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Memory through Metamorphosis in Normal and Mutant *Drosophila*

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To establish that a stable, long-lasting form of memory exists in *Drosophila*, we trained third-instar larvae by electroshocking them in the presence of a specific odor using a Pavlovian conditioning procedure. We show that conditioned odor avoidance produced in larvae still was present in adults 8 d later. Such memory through metamorphosis was specific to the temporal pairing of odor and shock; presentations of odors alone or shock alone did not produce a change. Thus, the memory involved associative processes. We also show that similar training of the single-gene memory mutants *dunce* and *amnesiac* did not yield any detectable learning in larvae or memory retention in adults, suggesting that these mutations interfere with long-term memory (LTM) formation even if LTM is induced independently of earlier memory retention processes.

[Key words: learning, memory, metamorphosis, long-term memory,* Drosophila, *larvae, Pavlovian conditioning]

A primary goal in the study of learning and memory is to define the "engram"—the biological substrate(s) of long-term memory (LTM; Lashley, 1950). Work from several vertebrate and invertebrate model systems has revealed the involvement of neurotransmitters, their receptors, ion channels, G-proteins, adenylyl cyclase, phosphodiesterase, and protein kinases within the first few hours after an animal learns something new about its environment (Kandel et al., 1987; Crow, 1988; Madison et al., 1991; Tully, 1991). By biochemical and molecular analyses, the *Drosophila* learning/memory genes *dunce* and *rutabaga,* in particular, were found to encode a cAMP phosphodiesterase and an adenylyl cyclase, respectively (Chen et al., 1986; Levin et al., 1992)—lending support to the notion that the basic molecular mechanisms of (some of) these early events may be evolutionarily conserved. Much less is known, however, about how these early biochemical changes give rise to longer-lasting neuronal changes. One persistent view is that LTM ultimately is encoded as a change in the number or pattern of synaptic connections (Greenough and Bailey, 1988; Montarolo et al., 1986; Bailey et al., 1992; Mayford et al., 1992).

Potentially, a genetic dissection of LTM formation in *Drosophila* could expedite our understanding of the process—primarily because genes involved in the behavior can be identified without any a priori knowledge of the underlying biochemistry or anatomy. Existence in fruit flies of a bona fide LTM of an associative task, however, has not yet been reported. Experience-dependent modifications lasting several days have been reported for mating behavior in *D. pseudoobscura* (Pruzan and Ehrman, 1974; Pruzan, 1976) and for phototaxis in *D. melanogaster* (Willmund and Fischbach, 1977), but these behavioral tasks have not been shown to be associative in nature. Some memory was detected 24 hr after sucrose-approach learning (Tempel et al., 1983) or shock-avoidance learning (Tully and Quinn, 1985)—which clearly are associative tasks—but memory of the latter is gone after 72 hr (Dudai et al., 1988) and that of the former has not been reported for longer retention intervals.

Here, we demonstrate a clear case of stable, long-lasting memory after associative learning: by using a larval olfactory conditioning procedure (Aceves-Pina and Quinn, 1979), we show that trained larvae remember an odor–shock association 8 d later as adults. The existence of such memory seems remarkable, since the larval PNS and CNS undergo considerable degeneration, reorganization, and growth during metamorphosis.

Materials and Methods

Fly stocks. Wild-type Canton-S flies and the learning/memory mutants *dunce*^{1} and *amnesiac* were used in this study, along with the X chromosome balancer *FM7[In(1)sc*^{8}, *y*$^{+d}$ *sc*^{8} *w*^{a} *lz*^{vp} *B*]. The genetic background of the *FM7* stock was "equilibrated" with that of the wild-type stock by backcrossing +/*FM7* females to Canton-S males for at least five generations. The learning/memory stocks were maintained by backcrossing *mut*/*FM7* females to *FM7* males. This served to equilibrate the genetic backgrounds of the mutants to that of Canton-S and to prevent the accumulation of genetic modifiers that ameliorate the mutant phenotypes (Tully and Quinn, 1985; Boynton and Tully, 1992). A few weeks before experiments began, homozygous mutant flies were bred from the heterozygous stocks. Learning or memory were assayed in such homozygous mutants with the standard Pavlovian conditioning procedure (Tully and Quinn, 1985) to verify that the stocks, in fact, yielded the expected phenotypic deficiencies. These homozygous stocks never were maintained for more than 3 months.

All stocks were grown at 23 ± 2°C in a 16 hr:8 hr light/dark cycle with lights on at 8 A.M. Flies were raised on a food medium consisting of 84.0 gm/liter agar, 31.9 gm/liter yeast, 62.8 gm/liter dextrose, 31.4 gm/liter sucrose, 8.7 gm/liter potassium tartrate, 7 gm/liter $CaCl_2$, 76.1 gm/liter cornmeal, and 2 gm/liter Tegosept M mold inhibitor. This food medium and other rearing conditions were identical to those used before (Tully and Quinn, 1985; Gailey et al., 1991; Boynton and Tully, 1992; Dura et al., 1993; Tully and Gold, 1993). We have tried other food media, in particular one containing molasses, but have obtained lower learning scores with Canton-S flies (not shown).

