Memory Consolidation in *Drosophila* Operant Visual Learning

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Abstract

A new conditioning procedure, developed for the operant learning paradigm at the flight simulator, leads to stronger learning scores in wild-type flies. This procedure produces mean learning indices up to $LI = 0.52$. The heat-avoidance behavior acquired during training is very difficult to extinguish during extinction. Memory decays quickly during the first 3 hr after training and still is measurable 48 hr later. Disruption experiments demonstrate that memory can be disrupted by cold anesthesia within the first ~20 min after training, whereas KCl- and CXM-feeding regimens abolish memory soon after training and 3 hr later, respectively. These results initially suggest at least three distinct memory phases involved in memory consolidation in *Drosophila* after operant conditioning.

Introduction

Fruit flies can learn a variety of associative tasks (for review, see McGuire 1984; Tully 1984; Heisenberg 1989). Many studies have employed discriminative conditioning procedures, in which two stimulus cues are presented sequentially or simultaneously during training, whereas one of them is paired temporally with negative or positive reinforcement. With these conditioning procedures, flies can learn to associate odor cues with negative reinforcement such as electric shock (Quinn et al. 1974; Dudai 1977, 1983; Tully and Quinn 1985) or with positive reinforcement such

as sucrose (Tempel et al. 1983). They also can learn that a specific light color is paired with negative or positive reinforcers (Folkers and Spatz 1981; Heisenberg 1989). Furthermore, flies show associative learning ability to modulate the proboscis extension reflex (DeJianne et al. 1985). Finally, associative learning has been assayed in experience-dependent components of *Drosophila* courtship (Siegel and Hall 1979; Tompkins et al. 1983; Cowan and Siegel 1984).

Operant behavior has been investigated on many kinds of animals and with various tasks (Skinner 1950; Horridge 1962; Heisenberg and Wolf 1979; Hoyle 1979; Booker and Quinn 1981; Mariath 1985; Guo and Goetz 1997). Based on the flight orientation behavior of *Drosophila*, which has been proven to be operant (for review, see Wolf and Heisenberg 1986, 1990; Heisenberg and Wolf 1993), a new learning paradigm has been introduced (Wolf and Heisenberg 1991), which allows the measurement of individual performance. In our setup, the original conditioning procedure (Wolf and Heisenberg 1991) leads to comparatively low learning indices ($LI = 0.35$). After modification of the conditioning procedure, much stronger learning indices are obtained (see Results).

In this study we (1) characterize acquisition and extinction of operantly conditioned behavior, (2) demonstrate memory retention of the conditioned heat avoidance behavior for at least 48 hr, and (3) delineate memory disruption of the heat avoidance behavior and the memory phases.

Materials and Methods

FLIES

Drosophila melanogaster of the wild-type strain Berlin is used throughout in this study. Flies are grown at $24 \pm 1^\circ\text{C}$ in a 14:10-hr light/dark

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cycle with the light cycle beginning at 7 a.m. Flies are bred on standard corn meal/molasses food medium (Wuerzburg recipe, see Guo et al. 1996).

Experimental flies are transferred to fresh food vials 0–24 hr after hatching, where they are raised for ~36 hr. Single flies are prepared with a small hook of copper wire (0.05 mm diam.) glued to their head and thorax (Heisenberg and Wolf 1984). These flies are put into transparent chambers ($\pi \times 8^2 \times 30 \text{ mm}^3$) individually where they remain overnight so that they become accustomed to the situation with the triangle hooks on their back. Experiments are carried out on single flies subsequently between 8 a.m. and 8 p.m. the following day. Most flies are 48–72 hr old when training begins, but occasionally, flies that are 72–96 hr old are also used. Within these ranges of age, no differences have been detected in conditioned performance of flies. No sex-related differences in learning ability are apparent. However, male flies appear to have a more persistent flight behavior. Thus, male flies are used in all experiments.

LEARNING APPARATUS

The flight simulator (Fig. 1) establishes normal negative feedback between the fly's yaw torque and angular velocity of the visual panorama surrounding it (closed loop). The coupling coefficient (for details, see Heisenberg and Wolf 1988; Wolf

and Heisenberg 1991) between yaw torque and angular velocity of the panorama is set to $K = -11^\circ/(10^{-10} \text{ Nm/sec})$. The torque meter transduces the fly's yaw torque into a DC voltage that is stored in the computer continuously. The DC voltage is used to rotate the visual panorama with help of a DC servomotor (Faulhaber motor-tacho-combination 3557/220, 20 V, Germany). The angular position of the panorama (i.e., the fly's flight orientation with respect to the visual landmarks at the wall of the cylindrical panorama) is detected continuously by a ring potentiometer (Novotechnik P4102 a 103, 10 k Ω , Germany) and also stored continuously in the same computer for the purpose of further evaluation of the learning scores and for control of the reinforcer. The visual landmarks consist of four equally sized [width (max.) 40° height (max.) 40°], T-shaped black patterns; two of them (opposing quadrants) are inverted. Negative reinforcement is provided by a microscope lamp (Zeiss, 6 V, 15 W) which is lit during training but not during testing. The light beam is focused onto the fly from above by a lens ($f = 60 \text{ mm}$) and can be intercepted by a computer-controlled shutter. The heat spot is ~3 mm in diameter and covers the whole abdomen of the animal.

