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# Genetic Dissection of Memory Formation in *Drosophila melanogaster*

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Over a decade ago, the last mutagenesis to generate mutations affecting associative learning in *Drosophila* ended. In all, about 3500 strains, mutagenized with ethylmethane sulfonate (EMS), were screened first in S. Benzer's laboratory at CalTech and then in W.G. Quinn's laboratory at Princeton (for review, see Aceves-Pina et al. 1983). Associative learning was assayed with an olfactory shock-avoidance conditioning procedure specifically designed to produce an average learning index for a population of flies, which were trained and tested en masse (Quinn et al. 1974). The conditioning procedure employed two conditioned stimuli (CSs): Flies received electric shock (US) if they approached one odor ($CS^+$), but not if they approached a second odor ($CS^-$). In a subsequent test trial, one could conclude that associative learning occurred only if more flies avoided the $CS^+$ than the $CS^-$, and only in this case would the learning index be greater than zero (for more details, see Tully 1984). Significantly, this discriminative, group conditioning procedure precluded the time-consuming process of assaying learning in individuals, thereby increasing the efficiency of screening thousands of mutant strains (see also Tully 1986).

When the mutagenic dust had settled, six mutant strains showed no shock-avoidance conditioning but normal olfactory acuity and shock reactivity. Initial genetic complementation tests suggested that five genes on the X chromosome were mutated. Two EMS-induced alleles of the *dunce* gene were isolated by D. Byers in Benzer's laboratory (Dudai et al. 1976; Byers et al. 1981), while P. Sziber in Quinn's laboratory isolated one mutant allele for each of the *rutabaga, cabbage, turnip,* and *radish* genes (Aceves-Pina and Quinn 1979; Duerr and Quinn 1982; Aceves-Pina et al. 1983). P. Sziber also identified a sixth X-linked gene, *amnesiac,* by conditioning flies from mutant strains with a modified shock-avoidance procedure in which conditioned responses were assayed 45 minutes after training. Under these conditions, *amnesiac* mutants learned normally but showed abnormally rapid memory loss (Quinn et al. 1979). Flies from each of these six mutant strains subsequently were shown to perform poorly in several other behavioral tasks thought to involve some aspect of learning (Siegel and Hall 1979; Booker and Quinn 1981; Duerr and Quinn 1982; Folkers 1982; Gailey et al. 1982, 1984; Tempel et al. 1983; Kyriacou and Hall 1984).

A fundamental genetic issue that arises from such mutagenesis is whether the aberrant phenotype of a mutant strain is produced by one or more than one mutation. The EMS procedure employed was designed to produce one lethal mutation in about 30% of X chromosomes (Lewis and Bacher 1968). Two or more viable mutations on the same chromosome can be produced, however. Moreover, mutagenized flies already may carry spontaneous mutations, which then might be isolated in the phenotypic screen. Evidence for two (complementing) mutations on the same chromosome frequently has been documented in mutagenesis for other traits. Sometimes the mutations affected unrelated phenotypes (Johnson et al. 1981); sometimes they affected similar phenotypes (Baker and Carpenter 1972; Kulkarni and Hall 1987).

Examples of each outcome also exist for the learning mutants. The *dunce* gene now is known to be involved with both associative learning and female fertility. Unlike $dunce^2$ mutants, however, $dunce^1$ flies were not sterile. Subsequent genetic analyses later revealed that $dunce^1$ flies carried a second mutation, a dominant suppressor of sterility, in another X-linked gene (Saltz et al. 1982); *rutabaga* flies also carry a second, X-linked mutation that produces "blistered" wings (T. Tully, unpubl.). Mutant *turnip* flies appear to be even more complicated genetically: The *turnip* X chromosome carries a lethal mutation and a suppressor of lethality, both of which map distal to the *turnip* locus (R.F. Smith et al., unpubl.), and a third mutation that affects protein kinase C (PKC) activity (Smith et al. 1986). The PKC-disrupting mutation most likely is not solely responsible for abnormal learning in *turnip* flies. This phenotype may result from an interaction between the "PKC" mutation and at least one other autosomal gene (Tully 1986). Given such caveats, it is important to note that genetic analyses only with *dunce, rutabaga,* and *amnesiac* flies have provided clear evidence that their mutant behaviors result from single-gene mutations (Byers 1980; Byers et al. 1981; Livingstone et al. 1984; Dudai et al. 1985; Livingstone 1985; Tully and Gergen 1986). Convincing evidence for the *cabbage, radish,* and *turnip* strains still is lacking.

Of the three bona fide single-gene learning/memory

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mutations, biochemical abnormalities have been identified for two (for reviews, see Kiger and Saltz 1985; Heisenberg 1989). The *dunce* mutations disrupt a cAMP-specific phosphodiesterase (Byers et al. 1981; Kiger et al. 1981; Kauvar 1982; Shotwell 1983), and the *rutabaga* mutation disrupts several biochemical properties of adenylate cyclase (Livingstone et al. 1984; Dudai et al. 1985; Livingstone 1985). Davis and Davidson (1984) capitalized on the aberrant phosphodiesterase activity of *dunce* mutants to obtain DNA clones from the *dunce* region via linkage analyses with restriction site polymorphisms. Subsequent DNA sequence analysis has confirmed that the *dunce* gene encodes a phosphodiesterase (Chen et al. 1986).