Third-instar larvae for behavioral experiments were produced by letting about 50 adult females lay eggs at 25°C in standard bottles of food medium. After 10–12 hr, adults were cleared, and the bottles were placed at 18°C for 5–6 d. Thirty to sixty minutes before an experiment, a bottle containing several hundred larvae was filled with a solution of 15% sucrose, and the surface of the medium was gently agitated with a camel's hair brush, causing the larvae to dislodge from the medium and float to the surface. Immediately before an experiment, 50–100 larvae were

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scooped from the bottle, placed on a nylon mesh screen, and gently rinsed with distilled water. For adult experiments, about 400 imagoes 2–4 d old were transferred without anesthesia from their culture bottles to fresh, dry bottles the night before.

Larval conditioning apparatus. A 19.2 × 11.8 × 2.5 cm Plexiglas chamber was designed with three internal chambers through which air currents were drawn. The two distal chambers (9.5 × 4.5 × 1.0 cm inside) served as air baffles, while the central chamber (8.8 × 10.2 × 2.0 cm inside) contained the larvae crawling on top of a 0.3-cm-thick layer of 1.5% agarose (FMC Seakem LE) made conductive with 2 mM $LiCl_2$. Two copper electrodes were fixed along the lateral walls of the central chamber. The two distal chambers were separated from the central one by nylon mesh (Nitex #3-180/43). This design optimized the laminar flow of air through the larvae chamber during conditioning (training) and testing of odor responses (see below). Some of our data were generated in earlier versions of the conditioning apparatus, which most likely did not produce such laminar currents of air. These deviations from our standard conditions will be delineated in Results.

Larval olfactory acuity. Different groups of about 50 naive, third-instar larvae were placed in the center of the larval chamber of the conditioning apparatus (see Fig. 1*B*) and were exposed to converging currents (each at 35 ml/min) carrying ethyl acetate (EA; Fluka) versus air or isoamyl acetate (IA; Aldrich) versus air at each of five dilutions in heavy mineral oil (Sigma). After 5 min, the numbers of larvae in each side of the chamber were counted. A performance index (PI) then was calculated (as for adult behavior) by normalizing the percentage "correctly" *avoiding* the odor (Gailey et al., 1991; Boynton and Tully, 1992; Dura et al., 1993):

$$\mathrm{PI} = \frac{\left(\frac{\mathrm{COR}}{\mathrm{COR} + \mathrm{INCOR}}\right) - 0.5}{0.5} \times 100$$

A PI would be zero if flies did not prefer either side of the chamber (COR vs INCOR), and would be ±100 if all flies preferred one side. Positive values indicate that more larvae were on the "air" side than on the "odor" side of the dish; negative values indicate the converse.

Larval conditioning. To train larvae in classical conditioning experiments, air was drawn unidirectionally through the central chamber by applying a vacuum to one end of the apparatus. Room air was drawn (bubbled) at 35 ml/min first through a 20 × 150 mm side-arm test tube containing 20 ml of distilled water, then through another such test tube containing 20 ml of an odorant (either EA or IA undiluted or diluted in heavy mineral oil), and finally through the conditioning apparatus. About 80–100 larvae were placed in the central chamber of the conditioning apparatus (see Fig. 1*A*) and then were conditioned by exposing them sequentially to two undiluted odors for 60 sec each with a 90 sec rest period after each. During the latter 30 sec of exposure to the first odor (CS+), larvae received 90 V (AC) of continuous shock (US), while no shock was applied during exposure to the second odor (CS−). This 300 sec training cycle was repeated eight times unless otherwise noted (see Results). To prevent confusion from residual odors, larvae gently were transferred back and forth during the rest periods to different apparatuses dedicated to one particular odor. Such "odor–shock paired" groups were run for Canton-S, *dunce,* and *amnesiac* larvae. "Odors-alone" and "shock-alone" control groups also were run with Canton-S flies. The stimulus schedules for each control group were identical to that of the odor–shock paired group, except that exposure to one of the two stimuli was omitted. For one complete experiment, two reciprocal groups of larvae were tested; the CS+ and CS− were EA and IA for the first group and were IA and EA for the second. For the control groups, two successive runs arbitrarily were designated as reciprocal groups.

To test larval odor avoidance responses after training, air was drawn bidirectionally through the central chamber by applying a vacuum along the midline of the cover of the central chamber (see Fig. 1*B*). In this manner, two converging currents of air were drawn into the central chamber from each end at 35 ml/min, thereby providing larvae with a choice between EA and IA. The numbers of larvae on each side of the chamber were counted after 5 min, and PI values were calculated as for olfactory acuity above. The PI for one complete conditioning experiment ($N = 1$), however, was the average PI of the two reciprocal groups. To test for memory retention after metamorphosis, half of the larvae were trained but not tested. Instead, they were transferred immediately to food vials. Their odor avoidance responses were tested as 2–3-d-old adults 8 d later (see below).

