latheo, a New Gene Involved in Associative Learning and Memory in *Drosophila melanogaster*, Identified from *P* Element Mutagenesis

Susan Boynton and Tim Tully[1]

Department of Biology, Brandeis University, Waltham Massachusetts 02254

Manuscript received January 3, 1992
Accepted for publication March 28, 1992

ABSTRACT

Genetic dissection of learning and memory in Drosophila has been limited by the existence of ethyl methanesulfonate (EMS)-induced mutations in only a small number of *X*-linked genes. To remedy this shortcoming, we have begun a *P* element mutagenesis to screen for autosomal mutations that disrupt associative learning and/or memory. The generation of "*P*-tagged" mutant alleles will expedite molecular cloning of these new genes. Here, we describe a behavior-genetic characterization of $latheo^{P1}$, a recessive, hypomorphic mutation of an essential gene. $latheo^{P1}$ flies perform poorly in olfactory avoidance conditioning experiments. This performance deficit could not be attributed to abnormal olfactory acuity or shock reactivity–two task-relevant "peripheral" behaviors which are used during classical conditioning. Thus, the $latheo^{P1}$ mutation appears to affect learning/memory specifically. Consistent with chromosomal *in situ* localization of the *P* element insertion, deficiencies of the 49F region of the second chromosome failed to complement the behavioral effect of the $latheo^{P1}$ mutation. Further complementation analyses between $latheo^{P1}$ and lethal alleles, produced by excision of the $latheo^{P1}$ insert or by EMS or γ-rays, in the 49F region mapped the *latheo* mutation to one vital complementation group. Flies heterozygous for $latheo^{P1}$ and one of two EMS lethal alleles or one lethal excision allele also show the behavioral deficits, thereby demonstrating that the behavioral and lethal phenotypes co-map to the same locus.

MUTANT analysis in Drosophila has allowed genetic dissection of behaviors ranging from leg shaking to courtship (review: HALL 1985). Associative learning is one such behavior for which a genetic approach has been amenable (review: TULLY 1987). Wild-type flies are capable of learning to avoid an odorant which previously was paired with electric shock (QUINN, HARRIS and BENZER 1974; TULLY and QUINN 1985). Five ethyl methanesulfonate (EMS)-induced *X*-linked mutant strains that consistently failed to learn or remember an instrumental shock avoidance task have been isolated (DUDAI *et al.* 1976; ACEVES-PINA and QUINN 1979; QUINN, SZIBER and BOOKER 1979). These mutant strains subsequently were shown to perform poorly in other behavioral assays thought to involve some aspects of learning (SIEGEL and HALL 1979; BOOKER and QUINN, 1981; DUERR and QUINN 1982; FOLKERS 1982; GAILEY, JACKSON and SIEGEL 1984; KYRIACOU and HALL 1984; WITTEKIND and SPATZ 1988; CORFAS and DUDAI 1989), suggesting that these gene products act centrally in a general learning process.

Behavioral deficits in the mutant strains *dunce*, *rutabaga* and *amnesiac* are known to derive from single-gene effects (BYERS, DAVIS and KIGER 1981; LIVINGSTONE, SZIBER and QUINN 1984; LIVINGSTONE 1985; DUDAI *et al.* 1985; TULLY and GERGEN 1986). Underlying biochemical lesions, however, are known only for *dunce* and *rutabaga*. Biochemical assays on aneuploid flies originally suggested that the *dunce* gene encoded the structural gene for a cAMP-specific phosphodiesterase and that the *rutabaga* gene encoded a structural gene for Ca^{2+}-stimulated adenylyl cyclase (BYERS, DAVIS and KIGER 1981; KIGER *et al.* 1981; KAUVAR 1982; SHOTWELL 1983; LIVINGSTONE, SZIBER and QUINN 1984; DUDAI *et al.* 1985; LIVINGSTONE 1985). Subsequent molecular cloning of these genes has confirmed the biochemical data and, more interestingly, has shown that both gene products are preferentially expressed in adult mushroom bodies (CHEN, DENOME and DAVIS 1986; LEVIN *et al.* 1992; NIGHORN, HEALY and DAVIS 1991; P. L. HAN, L. R. LEVIN, R. R. REED and R. L. DAVIS, unpublished data–structures thought to be involved in olfactory learning (HEISENBERG *et al.* 1985; MENZEL *et al.* 1991). These results are generally consistent with a model in *Aplysia* of associative learning, in which the cAMP cell-signaling pathway plays a crucial role (KANDEL *et al.* 1987). Recent results from transgenic Drosophila also are consistent with this model: inhibition of one of the targets of cAMP signaling–cAMP-dependent protein kinase–appears to interfere with associative learning (DRAIN, FOLKERS and QUINN 1991).

Historically, molecular genetic identification of

[1] Current address: Beckman Neuroscience Center, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724.

learning/memory genes has been hampered by the technical problems associated with genetic mapping and subsequent molecular cloning of EMS-induced point mutations that affect only behavioral phenotypes. The *dunce* and *rutabaga* genes were cloned, in fact, because other pleiotropic effects for the mutations first were identified. The effect of *dunce*^{2} on female sterility facilitated recombinant mapping of the gene, which in turn led to its identification as a gene encoding a phosphodiesterase (BYERS, DAVIS and KIGER 1981). Restriction fragment length polymorphisms then were used along with the biochemical phenotype to clone the gene (DAVIS and DAVIDSON 1984). Biochemical results with *dunce* led to a search for lesions in the cAMP cell-signaling pathway in the other learning/memory mutants. In this manner, the *rutabaga* mutation was discovered to affect adenylyl cyclase. The biochemical phenotype then was used to deletion-map the mutation to region 12E-F of the *X* chromosome. Finally, the *rutabaga* gene was cloned by using heterologous DNA probes from bovine or rat genes encoding adenylyl cyclase (KRUPINSKI *et al.* 1989; FEINSTEIN *et al.* 1991), followed by chromosmal *in situ* hybridization to identify a Drosophila DNA clone from the 12E-F region (LEVIN *et al.* 1992). Pleiotropic effects for the other extant learning/memory genes are not known (except possibly for *turnip*; see CHOI *et al.* 1991), and, consequently, molecular cloning of these genes has not yet been accomplished.

To extend and to expedite this genetic dissection, we have begun a screen for new, autosomal mutations affecting learning or memory using *P* element mutagenesis (KIDWELL 1986; O'KANE and GEHRING 1987; COOLEY, KELLY and SPRADLING 1988; BIER *et al.* 1989). To date, we have generated more than 1000 mutant strains homozygous for single *P* element insertions in autosomes. These mutant strains were screened for memory deficits three hours after olfactory avoidance conditioning. In this manner, mutations affecting later memory phases, as well as those affecting acquisition and early memory phases, could be isolated (see TULLY 1991). Below we describe the behavioral and genetic characterization of *latheo*^{P1}, one of the mutations isolated in this mutagenesis.

MATERIALS AND METHODS

Stocks: Ample evidence exists for polygenic variation in olfactory responses and in associative learning in Drosophila (ALCORTA and RUBIO 1989; MONTE *et al.* 1989; FUYAMA 1976; GAILEY, VILLELLA and TULLY 1991). As a result, we replaced the chromosomes of our stocks with those from Canton-S (labeled "+" in our figures and text) whenever possible. Morphological phenotypes of dominant markers (*Sb* and *Sp*), balancer chromosomes (*CyO*, *TM6B* and *SM5*) and a "cosegregator" stock (*Xa*) are described by LINDSLEY and ZIMM (1992).

The following stocks were used in the generation of *P* element insertion strains: *w*(C-S4) was derived from a stock carrying the *white*^{1118} deletion allele (HAZELRIGG, LEVIS and RUBIN 1984). It was "Cantonized" by crossing *w*^{1118} males to virgin Canton-S females. Heterozygous virgin female F_1 progeny then were mated to Canton-S males, and *white* male F_2 progeny were collected. This two-generation mating scheme was done twice, resulting in four generations of outcrossing for the autosomes and two generations of outcrossing via recombination for the (*w*^{1118}) *X* chromosome. The mutator *P* element stock was *9.3*, containing a single *w*$^+$ *P* element on the *X* chromosome (COEN 1990). The +/+; *Sb*Δ2-3/*TM6*, *Ubx* stock contained a stable, constitutive transposase source inserted at chromomere 99B (ROBERTSON *et al.* 1988). Flies homozygous for a given *9.3* transposition to an autosome ultimately were bred from heterozygous flies using a *w*^{1118}; +/*CyO*; *Sb*/*TM6B* balancer stock.

Excisions of the *9.3 P* element inserts were generated using *Sp*/*CyO*; *Sb*Δ2-3/*TM6*, *Ubx* as a transposase source. Stocks homozygous for particular excisions were bred using the *w*^{1118}; *CyO*; +/*Xa* strain. The *Xa* chromosome is a 2;3 translocation, which forces the *CyO* second and + third chromosomes to cosegregate during meiosis.

The *latheo*^{P1} mutation was mapped genetically via crosses with various γ-ray- and EMS-induced chromosomal deficiencies and point mutations. The *Df*(*2R*)*vg*^{b}/*SM5* stock, carrying a deficiency of the *vestigal* region (49E-F) of the second chromosome, was obtained from the National Drosophila Stock Center (Bloomington, Indiana). Deficiency stocks *Df*(*2R*)*vg*^{56}/*SM5* and *Df*(*2R*)*vg*^{107}/*SM5* were provided by M. L. PARDUE, along with stocks carrying several other lethal mutations that do not have any detectable cytological abnormalities (*vr3.2*, *vr3.4*, *vr3.16*, *vr3.55*, *vr3.56*, *vr4.57*, *vr5.48*, *vr6.6*, *vr6.7*, *vr6.35*, *vr6R6*, *vr9.11*, *vr9.22*, *vr9R2*, *vr11.14*, *vr13.24*, *vr13.47*, *vr14.33* and *vr19.41*; see LASKO and PARDUE 1988). The lethal mutations *vr9.23* and *vr9.43* were provided by T. WU. Each of these mutant stocks also carried the *SM5* balancer chromosome. Stocks carrying these lethal mutations were crossed *inter se* to verify lethal complementation groups. Results from our experiments differed from those of LASKO and PARDUE (1988) (see Table 2). Stocks of the genotype *w*/*w*; *Df*/*CyO* were generated by crossing males from each *Df*/*SM5* stock to *w*/*w*; *CyO* +/*Xa* females. F_1 *w*/*Y*; *Df*/*CyO* progeny were mated with *w*/+; *Df*/*CyO* siblings to produce *Df*/*CyO* F_2 progeny that were homozygous or hemizygous for *w*; these flies then were interbred.

All strains were maintained at 25° on a 16/8 hr light/dark cycle with lights on at 7:00 a.m. Flies were raised on a food medium consisting of 8.4 g/liter agar, 31.9 g/liter yeast (Nutrex #540), 94.2 g/liter dextrose, 8.7 g/liter potassium tartrate, 7 g/liter $CaCl_2$, 76.1 g/liter cornmeal and 2 g/liter Tegosept M mold inhibitor. This food medium and other rearing conditions were identical to those used in TULLY and QUINN (1985). We have tried other food media, in particular a medium containing molasses, but have obtained lower learning scores with Canton-S flies (data not shown).

Generation of *P* element insertion and excision strains: Mutant strains were generated using *P* element transposition as depicted in Figure 1A. Females homozygous for a single *9.3 P* element on the *X* chromosome were mated to males carrying the Δ2-3 *P* element transposase source. The *9.3 P* element contains the *w*$^+$ gene sequence, thereby conferring red eye color to flies homozygous for *w*^{1118}. Transposase activity in somatic tissue was apparent in *Sb* F_1 males; their eyes were mosaics of red and white tissue. Two or three of these dysgenic (mosaic) males were mated to 5–10 *w*(C-S4) females per vial in cross II. All male progeny from this second cross carried a *w*^{1118} *X* chromosome; those with red eyes also carried a *9.3 P* element that had transposed from the *X* chromosome to one of the autosomes. In cross III, a

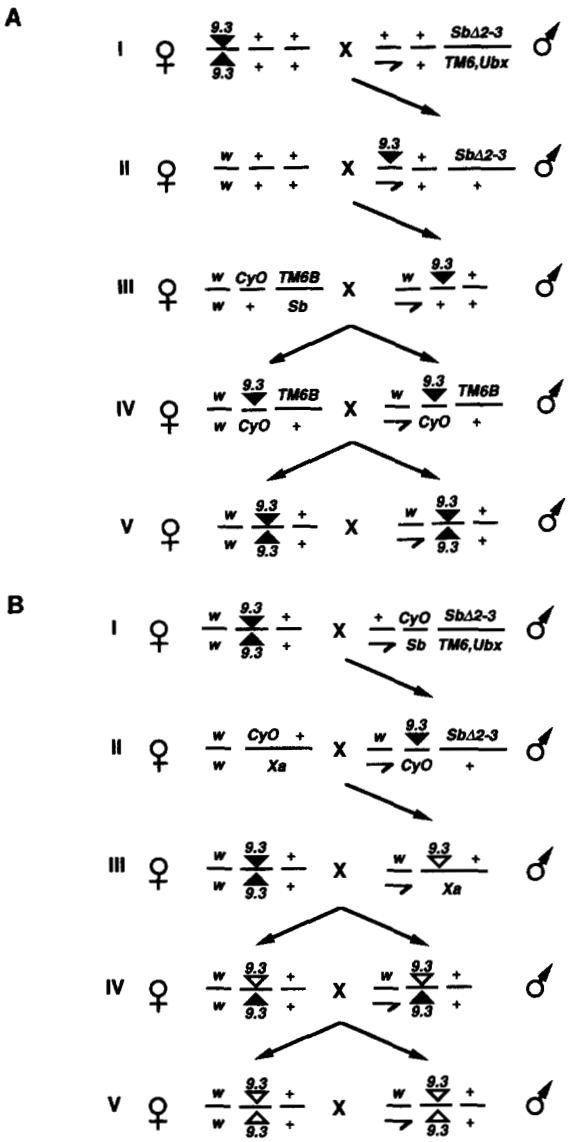

FIGURE 1.—Generation of mutant and excision strains via *P* element transposition. **A**, *P* element transpositions were generated using *9.3*, a *P* element containing a wild-type copy of *white*, and using the stable transposase source Δ2-3. A 15-min retention was assayed in flies homozygous for single *P* element insertions. **B**, Excision strains were generated by mobilizing the *P* element out of the second chromosome with Δ2-3. Flies carrying an "excision" chromosome were mated to *latheo*^{P1} mutants in cross III to minimize differences in genetic backgrounds between the excision strains and *latheo*^{P1} mutant. Solid triangles refer to the *latheo*^{P1} *P* element insertion. Open triangles refer to excisions of the *latheo*^{P1} *P* element. See text for details.

single "transposition" male, which did not carry the transposase source and, therefore, contained a stable new *P* element insertion, from each vial was mated to 5–10 w^{1118}; *+/CyO; Sb/TM6B* females per vial. Strains homozygous for a given, independent *P* element insert on an autosome then were generated in crosses IV and V.