Although these biochemical discoveries, implicating the cAMP cell-signaling pathway with associative learning in *Drosophila*, are impressive, one can argue that little progress toward a molecular understanding of the phenotype has been made. Compared to analyses of many other phenotypes in fruit flies, in fact, this behavior-genetic analysis has proceeded at glacial speed. In hindsight, however, we can attribute such slow progress to two technical problems:

1. Measuring differences among individuals (or genotypes) for behavioral phenotypes is time-consuming. Learning and memory, in particular, require excessive time to assay. Furthermore, since behavioral responses are ephemeral in design, many environmental factors conspire to make individual scores vary, thereby demanding larger sample sizes to obtain accurate estimates of genotypic effects. Thus, routine genetic experiments to generate new mutations or to map single-gene effects require such an investment of time as to make even the most resolute hesitate.
2. In addition to frequently isolating more than one mutation per chromosome, EMS mutageneses also usually produce single base-pair substitutions or small deletions, which are difficult to detect at the DNA level with existing molecular techniques. Therefore, further genetic experiments are required to isolate (molecularly visible) chromosomal breakpoints in or near the gene of interest. Even then, a tedious chromosomal walk often is necessary to clone the relevant stretch of genomic DNA. Importantly, identification of a second, pleiotropic phenotype that is easier to assay than the behavioral one can speed up genetic experiments. In fact, such was the case for the *dunce* and *rutabaga* genes. Usually, however, looking for pleiotropic effects is akin to looking for a needle in a haystack. A case in point is work on the *amnesiac* gene. Extensive biochemical experiments have detected no pleiotropic effects, and a chromosomal walk is mired in repetitive DNA (C. Brandes and T. Tully, unpubl.).

To remedy this situation, we at Brandeis have begun a mutagenesis in *Drosophila* to isolate new mutations affecting associative learning and memory. Our mutagens are genetically engineered P-element transposons (see Bier et al. 1989), which first can be mobilized to jump randomly into genes, thereby disrupting them. Then, further transposition can be stabilized. Significantly, these P-element mutators represent molecular "tags," which are used to identify adjacent DNA sequence from the disrupted gene, thereby expediting its cloning. In addition, these mutators contain functional DNA sequences from other *Drosophila* genes, which encode products involved with eye pigmentation. Thus, in an appropriate genetic background, the mutator provides a "pleiotropic" morphological tag, which greatly facilitates subsequent genetic analyses of the behavioral phenotype. We describe behavioral and genetic work on the first P-element insertion mutant we have isolated.

To interpret the phenotype of our new mutant properly, we first describe behavioral experiments on normal (wild-type) and *amnesiac* flies. We have focused our attention on memory formation after an olfactory classical conditioning procedure (Tully and Quinn 1985). Our results suggest that three behaviorally distinct phases, or components, of memory underlie normal memory retention during the first 7 hours after training. Moreover, the *amnesiac* mutation may disrupt one of these components. We also introduce a new training procedure that substantially improves 24-hour memory in wild-type flies.

## METHODS

***Subjects.*** *Drosophila melanogaster* were of the wild-type Canton-S (Can-S) strain, a *white*$^{1118}$ strain, and the *amnesiac* memory mutant strain (Quinn et al. 1979). Over the years, we have observed that the abnormal behavioral phenotypes of many of the extant learning/memory mutants become more normal with time. Presumably, flies carrying the mutant phenotype are less fit than wild-type flies, and natural selection over generations produces an accumulation of phenotype-ameliorating modifying alleles in the genetic background of mutant flies. To minimize this effect, we (1) replaced the second and third chromosomes in the original *amnesiac* strain, using the double balancer strain *y*; *Pm*/*CyO*; *Sb*/*TM6*, (2) maintained the *amnesiac* X chromosome over the *FM7a* X-chromosome balancer, which itself was outcrossed repeatedly to the Can-S strain to "cantonize" the autosomes, (3) only bred heterozygous *amn*/*FM7a* females (*amn* is recessive to the *amn*$^{+}$ allele in *FM7a* flies) to *FM7a* males every generation, and (4) bred homozygous *amn* flies every few months to use in behavioral experiments.