Adult conditioning apparatus. Adult behavior was assayed in the conditioning apparatus of Tully and Quinn (1985), which consists of a training chamber, a T-maze choice point, and an "elevator" to transfer flies from one chamber to another. Odors were delivered to the training chamber or to the arms of the T-maze on currents of air (750 ml/min). Pulses of electric shock (DC) were delivered to flies via a copper grid covering 90% of the inside surface of the training chamber.

Adult olfactory acuity. Different groups of about 100 3 d-old adults were transferred in the elevator to the choice point of conditioning apparatus's T-maze and then were exposed to either EA versus air or IA versus air at each of five dilutions (in heavy mineral oil). After 2 min, flies in each arm of the T-maze were trapped (by pulling the elevator up out of register with the T-maze arms), anesthetized, and counted. A PI then was calculated as for larval olfactory acuity (see above).

Adult conditioning. Adult flies were trained with a discriminative Pavlovian conditioning procedure using odors as the conditioned stimuli (CSs) and electric shock as the unconditioned stimulus (US; see Tully and Quinn, 1985). Briefly, 50–150 flies were transferred to the training chamber (without anesthesia), allowed 90 sec to acclimate and then exposed sequentially to EA and IA for 60 sec each with a 45 sec rest interval after each odor presentation. During exposure to the first odor (the CS+), flies received 12 1.25 sec pulses of electric shock (DC) at 0.2 Hz. Only one such training "cycle" was done for this study.

To test for conditioned odor avoidance responses immediately after training, flies were tapped gently into the elevator and transferred to the T-maze choice point within 90 sec. The test trial began by lowering the elevator into register with the arms of the T-maze, thereby initiating the flow of converging air currents carrying EA or IA. After 120 sec, the elevator was pulled up out of register with the T-maze arms, trapping the flies in their respective arms. The flies then were anesthetized and counted, and PIs were calculated as for larval conditioning. Here again, one complete experiment consisted of the average PIs from two reciprocal groups. To test for memory retention through metamorphosis, 2–3-d-old adults trained as larvae were tested by transferring them directly to the elevator of the T-maze and then proceeding with a test trial.

Statistics. We have shown that performances indices are distributed normally (Tully and Gold, 1993); thus, raw data were analyzed here using JMP 2.0 statistical software for the Macintosh (SAS Institute, Cary, NC). Unplanned pairwise comparisons among means were done according to the Tukey-Kramer method with $\alpha = 0.05$ after initial one-way ANOVAs indicated significant differences among groups.

Results

Olfactory acuity is qualitatively different between larvae and adults

To improve our understanding of the larval olfactory learning procedure reported by Aceves-Pina and Quinn (1979; cf. Heisenberg et al., 1985), we first studied olfactory responses in naive larvae. We designed a larval conditioning apparatus to deliver odor cues in converging laminar air currents across an agarose surface (see Fig. 1). We obtained from naive (untrained) larvae only moderate responses to the odorants 3-octanol (OCT) and 4-methylcyclohexanol (MCH) used in previous studies (data not shown). In contrast, we observed more robust olfactory responses to the odorants EA and IA. Third-instar larvae were strongly *attracted* to these odors, while adult flies were strongly *repelled* (Fig. 2). This qualitative switch in naive olfactory responses between third-instar larvae and adults has been noted before (Rodrigues, 1980) and appears to be a more general property of chemotactic behavior in *Drosophila* (cf. Lilly and Carlson, 1989; Alcorta, 1991).

Normal but not mutant larvae show associative learning

In our apparatus, pairing one odor (CS+) with electrical shock (US) and presenting the second odor (CS−) without shock for eight training cycles produced a mean PI (see Materials and Methods) of 32 ± 4 for wild-type (Canton-S) larvae (Fig. 3*A*). The magnitude of this conditioned response was similar to those