STANDARD CONDITIONING PROCEDURE

In the standard conditioning procedure (SCP) at the start of each experiment, a single fly is fixed to the torque meter and placed in the center of a vertical panorama, where it is allowed to control the angular velocity of the panorama with its yaw torque. Training and testing are performed on the fly in the following order: the pretraining session, the massed training session, the spaced training session, and the test session. The pretraining session comprises three consecutive 2-min test periods during which the animal flies in the closed loop without heat reinforcement. It has been shown earlier (Guo et al. 1996), that learning experiments without any pretraining lead to lower learning scores. It is likely that a fly has to become familiar with the flight conditions in the flight simulator, that is, learn how to stabilize the panorama in the closed loop. At the same time, it is tested for its spontaneous preference with respect to the two visual patterns. The massed training session is composed of three consecutive 6-min training cycles that are subdivided into three 2-min training periods. During these periods, the com-

Figure 1: Simplified diagram of the flight simulator as conditioning apparatus in all experiments.

puter-controlled infrared beam, focused onto the fly, is switched on whenever the fly flies toward a quadrant that contains the upright T. When the inverted T is in its frontal visual field, the beam is intercepted by the computer-controlled shutter. Following this session, in a 1-hr interval during which the torque meter with the fly is lifted above the panorama, the fly is provided with sugar solution soaked in a small piece of tissue. Subsequently, the fly is lowered in the center of the panorama ~2 min before the spaced training session begins. This session consists of three training-test cycles and one 6-min training cycle, as mentioned above. One training-test cycle comprises two 2-min training periods and one 2-min test period. Immediately after conditioning, the fly is tested for learning acquisition in one test session of three 1-min test periods in which it can make its own choice between both pattern types without being heated. Before testing, the panorama is set to a new random position.

As for the first fly, it is always conditioned to avoid the upright T paired with heat. Half of the flies are trained and tested, as described above, with the upright T and the other half with the inverted T as the heat-associated pattern. All experiments are performed at 24°C–25°C and 40% relative humidity to rule out possible effects of these environmental factors on the performance of the flies.

ORIGINAL CONDITIONING PROCEDURE

The original conditioning procedure (OCP) designed for this learning paradigm consists of one 6-min pretraining session and two consecutive 6-min training-test cycles as described above (Xia et al. 1997a; cf. Wolf and Heisenberg 1991 and Dill et al. 1993). The fly is tested for its spontaneous pattern preference during the pretraining session and trained to avoid the heat-associated pattern in the training-test cycles. After conditioning, the fly is tested for learning acquisition or memory retention during one test session, which comprises three 2-min test periods.

TESTING FOR MEMORY

In these experiments the flies are not tested immediately after training but only after an appropriate retention interval. For intervals not longer

than 12 hr the flies are left at the torque meter and lifted above the panorama where they are provided with sugar solution soaked in a small piece of tissue before the test session begins. For intervals longer than 12 hr, the flies are removed from the torque meter and put back into the chambers. About 10 min before the test begins, the flies are fixed to the torque meter by the same experimenter to reduce possible effects caused by individual handling. Additionally, by masking labels on the fly chambers, the experimenter has no knowledge about the pretreatment of the single flies. About 3 min before the test begins, the flies are replaced in the center of the panorama where they remain for about 2 min. Then they perform their usual 3-min choice between the upright T and the inverted T in one test session. Each fly is tested only once at some retention interval to avoid “active” memory decay caused by extinction.

DRUG FEEDING

CYCLOHEXIMIDE FEEDING

In experiments involving cycloheximide (CXM; Sigma) feeding, the flies with hooks glued to their head and thorax are fed with 35 mM CXM (CXM+) in 5% sugar solution (wt/vol) or 5% sugar solution alone at 24°C–25°C for 12–16 hr before training. Flies are placed singly in small transparent chambers with filter paper on the bottom, which has been soaked in one of the above solutions. Before training begins, a single fly is fixed to the torque meter and allowed to groom itself on a small piece of tissue soaked with distilled water. Immediately following training, the flies are lifted out of the panorama and fed with the solution again. After 10 min, the tissue is exchanged with a new wet tissue soaked with distilled water, which the flies keep for various retention intervals tested. This feeding regimen and the drug concentration are basically the same as reported previously (Tully et al. 1994), except that the size of the filter paper and the volume of solution used to soak the paper are not well controlled.

For experiments testing avoidance indices and learning acquisition, the groups are fed with 5% sugar solution alone or solution laced with 35 mM CXM for 12–16 hr before training, as described above, but not after training. They are tested for learning acquisition immediately after training.

KCI FEEDING

In the KCI feeding experiments, the flies with their heads and thoraxes glued to a wire are placed in small chambers and fed on 154 mM KCl (KCl+) in 5% sugar solution (wt/vol) or 5% sugar solution alone for >12 hr before training, following the same feeding regimen as in the CXM experiments, described above.