The mutant *latheo (lat)* was generated in our laboratory from an ongoing P-element insertional mutagenesis, the general details of which have been published elsewhere (Cooley et al. 1988; Tully 1990). We used a "mutator" strain, *w*,*9.3*, which contained a single, genetically engineered P-element transposon on the X chromosome (D. Coen and D. Anxolabehare, in prep.) in conjunction with the *Sb*Δ*2-3*, *ry*/*TM6* "transposase donor" strain (Robertson et al. 1988). Indepen-

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dent transposition events were isolated first in heterozygous form, and then strains homozygous for each single P-element insertion were bred. Mutant *latheo* flies were the first to satisfy our behavioral and genetic criteria (see below). The appropriate control strain for behavioral comparisons with *latheo* was $white^{1118}$ *[w(H)]*, which was cantonized by 4 generations of outcrossing to Can-S flies. Heterozygous, *w, lat/w,* + flies were outcrossed to *white* flies each generation to minimize any possible accumulation of genetic modifiers. Homozygous *w, lat/w, lat* flies then were bred regularly from the heterozygous strain for use in behavioral experiments.

***Classical conditioning.*** Tully and Quinn (1985) modified the T-maze chamber of Dudai et al. (1976) so that carefully controlled currents of air could be drawn through it (Fig. 1). In this manner, the instrumental shock-avoidance conditioning procedure of Quinn et al. (1974) was adapted to a classical conditioning procedure, in which flies always received negative reinforcement in the presence of the $CS^+$. About 100 flies were sequestered in a closed chamber and were trained by exposing them sequentially to two odors (either 3-octanol [OCT] or 4-methylcyclohexanol [MCH]) delivered in air currents. In the standard procedure, flies received a 60-second presentation of the first odor ($CS^+$) along with 12 1.25-second 60-V (DC) pulses of electric shock every 5 seconds (US), a 30-second rest, a 60-second presentation of the second odor ($CS^-$) without shock, and finally another 30-second rest.

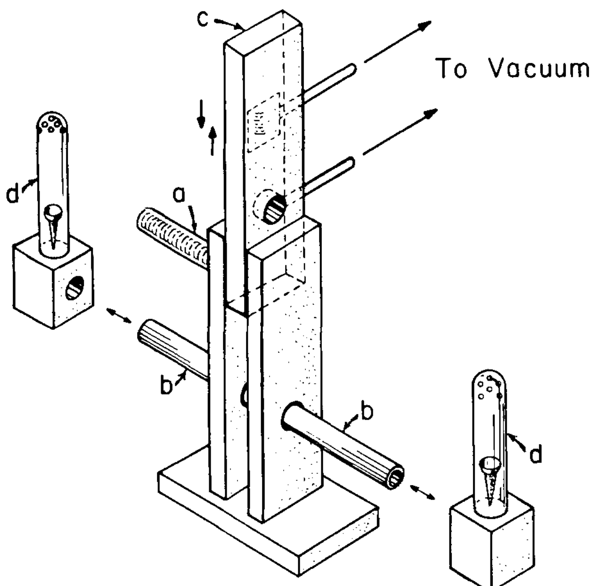


**Figure 1.** Classical conditioning apparatus of Tully and Quinn (1985). A group of about 100 flies are sequestered in the training chamber (*a*) and exposed sequentially to two different odors (contained in *d*)—one presented alone and the other presented along with electric shock. After training, flies are transferred via a miniature elevator (*c*) to the choice point of a T-maze, where they are exposed to the same two odors simultaneously in converging currents of air. After the test trial, flies are trapped in the arms of the T-maze (*b*), anesthetized, and counted (see text for more details).

To test for conditioned avoidance, flies were tapped gently into an elevator-like sliding compartment and were transported to the T-maze choice point of the teaching machine, between converging currents of OCT and MCH. After 120 seconds, the center compartment was slid up, trapping flies in the arms of the T-maze. Typically, 90% of wild-type (Can-S) flies avoided the $CS^+$, 5% avoided the $CS^-$, and 5% remained at the choice point.

After conclusion of the test trial, flies were anesthetized and counted. A "half lambda" was calculated as the fraction of flies avoiding the $CS^+$ (they ran toward $CS^-$) minus the fraction of flies avoiding the $CS^-$ (they ran toward $CS^+$). Flies remaining at the choice point were included in the total used to compute fractions. A second group of flies then was trained and tested as above using reciprocal odors for $CS^+$ and $CS^-$ (i.e., if OCT was $CS^+$ for the first group, then MCH was $CS^+$ for the second). A half lambda was calculated for the second group of flies, and then the two half lambdas were averaged to yield one learning index. In this manner, nonassociative changes in odor avoidance and any slight odor biases were eliminated arithmetically from the learning index. Concentrations of OCT and MCH were adjusted so that naive flies distributed themselves 50:50 in the T-maze during the test trial. Thus, if no associative learning occurred, then the learning index would be zero. Conversely, if all flies learned perfectly to associate a specific odor with electric shock, then all flies would avoid the $CS^+$ and the learning index would be one. Typically, wild-type flies yielded an average learning index of 0.85 (for more details, see Tully and Quinn 1985).