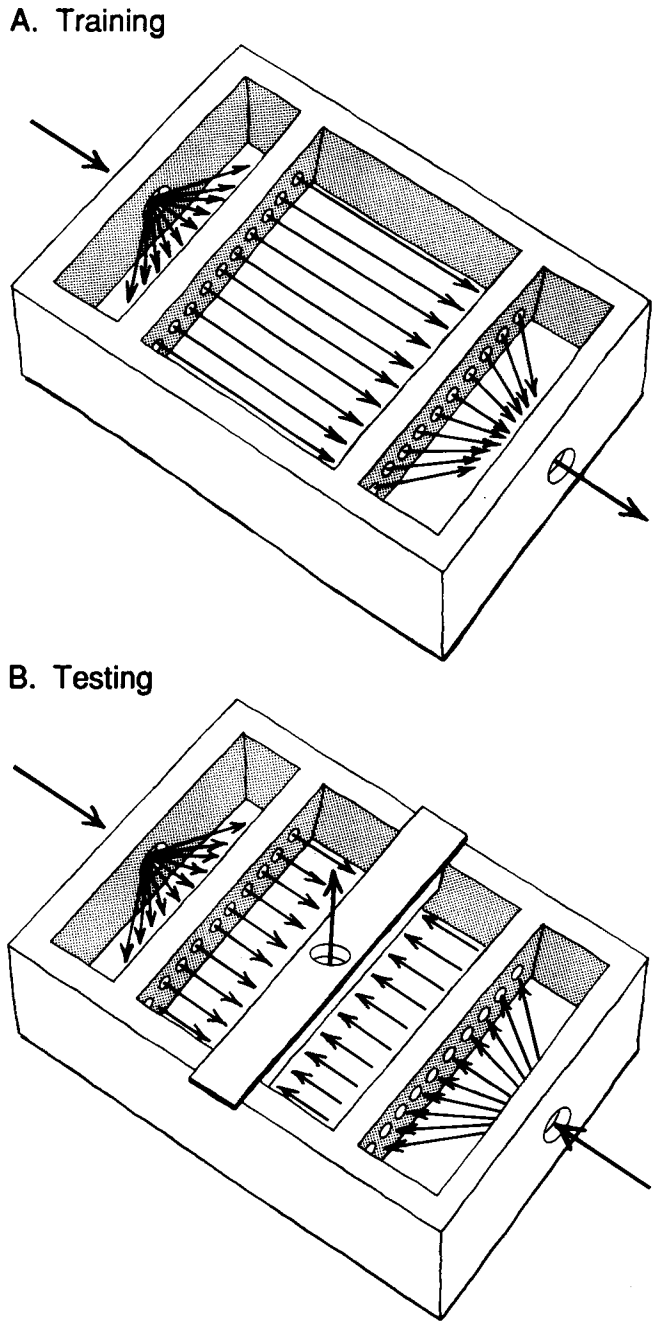

Figure 1. Schematic drawing of larval conditioning apparatus. A 19.2 × 11.8 × 2.5 cm Plexiglas chamber was designed with three internal chambers through which air currents were drawn. The two distal chambers (9.5 × 4.5 × 1.0 cm inside) served as air baffles, while the central chamber (8.8 × 10.2 × 2.0 cm inside) contained the larvae crawling on top of a 0.3-cm-thick layer of 1.5% agarose (FMC Seakem LE) made conductive with 2 mM $LiCl_2$. Two copper electrodes were fixed along the lateral walls of the central chamber. The two distal chambers were separated from the central one by nylon mesh (Nitex #3-180/43). *Arrows* indicate air flow. *A*, To train larvae in classical conditioning experiments, air was drawn unidirectionally through the central chamber by applying a vacuum to one end of the apparatus. Room air then bubbled at 35 ml/min first through a 20 × 150 mm side-arm test tube containing 20 ml of distilled water, then through another such test tube containing 20 ml of an odorant [either ethyl acetate (EA; Fluka) or isoamylacetate (IA; Aldrich), undiluted or diluted in heavy mineral oil], and finally through the conditioning apparatus. *B*, To test odor avoidance responses (in trained or naive larvae), air was drawn bidirectionally through the central chamber by applying a vacuum along the midline of the cover of the central chamber. In this manner, two converging currents of air were drawn into the central chamber from each end at 35 ml/min, thereby providing larvae with a choice between odor and fresh air for olfactory acuity experiments (see Fig. 2*A*) or between EA and IA for conditioning experiments (see Fig. 3*A*).

previously reported (Aceves-Pina and Quinn, 1979; Heisenberg et al., 1985) and indicated that about 65% of trained larvae *avoided* an odor (CS+) to which they normally were attracted (see Fig. 2*A*). Such conditioned avoidance was due to associative processes, since mean PIs from nonassociative control groups (odors alone or shock alone) were not greater than zero (cf. Tully, 1984; Tully and Quinn, 1985).

We detected no initial learning in mutant *dunce* and *amnesiac* larvae (Fig. 3*A*). A similar result for *dunce* larvae was reported by Aceves-Pina and Quinn (1979), but *amnesiac* larvae showed near-normal retention of conditioned responses 15 min after training in their experiments (PI = 11 ± 1 for wild-type Can-S vs 9 ± 1 for *amnesiac*). We did not assay 15 min retention, and Aceves-Pina and Quinn (1979) did not assay immediate learning, in *amnesiac* larvae; thus, our results cannot be compared directly. Nevertheless, we presume that 15 min retention in *amnesiac* larvae most likely would be zero in our experiments, since such mutants showed no initial learning. Importantly, *amnesiac* mutants can accumulate over generations genetic modifiers (suppressors) that ameliorate the mutant learning/memory deficit (T. Tully, unpublished observations; cf. Boynton and Tully, 1992). Consequently, we outcrossed our mutant stocks and verified before this study that mutant stocks yielded the expected mutant phenotypes (cf. Tully and Quinn, 1985; see Materials and Methods). Aceves-Pina and Quinn (1979) did not report doing either control procedure. Thus, another possible explanation for the apparent discrepancy between their results and ours is that their *amnesiac* stock had accumulated modifiers and, therefore, did not display a mutant behavioral phenotype in their larval learning experiments.