COLD ANESTHESIA

In the experiments involving cold anesthesia, the subjects are lifted by the hook and then floated on the surface of 0°C ice water so as not to immerse their wings, immediately after training or at a specific interval. The flies stop flying immediately after exposure to the cold, and their legs become immobile within 10 sec. Five minutes later the water is removed and a small piece of tissue is used to absorb residual condensed water from the flies. Within 2 min, they begin to move their legs and groom themselves with their legs. Their flight appears normal after an additional 3–5 min. Then they are provided with sugar solution soaked in small tissue and will “hold” the tissue for various intervals to be tested for memory retention. During these intervals, they are provided with new pieces of wet tissue when losing the old ones.

PERFORMANCE INDEX

As demonstrated in Wolf and Heisenberg (1991), a performance index (PI) is calculated for each 1- or 2-min flight period as $(t_1 - t_2)/(t_1 + t_2)$, with t_1 and t_2 indicating the time the fly spends fixating the no-heat- and heat-associated quadrants, respectively. Here, to rule out any possible spontaneous pattern preferences and asymmetries in the system (Rescorla 1967; Quinn et al. 1974; Carew et al. 1983; Tully and Quinn 1985), the PI is redefined as the average PI of two flies, one of which has the upright T, the other the inverted T associated with heat. For convenient analysis of the behavior of flies during training and testing, two quantitative indices are used in numerical statistics based on the PI as defined above.

LEARNING INDEX

The learning index (LI) is a measure for the pattern-specific avoidance behavior that is ac-

quired from training; that is, the fraction of time for subjects to avoid the heat-associated pattern minus that for them to avoid the alternative pattern during a test period. In the training–test cycle, the LI is the PI for the 2-min test period. When flies are tested for their learning acquisition or memory retention, LI is defined as the average of PIs during the three 1-min test periods. When testing extinction, the LI is the average of the PIs of three 2-min test periods of the extinction cycle. Values of LIs range from –1 to 1, with 0 indicating no learning, 1 indicating perfect learning, and –1 indicating perfect “wrong decision.” However, perfect learning or wrong decision will never occur in whole populations of flies, because the panorama is always set to a random position for flies to begin their choice before testing.

AVOIDANCE INDEX

The avoidance index (AI) is the measure of the pattern-specific avoidance behavior shown by flies to avoid heat punishment during training. Here only behavioral performance during the massed training session or the training cycle in the spaced training is used to analyze their avoidance behavior. Thus, AI is defined as the average of PIs of the three 2-min training periods. An index value of 1 indicates complete avoidance of the heat-associated pattern and –1 indicates complete fixation of the heat-associated pattern.

STATISTICS

Numerical confidence limits and error bars indicate standard errors of the mean (S.E.M.s). Samples (n) for experiments using LI or AI indicate the number of complete experiment runs, that is, the number of the paired measures from two flies (one with the upright T and the other with the inverted T associated with heat). Because PIs calculated as $(t_1 - t_2)/(t_1 + t_2)$ have been determined empirically to distribute normally (data not shown), LIs and AIs as defined above should also distribute normally (Sokal and Rohlf 1981). Thus, statistical significance of the differences among two or more means of untransformed (raw) data is assessed with analysis of variance (ANOVA); if necessary, Tukey’s honestly significant difference method (T-method) is used to assess unplanned pairwise comparisons between group means (So-

kal and Rohlf 1981). Comparisons between the two means are also assessed with *t*-tests.

Results

ACQUISITION AND DEVELOPMENT OF CONDITIONING PROCEDURES

The OCP produces a somewhat low LI, and even by increasing the number of training-test cycles to nine (the total training time of 36 min amounts to that in the SCP) induces no stronger learning acquisition level. To determine the appropriate conditioning procedure, different groups of flies ($n = 10$ for each group) are trained in parallel (i.e., alternating with individuals of the same population of flies) with a variety of conditioning procedures and then tested for learning acquisition. In all of these procedures, the training sessions include (1) one massed training session, a 1-hr intersession interval, and a spaced session (SCP), (2) two or three consecutive massed sessions (interval-), (3) two or three massed sessions with a 1-hr rest interval after the first and second sessions (interval+), and (4) two massed ones and a spaced session with a 1-hr rest interval after the first and second sessions (M + SCP). All other training and testing conditions remain unchanged. With the SCP, the flies avoid the heat-associated pattern during ~76% of test time; thus, a learning index of 0.52 ± 0.031 for SCP is obtained. Increasing the number of the massed sessions from one to two induces no higher learning index (0.52 ± 0.044 for M + SCP). Changing the spaced training session into the massed one (interval+) in the two procedures above reduces the LI to 0.46 ± 0.052 and 0.48 ± 0.067 , respectively. Two interval- procedures lead to further decrement in learning acquisition (0.041 ± 0.061 , 0.046 ± 0.038). These results indicate that the standard conditioning procedure may yield the maximal learning scores.