Excisions of the *latheo*^{P1} *P* element insert were generated as in Figure 1B. The *latheo*^{P1} insertion first was mobilized using the Δ2-3 transposase in cross I, and 2–3 dysgenic F_1 male progeny were mated to 5–10 *w; CyO; +/Xa* females in cross II. One white-eyed, *Cy*$^+$ male progeny (in which the *9.3 P* element was excised) from each vial was mated to 5–10 virgin *latheo*^{P1} females in cross III. Crosses IV and V then were necessary to establish white-eyed excision strains, each of which was homozygous for independent excision events. Flies carrying excisions of the *latheo*^{P1} *P* element first were backcrossed to mutant *latheo*^{P1} flies to minimize any genetic background differences between the excision strains and their parental mutant strain. For similar reasons, the cosegregator *Xa* was used (in cross III males) to force an unmarked third chromosome in *latheo*^{P1} flies to segregate with the *latheo*^{P1} excision (second) chromosome. After homozygous excision strains were maintained for more than eight generations, they were outcrossed again to *latheo*^{P1} flies, which themselves had been outcrossed to *w*(C-S4) for several generations. Heterozygous excision/*latheo*^{P1} virgin female progeny then were mated to *latheo*^{P1} males. Finally, the progeny from this cross were mated en masse to generate (white-eyed) flies homozygous for the excision allele.

Lethal excisions first were identified when no white-eyed progeny resulted from cross IV. The red-eyed male progeny then were mated to *w; CyO+/Xa* females, and *w; lE/CyO; +/+* progeny from this cross were interbred to establish each lethal excision line. Within each lethal excision line, no *Cy*$^+$ flies ever have been observed. These lethal excision lines were not outcrossed.

Associative learning: Flies were trained and tested with the classical conditioning procedure of TULLY and QUINN (1985). To begin training, a mixture of roughly 50 mutant and 50 control flies, 1–4 days old, was trapped inside a chamber which contained an electrifiable grid on 90% of its inner surface. Flies were exposed sequentially to two odors, 3-octanol (OCT; ICN-K&K Labs) and 4-methylcyclohexanol (MCH; ICN-K&K Labs), carried through the training chamber on a current of air (750 ml/min). Flies first were exposed for 60 sec to the conditioned stimulus (CS$^+$; either OCT or MCH), during which time they received the unconditioned stimulus (US), 12 1.25-sec pulses of 60 V DC electric shock at 5-sec interpulse intervals. After presentation of the CS$^+$, the chamber was flushed with fresh air for 30 sec. Then, flies were exposed for 60 sec to a second, control stimulus (CS$^-$; either MCH or OCT), which was not paired with electric shock. After presentation of the CS$^-$, the chamber again was flushed with fresh air for 30 sec. After training, flies were tapped gently from the training chamber into an elevator-like compartment that transported them to the choice point of the T-maze, where relative (conditioned) odor avoidance responses were assayed by exposing the flies to two converging currents of air (1500 ml/min at the choice point)–one carrying OCT, the other MCH, from opposite arms of the T-maze. Flies were allowed to choose between the CS$^+$ and CS$^-$ for 120 sec, at which time they were trapped inside their respective arms of the T-maze (by sliding the elevator out of register), anesthetized and counted. Flies that chose to avoid the CS$^+$ ran into the T-maze arm containing the CS$^-$, while flies that chose to avoid the CS$^-$ ran into the arm containing the CS$^+$. A small percentage of flies (< 5%) usually remained at the choice point.

Relative concentrations of OCT and MCH were adjusted so that naive flies distributed themselves 50:50 in the T-maze. The absolute concentrations of these odors were relatively high; more than 80% of naive flies avoided OCT or MCH when given a choice between either one of the odors and fresh air (see RESULTS). All training and testing was done at 25° and 70% relative humidity in dim red light. For more details on the conditioning procedure and the conditioning apparatus, see TULLY and QUINN (1985).

Two groups of flies were conditioned in one complete experiment. The CS$^+$ was OCT and the CS$^-$ was MCH for

one group; the CS^+ was MCH and the CS^- was OCT for the second group. For each reciprocal group, the "probability correct" (COR) was calculated as the number of flies avoiding the CS^+ divided by the total number of flies in the T-maze arms:

$$COR = [CS^+]/([CS^+] + [CS^-]).$$

These two COR values then were averaged, and that average was normalized to produce one performance index (PI):

$$PI = \frac{[(COR_{OCT} + COR_{MCH})/2] - 0.5}{0.5} \times 100$$

$$= [(COR_{OCT} + COR_{MCH}) - 1] \times 100.$$

This index creates scores ranging from 0 (no learning) to 100 (perfect learning). Calculation of a PI in this manner is algebraically equivalent to the learning index (λ) of TULLY and QUINN (1985), except that the number of flies left at the choice point is included in calculation of the latter. Like the learning index, the PI is a measure of associative learning unaltered by any nonassociative changes in odor avoidance that may occur during classical conditioning. [Performance indices were calculated with data from the nonassociative control experiments of TULLY and QUINN (1985). As is the case with learning indices, mean PIs for the nonassociative control groups did not differ significantly from zero (T. TULLY, unpublished).]

Memory retention: Groups of flies were trained as above, then removed from the training chamber and stored in the dark for 15, 30, 60, 180 or 360 min in test tubes containing food medium. Seventy seconds before the usual 2-min test trial, flies were transferred to the choice point of the T-maze. For retention intervals of 0 min, flies were transferred to the T-maze 1.5 min after training (3.5 min after the CS^+ presentation). An equal number of performance indices were collected for each retention interval on a given day to minimize spurious differences among groups due to daily variation in the functioning of the teaching machines and other uncontrolled variables.

Olfactory acuity: Absolute odor avoidance responses were quantified by exposing a mixture of 50 naive mutant and 50 naive control flies to each odor (either OCT or MCH) *vs.* air in the T-maze. After 2 min, the number of flies in each arm of the T-maze was counted, and a performance index was calculated for each odor individually:

$$PI_{OCT} = \frac{COR_{OCT} - 0.5}{0.5} \times 100$$

$$= [2COR_{OCT} - 1] \times 100$$

$$PI_{MCH} = \frac{COR_{MCH} - 0.5}{0.5} \times 100$$

$$= [2COR_{MCH} - 1] \times 100$$

Olfactory acuity was assayed by measuring absolute odor avoidance responses over a range of odor concentrations, from pure, undiluted odorant to a three-log dilution in heavy mineral oil (Fisher). The odor and air were presented in the left and right arms of the T-maze, respectively, for half of the experiments; the reciprocal arrangement was used for the other half. In this manner, any environmental cues that might have produced small "side biases" were eliminated algebraically from the mean PI for the group.

Shock reactivity: The ability to sense electric shock and to escape from it was quantified by inserting electrifiable grids into both arms of the T-maze. A mixture of about 100 mutant and control flies were aspirated into one arm and exposed to one 1.25-sec shock pulse before the center compartment was opened, thereby allowing flies to escape into the opposite, unshocked arm of the T-maze. Thereafter, shock pulses continued to be delivered every 5 sec, and flies were allowed to escape into the opposite T-maze arm. After 60 sec, the center compartment was closed, trapping flies in their respective arms. Flies in the center compartment or in the unshocked T-maze arm were considered to have escaped electric shock. Percent avoidance (PA) was calculated as the number of flies in the unshocked T-maze arm and in the center compartment divided by the total number of flies (times 100). During this test, amenotactic cues were provided by drawing air through the arms of the T-maze and out the center compartment (1500 ml/min). Again to eliminated any side biases in mean scores, equal numbers of groups of flies were shocked in either the left or right arm of the T-maze.

Unpaired control: Changes in olfactory avoidance responses occur when flies are exposed to electric shock (TULLY and QUINN 1985). To assess this type of nonassociative change, mixtures of mutant and control flies first were conditioned in the usual way (see above). During the 2-min test trial, however, absolute–rather than relative–odor avoidance responses were assayed by presenting flies with the CS^- in one T-maze arm and fresh air in the other. PIs were calculated for each odor individually as in olfactory acuity experiments.

Locomotor activity: Locomotor activity was measured using the countercurrent phototaxis apparatus of BENZER (1967)–in the absence of light. Under these conditions, tapping flies into the "start tube" between trials produces an escape response, generally referred to as locomotor reactivity, which decays with time until only spontaneous locomotor activity is expressed (MEEHAN and WILSON 1987). Roughly 100 flies, half mutant and half control, were loaded into a start tube which was aligned with a second, distal tube. The flies were tapped to the bottom of the start tube, which then was laid horizontally on a table. The flies were allowed to distribute themselves between the start and distal tube(s) for 30 sec. The two tubes then were shifted out of alignment, separating those flies that ran into the distal tube from those that remained in the start tube. The flies in the distal tube were tapped into a second start tube, and then both groups again were allowed to run into distal tubes for 30 sec. This procedure to separate flies into distal and start tubes was done five times, ultimately fractionating flies into six groups based on the number of times a fly ran into a distal tube. The most active flies ran into distal tubes five times and received a score of five; the least active flies stayed in the original start tube throughout the experiment and received a score of zero. Locomotor activity scores of mutant flies were normalized to the mean score of *w*(C-S4) control flies each day to minimize daily variations in overall activity.

Habituation: Based on an olfactory jump assay developed by CARLSON and coworkers (MCKENNA *et al.* 1989), we have shown a waning of the jump reflex over repeated trials (S. KOSS and T. TULLY, in preparation), which shows many properties of habituation as defined by THOMPSON and SPENCER (1966). Single males were housed in chambers consisting of a Lucite base and an inverted plastic test tube (Falcon #2017). A vacuum source was connected to the base of the chamber, drawing air (1000 ml/min) through distilled water, in a 3-mm hole at the top of the test tube, through the chamber and out nylon mesh-covered holes in the base. At regular intervals, a computer controlled, 3-way solenoid valve switched from the stream of "fresh" air to one that was bubbled first through distilled water and then through 10% benzaldehyde (Fluka) in heavy mineral oil (Fisher).

Each fly was habituated by exposing it repeatedly to 10% benzaldehyde for 4 sec at 10-min intertrial intervals. Each fly was judged to have habituated when it failed to jump during the 4-sec odor presentation in four consecutive trials (4 no-jumps). A fly's habituation score was the number of trials needed to obtain a block of 4 no-jumps (trials to criterion). After reaching criterion, each fly was vortexed in its test tube for 90 sec to dishabituate the jump reflex. Dishabituation was monitored 2 min after a fly reached criterion by exposing it one more times to a 4-sec presentation of 10% benzaldehyde. The percentage of flies that failed to jump during this test trial reflected the average amount of dishabituation for each genotype. A level of dishabituation lower than wild-type can result from sensory or motor fatigue (see RESULTS).

Males were collected under CO_2 anesthesia on the day of eclosion and then were stored in food vials at 25° for 2 days. Locomotor activity in 2–3-day-old males was assessed the day of the habituation experiments (see above). Only males with an activity score of 0 were discarded. Usually 8 or 16 flies were habituated in one experiment. In either case, equal numbers of mutant and control flies were tested. These experiments were run at room temperature in ambient relative humidity (although the air currents were bubbled through water) between 8 a.m. and midnight.