Memory retention survives metamorphosis in normal but not mutant flies

Before assaying memory through metamorphosis, several essential control experiments were performed. First, we verified that adults could be conditioned to EA and IA, since we normally train and test adults with the odorants OCT and MCH (Tully and Quinn, 1985; Tully and Gergen, 1986; Gailey et al., 1991; Boynton and Tully, 1992; Dura et al., 1993; Tully and Gold, 1993; cf. Cowan and Seigel, 1986; Asztalos et al., 1991, 1993). With undiluted EA and IA, wild-type flies produced a mean PI of 80 ± 1 (N = 13) immediately after training, the level of which is comparable to experiments using the alcohols. We also conditioned adults using 1000-fold dilutions (in heavy mineral oil) of EA and IA (see Fig. 2*B*), since learning has been shown to be a function of odor concentration (Tully and Quinn, 1985). With such odor concentrations, wild-type, *amnesiac*, and *dunce* flies yielded mean PIs immediately after training of 57 ± 2 (N = 7), 32 ± 9 (N = 6), and 27 ± 2 (N = 5), respectively. For mutant flies, the decrement in learning scores when using a lower odor concentration was not greater than that observed for wild-type flies (cf. Tully and Quinn, 1985). Such results verified that our mutant stocks could smell the diluted odors (cf. Dudai et al., 1976; Dudai, 1979; Quinn et al., 1979).

Half of the larvae that we conditioned were not tested immediately after training. Instead, they were transferred to food

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vials to test as 3-d-old adults—8 d after training—in the adult T-maze. Only the mean PI for wild-type adults of the odor–shock paired group was significantly greater than zero, and it differed from all other groups, which did not differ from each other (see Fig. 3*B*). "Training" wild-type larvae with odors alone or with shock alone did not produce mean PIs greater than zero in adults. These results indicate that *associative* learning was retained through metamorphosis. Memory through metamorphosis was not detected in *dunce* or *amnesiac* mutants (Fig. 3*B*).

The data presented here represent results from one balanced experiment done under one set of experimental conditions. We also have reproduced the memory-through-metamorphosis result in two previous experiments, each using somewhat different procedures and apparatuses. We first documented the phenomenon in 1985 at Princeton University (cf. Tully, 1988). We used a larval *training* apparatus that delivered OCT or MCH to larvae in laminar air currents at 30 ml/min in an environment room at 25°C and 40% relative humidity. Pure solutions of OCT or MCH were contained in "odor tubes," which normally were used in the adult conditioning experiments of Tully and Quinn (1985). The odorants, shock intensity, and larval *testing* apparatus were similar to those of Aceves-Pina and Quinn (1979). Third-instar larvae were trained by exposing them to the CS+ paired with 90 V (AC) shock (US) for 30 sec, followed by a 30 sec rest, 30 sec exposure to the CS−, and 30 sec rest during one training cycle. Sixty seconds after eight successive training cycles, larvae were transferred to the center of a 150-mm-diameter petri dish, in which they were exposed for 90 sec simultaneously to OCT and MCH on filter disks. Then, the larvae on either side of the petri dish were counted, and a learning index was calculated. (A learning index × 100 is algebraically equivalent to a PI in these experiments.) With such conditions, the odor–shock paired group of third-instar larvae yielded a mean PI ± SEM of 23 ± 5 ($N = 8$), while naive, odors-alone, shock-alone, and *amnesiac* groups yielded scores of 2 ± 1 ($N = 3$), 0 ± 2 ($N = 5$), −3 ± 4 ($N = 5$), and 3 ± 6 ($N = 6$), respectively. Adult retention scores for trained larvae were 15 ± 2 ($N = 8$), −1 ± 2 ($N = 5$), 0 ± 4 ($N = 5$), −5 ± 1 ($N = 5$), and −5 ± 5 ($N = 5$) for the odor–shock paired, naive, odors-alone, shock-alone, and *amnesiac* groups, respectively. Furthermore, we detected no memory through metamorphosis in adults after only three larval training cycles (PI = 1 ± 2; $N = 5$).

We ran the experiment a second time in 1990 at Brandeis University, using a new apparatus designed to train *and* to test larvae with odors delivered in laminar air currents (although laminar flow was not as strong in it as in the apparatus depicted in Fig. 1). Experiments were not done in an environment-controlled room, but humid air currents were drawn through the conditioning apparatus (35 ml/min) first by bubbling room air (20–25°C) through distilled water and then through odorants diluted (10^{-3}) in mineral oil. IA and EA were used instead of OCT and MCH. Third-instar larvae were trained by exposing them for 60 sec to CS+, 90 sec rest, 60 sec to CS−, and 90 sec rest. During the latter 30 sec of CS+ exposure, the larvae received shock (90 V AC). After eight cycles of training, larvae were exposed to converging currents of IA and EA for 5 min. These conditions yielded larval mean PIs of 37 ± 10 ($N = 8$), 1 ± 4 ($N = 5$), and −2 ± 7 ($N = 5$) for the odor–shocked paired, odors-alone, and shock-alone groups, respectively. Adult retention scores for trained larvae were 37 ± 10 ($N = 8$), −13 ± 8 ($N = 5$), and −3 ± 9 ($N = 5$) for the odor–shock paired, odors-alone, and shock-alone groups, respectively.