To assay acquisition performance in more detail in the standard conditioning procedure, Figure 2 shows the means of the three LIs during test periods in training-test cycles of the spaced training session and the one measured in the test session immediately after the last training session. Learning acquisition derives mainly from the massed training session (0.36 ± 0.046). Then it rises quickly to an asymptote within two to three training-test cycles (0.51 ± 0.055). The additional training cycle induces almost no increment in

Figure 2: Learning acquisition as a function of training-test cycles. Twenty flies ($n = 10$) are tested for learning acquisition during the spaced training session and immediately after the last training.

learning acquisition (0.52 ± 0.031). These observations indicate that the acquired heat avoidance behavior is maximal in the SCP.

BEHAVIORAL PERFORMANCE DURING TRAINING

Behavioral performance of the flies during training is highly variable, and some individuals never seem to avoid the flight directions with respect to the heat-associated pattern so that they can “free” themselves from heat punishment efficiently (Guo et al. 1996). Nevertheless, averaged AIs indicate that the flies learn quickly to avoid the heat-associated pattern during training and their performance continues to improve over the training time (Fig. 3). However, the fact that a fly does not change its flight direction from the “cold” quadrants to the “hot” ones during training could also be attributable to other reasons (e.g., inactivity, spontaneous pattern preference, or spontaneous change of pattern preference, etc.). The principal difficulty in interpreting the fly’s behavioral performance during training leads us to the differences between avoidance behavior (during training) and learning (during testing). The avoidance behavior is an operant behavior (Wolf and Heisenberg 1991), but there is no evidence that learning is involved in operant behavior itself (see also Wolf and Heisenberg 1986). However, if a fly increases its AIs over the training time, this increment may be interpreted as a reference to learning.

A one-way ANOVA, with the *training* cycle as a main effect, produces a significant between cycle effect (see control flies in Fig. 3; $F_{[3,28]} = 22.8$;

Figure 3: Avoidance indices of flies subjected to different disruptive treatments. The flies are fed 5% sugar solution alone (control), or solution laced with 154 mM KCl (KCl+), 35 mM CXM (CXM+) for 12–16 hr before, or subjected to cold anesthesia (cold+) ~10 min before training. $n = 8$ (2×8 flies) for each group.

$P < 0.001$). The T method ($\alpha = 0.05$) confirms that (1) the first mean AI is significantly lower than the other three and the second AI is significantly lower than the last, and (2) the remaining pairwise comparisons between group means do not show statistical significances. This indicates that learning itself may be involved in operant training so that the flies continue to improve their behavioral performance as the training proceeds. Therefore, behavioral performance of flies during training represents their avoidance behavior of heat and learning performance (referred to the increment of AIs with the training time).

EXTINCTION

To measure extinction, 20 flies are subjected to the SCP followed by ten 6-min extinction cycles, which begin immediately after training. These extinction cycles are consecutive except that there is a 10-min rest interval between the fourth and fifth extinction cycles. In this rest interval, the flies are lifted out of the panorama and provided with sugar solution soaked in tissue. The extinction cycle is identical to the pretraining session (i.e., without heat reinforcement). Figure 4 shows the mean LI as a function of the number of extinction cycles; the mean LI decreases with time. At the fifth cycle the residual memory retention amounts to ~50% of the original acquisition of the fly, and it is still measurable ($t_{(9)} = 2.47$; $P < 0.05$) after 10 cycles. It is also observed that all flies increase their LIs immedi-

Figure 4: Extinction of the heat avoidance behavior. A group of 20 flies ($n = 10$) receives, consecutively, the numbers shown of extinction cycles after training. The smooth curve is the best fitting exponential function.

ately after training as compared with the mean PI in the pretraining session, indicative of acquisition of the conditioned heat avoidance behavior. However, an increasing number of flies restore their spontaneous pattern preference as the test proceeds (data not shown).

MEMORY RETENTION

Different groups of flies receive the SCP or the OCP and then are tested for learning acquisition immediately after training (0 hr) or memory retention at 1, 2, 3, 12, 24, 48 hr after training (Fig. 5). These experiments are all performed under the same general conditions, and the only difference is the respective conditioning procedure. In both

Figure 5: Memory retention. Populations of flies are measured for memory retention level at 0-, 1-, 2-, 3-, 12-, 24-, and 48-hr rest intervals after receiving the SCP (●) or the OCP (○) (see text). $n = 8$ for each point.

cases, memory retention attenuates more quickly during the first hour after training and then somewhat slower, especially after 3 hr when the flies retain about half of their learning acquisition. The acquired heat avoidance behavior is still present even at 48 hr for the SCP ($t_{[7]} = 2.96$; $P = 0.02$ for SCP vs. zero). The results for OCP confirm the previous reports about memory retention within 12 hr after training (Xia et al. 1997a), but 24 hr retention is not significantly different from zero ($t_{[7]} = 1.46$; $P = 0.2$). This indicates that memory may not be detected 24 hr after the conditioning procedure. A two-way ANOVA, with *procedure* and *time* (memory retention at 0, 1, 3, 12, 24 hr) as main effects, indicates that (1) the two conditioning procedures produce significantly different effects on memory retention ($F_{[1,70]} = 11.6$; $P < 0.001$), and (2) *procedure* and *time* do not interact ($F_{[4,70]} = 2.24$; $0.05 < P < 0.1$). The T method ($\alpha = 0.05$) from separate one-way ANOVA confirms that SCP produces significantly higher learning acquisition ($F_{[1,14]} = 8.7$; $P < 0.05$) than OCP. It has also been observed that some flies make consistent choices between the two patterns in all three 1-min periods irrespective of the original position of the panorama set randomly by the computer, but some of them always fixate the heat-associated pattern, maybe because of confusion between the two patterns or as an effect of their spontaneous pattern preference. Other flies fail to make consistent choices between the two patterns through the test session, and their choice largely depends on the original position of the panorama (data not shown). This may reflect a decay of pattern memory or amnesia.