***Memory retention.*** Groups of flies were trained as above, except that the $CS^-$ was presented first, followed by the $CS^+$. Within 90 seconds after training, the flies were removed from the training chamber and stored at 25°C in the dark for 10, 15, 20, 30, 60, 120, or 180 minutes in plastic test tubes (Falcon no. 2017) containing pieces of filter paper soaked in 4% sucrose solution. Flies were aspirated from the test tubes to the choice point of the T-maze 70 seconds before the usual 120-second test trial. At retention time 0 in Figure 2, flies were transferred from the training chamber to the T-maze choice point 120 seconds after the shock stimulus ended. In memory retention experiments with *latheo* flies, equal numbers of mutant and *w(H)* control flies were mixed and then were trained, stored in glass shell vials with food, and tested together. Afterward, learning indices for each strain were obtained by separating the two genotypes according to eye color (*latheo* flies were red-eyed; *w(H)* flies were white-eyed).

***Retrograde amnesia.*** Groups of flies were transferred 0, 10, 20, 30, 60, or 120 minutes after training (second odor shocked as above) to a 3.5 × 1.2-cm glass test tube, and the test tube was submerged in salted ice water (0°C) for 2 minutes. Flies stopped moving and fell to the bottom of the test tube within 30 seconds

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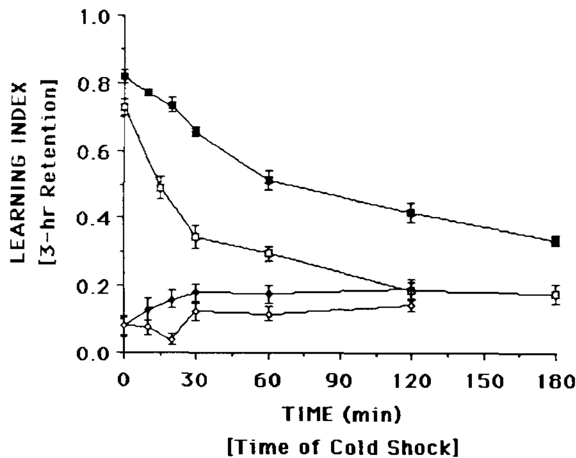


**Figure 2.** Memory retention and anesthesia-resistant memory in wild-type (Canton-S) and *amnesiac* flies. Retention was determined by training flies in the standard classical conditioning procedure and then by assaying conditioned avoidance responses (LEARNING INDEX) at various intervals (TIME) afterward. Memory decays with time in wild-type flies (■) but decays more quickly in *amnesiac* flies (□). Anesthesia-resistant memory was inferred from retrograde amnesia experiments, in which 3-hr retention was assayed in flies that received cold-shock anesthesia at various intervals (Time of Cold Shock) after training. Flies showed progressively higher 3-hr memory scores as the interval of time between training and cold-shock anesthesia increased. This result implies that an anesthesia-resistant phase of memory begins to form during training and reaches maximal levels 1–2 hr later. Interestingly, anesthesia-resistant memory levels were similar in wild-type (◆) and *amnesiac* (◇) flies. $n = 18$ and 8 learning indices at each memory retention interval for wild-type and *amnesiac* flies, respectively. $n = 8$ and 12 learning indices at each cold-shock interval for wild-type and *amnesiac* flies, respectively.

after being placed in ice water. Flies recovered from this "cold-shock" within 30 seconds after being removed to 25°C. Three-hour retention was assayed for each of these posttraining cold-shock groups, as well as for a pretraining control group that was cold-shocked 60 minutes before training. During the time intervals between training and cold-shock, and between cold-shock and testing, flies were stored in plastic test tubes containing a strip of filter paper soaked with 4% sucrose.

***Reversal learning.*** After one training cycle, during which the second odor was paired with shock, groups of flies were retrained 0, 10, 20, 30, 60, or 180 minutes later by pairing the reciprocal odor with shock, i.e., if OCT was $CS^+$ in cycle 1, then MCH was $CS^+$ in cycle 2. Conditioned avoidance responses were measured immediately after cycle 2 training by transferring the flies to the T-maze for 120 seconds. To calculate a learning index, "$CS^+$" was the $CS^+$ of cycle 2. During the time intervals between cycle 1 and cycle 2 training, flies were stored in glass shell vials containing their usual food medium.

***Long-term memory.*** Groups of flies received ten, instead of one, training cycles, with a 15-minute intercycle interval. Odor concentrations were a $10^{-3}$ dilution in mineral oil. Conditioned avoidance responses were tested 24 hours after training in the usual manner. During the retention interval, flies were stored in glass shell vials with food. They then were transferred to the T-maze choice point 90 seconds before the 120-second test trial began.

## RESULTS

### Memory Retention and Retrograde Amnesia in Wild-type and *amnesiac* Flies

Figure 2 compares memory retention curves between wild-type (Can-S) and *amnesiac* flies. As originally reported by Tully and Quinn (1985), *amnesiac* flies showed near-normal learning followed by a more rapid memory decay during the first hour after training. Thereafter, the *amnesiac* memory decay rate was similar to that of wild-type flies, suggesting that the *amnesiac* mutation interfered with early memory formation.

