The *Drosophila* Mutation *turnip* Has Pleiotropic Behavioral Effects and Does Not Specifically Affect Learning

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Abstract

The *Drosophila* mutant *turnip* (*tur*) was isolated on the basis of its poor performance in an olfactory learning task, and also has a reduction in protein kinase C (PKC) activity. PKC has been found in the nervous systems of a wide range of organisms and appears to have an important role in learning and memory-related processes. Unfortunately, previous reports documenting the learning defect of *tur* lacked the controls required to assess the origins of the poor performance of the mutant. We have analyzed the effects of the *tur* mutation on both associative and nonassociative learning as well as on PKC activity. Additionally, the effects of the mutation on the task-relevant sensorimotor abilities of the flies were assessed. Although we were able to replicate previous behavioral and biochemical results obtained with *tur*, we discovered that the *tur* mutation also affected response to electric shock and caused a drastic reduction in the locomotor ability of the flies. Because locomotion is an essential component of the learning assays, this result makes it impossible to conclude that *tur* specifically affects learning and demonstrates the crucial importance of sensorimotor controls in conditioning experiments.

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Introduction

Associative learning in the fruit fly *Drosophila melanogaster* was reported by Benzer's lab over 20 years ago (Quinn et al. 1974). The isolation and characterization of numerous single-gene mutants defective in learning and memory followed quickly thereafter. Two of these original mutants, *dunce* (*dnc*) and *rutabaga* (*rut*), have been characterized extensively both biochemically and behaviorally. Both mutations affect central components in the cyclic AMP (cAMP) signal transduction pathway: *dnc* encodes a cAMP phosphodiesterase (Byers et al. 1981; Chen et al. 1986; Qiu et al. 1991), and *rut* is the gene for Ca²⁺/calmodulin-stimulated adenylyl cyclase (Livingstone et al. 1984; Levin et al. 1992). In addition, protein kinase A (PKA) activity in *Drosophila* has been disrupted using inducible transgenes expressing an enzyme-specific pseudo-substrate peptide (Drain et al. 1991), as well as by mutations in a PKA catalytic subunit (Skoulakis et al. 1993; Li et al. 1996) and an RI regulatory subunit (S.F. Goodwin, M. Del Vecchio, C. Hogel, T. Tully, and K. Kalser, in prep.). Interfering with normal PKA activity in each case impaired olfactory associative learning or memory. The importance of the cAMP/PKA pathway to normal learning and memory in flies has been bolstered further by recent data implicating the cAMP-responsive transcription factor CREB in the formation of long-term memory in *Drosophila* (Tully et al. 1994; Yin et al. 1994, 1995).

The early genetic identification of cAMP as required for learning and memory in flies was particularly exciting in view of physiological results. cAMP also had been identified as an important factor in normal learning and memory processes using whole animal, dissociated ganglia, and cell culture

preparations of *Aplysia californica* (Cedar et al. 1972; Kandel et al. 1987). In addition, electrophysiological studies pointed to a role for the cAMP second messenger system in the formation and maintenance of long-term potentiation, which is generally thought to be a cellular correlate of memory in mammals (Huang and Kandel 1994; Huang et al. 1994; Weisskopf et al. 1994). Our belief that findings in *Drosophila* will be relevant to learning and memory in other systems has been justified to a large extent by this convergence of results.

Many other signal transduction systems intersect with the cAMP pathway, and so it is not surprising that other molecules, notably kinases, have been shown to be required for normal learning and memory in a variety of organisms. One of the first of the so-called gene knockout mice was specifically engineered to lack the α -isoform of calmodulin-responsive kinase II (α CaM-k II; Silva et al. 1992a). These mice were shown to be defective not only physiologically, but also behaviorally (Silva et al. 1992b). α CaM-k II activity in *Drosophila* was reduced by an inducible transgene expressing an α CaM-k II-specific peptide inhibitor (Griffith et al. 1993). The effect of this was to reduce performance in both nonassociative acoustic priming (Kyriacou and Hall 1984) and associative courtship conditioning tasks (Siegel and Hall 1979).