MEMORY DISRUPTION

Two common behavioral features in memory formation are that (1) memory immediately after training exists as a short-lived disruptable form, which then is consolidated into a longer-lasting stable form; (2) administration of anesthetic or chemicals blocks memory consolidation so as to induce retrograde amnesia (McGaugh and Herz 1972; Quinn and Dudai 1976; Menzel et al. 1990; Allweis 1991; Ng and Gibbs 1991; Tully et al. 1994; DeZazzo and Tully 1995). Here several agents have been employed to prevent memory formation, which include cold anesthesia, KCl, and CXM (Watts and Mark 1971; Quinn and Dudai 1976; Dudai 1977; Gibbs and Ng 1977, 1978; Tully et al.

1994; Yin et al. 1994). The controls are always carried out in parallel with the respective experiments.

DISRUPTIVE EFFECT OF KCl

Populations of flies are fed isotonic concentrations of KCl as described in Materials and Methods for 12, 2, 1 hr, or just before training, trained for the SCP, and then tested for memory retention at 1 hr after training. The results indicate that drug feeding <1 hr prior to the training exerts no effect on memory retention (data not shown). So the following experiments are all performed on flies fed KCl for >12 hr. Different groups of flies are subjected to operant training and then tested for learning acquisition or memory retention at various intervals (Fig. 6A); they are fed with 5% sugar solution alone or solution laced with 154 mM KCl (KCl+). A two-way ANOVA, with *feeding* regimen and *time* as main effects, indicates that (1) KCl feeding and sugar feeding produce different effects on memory retention ($F_{[1,56]} = 29.3$; $P < 0.001$), and (2) *feeding* regimen and *time* do not interact ($F_{[3,56]} = 1.58$; $P > 0.1$). T methods ($\alpha = 0.05$) from separate one-way ANOVAs confirm that (1) KCl feeding significantly reduces memory retention at 0.5, 1, and 2 hr after training when compared with control regimen, and (2) such feeding regimen exerts no diminishing effect on learning acquisition. In addition, KCl feeding abolishes memory soon after training, and no recovery of memory is detected 2 hr later based on *t*-tests ($t_{[7]} \leq 1.11$; $P \geq 0.3$ for KCl+ versus zero at 0.5, 1, and 2hr, respectively). Along with the fact that the KCl-fed flies show normal behavioral performance during training [Fig. 3; two-way ANOVA, with *feeding* regimen and *training* cycle as main effects, indicating no different effects between the KCl and control feeding regimens ($F_{[1,56]} = 0.41$; $P > 0.2$); these results suggest that such a KCl-feeding regimen exerts no effect on heat avoidance and learning performance during training, and also not on learning acquisition, but disrupts memory formation soon after training.

COLD ANESTHESIA-INDUCED RETROGRADE AMNESIA

The disruption of memory is also studied by applying cold anesthesia, as described in Materials

Figure 6: (A) Disruption of memory by KCl-feeding regimen. Different groups of flies are tested for memory retention at 0-, 0.5-, 1-, and 2-hr rest intervals after training; flies have been fed sugar solution alone (control; ●) or solution laced with isotonic KCl (KCl+; ○) for at least 12 hr prior to the training. $n = 8$ for each point. (B) Susceptibility of memory to cold anesthesia. Different groups of flies, subjected to cold anesthesia, are tested for 1-hr memory retention 0, 10, 20, 25, and 30 min after training (○). Among them, the measurement at 25 min is introduced later. Another group of flies is tested for 1-hr retention without introducing cold anesthesia (●). $n = 8$ for each point. (C) Memory disruption by applying CXM-feeding regimen. Different groups of flies, fed sugar solution alone (●) or solution laced with 35 mM CXM (CXM+; ○) for >12 hr before training, are tested for memory retention at 0-, 2-, 3-, and 12-hr rest intervals after training. $n = 8$ for each point.

and Methods, immediately after training (0 min) or at various intervals after training, and in each case 1 hr memory retention is measured (Fig. 6B). A one-way ANOVA, with *group* as the main effect, produces a significant between-group effect ($F_{[5,42]} = 7.5$; $P < 0.001$). The T method ($\alpha = 0.05$) confirms that (1) mean LIs at 0 and 10 min differ from the ones at 25, 30 min, and the control, which do not differ from each other, (2) the LIs at 0 and 10 min do not differ from each other, and (3) the one at 20 min does not differ from the remaining ones. In addition, *t*-tests indicate that LIs at 0 and 10 min are near zero ($t_{[7]} = 1.2$, 0.6 ; $P = 0.3$, 0.6 , respectively), and the one at 20 min is significantly higher than zero ($t_{[7]} = 3.76$; $P < 0.01$) but lower than that of control group ($t_{[14]} = 2.84$; $P < 0.02$). This may suggest that, when introduced at 0 and 10 min after training, cold anesthesia abolishes memory completely; when introduced later than 20 min, it exerts no effects on memory formation. Furthermore, a consolidation “gradient” is observed, indicating that cold sensitivity decreases gradually between 0 and 25 min after training.