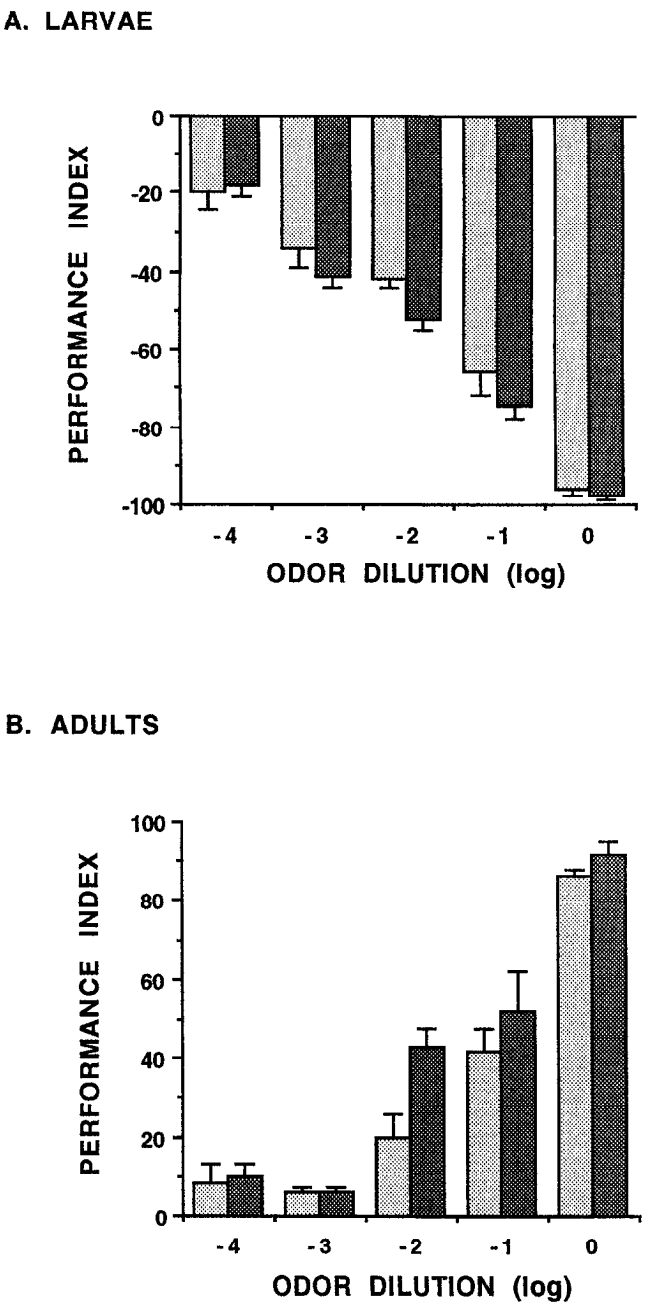

Figure 2. Olfactory acuity in wild-type (Canton-S) larvae and adults. *A*, Different groups of about 50 naive, third-instar larvae were placed in the central chamber of the conditioning apparatus (see Fig. 1*B*) and then exposed to converging currents (each at 35 ml/min) carrying EA versus air (*lighter shading*) or IA versus air (*darker shading*) at each of five dilutions (in heavy mineral oil). After 5 min, the numbers of larvae in each side of the chamber were counted. A performance index (PI) then was calculated (see Materials and Methods). Third-instar larvae normally are *attracted* to EA and to IA, resulting in mean PIs that are negative and that decrease with higher odor concentrations. $N = 5$ PIs for each group, except $N = 4$ for EA-0. *B*, Different groups of about 100 adults 3 d old were placed at the choice point of a T-maze (see Tully and Quinn, 1985) and then exposed to converging currents carrying either EA versus air (*lighter shading*) or IA versus air (*darker shading*) at each of five dilutions (in heavy mineral oil). After 2 min, flies in each arm of the T-maze were trapped, anesthetized, and counted, and a PI then was calculated. These adult flies normally are *repelled* by EA and by IA, resulting in mean PIs that are positive and that increase with higher odor concentrations. $N = 5$ PIs for all groups, except $N = 3$ for IA at a 10^{-2} dilution.