Genetic mapping: The *latheo*^{P1} *P* element insert was localized in situ on polytene salivary chromosomes according to ENGELS *et al.* (1986), using DNA sequence from the *white*$^{+}$ gene as a probe. For complementation tests with deficiency chromosomes, *w/w; lat/lat* females were crossed to *w/Y; Df/CyO* males, thereby providing *w/w* (or *Y*); *lat/Df* progeny. These *lat/Df* flies were mixed in approximately equal numbers with *w/w* (or *Y*); *+/Df* flies, generated by crossing *w/w; +/+* females to *w/Y; Df/CyO* males. Such mixed groups of flies then were trained and tested for 15-min retention, olfactory acuity, shock reactivity and locomotor reactivity. Females homozygous for *white* and the viable *latheo*^{P1} excision allele, *lE16b* (see RESULTS), also were crossed to white-eyed *vr6.6/CyO* males to produce *w/w* (or *Y*); *lE16b/vr6.6* progeny. Immediate learning (retention time 0) was assayed in these flies. For these experiments, *+/vr6.6* control flies were trained and tested separately, since they too were white-eyed.

The *latheo* gene was localized further via complementation crosses between each of 10 lethal *latheo*^{P1} excision alleles and other γ-ray- or EMS-induced lethal mutations in the 49F region (see above). For each complementation cross, *lE/CyO* flies were crossed to *vr/CyO* flies. The absence of *Cy*$^{+}$ progeny indicated that the *lE* mutation failed to complement the *vr* mutation. Based on these results, *latheo*^{P1}/*latheo*^{P1} females were crosed to *lE49/CyO*, *vr6.6/CyO* and *vr6.35/CyO* males to verify that each of these three mutant alleles of *latheo* failed to complement the behavioral phenotype associated with the *latheo*^{P1} allele. Here again, *latheo*^{P1}/*vr6.6* or *latheo*^{P1}/*lE49* flies were trained and tested with *+/vr6.6* or *+/lE49* flies as an internal control in the behavioral experiments.

Anatomy: Neural anatomy of adult brains was examined in 6-μm paraffin sections. Heads were fixed overnight in FAAG (formalin:alcohol:acetic acid; 10:85:5 with 1% gluteraldehyde), cleared, and embedded in paraplast (CAMPUS, GROSSMAN and WHITE 1985). Sections were stained with hematoxylin before examination (HUMASON 1972).

Statistics: Since a performance index itself is an average of percentages, a sample of performance indices is distributed normally. Thus, most statistical analyses reported herein were based on analysis of variance (ANOVA) with unplanned pairwise comparisions among group means (SOKAL and ROHLF 1981). Computations were done on a VAX mainframe computer with SAS statistical software (SAS Institute, Inc., Cary, North Carolina). Since individual jump responses were scored as zero or one in the habituation experiments, the response decrement over trials for each genotype was subjected to a log likelihood analysis of covariance (ANCOVA) on a Macintosh II with JMP 2.0 statistical software (SAS Institute, Inc.). Mean habituation scores (which were distributed approximately binomially) for mutant and control flies were compared with Student's *t*-test (SOKAL and ROHLF 1981).

RESULTS

One thousand sixteen homozygous *P* element insertion strains initially were screened for memory deficits 3 hr after classical conditioning by generating one PI for each strain. One hundred and seventy strains yielded PIs less than 50% of the 3-hr memory score for *w*(C-S4) control flies. Three or more PIs then were generated for these 170 strains to obtain more reliable estimates of 3-hr memory. Thirty-nine mutant strains produced mean 3-hr memory scores that were 70% or less of the mean score for *w*(C-S4) control flies.

Olfactory acuity (see below) for OCT and MCH varied among these 39 putative mutant strains and, on average, was lower than that of *w*(C-S4) flies. Significant polygenic variability exists for olfactory responses in Drosophila (see ALCORTA and RUBIO 1989; MONTE *et al.* 1989; FUYAMA 1976). We also have obtained evidence recently for variability in genetic background that is specific for associative learning (GAILEY, VILLELLA and TULLY 1991). Thus, differences in olfactory acuity among our *P* element strains may have arisen in four different ways. (1) Differences in genetic backgrounds may have existed among the parental stocks, *9.3*, Δ2-3 and *w*(C-S4), which were used to generate the mutant strains. (2) Genetic background differences among mutant strains may have arisen from a "founder effect." Such sampling variation existed because each homozygous mutant strain originated from a *single* transposition male (see cross III in Figure 1A). (3) A *P* element may have inserted in a gene involved with olfaction. (4) Selection against (less fit) mutant phenotypes also may have occurred (*cf.* TULLY and QUINN 1985; TULLY and GERGEN 1986), producing allelic differences in "modifying" genes among the mutant strains in a stochastic manner. To the extent that olfactory acuity affects fitness, this phenomenon may have contributed to the observed variation among mutant strains.

To alleviate such differences in genetic background, the 39 low scoring mutant strains were outcrossed for five generations to *w*(C-S4) flies (see MATERIAL AND METHODS). Following outcrossing, the strains were tested again for 3-hr memory and for olfactory acuity. Mean 3-hr memory scores in 12 outcrossed mutant strains did not differ from that in wild-type controls. Mean 3-hr memory scores were significantly lower

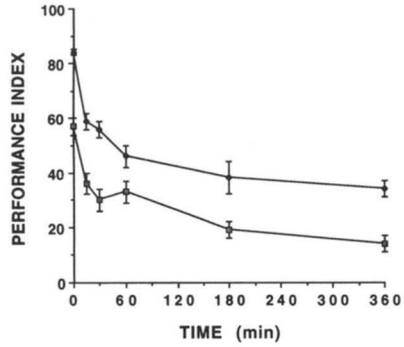

FIGURE 2.—Memory retention in mutant and control flies. Homozygous *latheo*^{P1} (open squares) and wild-type *w*(C-S4) (closed circles) flies were trained and tested together. Afterward, the two genotypes were distinguished based on the difference in eye color, and then separate PIs were calculated. Different groups of flies were used for each retention interval. Mutant *latheo*^{P1} flies show a consistent reduction in learning and memory at all retention intervals. $n = 8$ PIs for each genotype at each retention interval.

than controls in 18 of the remaining 27 outcrossed mutant strains, but they still showed lower-than-normal olfactory acuity. (Olfactory acuity in one strain, E_s152, showed no improvement after outcrossing; thus, it may be an olfactory mutant. We continue to outcross all 18 of these strains.) Nine outcrossed mutants maintained poor memory retention while displaying normal olfactory acuity. These strains now are outcrossed to *w*(C-S4) flies each generation to minimize any build-up of modifiers in the genetic background.

Here, we report the behavioral and genetic characterization of one of these nine mutant strains. It carries a single *P* element insertion that is solely responsible for the mutant phenotype. Thus, we have named the newly defined gene *latheo*, which is Greek "to cause a person not to know" (*Benet's Reader's Encyclopedia* 1987)

Behavioral characterization of *latheo*^{P1} flies

Memory retention: Different mixed populations of *latheo*^{P1} mutant and *w*(C-S4) control flies were tested at various retention intervals after classical conditioning. After the test trial, the two genotypes were sorted according to eye color, and PIs were calculated for each genotype separately. Figure 2 shows memory retention over the first 6 hr after training for *latheo*^{P1} mutants and *w*(C-S4) control flies. Mean PIs are lower in mutant *latheo*^{P1} flies at all retention intervals. A 2-way ANOVA yielded a significant effect of GENOTYPE ($F_{[1, 84]} = 118.8$, $P = 0.0001$), a significant effect of TIME ($F_{[1, 84]} = 44.9$, $P = 0.0001$) and an insignificant GENOTYPE × TIME interaction effect ($F_{[5, 84]} = 1.0$, $P = 0.3927$). These results indicate that the initial difference between *latheo*^{P1} and *w*(C-S4) flies remained constant during the first 6 hr of memory decay (*i.e.*, the memory decays curves are parallel). When averaged over all retention intervals, *latheo*^{P1} scores were 59% of those of *w*(C-S4) controls.

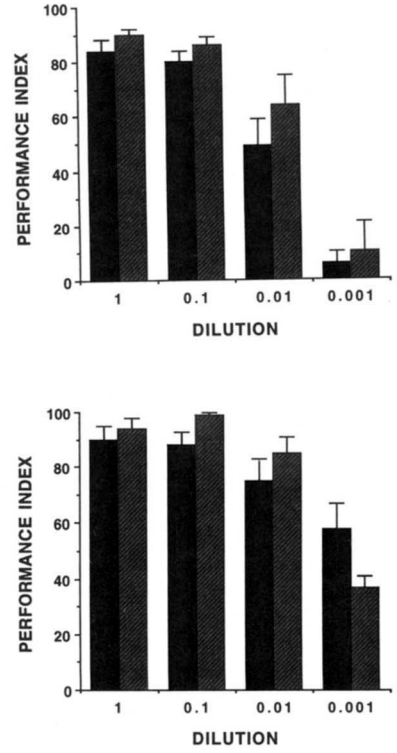

FIGURE 3.—Olfactory acuity in mutant and control flies. Homozygous *latheo*^{P1} (solid bar) and wild-type *w*(C-S4) (striped bar) flies were mixed together and then tested for olfactory avoidance over a range of concentrations by exposing them in the T-maze to converging currents of air–one of which delivered odorant of a given concentration and the other "fresh" air. The percentage of flies avoiding the T-maze arm containing the odor by running into the opposite arm was used to calculate a PI. **Top**, Avoidance responses over a three-log dilution of methylcyclohexanol (MCH). **Bottom**, Avoidance responses over a three-log dilution of OCT. Olfactory acuity in mutants flies did not differ significantly from that in wild-type controls. $n = 4$ PIs for each genotype at each dilution.

Olfactory acuity: Low performance of *latheo*^{P1} flies in conditioning experiments might have been a secondary result of a reduced ability to smell the odor cues. To distinguish such an effect, mixed populations of naive *latheo*^{P1} and *w*(C-S4) flies were given a choice between various concentrations of OCT or MCH *vs.* air in the T-maze. Figure 3 shows the odor avoidance of *latheo*^{P1} and control flies over a 3-log range of dilutions. Seperate 2-way ANOVAs for OCT and for MCH detected significant effects of CONCENTRATION ($F_{[3, 24]} = 27.05$, $P = 0.0001$ and $F_{[3, 24]} = 52.03$, $P = 0.0001$, respectively), no significant effect of GENOTYPE ($F_{[1, 24]} = 0.05$, $P = 0.8206$ and $F_{[1, 24]} = 2.58$, $P = 0.1214$, respectively) and one significant GENOTYPE × CONCENTRATION interaction ($F_{[3, 24]} = 3.43$, $P = 0.0330$ and $F_{[3, 24]} = 0.25$, $P = 0.8630$, respectively). The significant interaction effect for OCT resulted from a *higher* mean PI for *latheo*^{P1} flies at the 0.001 dilution. Taken together, these results suggest that olfactory acuity for the two

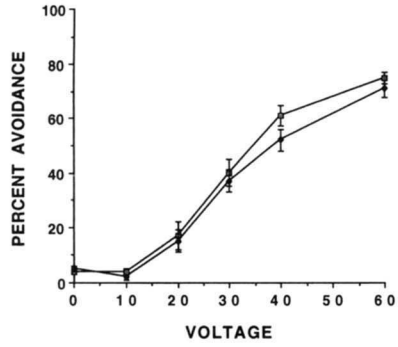

FIGURE 4.—Shock reactivity in mutant and control flies. Homozygous *latheo*^{P1} (open squares) and wild-type *w*(C-S4) (solid circles) flies were mixed together and then tested for shock reactivity over a range of voltages by placing them in one arm of the T-maze, delivering 1-sec pulses of electric shock to that arm for 60 sec while allowing the flies to escape to the opposite (unshocked) T-maze arm. Shock reactivity was quantified as the PA of flies avoiding (escaping from) the shocked T-maze arm. Here again, shock reactivity was normal in mutant flies. $n = 8$ PAs for each genotype at each voltage.

odors used in classical conditioning experiments was not defective in *latheo*^{P1} flies.

Shock reactivity: Poor performance of *latheo*^{P1} flies in conditioning also might result from an inability to sense and escape from electric shock. To assess this "peripheral" behavior, mixtures of *latheo*^{P1} and *w*(C-S4) flies were exposed in the T-maze to electric shock pulses over a range of voltages. Figure 4 shows the resulting PAs in normal and mutant flies at different voltages. A 2-way ANOVA detected a significant effect of VOLTAGE ($F_{[5, 84]} = 156.82$, $P = 0.0001$), no significant effect of GENOTYPE ($F_{[1, 84]} = 2.64$, $P = 0.108$), and no significant VOLTAGE × GENOTYPE interaction ($F_{[5, 84]} = 0.43$, $P = 0.800$). Thus, shock reactivity appeared to be normal in *latheo*^{P1} flies.