The flies subjected to cold anesthesia ~10 min before training produce normal learning acquisition (0.56 ± 0.052 for $n = 2 \times 7$ flies) and 1 hr memory retention (0.37 ± 0.071 for $n = 2 \times 8$ flies). They also show the same avoidance indices during training as control flies [Fig. 3; two-way ANOVA, with *treatment* and *training cycle* as main effects, producing no different effects between cold anesthesia and control groups ($F_{[1,56]} = 0.18$; $P \rightarrow 0.2$). These results indicate that cold anesthesia exerts no diminishing effect on flies’ ability to discriminate the visual patterns, perceive the heat, learn the heat association, and form memory when it is introduced before training.

CXM-INDUCED RETROGRADE AMNESIA

To test whether protein synthesis is required during and soon after training for the long-lasting memory formation under operant conditioning, groups of flies are fed the protein synthesis inhibitor cycloheximide (CXM+) in 5% sugar solution or fed sugar solution alone as control for 12–16 hr before and again immediately after training, and then their memory retention is assayed at different time intervals (Fig. 6C). Another group of flies is tested for learning acquisition (LI at 0 hr) when fed CXM for 12–16 hr before training. A two-way ANOVA, with *feeding regimen* and *time* as main

effects, indicates that (1) CXM feeding and sugar feeding produce different effects on memory retention ($F_{[1,56]} = 8.2$; $P < 0.001$), and (2) *feeding* regimen and *time* do not interact ($F_{[3,56]} = 1.55$; $P > 0.1$). T methods ($\alpha = 0.05$) from separate ANOVAs confirm that CXM feeding exerts no effects on learning acquisition and memory formation within the first 2 hr after training but significantly diminishes memory at 3 and 12 hr after training. In addition, *t*-tests indicate that CXM abolishes memory after 3 hr ($t_{[7]} = 1.0$; $P < 0.3$ for CXM+ vs. zero), and no recovery of memory is detected 12 hr later ($t_{[7]} > 0.31$; $P < 0.7$ for CXM+ vs. zero).

A two-way ANOVA, with *feeding* regimen and *training* cycle as main effects, indicates no different effects between both CXM-feeding and control groups on behavioral performance during training (Fig. 3; $F_{[1,56]} = 0.92$; $P > 0.2$). Along with the fact that CXM exerts no diminishing effect on learning acquisition and memory formation within the first 2 hr after training, this result suggests that memory disruption in CXM-fed flies is not induced by non-specific effects of the drug. Taken together, these observations demonstrate that there are at least two memory phases: One is protein synthesis insensitive, and the other is protein synthesis dependent.

Discussion

With the flight simulator, behavioral experiments are carried out on *Drosophila* under operant conditioning. During training, two equally attractive visual pattern types are used as visual landmarks; with one of the pattern types in the frontal quadrant of the panorama, the fly is punished with heat as negative reinforcement, and with the other, it is not. The animal is located in the center of the panorama, able to escape the heat “punishment” using its own control on the flight direction with respect to the landmarks. Afterward, with the fly in the same situation, it makes a choice between the two pattern types for testing learning acquisition or memory formation without heat reinforcement. During these test periods, the fly maintains avoidance of the previously punished flight directions and chooses to orientate toward the alternative ones in ~75% of instances.

The various (i.e. interval–, interval+, standard) conditioning procedures produce different learning acquisition. In addition, these procedures should yield different numbers of stimulus pairings

(thus, different amounts of training), as heat punishment is contingent on the flies’ behavioral performance during training. All of these conceptual limitations may indicate that direct comparisons among these procedures are not appropriate, but stronger long-lasting memory has been demonstrated than that reported previously (Fig. 5; Xia et al. 1997a), and 1- or 2-day memory has been demonstrated for the first time about this learning paradigm with the newly developed SCP. These results suggest that the improvement of learning performance occurs between training sessions or training-test cycles, and the stronger, longer-lasting memory can be induced by multiple training sessions or cycles with an appropriate rest interval between them. This agrees well with many reports published previously (Carew et al. 1972; Dudai 1977; Frost et al. 1985; Karni and Sagi 1993; Tully et al. 1994; Yin et al. 1995). It has been proposed that this improvement in learning performance may suggest a consolidation period (Karni and Sagi 1993), and the parameters of extended training (number of training sessions and the intersession rest interval) are determined by the opposing functions of cAMP-responsive element-binding (CREB) activators and repressors, which act as a “molecular switch” (Yin et al. 1995). Thus, further investigations might be needed to check whether the standard conditioning procedure is the optimal one and, if possible, to determine the optimal parameters of extended training, as we cannot determine whether the LIs shown here are maximal.