The use of kinase inhibitors in the chick (*Gallus domesticus*) has implicated α CaM-k II, PKA, and protein kinase C (PKC) in memory formation (Rosenzweig et al. 1993). In *Aplysia*, both PKA and PKC appear to mediate neurotransmitter-induced synaptic transmission at the sensory and motor neuron synapse controlling the gill-withdrawal reflex (Braha et al. 1993). The contribution of PKC in facilitating neurotransmitter release increased as synaptic transmission was reduced as a result of habituation, a form of nonassociative learning (Ghirardi et al. 1992).

Recent evidence has also indicated that PKC is involved in learning and memory processes in mice (Abeliovich et al. 1993a). Eliminating the function of one of the 10 known PKC isoforms in mice, PKC γ , slightly impaired the ability of mice to learn spatial and contextual cues. Both spatial and contextual conditioning in mammals have been shown to be dependent on normal hippocampal function (Sutherland et al. 1982; Squire and Zola-Morgan 1988; Sutherland and McDonald 1990; Squire 1992). Long-term potentiation (LTP) is also defec-

tive in the PKC γ -mutant mice (Abeliovich et al. 1993b), which is consistent with pharmacological evidence that PKC activity is required for induction (Malinow et al. 1989) and maintenance (Colley et al. 1990; Wang and Feng 1992) of LTP in rat hippocampal slices.

PKC is present in both the cytosol and membrane of most cells (Nishizuka 1992). In rabbits, Bank et al. (1988, 1989) demonstrated that, following associative learning, PKC was redistributed from cytosol to membrane; it was the membrane-bound form of PKC which was active (Burgoyne 1989). This finding also was corroborated using discrimination learning in rats (Olds et al. 1990; Olds and Alkon 1991; Paylor et al. 1992) and in the nudibranch *Hermisenda* (Farley and Auerbach 1986; McPhie et al. 1993). Further experiments with *Hermisenda* revealed that PKC inhibitors can block the changes in membrane currents produced by associative learning (Farley and Schuman 1991) and that PKC activators can produce the same changes in K⁺ conductance as associative learning does (Etcheberrigaray et al. 1992). The fact that PKC has been shown to be involved in learning and memory in both vertebrate and invertebrate systems suggests its role in these processes may be fundamental.

After *dnc* and *rut*, the most studied of the original *Drosophila* learning and memory mutants is *turnip* (*tur*). *tur* flies have been tested in a variety of behavioral assays, including visual and olfactory associative learning tasks (Aceves-Piña and Quinn 1979; Quinn et al. 1979; Booker and Quinn 1981; Folkers 1982; Tempel et al. 1983; Tully and Quinn 1985), courtship conditioning (Gailey et al. 1982, 1984), and sensitization and habituation of the proboscis extension reflex (Duerr and Quinn 1982). The results of many of these experiments have not been particularly convincing primarily because the necessary control experiments were not performed (see Discussion). For example, the first report that mentions *tur* (Quinn et al. 1979) contains no data on the mutant's ability to sense or respond to any of the stimuli used during the training procedure. Similarly, stimulus perception by *tur* flies was not assessed quantitatively in any of the associative learning studies.

Numerous biochemical phenotypes have been linked to the *tur* mutation over the years: an altered G_s protein (R.F. Smith, K.-W. Choi, T. Tully, and W.G. Quinn, unpubl.), reduced binding affinity by a serotonin receptor (Aceves-Piña et al. 1983), and, most recently, reduced PKC activity

(Choi et al. 1991). In view of these apparent pleiotropic effects of the *tur* mutation and the possibility that *tur* might represent an entry point to further analysis of the role of PKC in learning and memory, we have undertaken a careful examination of the levels of PKC activity in *tur* flies as well as assaying this mutant's performance in Pavlovian (associative) and habituation (nonassociative) learning tasks. Our results show that although the *tur* mutation clearly caused poor performance during both Pavlovian olfactory learning and olfactory habituation, as well as reducing PKC activity, behavioral control experiments revealed that *tur* also disrupted general locomotor activity, making it impossible to conclude that these animals have a specific defect in learning.