Results from retrograde amnesia experiments seem to support this notion. Cold-shock anesthesia administered to wild-type flies immediately after training served to diminish 3-hour retention levels. This amnestic effect was less severe, however, when cold-shock was administered at later intervals after training (see Fig. 2; cf. Quinn and Dudai 1976; Tempel et al. 1983; Tully 1988). Interestingly, the effect of retrograde amnesia in *amnesiac* flies was similar to that in wild-type flies.

Traditionally, retrograde amnestic effects have been interpreted to indicate that an anesthesia-resistant phase of memory begins to form during training, or immediately thereafter, reaching maximal levels within a few hours (cf. Andrew 1980). Thus, memory formation after classical conditioning in *Drosophila* appears to be composed of anesthesia-sensitive and anesthesia-resistant components. This idea is visualized in Figure 2 by plotting results from the retrograde amnesia experiments along with the memory retention curves of wild-type (Can-S) and *amnesiac* flies. Comparisons of memory retention curves with the anesthesia-resistant memory (ARM) curves suggest that a cold-shock-sensitive phase of memory still is present 2 hours after training in wild-type flies. In contrast, no such memory phase is detectable 2 hours after training in *amnesiac* flies. In other words, ARM can account entirely for memory levels 2 (or more) hours after training in *amnesiac* flies but not in wild-type flies. So what is the nature of the cold-shock-sensitive phase of memory in wild-type flies?

### Components of Memory

One approach to answering the question above is to decompose the wild-type memory retention curve into additive components. First, the ARM curve (wild-type and *amn* data combined) can be subtracted from the *amnesiac* retention curve to reveal a short-term compo-

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nent presumably present in both wild-type and *amnesiac* flies. Second, the *amnesiac* retention curve can be subtracted from the wild-type retention curve to reveal another component presumably present in wild-type flies but missing in *amnesiac* flies. Thus, Figure 3 shows the three resulting hypothetical components of memory, which we refer to as short-term memory (STM), middle-term memory (MTM), and anesthesia-resistant memory (ARM). The kinetics of these three memory components are surprisingly similar to a model of memory formation proposed earlier (Tully 1988) and to results from pharmacological experiments on memory formation in chicks and on long-term potentiation (Gibbs and Ng 1976; Patterson et al. 1986; Matthies 1989).

At this stage of model building, we must emphasize two important points: (1) The components of memory that we have derived are based on the assumptions that memory phases act additively to produce overall memory retention and that the *amnesiac* mutation eliminates a specific component. (2) Although we empirically can distinguish the ARM component from earlier components (STM and MTM), we have not yet provided experimental evidence for the existence of separate STM and MTM components. These memory components exist only by assuming that the *amnesiac* mutation disrupts MTM specifically. An alternative hypothesis is that classical conditioning produces only one early (cold-shock-sensitive) phase of memory and therefore the *amnesiac* mutation produces a quantitative effect on this early phase (STM), rather than eliminating a qualitatively different component (MTM). Interpretation of results from the next experiment begins to shed light on these two alternative hypotheses.

### Reversal Learning in Wild-type and *amnesiac* Flies

Although reversal learning per se has been done before with olfactory conditioning in *Drosophila*

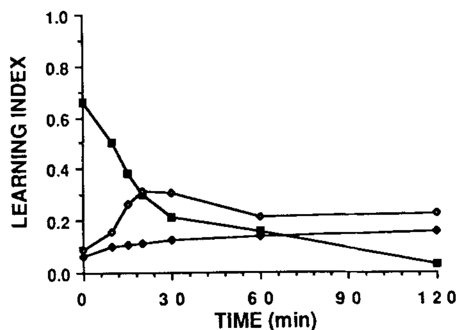


**Figure 3.** Hypothetical components of memory in wild-type flies. Anesthesia-resistant memory (ARM) component (◆), the average of wild-type and *amnesiac* data, was derived empirically from retrograde amnesia experiments. The short-term memory (STM) component (■) was obtained by subtracting ARM from the *amnesiac* retention curve in Fig. 2. The middle-term memory (MTM) component (◇) was obtained by subtracting the *amnesiac* retention curve from the wild-type retention curve. See text for more details.

(Quinn et al. 1974, 1979; Dudai 1983; Tully and Quinn 1985), we extended the experiment to determine the interaction of reversal learning (but not memory induced by it) with memory present at *several* retention intervals after cycle 1 training (see Methods). Much to our surprise, the resulting "reversal retention" curves of wild-type and *amnesiac* flies were not significantly different (Fig. 4). One way to interpret these results is that an environmental manipulation (reversal learning) has eliminated the phenotypic difference in (cycle 1) memory between wild-type and *amnesiac* flies (compare Figs. 2 and 4).