When testing for memory >12 hr after training, it has been observed that whether the flies are removed from the torque meter or not, they become inert and stop flying more often than during conditioning, probably because of malnutrition (i.e., they were fed on sugar solution; see Materials and Methods). Therefore, feeding subjects with sugar solution all through the retention intervals may alter the results by affecting flies’ motor behavior. In addition, as the flies store the visual image of the panorama as a memory template and then superimpose the actual image on the memory template through pattern recognition during test periods (Dill et al. 1993), this matching process may be affected by removing the flies from the setup, which may displace the animals or change their flying posture. Although it has been observed that 3- or 12-hr memory retention is not significantly affected by such handling (data not shown), further efforts are needed to solve this problem. As for the low value of the 24-hr memory retention for

OCP, we still have no data to indicate whether it is caused by this handling problem.

Cold anesthesia, if carefully applied, is a non-invasive procedure to induce retrograde amnesia. It does not interfere with subsequent neural function such as sensory perception or capacity to learn and form memory itself (see also Quinn and Dudai 1976). In addition, flies recover from cold anesthesia quickly, and their locomotion seems normal within 5–10 min after treatment. Thus, this treatment appears to be a very useful way for further assessing memory disruption induced by anesthesia. The result that cold anesthesia abolishes memory formation when introduced within 20 min after training but exerts no effect on memory when introduced later than 25 min suggests that there are at least two forms in consolidation process of memory formation—an early one sensitive to cold anesthesia and a later one insensitive to such treatment. These results are consistent with those reported by other researchers (Quinn and Dudai 1976; Dudai 1977; Yamada et al. 1992; Tully et al. 1994).

In KCl-feeding experiments, considering that the regimen has no effect on behavioral performance during training and on learning acquisition, memory deficits soon after training should not result from nonspecific effects of the drug. Work has been done to investigate the effects of the other depolarizing drugs such as lithium chloride (LiCl) and monosodium glutamate on memory formation in flies (Xia et al. 1997b). The effects of these drugs on memory parallel that of KCl in most respects, and it has also been demonstrated that KCl disrupts memory formation rather than memory retrieval by reversal training of flies. This result, along with cold anesthesia experiments, suggests that there is a cold anesthesia-sensitive memory form immediately after training, which is also susceptible to inhibition of KCl. This short-lived disruptable memory form is called anesthesia-sensitive memory (ASM).

The level of protein synthesis inhibition in brain tissue by the CXM-feeding regimen described above is estimated to be 50% (Tully et al. 1994). In addition, the feeding regimen produces no effects on flies' behavioral performance and on learning acquisition. Finally, it exerts no effect on memory within 2 hr after training, and thus the inhibitive action of the drug cannot be attributed, at least solely, to an effect on memory retrieval. Yet, this moderate level of protein synthesis inhibition during and soon after training is enough to block con-

solidation of long-lasting memory (>2 hr after training). This result agrees well with that reported for olfactory-associative learning in flies (Tully et al. 1994; Yin et al. 1994), and with the notion that the appearance of long-term memory is protein synthesis dependent (Flood et al. 1977; Andrew 1980; Ng and Gibbs 1991; Tully et al. 1994). The dependence of long-term memory on protein synthesis is consistent with the neurobiological view that memory consolidation reflects the establishment of long-lasting structural changes in synaptic morphology (Greenough 1984; Stewart 1991; Bailey and Kandel 1994). Considering that cold anesthesia has no effect on memory when introduced >25 min after training and CXM disrupts memory 3 hr later (Fig. 6B,C), the cold anesthesia-insensitive phase may be subdivided into at least two categories—an early CXM-insensitive phase, and a later, protein synthesis-dependent one. The early memory phase is called anesthesia-resistant memory (ARM) and the later, long-term memory (LTM). Such CXM feeding regimen does not disrupt the formation of ARM, but as argued previously (Tully et al. 1994), only 50% inhibition of protein synthesis is not enough to suggest that the appearance of ARM is independent of protein synthesis. Thus, ARM is characterized as CXM insensitive.

Memory disruption experiments suggest at least three phases in memory formation under operant conditioning: (1) an ASM phase that lasts ~20 min after training and can be disrupted by KCl-feeding regimen and cold anesthesia, (2) an ARM phase that is cold anesthesia-insensitive and CXM-insensitive, and (3) a LTM phase that is protein synthesis-dependent and can be disrupted by CXM feeding. This multiple-phase model of memory consolidation after operant conditioning agrees broadly with the hypothesis that memory formation involves an intricate, multiple-phase consolidation pathway, which has been suggested independently by many other researchers using different species and tasks. In both chicks and rats, injections of a wide variety of amnesic agents after training reveal three distinct periods of amnesia, corresponding to the temporal appearances of short-, middle-, and long-term memory (Gibbs and Ng 1977; Allweis 1991). In bees, a similar three-phase model of memory formation has been suggested by Hammer and Menzel (1995). In *Drosophila* an intricate, multiple-phase consolidation model has been proposed for the olfactory learning paradigm based on behavioral, pharmacological,

and genetic analyses (Tully et al. 1994; DeZazzo and Tully 1995). The similarity in the consolidation of memory formation greatly extends the argument that the basic mechanisms underlying learning and memory broadly appear to be common among different tasks in flies and among different species across the animal kingdom.