Materials and Methods

STOCKS

The *tur* lines used for these experiments all derived from a subline of the original *tur* mutant stock maintained by P. Sziber in W.G. Quinn's laboratory. The recombinant lines described here were constructed by R. Smith, K. Choi, and T. Tully in Quinn's laboratory using the multiply marked X chromosome *y v f car* (*yellow*, *vermillion*, *forked*, *carnation*) and were identical to those used by Choi et al. (1991) except that ours were continually outcrossed (see below). The marked chromosome did not carry *crossveinless* (*cv*), contrary to the statement of Choi et al. (1991). Whereas we tested these recombinant lines for immediate learning in an associative odor-discrimination task (Tully and Quinn 1985) as quickly as possible after the recombinant chromosomes were first identified (~2 months; see Fig. 2a,b), similar data presented by Choi et al. (1991) were generated after these lines had been maintained as homozygous stocks for at least 3 years, allowing the possible accumulation of genetic modifiers in the backgrounds of the behaviorally mutant recombinant lines (see Discussion). The two recombinant lines that were more extensively characterized in this study (*y v f tur* #87, low scoring; *y v f tur* #64, high scoring) represented the most extreme ends of the behavioral spectrum seen in our recombinant mapping experiments (Fig. 2a). Throughout most of this report (Figs. 3–8) we will refer to *y v f tur* #87 simply as *y v f tur* and *y v f tur* #64 as *y v f tur*⁺.

Flies carrying the X chromosome balancer *FM7a* were kept continually outcrossed to our

wild-type strain, Canton-S (Can-S). (The *FM7a* balancer chromosome is incapable of recombination with its normal homolog, and thus ensures that the recombinant *tur* chromosome remains intact in females.) To prevent the accumulation of modifiers in the genetic background of the *tur* recombinant stocks (Gailey et al. 1991; Boynton and Tully 1992; Dura et al. 1993; deBelle and Heisenberg 1996), these stocks were continually outcrossed to this "cantonized" *FM7a* background by mating heterozygous *y v f tur* recombinant/*FM7a* females to *FM7a/Y* males. In most of the experiments described here (Figs. 3–8) the chromosome bearing the *Df(1)JA27* deficiency (hereafter referred to as *JA27*) also carried the markers *y*, *v*, and *f*, which were recombined onto it by K.-W. Choi (pers. comm.); this *y v f JA27* strain was also continually outcrossed to our cantonized *FM7a* stock. All markers, the *FM7a* balancer chromosome, and the *Df(1)JA27* deletion are described in Lindsley and Zimm (1992).

Flies homozygous for a given *tur* recombinant chromosome were generated by mating heterozygous *y v f tur* recombinant/*FM7a* females to *y v f tur* recombinant/*Y* males. Similarly, *y v f tur* recombinant/*y v f JA27* females were generated by crossing *y v f JA27/FM7a* females to the appropriate *y v f tur* recombinant/*Y* males. These crosses were set up on a large scale and only first-generation flies were used for the associative conditioning and PKC experiments. The progeny of such first-generation homozygotes were used for the habituation and dark reactivity experiments.

All lines were maintained at 25°C on a 16/8-hr light/dark cycle with lights on at 8 a.m. Flies were raised on food consisting of (per liter) 94.2 grams of dextrose, 76.1 grams of cornmeal, 31.9 grams of yeast (Nutrex #540), 8.7 grams of sodium potassium tartrate, 8.4 grams of agar, 7 grams of CaCl₂, and 2 grams of Tegosept M mold inhibitor. This food medium and other rearing conditions are similar to those of Boynton and Tully (1992).