More detailed analysis of results from the reversal learning experiment also supports the hypothesis that STM and MTM are functionally distinct phases of memory. Figure 5 plots the observed reversal retention curve for wild-type and *amnesiac* flies combined (open diamonds connected by solid line), along with other "retention" curves representing hypothetical outcomes of the experiment. First, if reversal learning had no effect whatsoever, then the lowest curve in Figure 5 would represent memory retention induced by cycle 1 training. The learning indices are negative because of the way they are calculated in the reversal learning experiment (see Methods). Second, if reversal learning always was of the same magnitude (represented by arrows in Fig. 5) and *interacted additively* with cycle 1 memory at all retention intervals, then one expected reversal retention curve would resemble the curve at the top of the arrows in Figure 5. It follows that the difference between the observed reversal retention curve and this expected reversal retention curve (hatched area in Fig. 5) represents the *non*additive interaction of reversal learning with cycle 1 memory. Finally, if reversal learning completely disrupted cycle

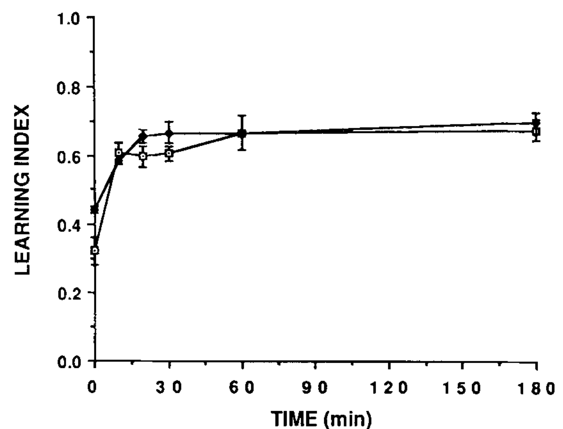


**Figure 4.** "Reversal retention" in wild-type (□) and *amnesiac* (◆) flies. Groups of flies were trained in the standard classical conditioning procedure in cycle 1. At various time intervals after cycle 1 training, the flies then were retrained (cycle 2) to the reciprocal odor combination, i.e., the odor that was $CS^+$ in cycle 1 was $CS^-$ in cycle 2 and vice versa (reversal learning). Conditioned avoidance responses (learning index) were assayed immediately after cycle 2 (re)training. The resulting reversal retention curves were similar for wild-type and *amnesiac* flies. $n = 4$ at 0, 10, 20, 30, and 60 retention intervals for wild-type and *amnesiac* flies. $n = 2$ for both genotypes at the 180-min interval.

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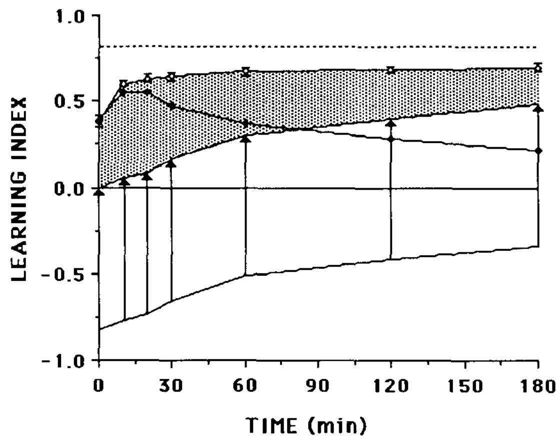


**Figure 5.** Reversal retention (for wild-type and *amnesiac* flies combined) in relation to hypothetical outcomes. If reversal learning did not occur, then the lowest line would be expected. If, however, reversal learning (*arrows*) interacted additively with memory of cycle 1 training, then the line on top of the arrows would be expected. The difference (*hatched area*) between this line and the observed reversal retention curve (◇) represents the nonadditive interaction of reversal learning with cycle 1 memory. This nonadditive effect (◆) can be considered a component of cycle 1 memory that is disrupted or "erased" by reversal learning and is similar to the hypothetical MTM component in Fig. 3. If reversal learning completely disrupted cycle 1 memory, then the dotted line would be expected. The difference between this line and the observed reversal retention curve represents cycle 1 memory that is not disrupted by reversal learning. Interestingly, this reversal-resistant memory appears to be composed of two components, perhaps corresponding to STM and ARM (see Fig. 3).

1 memory at all retention intervals, then a second expected reversal retention curve would be the dotted line in Figure 5.

A more meaningful interpretation of this analysis is that reversal learning disrupts or "erases" a component of cycle-1-induced memory. The kinetics of this phase of memory (solid diamonds connected by solid line in Fig. 5) are strikingly similar to those of the MTM component in Figure 3. These observations suggest that MTM in wild-type flies may be disrupted by reversal learning. Moreover, the existence of such a reversal learning-sensitive (MTM) phase in wild-type flies supports the hypothesis that the *amnesiac* mutation completely disrupts the same component of memory.