Unpaired control: The data presented above suggested that the memory deficit in mutant *latheo*^{P1} flies cannot be attributed to abnormal olfactory acuity or shock reactivity. The possibility remained, however, that exposure to both electric shock and odor during classical conditioning experiments might have altered olfactory responses differentially in *latheo*^{P1} *vs.* control flies. To control for this, olfactory acuity was measured after an unpaired conditioning procedure (see MATERIALS AND METHODS). Mixtures of *latheo*^{P1} and *w*(C-S4) flies were trained as usual, but afterward the 120-sec test trials consisted of a choice between the CS$^-$ in one arm of the T-maze and air in the other. Figure 5 shows olfactory avoidance behavior after such "unpaired" training, compared to that of naive flies, in normal and mutant genotypes. Separate 2-way ANOVAs detected a significant TREATMENT (naive *vs.* unpaired) effect for OCT ($F_{[1, 36]} = 72.54$, $P = 0.0001$) and for MCH ($F_{[1, 36]} = 65.41$, $P = 0.0001$), no significant effect of GENOTYPE for OCT ($F_{3[1, 36]} = 3.86$, $P = 0.0572$), a significant effect

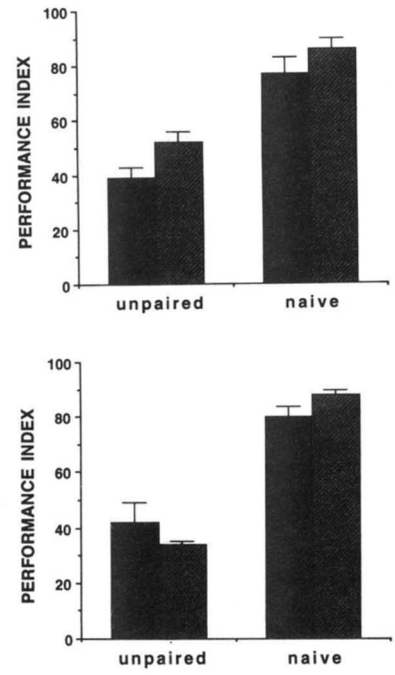

FIGURE 5.—Avoidance of the unpaired odor (CS$^-$) in normal and mutant flies. Homozygous *latheo*^{P1} (solid bars) and wild-type *w*(C-S4) (striped bars) flies were mixed together and trained with the usual classical conditioning procedure. Immediately afterward, flies were exposed to the CS$^-$ (the odor NOT paired with shock) *vs.* air in the T-maze. **Top**, Avoidance of MCH. **Bottom**, Avoidance of OCT. The nonassociative reduction in odor avoidance is seen in the unpaired groups; this effect is normal in mutant flies. $n = 4$ PIs for each genotype in each treatment group.

of GENOTYPE for MCH ($F_{[1, 36]} = 4.53$, $P = 0.0403$), and no significant GENOTYPE × TREATMENT interaction for either OCT or MCH ($F_{[1, 36]} = 0.66$, $P = 0.4215$ and $F_{[1, 36]} = 0.87$, $P = 0.3571$, respectively). These results corroborated the observation of TULLY and QUINN (1985) that an explicitly unpaired procedure produces a decrement in olfactory avoidance responses. More important, however, this nonassociative change in odor avoidance is normal in mutant *latheo*^{P1} flies.

Genetic characterization of the latheo^{P1} mutation

Excision lines: The *latheo*^{P1} *P* element was excised and strains homozygous for independent excision events were established via the breeding scheme outlined in Figure 1B. Fifteen-minute memory retention was assessed in 13 randomly chosen homozygous-viable excision strains; the results are shown in Figure 6 (stippled bars). Unplanned pairwise comparisons among mean scores revealed that lines *E8*, *E17*, *E9*, *E14* and *E16b* were significantly higher than *latheo*^{P1}, while lines *E12*, *E13*, *E4*, *E6*, *E11*, *E16a*, *E5*, *E1*, *E8*, *E17* and *E9* were significantly lower than *w*(C-S4). Although the statistical analysis permits us to lump the excision lines only into these two groups (with some overlap), the distribution of memory scores–ranging from *latheo*^{P1}-like to *w*(C-S4)-like–and addi-

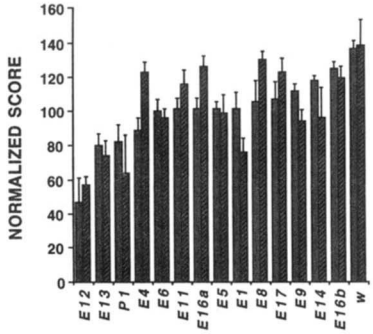

FIGURE 6.—Fifteen-minute memory retention in homozygous *latheo*^{P1} *P* element excision lines. Flies in one set of excision lines (stippled bars) had been maintained as homozygotes for eight or more generations and, as a result, may have accumulated genetic modifiers; flies in the other set (striped bars) had been outcrossed for two generations to "cantonized" *latheo*^{P1} flies. PIs for each group were normalized to the mean PI for control flies tested on the same day; this was done to remove daily variation from mean scores (and SEM). Outcrossing produced both higher and lower mean PIs, indicating the existence of (a) differences in genetic background among the lines due to the sampling variation introduced by selecting single excision males to establish each excision line and/or (b) the accumulation over generations of genetic modifiers in *latheo*^{P1} homozygotes. $n = 4$ PIs for all lines.

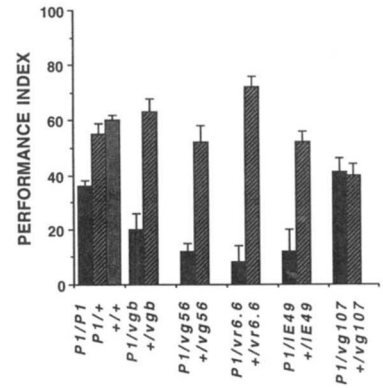

FIGURE 7.—Complementation analysis for 15-min retention among various deficiencies and EMS-, γ-ray- or *P* element excision-induced mutations in the *vestigal* region. Flies heterozygous for *latheo*^{P1} and *vgb*, *vg56*, *vr6.6* or *latheo*^{lE49} showed significantly lower 15-min retention compared to control flies that were heterozygous for + [*w*(C-S4)] and the same genetic variants. Homozygous *latheo*^{P1} flies also yielded 15-min retention scores lower than those of +/+ [*w*(C-S4)] wild-type controls. In contrast, 15-min retention in flies heterozygous for *lat*^{P1} and *vg107* or + was similar to their corresponding controls. These data indicate that the memory deficit maps to the 49Fd complementation group. $n = 18$ PIs for *latheo*^{P1} homozygotes and *w*(C-S4) flies; $n = 10$ PIs for *latheo*^{P1}/*vgb*, +/*vgb* and *latheo*^{P1}/+ flies; and $n = 4$ PIs for each of the other genotypes.

tional behavioral work with *E16b* (see below)–suggest that the *latheo* gene can be mutated to various viable, hypomorphic states. We also recovered 10 lethal excisions of the *latheo*^{P1} *P* element insertion, which suggested that *latheo* is an essential gene. These lethal excisions were used in complementation analyses with other lethal mutations from the region to verify the essential nature of *latheo* (see below).

To examine the effects of genetic background, we assayed 15-min retention twice in each of these 13 excision lines. The first time was eight generations after the lines were made homozygous (Figure 6, striped bars). The excisions lines then were outcrossed to *latheo*^{P1} flies for two generations to equilibrate their genetic backgrounds with that of the *latheo*^{P1} strain (see MATERIALS AND METHODS). After outcrossing, homozygous *latheo* excision flies were tested again for 15-min retention (Figure 6, hatched bars). Each day of these behavioral experiments, equal numbers of PIs were generated for each of the 15 lines [13 excision lines, *w*(C-S4) and *latheo*^{P1}]. Then, to minimize variability in memory scores due to daily fluctuations in teaching machine function, each PI was normalized by dividing it by the average PI of all the genotypes tested on that day. In general, outcrossing seemed to affect memory scores of several excision strains. Planned pairwise comparisons of the normalized scores indicated that outcrossing significantly increased the mean memory score of *latheo*^{E1} flies ($F_{[1, 96]} = 4.21$, $P = 0.043$), while it significantly decreased the mean score of *latheo*^{E4} flies ($F_{[1, 96]} = 6.84$, $P = 0.010$). In each of these cases, the genetic background of the excision line apparently was not "equilibrated" with that of the Cantonized *w*(C-S4) line. That this effect was due to genetic, rather than environmental, factors is shown by the stability of mean scores in the *w*(C-S4) strain. Sampling variation in genetic background (for genes affecting associative learning or for those affecting peripheral behaviors, such as olfaction) during the mutagenesis might produce such a "disequilibrium." Furthermore, even if the genetic background of a phenotypically mutant excision line (such as *latheo*^{E4}) was equilibrated initially, selection against the mutant phenotype might produce an accumulation of modifiers (suppressors) over generations (*cf.* TULLY and QUINN 1985), thereby leading to the observed results.

Deletion mapping and lethal complementation: *In situ* hybridization of *white*$^{+}$ DNA to polytene chromosomes from *latheo*^{P1} flies localized the *latheo P* element insertion to chromomeres 49F7-8 on the second chromosome (data not shown). We also determined that the mean 15-min retention score of *latheo*^{P1}/+ heterozygous flies was not significantly different from that of +/+ [*w*(C-S4)] flies (see Figure 7). These results indicated that the *latheo*^{P1} mutation acts as a recessive to the wild-type allele. Accordingly, three chromosomal deficiencies of this region, *Df(2R)vg*^{b}, *Df(2R)vg*^{56} and *Df(2R)vg*^{107} (which hereafter will be referred to as *vgb*, *vg56* and *vg107*), then were used to verify that the behavioral deficit of *latheo*^{P1} flies comaps to the region of the *P* element insert. Based on breakpoint analyses of these deficiencies by LASKO and PARDUE (1988), *vg107* was expected to complement *latheo*^{P1}, whereas *vgb* and *vg56* were not. Equal

numbers of flies heterozygous for the *latheo*^{P1} chromosome and one of the deficiency chromosomes were mixed with control flies, which were heterozygous for a wild-type *w*(C-S4) chromosome and the corresponding deficiency chromosome. These mixed groups were trained and tested for 15-min retention; afterward, the two genotypes were distinguished by eye color, and separate performance indices were calculated.

As shown in Figure 7, mean 15-min retention scores were significantly lower in *lat*^{P1}*/vgb* and *lat*^{P1}*/vg56* flies than in their +/*Df* controls ($F_{[1, 85]} = 43.3$, $P = 0.0001$ and $F_{[1, 85]} = 40.5$, $P = 0.0001$, respectively). In contrast, mean 15-min retention scores for *lat/vg107* flies did not differ from their +/*vg107* controls. (The lower memory score in +/*vg107* heterozygotes, as compared to +/+ controls, suggests either a genetic background effect of the *vg107* deficiency stock or a dominant effect of the *vg107* deficiency.) Thus, the former two deletions "uncovered" the *latheo*^{P1} mutation, while the latter deletion did not. Interestingly, mean memory scores in *latheo*^{P1}*/vgb* and *latheo*^{P1}*/vg56* heterozygotes were significantly lower than *latheo*^{P1} homozygotes (Tukey-Kramer unplanned comparisons, $\alpha = 0.05$), suggesting that *latheo*^{P1} is a hypomorphic allele of an essential gene–an observation consistent with results from behavioral experiments on the excision lines (see above).

LASKO and PARDUE (1988) saturated the *vestigal* region for EMS- and γ-ray-induced lethal mutations. Their lethal complementation groups 49Fa–49Fh map between the distal breakpoints of *vg107* and *vg56*. Recovery of lethal *latheo* excisions, then, allowed a finer localization of the *latheo* gene via lethal complementation analysis. We first crossed several of the extant lethal mutations *inter se* to verify the lethal complementation groups (see Table 1). In general, we corroborated the results of LASKO and PARDUE (1988). Two discrepancies existed, however. The *vr9.22* and *vr9r2* mutations appeared to be deletions, rather than point mutations, and *vr6.7* complemented the other *vr6* mutations. Instead, we found that *vr6.7* failed to complement *vr8* and *vr4*, which also failed to complement each other, placing these three mutations into a single complementation group.

We then crossed each of 10 lethal *latheo* excisions to one or more mutations from each of the extant lethal complementation groups. Table 2 shows that lethal *latheo* excision alleles *lE101*, *lE107* and *lE388* failed to complement mutations from two or more complementation groups. In contrast, lethal *latheo* excision alleles *lE34*, *lE49*, *lE311*, *lE344*, *lE352*, *lE357* and *lE372* failed to complement mutation(s) only in the *vr6* (49Fd) complementation group. Figure 8 summarizes these additions and corrections to the genetic organization of the *vestigal* region.

Based on these results, we assayed 15-min retention in flies heterozygous for one of the lethal alleles (*vr6.6*, *vr6.35* or *lE49*) and *latheo*^{P1}. Control flies were heterozygous for one of the lethal alleles and *lat*$^{+}$ *[w*(C-S4)]. Figure 7 shows the results for flies carrying *vr6.6* and for those carrying *lE49*. In both cases, mean 15-min retention scores were significantly lower in heterozygous flies carrying the *latheo*^{P1} allele ($F_{[1, 85]} = 7.54$, $P = 0.0001$ and $F_{[1, 85]} = 4.77$, $P = 0.0001$, respectively). Similar results were obtained for flies carrying the *vr6.35* allele: The mean 15 min PI (± SEM) for *vr6.35/lat*^{P1} flies was 13 + 4, and that for *vr6.35*/+ controls was 46 ± 7 ($t_{[6]} = 4.0$, $P = 0.008$, $n = 4$ for each genotype). Taken together, these data indicate clearly that both the *latheo*^{P1}-induced behavioral deficit and the lethal phenotype co-map to the *vr6* (49Fd) complementation group.

