially or intracisternally in these investigations. As for ouabain disrupting both ARM and LTM, it may interfere with LTM through other unknown mechanisms. It has been shown, for example, that ouabain has inhibitory action on protein synthesis presumably by affecting ionic conditions (53). This action may cause that ouabain prevents the formation of LTM. On the other hand, independence of ARM and LTM does not logically imply that the formation process underlying LTM occurs completely in parallel with that underlying ARM. Thus, though a valid explanation of the different effects by ouabain and EA is missing and requires further experiments, we would like to argue that EA disrupts ARM specifically and the formation of LTM is independent of ARM. This idea agrees well with the findings drawn from the classical olfactory experiments where at least two genetically distinct, functionally independent components of the long-lasting memory have been suggested: a CXM-insensitive ARM, and a CXM-sensitive LTM (52).

Results about CXM-induced retrograde amnesia agree with the notion that the appearance of consolidated (i.e., long-term) memory is protein synthesis dependent (2,4,17,39,44,48,52). The dependence of LTM formation on protein synthesis is consistent with the neurobiological view that memory consolidation reflects the establishment of long-lasting structural changes in synaptic morphology (5,6,27,38,49). It can also be deduced from these experiments that LTM may be activated not later than 150 min after training. These data demonstrate that flies may relay on protein synthesis-dependent processes to remember the operant "experience" acquired from training for longer time.

We have discussed that STM is normally available about 3 min after training, and susceptible to inhibition of the depolarizing drugs such as KCl (58). When analyzed in more detail, KCl exerted no effect on learning indices measured within the first 3 min, but disrupted memory later than 3 min after training. In addition, hypoxia disrupted memory only when it was introduced within the first 2 min after training (59). These observations appear to suggest a very short-living sensory buffer or memory phase that precedes STM and lasts only about 2 min following training. The idea is not entirely new that there may exist a pre-STM. Allweis and Rosenzweig et al. have postulated its existence in their multiple-phase models of memory consolidation independently by using different species, tasks, and inhibitive agents (3,45). In honeybee proboscis extension response conditioning, STM has been identified in part with a nonassociative memory component, which may be another "version" of pre-STM (30).

KCl interfered with STM (58), but left learning indices measured within the first 3 min after training unaffected. Both ouabain and EA disrupted ARM, and the former also inhibited LTM. CXM abolished LTM with no effect on ASM and ARM. Our data obtained until now are consistent with a four-phase model of memory consolidation with approximate duration of each phase as follows: 1) a very short-living sensory buffer or memory phase (i.e., pre-STM), which lasts up to only about 2 min following training and is KCl insensitive; 2) STM, which lasts about 20 min after training and is susceptible to disruption of the depolarizing drugs such as KCl and lithium chloride; 3) ARM, which is normally available between 15 and 150 min after training and susceptible to disruption of sodium pump inhibitors such as ouabain and EA; and 4) LTM, which is activated at least 150 min after training and susceptible to inhibition of protein synthesis inhibitors such as CXM (Fig. 7). The demonstration of ARM and LTM is consonant with the multiphasic consolidation model proposed for olfactory learning (10,52). The result, that LTM formed even in the absence of ARM in EA-feeding experiments, is also consistent with the analyses of the single-gene mutant radish that disrupts ARM but leaves LTM intact (52).

In the case of olfactory learning, ASM is subdivided into STM and MTM based on behavioral analyses of single-gene mutants and on the disruptive effects of reversal training in wild-type flies (52). However, our results indicate that ASM is decomposed into two memory phases, and the later one can be disrupted by the depolarizing chemicals such as KCl. The later phase has been named STM (58) to be consistent with the similar pharmacological experiments in chicks where the depolarizing chemicals interfere with STM (22,23,39). Because the cellular components underlying learning and memory appear to be conserved across species [for reviews see (10)], it will be even more difficult to explain why the depolarizing chemicals disrupt MTM in flies, but STM in chicks if we choose to subdivide ASM into STM and MTM. Until now we have no direct experiments to explain this apparent discrepancy. Future experiments may help to clarify the issue with KCl to disrupt memory in *Drosophila* olfactory learning. The second discrepancy is that ouabain and EA disrupt ARM in

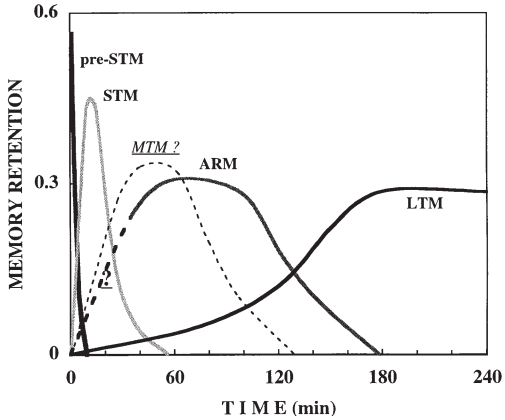

FIG. 7. The putative memory phases in flies assayed in operant visual learning. The consolidation processing involves several temporal phases: pre-STM, STM, ARM, and LTM. Among them, pre-STM, STM, and LTM may be derived directly from training [also see (59)]; ARM may be derived from the earlier memory phases (i.e., pre-STM and STM). A line indicting MTM (maybe present between 20 and 90 min) was dotted, for we had no direct experiments to identify its existence (see text).

this visual learning task (see above), but MTM in a visual discrimination paradigm in chicks (22,39). Finally, although our results suggest the existence of ARM and LTM, duration and time of onset of each phase are very different from those in olfactory learning (10,52). Whether these discrepancies are induced by the different training procedures and sensory modality remains to be determined.

Nevertheless, this multiple-phase model of memory consolidation broadly agrees with the notion that memory formation involves an intricate, multiple-phase consolidation pathway. In both chicks and rats, injection of a wide variety of amnestic agents after training reveals three distinct periods of amnesia, corresponding to the temporal appearances of STM, MTM, and LTM (3,39,46). The brief dips in memory retention present at the predicted phase transitions provide direct behavioral support for these pharmacological divisions (3,23,25,39). In *Aplysia* cell culture models of synaptic facilitation reveal short- and long-term phases of memory and suggest that they are independent functionally (16,37). In *Drosophila*, the behavioral, pharmacological, and genetic analyses demonstrate at least four distinct forms of memory for olfactory learning (10,28,52,60). The similarity in the consolidation of memory formation suggests that the basic mechanisms underlying learning and memory are broadly similar in invertebrates and vertebrates.

The present results lend new evidence to the consolidation hypothesis of memory formation [for reviews see (10)] and support our previous main findings (57,58). The appearance of distinct phases in the consolidation pathway of memory should open up new possibilities for understanding memory formation in flies. With this learning paradigm, we now investigate other inhibitive agents, and memory formation in mutants isolated in the olfactory learning paradigm (1,14,15,18,42,60), in structural mushroom body mutants (31), and in flies with chemically ablated mushroom bodies (9).

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