MAPPING

Unpublished results cited in Booker and Quinn (1981) placed *tur* between the markers *f* and *car* on the X chromosome. Several tester chromosomes bearing deletions in this region were used to further localize *tur* (Choi et al. 1991; T. Tully and J.P. Gergen, unpubl.). The only deletion chromosome that failed to complement the *tur* associative learning phenotype was *Df(1)JA27*.

In the initial deletion mapping experiments, we used the original version of the *JA27* chromosome, which lacked any morphological markers. The presence of *y*, *v*, and *f* on the *JA27* chromosome in our later experiments allowed us to compare directly the results with homozygous recombinant flies and recombinant/deficiency heterozygotes, because all carried the same markers. Previous results have indicated that some phenotypic marker genes, such as *y* (Tully and Gold 1993), can interfere with associative learning and memory. The wild-type Canton-S strain was used as an overall control to verify that the apparatuses used for behavioral testing were working properly and to provide an independent measure of wild-type PKC activity levels.

PKC ASSAY

PKC activity was determined essentially as described in Choi et al. (1991) except that we used the peptide substrate [Ser²⁵]-PKC (19–31) (Peninsula Laboratories) instead of histone H1, because of the increased specificity of PKC for the peptide (House and Kemp 1987). Each phosphorylation reaction was carried out in a final volume of 50 μ l containing 20 μ l of tissue homogenate and 10 μ l of 5 $\times$ assay cocktail, supplemented with 10 μ l of 5 mM CaCl₂ and 10 μ l of a 10 mg/ml phosphatidyl serine:1 mg/ml dioleoylglycerol mixture (Avanti Polar Lipids), as appropriate (see below).

One tissue homogenate was prepared from the heads of ~400 flies of each genotype. The heads were collected by placing the flies in a 50 ml conical-bottomed centrifuge tube and immersing the tube in liquid nitrogen for 5 min. The tube was removed from the liquid nitrogen and pounded on the benchtop ~20 times, separating the heads, wings, and legs from the bodies. The heads were isolated with a series of metal sieves (prechilled on dry ice).

The heads were homogenized in 16 ml of ice-cold buffer per gram of heads, where the buffer consisted of 20 mM HEPES at pH 7.0, 0.2 M sucrose, 2 mM EDTA, 1 mM EGTA, 25 mM benzamidine, 1 mM PMSF, and 1 mg/ml of leupeptin. To facilitate homogenization, the heads first were ground to a powder using a liquid nitrogen-chilled mortar and pestle, and then the powder was transferred to an ice-cold glass-on-glass homogenizer for homogenization. The homogenate was centrifuged at 2000g for 12 min at 2°C to pellet large particles and de-

bris. The supernatant then was centrifuged at 40,000g for 35 min at 2°C to separate the soluble (cytosolic) PKC from the particulate (membrane-associated) PKC.

The pellet containing particulate PKC was resuspended in 1.667 ml of buffer per gram of heads, this buffer being 25 mM Tris-acetate at pH 7.6 containing the same complement and concentration of protease inhibitors as the initial homogenization buffer. This mixture then was diluted to a protein concentration of 1 mg/ml, brought to 5 mM EGTA, and placed on ice for 15 min to extract the membrane-associated PKC. After incubating on ice, the mixture was centrifuged at 150,000g for 35 min at 2°C. The supernatant contained the membrane-associated PKC released from the membrane by EGTA. This supernatant was used as the membrane-associated fraction in our experiments. The tissue homogenate fractions were adjusted to 2.5 mM EGTA, resulting in a final EGTA concentration of 1 mM in each assay. Serial dilutions of both the soluble and membrane-associated fractions were assayed to ensure linearity of enzyme activity versus protein concentration (data not shown).