Behavior-genetic characterization of more severe *latheo* genotypes

Retention in excision/lethal heterozygotes: Figure 7 shows that mean 15-min retention scores of flies heterozygous for *latheo*^{P1} and *vgb*, *vg56*, *vr6.6* or *lE49* all were significantly lower than that of *latheo*^{P1} homozygotes (Tukey-Kramer unplanned comparisons; $\alpha = 0.05$). This observation suggested to us a more stringent test to identify *latheo*^{P1} excision lines that were true revertants. The mean 15-min retention score of one homozygous excision line, *E16b*, did not differ significantly from that of *w*(C-S4) controls (planned comparison; $F_{[1, 48]} = 1.10$, $P = 0.30$), suggesting that the *latheo* allele in this strain had reverted to wild type (see Figure 6). We reasoned, however, that if *latheo*^{E16b} was a weak hypomorph rather than a true revertant, then *E16b/vr6.6* flies would score significantly lower than their +/*vr6.6* controls. On the other hand, if *latheo*^{E16b} were a reversion, then *E16b/vr6.6* and +/*vr6.6* flies would yield similar scores. In fact, we found that the mean learning score of *E16b/vr6.6* flies (44 ± 2, $n = 4$) was significantly lower than that of +/*vr6.6* flies (76 ± 5, $n = 4$; $t_{[6]} = 5.96$, $P < 0.001$), indicating that *latheo* excision allele *E16b* is a weak hypomorph. Most likely, then, none of the 13 excision lines that were tested for 15-min retention represented complete revertants.

One immediate interpretation of the results of Figure 7 is that *latheo*^{P1} is a hypomorphic mutation and the lower 15-min retention scores for the *lat*^{P1}*/Df* genotypes result from a gene dosage effect (see STEWART and MERRIAM 1980). Another possibility, however, is that these more severe genotypes might have produced pleiotropic effects on other task-relevant "peripheral" behaviors, which then would have affected preformance in learning/memory experiments secondarily. Accordingly, we addressed this notion by assaying olfactory acuity and shock reactivity in *lat*^{P1}*/Df* flies.

TABLE 1

Complementation analysis of EMS- or γ-ray-induced lethal mutations in the *vestigal* region

	vr6	*vr6.7*	*vr8.9*	*vr9*	*vr9.22*	*vr9r2*	*vr11.14*	*vr13*	*vr5.48*
vr14.33		+							
vr4.57	+	−	−	+	−	−			
	(6.6)			(9.11)					
				(9.43)					
vr3		+		+	−	−			
		(3.55)		(3.55×9.11)	(3.2)(3.16)	(3.2)(3.16)			
				(3.55×9.43)	(3.4)(3.56)	(3.4)(3.56)			
vr6	−	+	+	+	−	−			
	(6.6×6.35)	(6.35)	(6.6)	(6.6×9.11)	(6.6)	(6.6)			
	(6.6×6r6)	(6r6)		(6.6×9.43)	(6.35)	(6.35)			
	(6r6×6.35)	(6.6)		(6.35×9.11)	(6r6)	(6r6)			
				(6r6×9.11)					
vr6.7			−	+	−	−	+	+	+
				(9.43)				(13.24)	
				(9.11)					
vr8.9				+	−	−			
				(9.11)					
				(9.43)					
vr9				−	−	−	+	+	+
				(9.11×9.23)	(9.11)	(9.11)	(9.11)	(9.11×13.24)	(9.11)
				(9.11×9.43)		(9.22)	(9.43)	(9.43×13.24)	(9.43)
				(9.23×9.43)					
vr9.22						−	−	−	+
								(13.24)	
								(13.47)	
vr9r2							−	−	+
								(13.24)	
								(13.47)	

"+" denotes complementation of lethality; "−" denotes failure to complement; blank spaces represent crosses not done. Crosses with specific mutations from each complementation group are indicated in parentheses. Contrary to expectations, mutation *vr6.7* complements other mutations of the *vr6* locus and fails to complement mutations *vr4.57* and *vr8.9*. Thus, we have assigned these three mutations to complementation group *vr4*. Mutations *vr9.22* and *vr9r2* appear to be deletions spanning several complementation groups (also see Figure 8).

TABLE 2

Complementation analysis of lethal *latheo*^{P1} excision alleles with other EMS- or γ-ray-induced lethal mutations in the *vestigal* region

	vr14	*vr4*	*vr3*	*vr6*	*vr9*	*vr11*	*vr13*	*vr5*	*vr19*
lE34		+	+	−	+	+	+	+	
lE49		+	+	−	+	+	+	+	
lE101	−	−	−	−	−	−	−	+	+
lE107	+	+	−	−	+	+	+	+	+
lE311		+	+	−	+	+	+	+	
lE344		+	+	−	+	+	+	+	
lE352		+	+	−	+	+	+	+	
lE357		+	+	−	+	+	+	+	
lE372		+	+	−	+	+	+	+	
lE388	+	+	−	−	+	+	+	+	+

"+" denotes complementation of lethality; "−" denotes failure to complement; and blank spaces represent crosses not done. Based on the cytological breakpoints of *Df(2)vg107* and *Df(2)vg56*, LASKO and PARDUE (1988) found the *vr14* and *vr19* loci to be most proximal and distal, respectively. They were not able to assign a linear order to the remaining loci. Our results indicate, however, that *vr3* lies next to *vr6* and that *vr5* lies proximal to *vr19*. The linear order of the remaining loci is arbitrary and is arranged here simply in numerical order (also see Figure 8).

Olfactory acuity in *lat*^{P1}*/Df* heterozygotes: Figure 9, A and B, shows olfactory acuity (undiluted MCH and OCT, respectively) in several of these genotypes. Once again, *lat*^{P1}*/Df* and +/*Df*, *lat*^{P1}*/vr6.6* and +/*vr6.6* or *lat*^{P1}/+ and +/+ flies were tested together. Afterward, these genotypes were distinguished based on eye color differences, and PIs were calculated separately. As was the case for 15-min retention scores, mean olfactory acuity scores in flies heterozygous for *lat*^{P1} and *vg56*, *vgb*, *vr6.6* or *lE49* were significantly lower than their controls, while those heterozygous for *lat*^{P1} and *vg107* or + [*w*(C-S4)] were

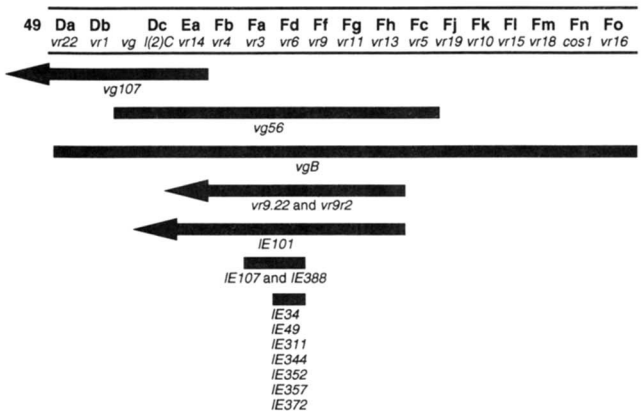

FIGURE 8.—Organization of lethal complementation groups and chromosomal deficiencies in the *vestigal* region. Lethal complementation groups (**Da**, **Db**, etc.), with corresponding names of mutations (*vr22*, *vr1*, etc.), and deleted regions (black bars; arrows indicate that deletions continue beyond tested complementation groups) of *Df(2)vg107*, *Df(2)vg56* and *Df(2)vgB* are arranged generally as in LASKO and PARDUE (1988). Our complementation analyses with *vr9.22* and *vr9r2* have shown that they, too, are deletions of several vital genes. Complementation analyses with the *latheo* lethal excisions (*lE101*, *lE107*, etc.) indicate that excisions *lE101*, *lE107* and *lE388* delete two or more vital genes in the region, while the remaining excisions only affect complementations with the **Fd** group. These results, along with those from behavioral experiments (see RESULTS) place the *latheo* mutations in complementation group **Fd**. In LASKO and PARDUE (1988), the linear order of vital genes **Fa–Fh**, between the distal breakpoints of *Df(2)vg107* and *Df(2)vg56*, was not determined. New results from our complementation analyses with the lethal *latheo* excisions indicate that **Fa** must be adjacent to **Fd** and **Fc** must be adjacent to **Fj** (see Tables 1 and 2).

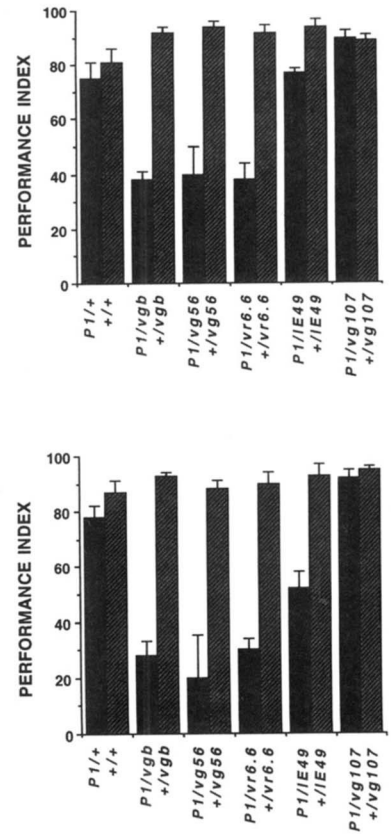

FIGURE 9.—Complementation analysis for olfactory acuity among various deficiencies and EMS-, γ-ray- or *P* element excision-induced mutations in the *vestigal* region. Genotypes are the same as those in Figure 7. **Top**, Avoidance of undiluted MCH. **Bottom**, Avoidance of undiluted OCT. Olfactory acuity is aberrant in more severe *latheo* genotypes. $n = 4$ PIs for each genotype.

not (planned comparisons, $\alpha = 0.05$). Interestingly, olfactory acuity in $lat^{P1}/lE49$ flies, which had a genetic background most similar to that of lat^{P1} homozygotes, was significantly higher than in flies heterozygous for lat^{P1} and *vg56*, *vgb* or *vr6.6* (unplanned comparisons, $\alpha = 0.05$; see below and DISCUSSION).

Shock reactivity in lat^{P1}/Df heterozygotes: Results from shock reactivity experiments with lat^{P1}/vgb and $+/vgb$ flies are shown in Figure 10. Here again, flies of both genotypes were tested together; the genotypes were distinguished afterwards by differences in eye color, and separate PAs were calculated. A two-way ANOVA indicated a significant effect of VOLTAGE ($F_{[5, 84]} = 62.99$, $P = 0.0001$), but no significant effects of GENOTYPE ($F_{[1, 84]} = 2.5$, $P = 0.16$) or the interaction of VOLTAGE and GENOTYPE ($F_{[5, 84]} = 0.66$, $P = 0.66$). Planned pairwise comparisons ($\alpha = 0.05$) of mean PAs also failed to reveal significant differences between the two genotypes at any particular voltage. Visual inspection of Figure 10, however, suggests a trend–that shock reactivity in lat^{P1}/vgb flies might be lower at higher voltages than that in $+/vgb$ flies. In fact, a Wilcoxon matched-pairs signed-ranks nonparametric test, which could be used because mutant and control flies were tested together, indicated that scores for lat^{P1}/vgb flies were significantly lower

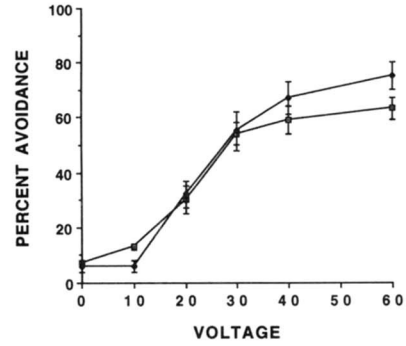

FIGURE 10.—Shock reactivity for heterozygous $latheo^{P1}/vgb$ flies (open squares) or for $+/vgb$ flies (solid circles). Behavior was measured as in Figure 4. Shock reactivity at 60 V is aberrant in more severe *latheo* genotypes. $n = 8$ for each genotype and voltage.

than those for $+/vgb$ flies at 60 V ($T = 0$, $N = 8$, $P < 0.05$)–which was the voltage used during conditioning experiments.

Locomotor activity in *lat* and lat^{+} flies

Poor performance in olfactory acuity and shock reactivity tests in $latheo^{P1}/Df$ and $latheo^{P1}/vr6.6$ flies suggested that these genotypes might suffer from a more general effect on behavior. The most obvious

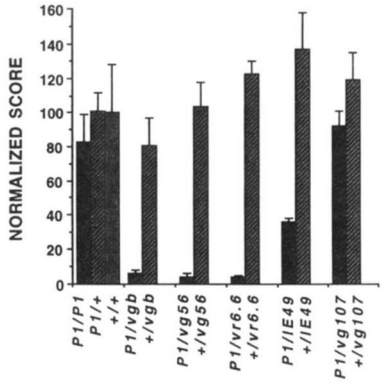

FIGURE 11.—Complementation analysis for locomotor activity among various deficiencies and EMS-, γ-ray- or *P* element excision-induced mutations in the *vestigal* region. Activity scores for 50–100 individual flies were obtained using a Benzer countercurrent apparatus in the dark. Scores ranged from 0 to 5, representing the number of trials in which the fly ran out of a start tube into a distal tube within 30 sec. Activity scores were normalized to the mean score of *w*(C-S4) flies run on the same day. Locomotor activity in more severe *latheo* mutants also is aberrant. $n = 4$ experiments (days) for each genotype.

component common to all of our behavioral assays is locomotion, so we assayed locomotor activity in the dark with a Benzer countercurrent apparatus (see MATERIALS AND METHODS). Because flies are repeatedly tapped into the start tube every 30 sec during this procedure, all three components of locomotor activity–spontaneous activity, reactivity and stimulated activity (MEEHAN and WILSON 1987)—probably are reflected in our measure. As usual lat^{P1}/Df, $lat^{P1}/vr6.6$ or $lat^{P1}/+$ flies were tested together with their appropriate controls and distinguished afterward based on eye color differences; then separate mean activity scores (which ranged from 0 to 5) were calculated for each genotype. In addition, activity scores for each mutant genotype were normalized to the mean score of *w*(C-S4) control flies each day to minimize daily variations in overall activity.