One final observation is that the difference between the second expected reversal retention curve (dotted line) and the observed reversal retention curve (open diamonds connected by solid line) represents reversal learning-resistant memory, which appears to be composed of two components—an early one lasting about 30 minutes and a later one lasting at least 180 minutes. These kinetics are similar to that of STM and ARM, suggesting that STM may be reversal learning-resistant, whereas MTM is reversal learning-sensitive (i.e., the two phases are functionally distinct). Taken together with the observation that the *amnesiac* mutation appears primarily to disrupt MTM, these data also suggest that STM, MTM, and ARM are genetically distinct components.

## Long-term Memory

Traditional views of memory consolidation would postulate that ARM (see Figs. 2 and 3) in *Drosophila* is a form of long-term memory. In fact, ARM levels in wild-type flies can account for overall retention levels 7 hours after classical conditioning (S. Boynton and T. Tully, data not shown), and ARM still is detectable 24 hours after similar training (Dudai et al. 1988). Moreover, memory retention in *amnesiac* flies can last 24 hours (Tully and Quinn 1985), and we have shown that ARM can account for overall retention levels in this mutant strain within 3 hours after training (see Fig. 2).

We have been perplexed, however, over the low levels of 24-hour memory displayed by wild-type flies. At best, our standard classical conditioning procedure yields 24-hour memory scores of $0.16 \pm 0.03$. Past attempts to increase 24-hour memory via extended training procedures failed, whether such training was massed or distributed (T. Tully and S. Boynton, unpubl.; cf. Woodworth and Schlosberg 1954). Recently, however, we have produced 24-hour memory scores of $0.44 \pm 0.01$ (a threefold improvement) by using a distributed training procedure with diluted odor concentrations (see Methods). In addition, conditioned avoidance responses still can be detected at least 4 days after such training (T. Preat and T. Tully, in prep.). Taken together, these data clearly demonstrate the existence in *Drosophila* of long-term memory with behavioral properties similar to long-term memories in other species (also see Tully and Quinn 1985 for additional behavioral properties of classical conditioned olfactory avoidance responses).

## Behavioral and Genetic Characterization of a New Mutant *latheo*

For the last 3 years, we have been generating autosomal mutations via a P-element insertional mutagenesis (see Methods). The breeding scheme was designed so that random transposition events were isolated on autosomes, and strains were made homozygous for each independent P-element insertion. Groups of flies from each of these mutant strains then were classically conditioned and tested 3 hours after training. Any strain that produced a mean 3-hour memory score reliably and significantly lower than that of control *w(H)* flies was subjected to further behavioral and genetic analyses. To date, we have screened over 2000 mutant strains, have identified one new mutant (described below), and still are "chasing" nine other putative mutants.

In Figure 6, we compare memory retention during the first 180 minutes after training in *latheo* flies with that in $lat^{+}$ control flies. In these experiments, the appropriate control strain was *w(H)*, instead of Can-S, since the P-element transpositions were induced in a *w(H)* genetic background (see Methods). As shown, memory retention in *latheo* flies was significantly lower than that in wild-type flies at every retention interval,

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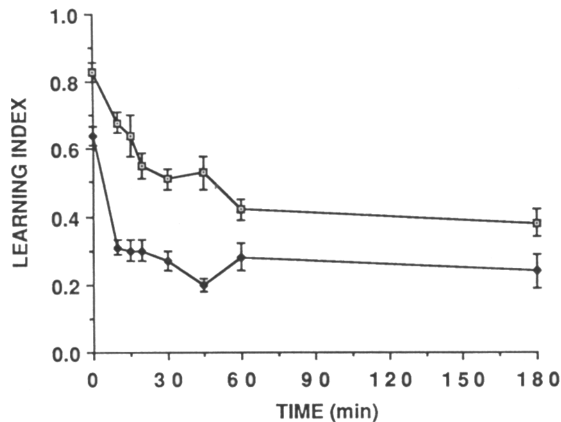


**Figure 6.** Memory retention in control *w(H)* (□) and mutant *latheo* (◆) flies, which were trained and tested together (see text). Mutant flies show less memory at all retention intervals. In addition, memory in *latheo* flies decays more rapidly during the first 15 min after training. $n$ = 6,4,4,4,8,4,8, and 6 learning indices for retention intervals 0,10,15,20,30,45,60, and 180, respectively, for both genotypes.

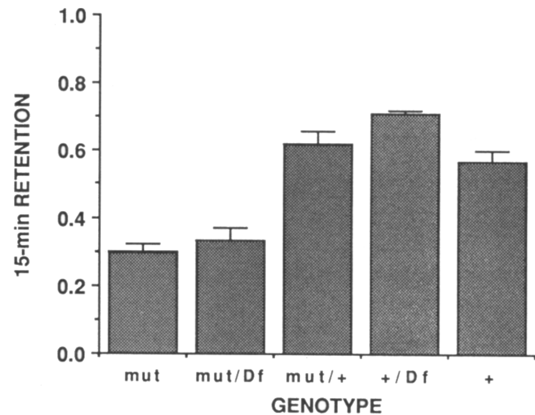


**Figure 7.** Cytological mapping of the mutant *latheo* phenotype. Flies from a strain carrying a small chromosomal deficiency (Df) of the *latheo* region were mated with *latheo* flies (mut), or with *w(H)* flies (+) to produce the genotypes shown here. Heterozygous mut/ + flies produce normal scores, indicating that the *latheo* mutation is recessive to its wild-type allele. More importantly, mut/Df flies produce mutant scores, and +/Df flies produce normal scores, indicating that the mutant phenotype maps to the region containing the P-element insertion. $n$ = 2 learning indices for each genotype.