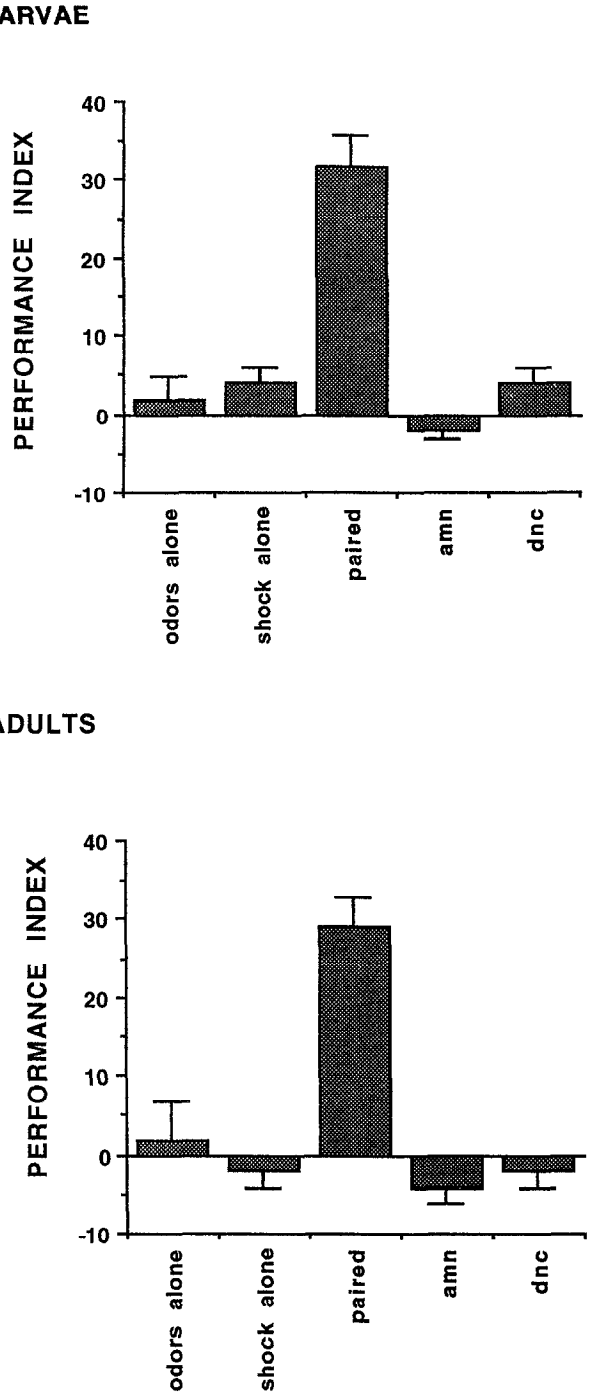

Figure 3. Classical conditioning of odor avoidance in normal and mutant third-instar larvae and retention in adults. $N = 6, 5, 9, 6$, and 5 PIs in each graph for the "odors-alone," "shock-alone," and "paired" wild-type (Canton-S) groups, *amnesiac* and *dunce,* respectively. About 80–100 larvae were placed in the central chamber of the conditioning apparatus (see Fig. 1*A*) and then were conditioned by exposing them sequentially to two undiluted odors for 60 sec each with a 90 sec rest period afterward (with the top of the central chamber removed). During the latter 30 sec of exposure to the first odor (CS+), larvae received 90 V (AC) of continuous shock (US), while no shock was applied during exposure to the second odor (CS−). This 300 sec training cycle was repeated eight times. To prevent confusion from residual odors, larvae gently were transferred back and forth during the rest periods to different apparatuses dedicated to one particular odor. For one complete experiment, two reciprocal groups of larvae were tested; the CS+ and CS− were EA and IA for the first group and IA and EA for the second. *A,* After training, odor avoidance responses for half of the trained larvae were assayed within 90 sec by presenting larvae both EA and IA simultaneously (see Fig. 1*B*). After 5 min, the numbers of larvae in each side of the central chamber were counted. PIs were calculated as in Figure 2, but one datum (N) represented the average PI of two reciprocal groups. *B,* Retention of conditioned odor avoidance was assayed in the other half of conditioned larvae as adult flies 8 d after training, using 1000-fold dilutions of EA and IA with an otherwise standard procedure in a T-maze (see Materials and Methods).

When the data from all three experiments are combined—which certainly is not entirely appropriate from an experimental design perspective—the mean PIs for the odor–shock paired, odors-alone, and shock-alone groups were 31 ± 4 ($N = 25$), 1 ± 2 ($N = 16$), and −1 ± 3 ($N = 15$) for larvae and 27 ± 5 ($N = 25$), −4 ± 3 ($N = 16$), and −3 ± 3 ($N = 15$) for adults, respectively. This observation emphasizes the reproducibility and robustness of the phenomenon.

Discussion

Retention of learning through metamorphosis has been reported in several other invertebrate and an amphibian species, but many earlier studies failed to distinguish between associative and nonassociative factors as causes of the behavioral change (Borsellino et al., 1970; Somberg et al., 1970; Jaffe, 1980; Punzo, 1980a,b, 1983, 1988; cf. Kim et al., 1992). Clear evidence for retention through metamorphosis of *associative* tasks has been reported for leg position learning in locusts, maze learning in grain beetles and shock avoidance learning in frogs (Alloway, 1972; Miller and Berk, 1977; Goldsmith et al., 1978). Early claims for "preimaginal conditioning" of food preference in fruit flies also lacked proper controls for nonassociative changes (Thorpe, 1939). In a definitive experiment, Manning (1967) showed that avoidance of geraniol-flavored food versus regular food by naive adults was changed to no preference (50% avoidance) in adults that were raised on geraniol-flavored food as larvae. Thus, habituation (nonassociative learning) could not be ruled out as a possible cause of food preference learning. In contrast, results from our odor–shock paired groups—when compared to the odors-alone and shock-alone nonassociative control groups—show clearly that preimaginal conditioning in *Drosophila* can be produced by associative processes and is retained through metamorphosis.