Results from the locomotor activity assays are summarized in Figure 11. No significant differences were detected among the mean (normalized) scores of lat^{P1}/lat^{P1}, $lat^{P1}/+$ or +/+ [*w*(C-S4)] flies (unplanned comparisons, alpha = 0.05), suggesting that $latheo^{P1}$ homozygotes have normal locomotor activity. More important, locomotor activity in flies that were heterozygous for lat^{P1} and *vg56*, *vgb*, *vr6.6* or *lE49* was significantly lower than that in their respective controls ($F_{[1, 39]} = 100.2$, $P = 0.0001$; $F_{[1, 39]} = 74.5$, $P = 0.0005$; $F_{[1, 39]} = 116.5$, $P = 0.0001$; and $F_{[1, 39]} = 101.0$, $P = 0.0001$). Mean scores of these mutant flies were quite low, indicating sluggish locomotor activity. These data suggest that poor performance of these same genotypes in the olfactory acuity and shock reactivity assays is caused, at least in part, by poor locomotor activity. Such sluggish behavior also is consistent with the notion that these mutant flies represent subviable genotypic variants of an essential gene.

Correlation between retention and olfactory acuity among *latheo* excision lines

The appearance of such a strong effect on olfactory acuity in the $lat^{P1}/null$ mutants prompted us to take a closer look at olfactory acuity in lat^{P1}/lat^{P1} homozygotes. Even though olfactory acuity scores did not differ significantly between lat^{P1}/lat^{P1} mutants and +/+ controls in Figure 3, we noticed that mean values of mutant flies were lower than those of wild-type flies in 7 of 8 groups. Since lat^{P1}/lat^{P1} and +/+ control flies were mixed together in olfactory acuity tests, we were able to apply a Wilcoxon matched-pairs signed-ranks nonparametric test (SIEGEL 1956) to reassess any differences between the two genotypes. Even with this more rigorous statistical test, olfactory acuity in mutant flies was significantly lower than that in controls in only 2 of the 8 groups in Figure 3. Thus, these data yield no evidence that olfactory acuity is lower than normal in $latheo^{P1}$ homozygotes.

This conclusion is reinforced from data on olfactory acuity in the 13 (outcrossed) excision lines (Figure 6). PIs for OCT and for MCH were generated by exposing flies to undiluted concentrations of each odor *vs.* air (see MATERIALS AND METHODS). Although olfactory acuity scores ranged from PI = 83 to 97 for OCT and from PI = 71 to 92 for MCH among these excision lines–a range larger than the observed difference between lat^{P1} homozygotes and +/+ flies–no significant correlation between olfactory acuity and 15-min retention was detected ($r_s = 0.122$, $P = 0.65$ for OCT; $r_s = 0.449$, $P = 0.08$ for MCH).

Habituation of the jump reflex to olfactory cues in *lat* and lat^{+} flies

A more rigorous demonstration that potential olfactory acuity, shock reactivity or locomotor deficits in lat^{P1}/lat^{P1} flies are not responsible for poor performance in learning tests was accomplished by studying habituation of the jump reflex (see MATERIALS AND METHODS). This form of nonassociative learning is produced by exposing single male flies to 4-sec pulses of 10% benzaldehdye in a constant current of air. Initially, 100% of flies will jump in response to this noxious stimulus, but the percentage of flies that jump decreases significantly with repeated presentations of the odor. Mutations that affect learning show a slower-than-normal response decrement over trials and normal levels of dishabituation, whereas mutations that affect sensory input or motor output systems show a faster-than-normal response decrement over trials and abnormally low levels of dishabituation (S. KOSS and T. TULLY, in preparation). Thus, potential pleiotropic effects of a mutation on sensory input or motor output systems and on learning produce op-

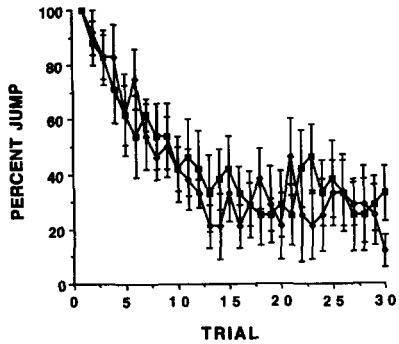

FIGURE 12.—Habituation of the jump reflex to olfactory cues in lat^{P1}/lat^{P1} (open squares) and $lat^{P1}/+$ (solid circles) flies. Individual flies of each genotype were exposed repeatedly to 4-sec pulses of 10% benzaldehyde delivered in a constant current of air. Habituation is shown as a decrement in the percentage of flies jumping during the 4-sec odor stimulus over 30 trials. Mutant flies habituated normally, suggesting that sensory input and motor output systems are normal (see text for more details). $n = 24$ flies for each genotype. Within each experiment, four flies of each genotype were tested; then the experiment was repeated six times. SEM for each trial and genotype were generated by calculating the percent jump for each experiment and then by averaging the six percentages.

posite effects in the habituation assay–unlike in the classical conditioning experiments.

Individual lat^{P1}/lat^{P1} or $lat^{P1}/+$ flies were exposed to 4-sec pulses of 10% benzaldehyde every 10 min for 30 trials. The percentages of flies that jumped within the 4-min odor presentation for each genotype are plotted as a function of trials in Figure 12. Log likelihood analysis of jump responses (0 or 1) with TRIAL as a (third-order polynomial) covariate revealed no significant effect of GENOTYPE ($X^2_{[1]} = 0.70$, $0.1 < P < 0.5$) and no significant GENOTYPE × TRIAL interaction ($X^2_{[1]} = 1.52$, $0.1 < P < 0.5$). In a separate experiment, we also determined a habituation score for individual flies as the number of trials required to reach a block of four consecutive no-jump trials (trials to criterion). Once again, mean habituation scores (± SEM) did not differ between lat^{P1}/lat^{P1} (15.4 ± 2.2, $n = 24$) and $lat^{P1}/+$ (13.1 ± 1.8, $n = 24$) flies ($t_{[46]} = 0.81$, $P = 0.42$). After reaching criterion, these flies were vortexed for 90 sec. Thirty seconds later, they then were presented with one last 4-sec stimulation of benzaldehyde to assess levels of dishabituation, which is another measure of sensory or motor fatigue (S. KOSS and T. TULLY, in preparation). Eighty-one percent and 79% of lat^{P1}/lat^{P1} and $lat^{P1}/+$ flies, respectively, jumped on the dishabituation test trial. [Usually, flies do not show any spontaneous recovery (jump), when left undisturbed for 2 min after reaching criterion.] Thus, these data also indicate that sensory or motor systems are normal in $latheo^{P1}$ homozygotes.

Results were quite different for lat^{P1}/Df heterozygotes. Whereas 100% of lat^{P1}/lat^{P1} and $lat^{P1}/+$ flies jumped in response to a 4-sec 10% benzaldehyde stimulus on the first trial (see Figure 12), only 18% of lat^{P1}/vgb flies did so (data not shown). Moreover, only 12% of these flies jumped in response to a 100% benzaldehyde stimulus. Since flies of this genotype also show severely reduced locomotor activity (see Figure 11), we surmised that locomotor ability is an important component of the jump reflex.

Gross anatomy of adult brain is normal in *latheo* mutants

Brain structure of $latheo^{P1}$ and $latheo^{P1}/vgb$ adult flies was examined in both frontal and horizontal paraffin sections to verify that missing or poorly formed brain structures were not at the root of the learning deficit in $latheo^{P1}$ flies. Six-micrometer slices showed no gross alteration in the mushroom bodies or central complex, two structures believed to be involved in learning (*cf.* HEISENBERG *et al.* 1985; NIGHORN, HEALY and DAVIS 1991, or in any other region of the brain (data not shown).

DISCUSSION

We have isolated, from a behavioral screen of 1016 homozygous *P* element insertion mutants, a new mutation, $latheo^{P1}$, which disrupts associative learning. Mutant flies, tested immediately after discriminative classical conditioning using odors as conditioned stimuli and electric shock as the unconditioned stimulus (negative reinforcement), on average show only 69% of wild-type levels of conditioned avoidance. This "learning" deficit remains constant during the first 6 hr of memory retention (see Figure 2), thereby producing a mutant memory curve that is parallel to that of wild-type flies. Such a memory decay curve for $latheo^{P1}$ mutants is qualitatively different from those for *dunce*, *rutabaga* and *amnesiac* mutants, all of which are thought to disrupt aspects of memory formation (see TULLY *et al.* 1990). Instead, the memory decay curve for $latheo^{P1}$ mutants is similar to that reported for flies heterozygous for a deletion of the *Ddc* (dopa decarboxylase deficient) gene [*Df(2L)TW130*] and Ddc^+ (TEMPEL, LIVINGSTONE and QUINN 1984). Combined with the fact that the *Ddc* gene is involved with the synthesis of two (putative) monoamine neurotransmitters, dopamine and serotonin (LIVINGSTONE and TEMPEL 1983), these behavioral results have led to the speculation that learning (acquisition), rather than memory, is disrupted in *Ddc* mutants. Thus, *latheo* may be involved in acquisition processes.

The performance deficit in homozygous $latheo^{P1}$ mutants does not result from aberrant olfactory acuity (Figure 3), shock reactivity (Figure 4) or locomotor activity (Figure 10). The former two "peripheral" behaviors were measured in a task-relevant manner. Behavioral responses to OCT and MCH (including the concentrations used for conditioning) and escape

from electric shock (including 60 V) were measured in the T-maze of the classical conditioning apparatus. In addition, the possibility that poor performance in mutant flies was due to an (nonassociative) interaction between olfaction and escape was excluded by results from the "unpaired" control experiments (Figure 5). Behavioral assays of olfactory acuity and shock reactivity themselves relied on locomotor activity, for which we obtained an independent measure as well. Furthermore, we did not detect a correlation between olfactory acuity and memory scores in flies homozygous for viable excision alleles of *latheo* (Figure 6). Consequently, these results lead us to believe that *latheo* is a gene involved with associative learning.

Although $latheo^{P1}$ homozygotes performed normally in this battery of behavioral tasks, we were able to detect aberrant peripheral effects in more severe *latheo* mutants (Figures 9, 10 and 11). The behavioral data suggest a general "pleiotropy" due to the sluggishness of subviable genotypes with too little functional *latheo* product. A similar observation was reported for the *cacophony* (*cac*) mutation, which affects components of the Drosophila courtship song. Homozygous *cac* mutants were slightly sluggish; *cac/Df* flies, however, showed much more severe effects on locomotor acitivity (KULKARNI and HALL 1987). More general pleiotropic effects also have been reported for a majority of mutations affecting behavior (GRIGLIATTI *et al.* 1973; HOMYK 1977; HOMYK and SHEPPARD 1977; HOMYK, SZIDONYA and SUZUKI 1980; KYRIACOU and HALL 1980; HOMYK and GRIGLIATTI 1983; ROYDEN, PIRROTTA and JAN 1987; KULKARNI, STEINLAUF and HALL 1988; MCKENNA *et al.* 1989; WHEELER *et al.* 1989; WOODWARD *et al.* 1989; LILLY and CARLSON 1990; KYRIACOU *et al.* 1990). Some genetic variants of the *optomotor blind* (*omb*) gene, for instance, have defective tergite coloration and wing development (BRUNNER *et al.* 1992). Mutants of the *pentagon* (*ptg*) gene show olfactory deficits, hyperpigmentation of the dorsal thorax and female sterility (HELFAND and CARLSON 1989). Even *dunce* memory mutants are female sterile and show severe morphological defects (BYERS, DAVIS and KIGER 1981; BELLEN and KIGER 1987; BELLEN *et al.* 1987).

Further consideration of the potential pleiotropic effects of a mutation on olfactory acuity and on associative learning of olfactory cues raises an important conceptual issue. Mutations that affect olfactory acuity or shock reactivity will produce low scores in classical conditioning tests, as will mutations that affect associative learning. Thus, one cannot use the olfactory classical conditioning procedure to determine whether associative learning also is disrupted in mutants with aberrant peripheral behaviors. One potential solution to this problem is to assay learning in a task that requires input from a different sensory modality. Different sensory modalities, however, may use different molecular mechanisms to produce learning (HEISENBERG and WOLF 1984; HEISENBERG *et al.* 1985; *cf.* STAUBLI, FARADAY and LYNCH 1985). So, a mutation that disrupts learning in one sensory modality necessarily may not disrupt learning in another.