The 5 $\times$ assay cocktail consisted of 100 mM Tris-acetate at pH 7.6, 50 mM magnesium acetate, 25 mM dithiothreitol, 10.0 μ M [Ser²⁵]-PKC (19–31), 100 μ M ATP, and 0.5 μ Ci [γ -³²P]ATP. Assays were initiated by adding tissue homogenate to tubes containing the assay cocktail with or without activators and were allowed to proceed for 10 min at 26°C. Each reaction was terminated by the addition of 20 μ l of glacial acetic acid. A 2 $\times$ 2-cm piece of Whatman P81 phosphocellulose filter paper was spotted with 20 μ l of the assay mixture and dried. Dried filters were washed three times in a large excess of 75 mM orthophosphoric acid; 2 min for the first wash and 10 min for each of the next two. Filter squares were dried again and counted in aqueous scintillation fluid.

Three sets of assays were run, in triplicate, for each homogenate: no activators, 1 mM Ca²⁺, and 1 mM Ca²⁺ with phosphatidyl serine and dioleoylglycerol (these lipids were sonicated for 1 min, on ice, immediately before use). Background phosphorylation was taken to be the counts incorporated in the assay with no activators added. This value was subtracted from the counts incorporated in both the Ca²⁺ and the Ca²⁺-phosphatidyl serine-dioleoylglycerol-stimulated assays. PKC activity was reported as the increase in ³²P incorporation (measured in counts per minute) attributable to the addition of the lipid activators and was typi-

cally five to seven times greater than the activity in the presence of Ca^{2+} alone.

ASSOCIATIVE LEARNING

Flies of each genotype were trained and tested using the Pavlovian conditioning procedure of Tully and Quinn (1985). On the evening prior to each day of associative conditioning tests, flies were placed in fresh half-pint glass bottles of food containing a dry, folded paper towel. This allowed the flies to clean themselves, helping to avoid the introduction of debris from the flies into the conditioning apparatus and preventing wet flies from short-circuiting the shock grid.

To begin training, ~100 flies were aspirated from their food bottle into the training tube of the conditioning apparatus, which contained an electrifiable copper grid covering 90% of its inner surface. After a 90-sec acclimation period, flies were exposed sequentially to two aversive odorants, 3-octanol (OCT; ICN) and 4-methylcyclohexanol (MCH; ICN), carried through the training tube on a current of air at 750 ml/min. Based on the results of previous experiments, the concentrations of the odors were adjusted by varying the surface area of each odorant exposed to the air stream so that naive flies would distribute themselves 50:50 in the T maze. During their 60-sec exposure to the first odor (the conditioned stimulus, CS+), flies received twelve 1.25-sec pulses of 60 V DC electric shock (the unconditioned stimulus, US) at 5-sec interpulse intervals. Following a 45-sec rest interval, the flies then were exposed to the second odor without shock (the CS-) for 60 sec. After presentation of the CS-, the flies received another 45-sec rest interval. During each rest interval the training tube was continually flushed with air. The flies then were transferred to the choice point of a T maze by tapping them into a small chamber at the end of the training tube and lowering this into register with the two arms of the T maze. At the choice point, flies were exposed to two converging currents of air (750 ml/min each) carrying the odors used during training. Flies were allowed to choose between the CS+ and the CS- for 120 sec, at which time they were trapped in each arm by sliding the small chamber out of register with the T maze. Flies from each tube were anesthetized, counted, and then released unharmed into the environment.

Each experiment consisted of two trials: in the

first, OCT served as the CS+ and MCH as the CS-, whereas in the second (using a different group of flies of the same genotype), MCH was the CS+ and OCT the CS-. For the flies tested in each trial, the probability of a correct response (COR) was calculated as the number of flies in the CS- tube divided by the total number of flies in the T maze arms: $\text{COR} = (\text{CS-}) / [(\text{CS+}) + (\text{CS-})]$. These two COR values were then averaged, and that average was normalized to produce one performance index (PI) for each experiment: $\text{PI} = \{[(\text{COR}_{\text{OCT}} + \text{COR}_{\text{MCH}}) / 2] - 0.5\} / 0.5 \times 100 = [(\text{COR}_{\text{OCT}} + \text{COR}_{\text{MCH}}) - 1] \times 100$. A PI can range from -100 to 100, where 0 represents no learning (50:50 distribution of flies at the choice point) and 100 represents perfect learning (all flies in the CS- tube). Calculation of a PI in this manner is algebraically similar to the calculation of the learning index (Λ) of Tully and Quinn (1985) except that the number of flies remaining at the choice point is not included. This change in calculation avoids the confounding of genotypic differences between mutant and wild-type flies by the number of animals remaining in the center compartment, as is the case when using Λ (Tully and Gold 1993). Because the PI was an average of reciprocal trials where the CS+ for one half of the experiment serves as the CS- for the second half, it is not biased by any nonassociative changes in odor avoidance that may have occurred during the conditioning (Tully 1984; Tully and Quinn 1985).