Interestingly, the wild-type adult memory through metamorphosis score is similar to the immediate learning score of larvae (see Fig. 3), suggesting no memory decay over 8 d of retention. Such a comparison probably is misleading, however, because of the different behavioral response systems and apparatuses used for larvae versus adults. Retention of adults-trained-as-larvae may be compared more appropriately to that of adults-trained-as-adults; the latter yield retention levels similar to that reported here 7 d after extended training (T. Tully, T. Preat, S. Boynton, and M. Del Vecchio, unpublished observations). Such long-lasting memory in adult flies is produced after 10, but not one or two, training cycles (Tully and Quinn, 1985; Dudai et al., 1988). Similarly, only three cycles of larval training did not produce any memory through metamorphosis (see above). Thus, the stable memory that persists through metamorphosis after extended training of larvae may represent the same long-lasting memory phase that is induced during extended training of adults.

At first glance, the fact that mutant *dunce* and *amnesiac* adults did not show any memory through metamorphosis seems obvious, since they also did not learn as larvae (see above). The

possibility existed, however, that some learning may have occurred but was more difficult to express for larvae crawling in their conditioning apparatus as opposed to adults walking in a T-maze. This notion is supported by the observation that *dunce* and *amnesiac* adults show moderate levels of conditioned avoidance (Tully and Quinn, 1985), while the mutant larvae show *no* conditioned avoidance, immediately after training. Furthermore, memory retention in wild-type adults decays to half of its initial value within 5 hr after one training cycle (Tully and Quinn, 1985), while memory in wild-type larvae decays to half of its initial value within 15 min after three training cycles (Aceves-Pina and Quinn, 1979). Finally, evidence now is accumulating to suggest that LTM may form independently of (i.e., parallel to) earlier phases of memory in rats, chicks, *Aplysia, Hermissenda,* and *Drosophila* (Tully et al., 1990; Allweis, 1991; Andrew, 1991; Emptage and Carew, 1992; Crow and Forrester, 1993; T. Tully, T. Preat, S. Boynton, and M. Del Vecchio, unpublished observations). Thus, our results from *dunce* and *amnesiac* formally exclude the possibility that a latent LTM was induced by larval training, retained through metamorphosis, and then expressed in mutant adults.

A more intriguing implication is that this form of LTM survives extensive reorganization of the nervous system during metamorphosis (cf. Bullock and Horridge, 1965; Norlander and Edwards, 1968; Edwards, 1969; Punzo and Malatesta, 1988). Sensory neurons originate from the periphery during embryogenesis or from imaginal disks during metamorphosis and send projections into the CNS, which itself contains only motoneurons and interneurons (Palka et al., 1984; Hartenstein, 1988). Near the end of larval development, the CNS is composed of two distinct classes of neurons—functional ones, which arose during embryogenesis, and nonfunctional "adult-specific" neurons, which accumulated throughout larval development (Truman and Booker, 1986; Truman, 1990). Although some neurons in abdominal regions of the ventral CNS die during the first hours after pupariation, most neurons in the brain and thoracic regions appear to survive metamorphosis. Many larval motoneurons, for instance, survive, some changing their peripheral targets and others innervating (adult) targets for the first time (Truman, 1990). Serotonergic, catecholaminergic, and some peptidergic larval interneurons also persist into adult stages with only a few cells added in the optic lobe or central brain regions during metamorphosis (White et al., 1986; Budnick and White, 1988; Valles and White, 1988). Thus, most of adult motor function and neuromodulation may derive from neurons of larval origin. Taken together, these observations may explain why conditioned *avoidance* is maintained through metamorphosis even though naive responses to the odorants change from attraction to repulsion.

Our data provide functional evidence consistent with the notion that some of these soma may serve as neural substrates of memory through metamorphosis. To this end, single-gene mutations that produce structural defects in two central brain regions—the mushroom bodies and the central complex—also disrupt olfactory learning in *Drosophila* (Heisenberg et al., 1985). Kenyon cells, which comprise at least half of the mushroom body neurons (Ito and Hotta, 1992), have been shown specifically to survive the pupal stage (Technau and Heisenberg, 1982). During metamorphosis, most of their projections first degenerate to a core of about 500 fibers and then regrow to make new connections (Technau and Heisenberg, 1982). Interestingly, the number of fibers projecting from these Kenyon cells in adult flies are affected by environmental stimulation and by the *dunce* and *rutabaga* memory mutants (Technau, 1984; Balling et al., 1987). Moreover, the $dunce^{+}$ and $rutabaga^{+}$ proteins have been shown to be expressed strongly in larvae and adult mushroom bodies and moderately (at least in *Dunce*) in adult central complex (Nighorn et al., 1991; Han et al., 1992). Using memory through metamorphosis as a relevant functional assay, we expect future work on structural brain mutants to reveal the anatomical sites involved with LTM.

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