Our solution was to study habituation of the jump reflex in $latheo^{P1}$ mutants. With this assay, waning of the jump reflex is abnormally fast in flies with mutations that affect sensory or motor (peripheral) systems, while it is abnormally slow in flies with mutations that affect learning (S. KOSS and T. TULLY, in preparation). Significantly, habituation and dishabituation of the jump reflex in $latheo^{P1}$ mutants were normal, again indicating that sensory and motor systems are normal in these mutants (Figure 12). Other forms of habituation have been shown to be aberrant in other extant learning/memory mutants (DUERR and QUINN 1982; WITTEKIND and SPATZ 1988; CORFAS and DUDAI 1989). Our results, along with those from the unpaired control experiment, suggest that *latheo* is involved with associative, rather than nonassociative, learning.

Convergent genetic evidence indicates that a *P* element insertion in 49F7-8 is solely responsible for the performance deficit in $latheo^{P1}$ mutants. (1) A single *P* element insertion in $latheo^{P1}$ flies was mapped *in situ* to cytological region 49F7-8 of polytene salivary chromosomes. Flies heterozygous for $latheo^{P1}$ and chromosomal deficiencies of this region (*vg56* and *vgb*) have 15-min retention deficits; flies heterozygous for $latheo^{P1}$ and a chromosomal deficiency next to, but not including, this region (*vg107*) show normal 15-min retention (Figure 7). (2) Although excision of the *P* element has not yet produced a revertant, a range of hypomorphic excision alleles was obtained (Figure 6). (3) Lethal excision alleles also were recovered; complementation mapping of some of these alleles with other EMS- or γ-ray-induced lethal alleles from the 49F region localized *latheo* to one lethal complementation group (Tables 1 and 2). Flies heterozygous for $latheo^{P1}$ and *vr6.6* or *vr6.35*–two EMS-induced lethal mutations in complementation group 49Fd–show a decrement in 15-min memory retention, as do flies heterozygous for $latheo^{P1}$ and $latheo^{lE49}$ (Figure 7 and RESULTS). (4) All mutations of *latheo* tested so far are recessive (Figure 7). Taken together, these results strongly suggest that $latheo^{P1}$ is a hypomorphic mutation of an essential gene. A possibility remains that *latheo* is a nonessential gene next to, or very near, *vr6*. For this to be true, however, the *vr6.6* and *vr6.35* EMS lethal mutations and the $latheo^{lE49}$ lethal excision each would have to affect both the essential and the nonessential genes, while the $latheo^{P1}$ mutation would affect only the nonessential one. We consider this possibility to be slight.

As was the case for pleiotropic effects, mutations in other vital genes have been identified from behavioral experiments. Both the *cac* and *omb* genes, mentioned above, are vital. The former has been cloned and shows (deduced) protein sequence homologies to DNA binding proteins (PFLUGFELDER *et al.* 1992). A *flightless* mutation (*fli 1*) turned out to be a viable allele of the ebryonic lethal abnormal visual system (*elav*) gene (HOMYK, ISONO and PAK 1980; CAMPOS, GROSSMAN and WHITE 1985; HOMYK, ISONO and PAK 1985). Molecular cloning of this gene suggests that it is an RNA-binding protein that is ubiquitously and specifically expressing in neurons of the central and peripheral nervous system (CAMPOS *et al.* 1987; ROBINOW and WHITE, 1991). The *technical knockout* (*tko*) gene was identified in a screen for "bang sensitive" mutants and encodes a protein homologous to a ribosomal S12 protein, thereby suggesting that this gene may affect mitochondrial function (ROYDEN, PIRROTTA and JAN 1987). The *optic ganglion reduced* (*ogre*) gene originally was identified in a screen for mutations affecting visual behavior (LIPSHITZ and KANKEL 1985), and a mutation in the *pentagon* gene was found in a screen for olfactory mutants (HELFAND and CARLSON 1989). No molecular insight is yet available for these latter two genes (but see WATANABE and KANKEL 1990).

A complicating factor during our mutant screen was the effects of genetic background primarily on olfactory acuity and associative learning. Polygenic variability for olfactory acuity and/or associative learning within and among wild-type strains has been reported (FUYAMA 1976; ALCORTA and RUBIO 1989; MONTE *et al.* 1989). In some instances, the magnitude of such background effects can be as large as the effects of single-gene mutations (see TULLY and QUINN 1985; GAILEY, VILLELLA and TULLY 1991). Thus, the sampling variation inherent to selecting single "transposition" or "excision" males most likely produced interstrain variability for these polygenic backgrounds when the homozygous *P* element insertion or excision lines were bred (see Figure 1). Outcrossing these lines to a wild-type "parental" strain [*w*(C-S4)] for several generations served to equilibrate these differences in genetic background, leading to the identification of false-positive mutant lines during the behavioral screen (see RESULTS) and to a few dramatic changes in 15-min retention scores in the excision lines (Figure 6). [In contrast, *latheo*^{P1} mutants still displayed their mutant phenotype now after more than 20 generations of outcrossing to *w*(C-S4) flies (see MATERIALS AND METHODS).] This genetic phenomenon has prompted us to breed isogenic parental stocks before continuing to screen for any new *P* element insertion mutants.

Work with homozygous *latheo*^{P1} or *latheo* excision lines over many generations during this study also revealed ample evidence for an amelioration of the mutant phenotype with time. This phenomenon, too, has been noted before in studies of other learning/memory mutants (TULLY and QUINN 1985; TULLY and GERGEN 1986) and apparently is due to the accumulation of modifiers in the (heterogeneous) genetic background because homozygous mutant flies are less fit (also see BRUNNER *et al.* 1992). As a result, we found it necessary to maintain mutant *latheo* alleles by outcrossing heterozygotes to "fresh" wild-type flies each generation. In this manner, we obtained the strongest mutant phenotype whenever *latheo* homozygotes were bred for behavioral experiments (see Figure 6).

Based on the facts that *latheo* is on the second chromosome and that all the other learning/memory genes are *X*-linked, *latheo* represents a new gene involved with associative learning in Drosophila. Since its memory decay curve is qualitatively different from those of *dunce* and *rutabaga*, both of which encode enzymatic components of the cAMP cell signaling pathway, we speculate that *latheo* may affect a different aspect of the underlying biological machinery. This new gene is *P* element "tagged," which will expedite its cloning and molecular identification. Finally, given its vital nature, studies of lethal genotypes may shed light on the nature of *latheo*'s role in essential developmental processes.

We thank A. SOUTHGATE, M. L. PARDUE and T. WU for providing stocks carrying lethal mutations and chromosomal deficiencies of the second chromosome, L. RESTIFO and K. WHITE for their expertise and advice on the anatomical studies, CH. BRANDES, J. M DURA, J. HALL, T. PREAT and R. MIHALEK for critical readings of this manuscript and M. DEL VECCHIO and A. VILLELLA for technical assistance. This work was supported by grant PO1 GM33205 from the National Institutes of Health, a Scholars Award from the McKnight Foundation and a John Merck Scholarship in the Biology of Developmental Disabilities in Children (to T.T.) and by grant F31 MH09946 from the National Institute of Mental Health (to S.B.).

LITERATURE CITED

ACEVES-PINA, E. O., and W. G. QUINN, 1979 Learning in normal and mutant *Drosophila* larvae. Science **206:** 93–95.

ALCORTA, E., and J. RUBIO, 1989 Intrapopulational variation of olfactory responses in *Drosophila melanogaster*. Behav. Genet. **19:** 285–300.

BELLEN, H. J., and J. A. KIGER, 1987 Sexual hyperactivity and reduced longevity of *dunce* females of *Drosophila melanogaster*. Genetics **115:** 153–160.

BELLEN, H. J., B. K. GREGORY, C. L. OLSSON and J. A. KIGER, 1987 Two *Drosophila* learning mutants, *dunce* and *rutabaga*, provide evidence of a maternal role for cAMP on embryogenesis. Dev. Biol. **121:** 432–444.

BENZER, S., 1967 Behavioral mutants of *Drosophila* isolated by countercurrent distribution. Proc. Natl. Acad. Sci. USA **58:** 1112–1119.

BIER, E., H. VAESSIN, S. SHEPHERD, K. LEE, K. MCCALL, S. BARBEL, L. ACKERMAN, R. CARRETTO, T. UEMURA, E. GRELL, L. Y. JAN and Y. N. JAN, 1989 Searching for pattern and mutation in the *Drosophila* genome with a P-*lacZ* vector. Gene Dev. **3:** 1273–1287.

BOOKER, R., and W. G. QUINN, 1981 Conditioning of leg position in normal and mutant *Drosophila*. Proc. Natl. Acad. Sci. USA **78:** 3940–3944.

BRUNNER, A., R. WOLF, G. O. PFLUGFELDER, B. POECK and M. HEISENBERG, 1992 Mutations in the proximal region of the *optomotor-blind* locus of *Drosophila melanogaster* reveal a gradient of neuroanatomical and behavioral phenotypes. J. Neurogenet. **8:** 43–53.

BYERS, D., R. L. DAVIS and J. A. KIGER, 1981 Defect in cyclic AMP phosphodiesterase due to the *dunce* mutation of learning in *Drosophila melanogaster*. Nature **289:** 79–81.

CAMPOS, A. R., D. GROSSMAN and K. WHITE, 1985 Mutant alleles at the locus *elav* in *Drosophila melanogaster* lead to nervous system defects. A developmental-genetic analysis. J. Neurogenet. **2:** 197–218.

CAMPOS, A. R., D. R. ROSEN, S. N. ROBINOW and K. WHITE, 1987 Molecular analysis of the locus *elav* in *Drosophila melanogaster*: a gene whose embryonic expression is neural specific. EMBO J. **6:** 425–431.

CHEN, C. N., S. DENOME and R. L. DAVIS, 1986 Molecular analysis of cDNA clones and the corresponding genomic coding sequences of the *Drosophila dunce* gene, the structural gene for cAMP-dependent phosphodiesterase. Proc. Natl. Acad. Sci. USA **83:** 9313–9317.

CHOI, K.-W., R. SMITH, R. M. BURATOWSKI and W. G. QUINN, 1991 Deficient protein kinase C activity in *turnip*, a *Drosophila* learning mutant. J. Biol. Chem. **266:** 15999–16006.

COEN, D., 1990 *P* element regulatory products enhance *zeste*^{1} repression of a *P[white*^{duplicated}*]* transgene in *Drosophila melanogaster*. Genetics **126:** 949–960.

COOLEY, L., R. KELLEY and A. SPRADLING, 1988 Insertional mutagenesis of the *Drosophila* genome with single P elements. Science **239:** 1121–1128.

CORFAS, G., and Y. DUDAI, 1989 Habituation and dishabituation of a cleaning reflex in normal and mutant *Drosophila*. J. Neurosci. **9:** 56–62.

DAVIS, R. L., and N. DAVIDSON, 1984 Isolation of *Drosophila melanogaster dunce* chromosomal region and recombinational mapping of *dunce* sequences with restriction site polymorphisms as genetic markers. Mol. Cell. Biol. **4:** 358–367.

DRAIN, P., E. FOLKERS and W. G. QUINN, 1991 cAMP-dependent protein kinase and the disruption of learning in transgenic flies. Neuron **6:** 71–82.

DUDAI, Y., Y. N. JAN, D. BYERS, W. G. QUINN and S. BENZER, 1976 *dunce*, a mutant of *Drosophila* deficient in learning. Proc. Natl. Acad. Sci. USA **73:** 1684–1688.

DUDAI, Y., B. SHER, D. SEGAL and Y. YOVELL, 1985 Defective responsiveness of adenylate cyclase to forskolin in the *Drosophila* memory mutant *rutabaga*. J. Neurogenet. **2:** 356–380.

DUERR, J. S., and W. G. QUINN, 1982 Three *Drosophila* mutations that block associative learning also affect habituation and sensitization. Proc. Natl. Acad. Sci. USA **79:** 3646–3650.

ENGELS, W. R., C. R. PRESTON, P. THOMPSON and W. B. EGGLESTON, 1986 *In situ* hybridization to *Drosophila* salivary chromosomes with biotinylated DNA probes and alkaline phosphatase. Focus **8:** 6–8.

FEINSTEIN, P. F., K. A. SCHRADER, H. A. BAKALYAR, W.-J. TANG, J. KRUPINSKI, A. G. GILMAN and R. R. REED, 1991 Molecular cloning and characterization of a Ca^{2+}/calmodulin insensitive adenylyl cyclase from rat brain. Proc. Natl. Acad. Sci. USA **88:** 10173–10177.

FOLKERS, E., 1982 Visual learning and memory of *Drosophila melanogaster* wild-type C-S and the mutants *dunce*^{1}, *amnesiac*, *turnip*, and *rutabaga*. J. Insect Physiol. **28:** 535–539.

FUYAMA, Y., 1976 Behavior genetics of olfactory responses in *Drosophila* I. Olfactometry and strain differences in *Drosophila melanogaster*. Behav. Genet. **6:** 407–420.

GAILEY, D., F. R. JACKSON and R. W. SIEGEL, 1984 Conditioning mutations in *Drosophila melanogaster* affect an experience-dependent behavioral modification in courting males. Genetics **106:** 613–623.