including 180 minutes. More interestingly, *latheo* memory decayed more rapidly during the first 15 minutes after training (this effect appeared as a significant interaction between TIME and STRAIN in a 2-way ANOVA). Importantly, olfactory avoidance responses to OCT versus Air or to MCH versus Air over a wide range of odor concentrations (a test of olfactory acuity) and escape responses to electric shock from 0 to 60 V (a test of shock reactivity), were normal in naive (untrained) *latheo* flies (S. Boynton and T. Tully, data not shown). Thus, the observed performance deficit in *latheo* flies most likely does not result from abnormal function of sensory or motor systems underlying conditioned avoidance responses.

Corroborative results from two sets of genetic experiments confirm that the mutant phenotype of *latheo* flies was produced by a single-gene mutation associated with a P-element insertion. In the first set of experiments, the cytological location of the autosomal P-element insert was mapped in situ using chromosome squashes from *latheo* flies and DNA sequence from the P-element mutator as a probe. Next, a strain carrying a chromosomal deficiency (Df) of the *latheo* region was obtained from the *Drosophila* stock center in Bloomington, Indiana, and these flies were mated with either *latheo* (mut) or *w(H)* (+) flies to produce mut/Df, +/Df, or mut/ + heterozygous offspring. Finally, 15-minute memory retention was assayed in these heterozygotes, as well as in mut/mut and + / + homozygotes. Figure 7 shows that mut/Df flies yielded a mutant memory score, whereas mut/ + and Df/ + flies yielded wild-type memory scores. These results indicate that the mutation responsible for the *latheo* phenotype maps to a region close to the P-element insertion and that the *lat* mutant allele is recessive to its wild-type counterpart.

In a second set of experiments, a breeding scheme was designed to produce excisions of the P-element insert in *latheo* flies. To date, nine independent excision events, visualized by loss of red eyes in some *latheo* progeny, have been isolated, and strains homozygous for each excision allele have been bred. Fifteen-minute retention scores produced by two of these excision strains were similar to those of *w(H)* flies (S. Boynton and T. Tully, data not shown). The appearance of wild-type "revertant" flies is expected only if the (previously disrupted) gene containing a P-element insertion actually is responsible for the mutant phenotype of *latheo* flies.

## DISCUSSION

We are encouraged by results from behavioral experiments on wild-type and *amnesiac* flies suggesting that memory formation after classical conditioning is composed of three distinct phases (STM, MTM, and ARM) with distinct properties. STM and ARM appear to be reversal learning-resistant, MTM is not; ARM is cold-shock anesthesia-resistant, MTM is not (we do not yet know if STM is cold-shock-sensitive). Most importantly, the *amnesiac* mutation specifically may disrupt MTM, leaving STM and ARM substantially intact. If this "components of memory" hypothesis withstands further behavioral and genetic scrutiny, then we will be able to conclude two important facts: (1) These memory phases are genetically distinct and (2) ARM, and possibly LTM, can be induced by STM in the absence of MTM. This latter conclusion would establish the notion that memory formation proceeds in a parallel rather than, or in addition to, a sequential fashion.

The behavioral experiments reported here, however, were neither perfect nor perfectly designed. Consequently, it is important to stress that our component model of memory formation must be considered pre-

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liminary. More behavioral and genetic work must be done, in particular, to determine whether ARM is completely normal in *amnesiac* flies (see Fig. 2), to establish a functional distinction between STM and MTM, and to assay each of these memory phases independently in *amnesiac* and other learning/memory mutants. Finally, we only have begun to study the relation of our distributed training-induced LTM to the other memory phases in normal and mutant flies. On the behavioral level alone, the next decade promises to be intriguing.

Memory retention in mutant *latheo* flies resembles that of *amnesiac* flies, suggesting that both genes are involved with MTM. We may confirm this notion by studying memory formation in *amnesiac, latheo* double mutants. Many such "phenogenetic" analyses of different genotypic combinations will be possible as additional P-element mutants are identified, contributing in yet another way to our understanding of the molecular basis of associative memory.

Molecular genetic analysis of our new P-element insertional mutants also holds great promise. These mutations contain both a morphological and a molecular "tag," which will expedite molecular cloning of these new learning/memory genes. In this manner, we can gain experimental access to gene products without making any assumptions about, or doing needle-in-haystack searches for, underlying biochemical, physiological, or structural mechanisms of memory formation. With the gene products in hand, we will be able to ask whether similar genes and protein are involved with learning and memory in other species.

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