A mechanism—that neural modifications of preexisting synapses following conditioning seem to be attributable to changing transmitter release from related neuron terminals by modifying the conductance of certain ion channels and lead to enhanced activity of a specific neural pathway—appears to operate in several invertebrate and vertebrate models.

In *Aplysia*, the relevant biochemical events after learning all occur in the presynaptic terminals made by sensory neurons onto motor neurons of the gill-withdrawal circuit. The spike activity associated with conditioned stimulus (CS) enhances the synthesis of cAMP induced by activation of adenylate cyclase, which in turn is activated by a related neuromodulator associated with unconditioned stimulus (US). The increased levels of cAMP cause greater closure of a particular class of potassium (K^+) channels. The closure of these channels produces a prolongation of action potential in the sensory neurons and causes an increased influx of Ca^{2+} ions and more transmitter release per action potential. The increased influx of Ca^{2+} is thought to modulate the adenylate cyclase complex in a temporally specific manner in association with the action of the neuromodulator released by US (Hawkins et al. 1983; Walters and Byrne 1983; Ocorr et al. 1985; T.W. Abrams, T.J. Carew, R.D. Hawkins, and E.R. Kandel; T.W. Abrams, L. Bernier, R.D. Hawkins, and E.E. Kandel, both unpubl.). In *Hermissenda*, persistent postsynaptic changes occur in the type B photoreceptor cell as a result of pairing a visual CS and vestibular US. These neural modifications include the enhanced spontaneous spike activity and the increased input resistance, thereby inducing a persistent and intrinsic change in membrane conductance of the photoreceptors. These changes appear to be attributable to reduction in two species of outward K^+ currents (i.e., an early, transient K^+ current and a later Ca^{2+} -activated K^+ current) in the type B cell (Crow and Alkon 1980; Alkon et al. 1982, 1985; J. Farley and D. Alkon; R. Forman, D.L. Alkon, M. Sakakibara, J. Harrigan, I. Lederhendler, and J. Farley, both unpubl.).

There is also some evidence for learning-in-

duced alterations in ion channels in vertebrate models. The increased excitability that apparently results from changes in conductance has been shown in neurons in the motor cortex of cats after eye-blink conditioning (Woody 1982). Other studies suggest that eye-blink conditioning in rabbits may decrease after hyperpolarization in pyramidal neurons by decreasing a Ca^{2+} -activated outward K^+ conductance (Disterhoft et al. 1986).

The mechanism also appears to exist in *Drosophila* after olfactory classical conditioning. Because of the alternated kinetics of action potential-dependent K^+ conductance (Salkoff and Wyman 1981), *shaker* mutants are characterized by increased neuronal excitability and multiple potential spikes, which therefore leads to enhanced terminal depolarization and elevated neurotransmitter release (Tanouye et al. 1981). A second K^+ -channel mutant, *eag*, is shown to primarily reduce the delayed rectification K^+ current, and the action potential-dependent K^+ conductance to a lesser degree (Wu et al. 1983). *Nap^{ts}* (no action potential, temperature sensitive) with nerve conductance partially reduced at room temperature ($23^{\circ}C$ – $25^{\circ}C$) is shown to decrease neuronal excitability most likely by specifically disrupting Na^+ -channel function (Wu and Ganetzky 1980). Previous studies have demonstrated that these *Drosophila* mutants all disrupt the formation of short-term memory after courtship or odor-avoidance conditioning due to the alterations in neuronal excitability (Cowan and Siegel 1984, 1986). Thus, we would assume a similar mechanism through which neural modifications are induced by changes of transmitter release as a result of a changed ion conductance based on the “homology of function” argument for a common memory formation process.

If this hypothesis is to be accepted, it may be deduced with due caution that the KCl-feeding regimen may disrupt memory formation by changing K^+ -channel function. There are at least two possible ways through which KCl would interfere with neuronal mechanisms underlying memory formation. First, KCl may directly interfere with some biochemical events associated with memory formation. High K^+ , for example, can reduce protein phosphorylation (Neary and Alkon 1983). Therefore, KCl might interfere with changes in ionic conductance following operant conditioning so as to disrupt memory formation by affecting phosphorylation of proteins associated with related ion channels. In addition, because depolariz-

ing drugs such as K^+ may cause neuron terminals to increase their permeability to Ca^{2+} , accumulate Ca^{2+} , and release transmitter under some physiological conditions (Blaustein 1975), KCl may induce some changes in neuronal function such as increasing membrane excitability or neuronal activity. These changes might lead to further enhancement of activity over the already elevated “baseline” levels by conditioning so that the encoded information about the temporal association of training would be meaningless. Considering that the KCl-fed flies can learn as well as control flies, the effect of the drug appears to be a level of physiological disruption at which normal learning can still occur.

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