GAILEY, D. A., A. VILLELLA and T. TULLY, 1991 Reassessment of the effect of biological rhythm mutations on learning in *Drosophila melanogaster*. J. Comp. Physiol. A **169:** 685–697.

GRIGLIATTI, T. A., L. HALL, R. ROSENBLUTH and D. T. SUZUKI, 1973 Temperature sensitive mutations in *Drosophila melanogaster*. XIV. A selection of immobile adults. Mol. Gen. Genet. **120:** 107–114.

HALL, J. C., 1985 Genetic analysis of behavior in insects, pp. 287–383 in *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, Vol. 9. edited by G. A. KERKUT and L. I. GILBERT. Pergamon Press, Oxford.

HAZELRIGG, T., R. LEVIS and G. M. RUBIN, 1984 Transformation of *white* locus DNA in *Drosophila*: dosage compensation, *zeste* interaction and position effects. Cell **36:** 469–481.

HEISENBERG, M., and R. WOLF, 1984 Vision in *Drosophila*, in *Studies of Brain Function XII*, edited by V. BRAITENBERG. Springer Verlag, Berlin.

HEISENBERG, M., A. BORST, S. WAGNER and D. BYERS, 1985 *Drosophila* mushroom body mutants are deficient in olfactory learning. J. Neurogenet. **2:** 1–30.

HELFAND, S. L., and J. CARLSON, 1989 Isolation and characterization of an olfactory mutant in *Drosophila* with a chemically specific defect. Proc. Natl. Acad. Sci. USA **86:** 2908–2912.

HOMYK, T., 1977 Behavioral mutants of *Drosophila melanogaster*. II. behavioral analysis and focus mapping. Genetics **87:** 105–128.

HOMYK, T., and T. A. GRIGLIATTI, 1983 Behavioral mutants of *Drosophila melanogaster*. IV. analysis of developmentally temperature-sensitive mutations affecting flight. Dev. Genet. **4:** 77–97.

HOMYK, T., K. ISONO and W. L. PAK, 1985 Developmental and physiological analysis of a conditional mutation affecting photoreceptor and optic lobe development in *Drosophila melanogaster*. J. Neurogenet. **2:** 309–324.

HOMYK, T., and D. E. SHEPPARD, 1977 Behavioral mutants of *Drosophila melanogaster*. I. Isolation and mapping of mutations which decrease flight ability. Genetics **87:** 95–104.

HOMYK, T., J. SZIDONYA and D. T. SUZUKI, 1980 Behavioral mutants of *Drosophila melanogaster*. III. Isolation and mapping of mutations by direct visual observations of behavioral phenotypes. Mol. Gen. Genet. **177:** 553–565.

HUMASON, G. L., 1972 *Animal Tissues and Techniques*, Ed. 3. W. H. Freeman, San Fransisco.

KANDEL, E. R., M. KLEIN, B. HOCHNER, M. SHUSTER, S. A. SIEGELBAUM, R. D. HAWKINS, D. L. GLANZMAN, V. F. CASTELLUCCI and T. W. ABRAMS, 1987 Synaptic modulation and learning: new insights into synaptic transmission from the study of behavior, pp. 471–518 in *Synaptic Function*, edited by G. M. EDELMAN, W. E. GALL and W. M. COWAN. John Wiley & Sons, New York.

KAUVER, L. M., 1982 Defective cyclic adenosine 3′5′ monophos-

phate phosphodiesterase in the *Drosophila* memory mutant *dunce*. J. Neurosci. **3:** 1347–1358.

KIDWELL, M. G., 1986 P-M mutagenesis, pp. 59–82 in *Drosophila: A Practical Approach*, edited by D. B. ROBERTS. IRL Press, Oxford.

KIGER, J. A., R. L. DAVIS, H. K. SALTZ, T. FLETCHER and M. BOWLING, 1981 Genetic analysis of cyclic nucleotide phosphodiesterase in *Drosophila melanogaster*. Adv. Cyclic Nucleotide Res. **14:** 273–288.

KRUPINSKI, J., F. COUSSEN, H. A. BAKALYAR, W. J. TANG, P. G. FEINSTEIN, K. ORTH, C. SLAUGHTER, R. R. REED and A. G. GILMAN, 1989 Adenylyl cyclase amino acid sequence: possible channel- or transporter-like structure. Science **244:** 1558–1564.

KULKARNI, S. J., and J. C. HALL, 1987 Behavioral and cytogenetic analysis of the *cacophony* courtship song mutant and interacting genetic variants in *Drosophila melanogaster*. Genetics **115:** 461–475.

KULKARNI, S. J., A. F. STEINLAUF and J. C. HALL, 1988 The *dissonance* mutant of courtship song in *Drosophila melanogaster*: isolation, behavior and cytogenetics. Genetics **118:** 267–285.

KYRIACOU, C. P., and J. C. HALL, 1980 Circadian rhythm mutations in *Drosophila melanogaster* affect short-term fluctuations in the male's courtship song. Proc. Natl. Acad. Sci. USA **77:** 6929–6933.

KYRIACOU, C. P., and J. C. HALL, 1984 Learning and memory mutations impair acoustic priming of mating behavior in *Drosophila*. Nature **308:** 62–65.

KYRIACOU, C. P., M. OLDROYD, J. WOOD, M. SHARP and M. HILL, 1990 Clock mutants alter developmental timing in *Drosophila*. Heredity **64:** 395–401.

LASKO, P. F., and M. L. PARDUE, 1988 Studies of the genetic organization of the *vestigal* microregion of *Drosophila melanogaster*. Genetics **120:** 495–502.

LEVIN, L. R., P.-L. HAN, P. M. HWANG, P. G. FEINSTEIN, R. L. DAVIS and R. R. REED, 1992 The *Drosophila* learning and memory gene, *rutabaga*, encodes a Ca^{2+}/calmodulin-responsive adenylyl cyclase. Cell **68:** 479–489.

LILLY, M., and J. CARLSON, 1990 *smellblind*: a gene required for Drosophila olfaction. Genetics **124:** 293–302.

LINDSLEY, D. L., and G. G. ZIMM, 1992 *The Genome of Drosophila melanogaster*. Academic Press, San Diego, Calif.

LIPSHITZ, H. D., and D. R. KANKEL, 1985 Specificity of gene action during central nervous system development in *Drosophila melanogaster*: analysis of the *lethal (1) optic ganglion reduced* locus. Dev. Biol. **108:** 56–77.

LIVINGSTONE, M. S., 1985 Genetic dissection of *Drosophila* adenylate cyclase. Proc. Natl. Acad. Sci. USA **82:** 5992–5996.

LIVINGSTONE, M. S., P. P. SZIBER and W. G. QUINN, 1984 Loss of calcium/calmodulin responsiveness in adenylate cyclase of *rutabaga*, a *Drosophila* learning mutant. Cell **37:** 205–215.

LIVINGSTONE, M. S., and B. L. TEMPEL, 1983 Genetic dissection of monoamine neurotransmitter synthesis in *Drosophila*. Nature **303:** 67–70.

MCKENNA, M., P. MONTE, S. HELFAND, C. WOODWARD and J. CARLSON, 1989 A simple chemosensory response in *Drosophila* and the isolation of *acj* mutants in which it is affected. Proc. Natl. Acad. Sci. USA **86:** 8118–8122.

MEEHAN, M. J., and R. WILSON, 1987 Locomotor activity in the *Tyr-1* mutant of *Drosophila melanogaster*. Behav. Genet. **17:** 503–512.

MENZEL, R., M. HAMMER, G. BRAUN, J. MAUELSHAGEN, and M. SUGAWA, 1991 Neurobiology of learning and memory in honeybees, pp. 323–353 in *The Behaviour and Physiology of Bees*, edited by L. J. GOODMAN and R. C. FISHER. CAB International, London.

MONTE, P., C. WOODWARD, R. AYER, M. LILLY, H. SUN and J. CARLSON, 1989 Characterization of the larval olfactory response in *Drosophila* and its genetic basis. Behav. Genet. **19:** 267–283.

NIGHORN, A., M. J. HEALY and R. L. DAVIS, 1991 The cyclic AMP phosphodiesterase encoded by the *Drosophila dunce* gene is concentrated in the mushroom body neuropil. Neuron **6:** 455–467.

O'KANE, C. J., and W. J. GEHRING, 1987 Detection *in situ* of genomic regulatory elements in *Drosophila*. Proc. Natl. Acad. Sci. USA **84:** 9123–9127.

PFLUGFELDER, G. O., H. ROTH, B. POECK, S. KERSCHER, H. SCHWARZ, B. JONSCHKER and M. HEISENBERG, 1992 The *l(1)optomotor-blind* gene of *Drosophila melanogaster* is a major organizer of optic lobe development. Isolation and characterization of the gene. Proc. Natl. Acad. Sci. USA **89:** 1199–1203.

QUINN, W. G., W. A. HARRIS and S. BENZER, 1974 Conditioned behavior in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **71:** 708–712.

QUINN, W. G., P. P. SZIBER and R. BOOKER, 1979 The *Drosophila* memory mutant *amnesiac*. Nature **277:** 212–214.

ROBERTSON, H. M., C. R. PRESTON, R. W. PHILLIS, D. M. JOHNSON-SCHLITZ, W. K. BENZ and W. R. ENGELS, 1988 A stable source of P-element transposase in *Drosophila melanogaster*. Genetics **118:** 461–470.

ROBINOW, S., and K. WHITE, 1991 Characterization and spatial distribution of the ELAV protein during *Drosophila melanogaster* development. J. Neurobiol. **22:** 443–461.

ROYDEN, C. S., V. PIRROTTA and L. Y. JAN, 1987 The *tko* locus, site of a behavioral mutation in *D. melanogaster*, codes for a protein homologous to prokaryotic ribosomal protein S12. Cell **51:** 165–173.

SHOTWELL, S. L., 1983 Cyclic adenosine 3′:5′-monophosphate phosphodiesterase and its role in learning in *Drosophila*. J. Neurosci. **3:** 739–747.

SIEGEL, S., 1956 *Nonparametric Statistics for the Behavioral Sciences*. McGraw-Hill, New York.

SIEGEL, R. W., and J. C. HALL, 1979 Conditioned responses in courtship behavior of normal and mutant *Drosophila*. Proc. Natl. Acad. Sci. USA **76:** 3430–3434.

SOKAL, R. R., and F. J. ROHLF, 1981 *Biometry: The Principles and Practice of Statistics in Biological Research*, Ed. 2. W. H. Freeman, New York.

STAUBLI, U., R. FARADAY and G. LYNCH, 1985 Pharmacological dissociation of memory: anisomycin, a protein synthesis inhibitor, and leupeptin, a protease inhibitor, block different learning tasks. Behav. Neural Biol. **43:** 287–297.

STEWART, B., and J. R. MERRIAM, 1980 Dosage compensation, pp. 107–140 in *The Genetics and Biology of Drosophila*, Vol. 2d, edited by M. ASHBURNER and T. WRIGHT. Academic Press, London.

TEMPEL, B. L., M. S. LIVINGSTONE and W. G. QUINN, 1984 Mutations in the dopa decarboxylase gene affect learning in *Drosophila*. Proc. Natl. Acad. Sci. USA **81:** 3577–3581.

THOMPSON, R. F., and W. A. SPENCER, 1966 Habituation: a model phenomenon for the study of neuronal substrates of behavior. Psychol. Rev. **173:** 16–43.

TULLY, T., 1987 *Drosophila* learning and memory revisited. Trends Neurosci. **10:** 330–335.

TULLY, T., 1991 *Drosophila*'s role in identifying the building blocks of associative learning and memory, pp. 46–64 in *Per-*

spectives in Cognitive Neuroscience, edited by R. G. LISTER and H. J. WEINGARTNER. Oxford University Press.

TULLY, T., and J. P. GERGEN, 1986 Deletion mapping of the *Drosophila* memory mutant *amnesiac*. J. Neurogenet. **3:** 33–47.

TULLY, T., and W. G. QUINN, 1985 Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. J. Comp. Physiol. A **157:** 263–277.

TULLY, T., S. BOYNTON, C. BRANDES, J. M. DURA, R. MIHALEK, T. PREAT and A. VILLELLA, 1990 Genetic dissection of memory formation in *Drosophila melanogaster*. Cold Spring Harbor Symp. Quant. Biol. **55:** 203–211.

WATANABE, T., and D. R. KANKEL, 1990 Molecular cloning and analysis of *l(1)ogre*, a locus of *Drosophila melanogaster* with prominent effects on the postembryonic development of the central nervous system. Genetics **126:** 1033–1044.

WHEELER, D. A., S. J. KULKARNI, D. A. GAILEY and J. C. HALL, 1989 Spectral analysis of courtship songs in behavioral mutants of *Drosophila melanogaster*. Behav. Genet. **19:** 503–528.

WITTEKIND, W. C., and H. CH. SPATZ, 1988 Habituation of the landing response of *Drosophila*, pp. 351–368 in *Modulation of Synaptic Transmission and Plasticity in Nervous Systems*, edited by G. HERTTING and H. CH. SPATZ. Springer-Verlag, Berlin.

WOODWARD, C., T. HUANG, H. SUN, S. L. HELFAND and J. CARLSON, 1989 Genetic analysis of olfactory behavior in *Drosophila*: a new screen yields the *ota* mutants. Genetics **123:** 315–326.

Communicating editor: A. CHOVNICK