Previous experiments have shown that even though flies of a given genotype are trained and tested as a group, individuals behave independently of one another in the T maze (Tempel et al. 1984; Tully and Gergen 1986) and each shows a similar probability of responding correctly (Tully et al. 1994).

OLFACTORY ACUITY

The ability of flies to perceive the odor stimuli presented during the conditioning experiment was measured by evaluating the avoidance of each odor versus air (Tully and Quinn 1985; Asztalos et al. 1991; Drain et al. 1991; Gailey et al. 1991; Boynton and Tully 1992; Dura et al. 1993; Folkers et al. 1993; Tully et al. 1994; Yin et al. 1994). Odor concentrations used for determining olfactory acuity were the same as those used for Pavlovian conditioning. A group of about 100 naive, female flies of each genotype were loaded into the small chamber

of the training apparatus, which was then lowered into register with the two tubes of the choice point; one tube contained the odorant and the other air. Flies were allowed to choose between odor versus air for 120 sec, then the flies in each arm of the T maze were counted and a performance index was calculated for each odor individually: $PI_{OCT} = [(COR - 0.5)/0.5] \times 100 = [(COR \times 2) - 1] \times 100$ and $PI_{MCH} = [(COR - 0.5)/0.5] \times 100 = [(COR \times 2) - 1] \times 100$. Experiments were balanced; the odor was in the left arm of the T maze for one half, and in the right arm for the other half.

SHOCK REACTIVITY

To measure the ability of the flies to sense and escape from the electric shock used during Pavlovian conditioning, shock reactivity was measured as described in Boynton and Tully (1992). A training tube containing an electrifiable copper grid was inserted into each side of the T maze. Flies were aspirated into the left arm of the T maze with the center post drawn up, blocking escape from that arm. Following 55 sec of rest, flies were given one 1.25-sec 60 V DC shock and the center post lowered, thereby allowing the flies to escape into the opposite, unshocked tube of the T maze. For 60 sec, the 60 V shock was delivered in 1.25-sec pulses every 5 sec, giving flies 1 min to escape from the shock. For the second half of the experiment, flies were aspirated into the right arm and had to escape the electric shock by moving into the left arm. The percent avoidance was calculated as the number of flies in the unshocked T-maze arm and in the center compartment (shocks cannot be delivered in this compartment) divided by the total number of flies and multiplied by 100.

HABITUATION

Habituation was modified somewhat from Boynton and Tully (1992). Briefly, 3- to 4-day-old male flies were contained individually in plastic chambers through which air (bubbled through mineral oil) was drawn. The flies were exposed to a 4-sec pulse of odorant [5% benzaldehyde (by volume) in mineral oil] delivered every minute, to which they initially responded by jumping off the chamber wall and attempting to fly (McKenna et al. 1989). After repeated presentations, each fly be-

came habituated to the odorant and stopped jumping. Our criterion for habituation was for the fly not to jump for four consecutive odor pulses. The number of jumps up to but not including the first of the four no-jumps was the TTC (trials-to-criterion) score.

Spontaneous recovery and dishabituation were both measured 2 min after the fly achieved criterion. For spontaneous recovery the fly was left undisturbed in the chamber and the odorant was again presented at the end of the 2 min period. For dishabituation, the chamber containing the fly was removed from the habituation apparatus, vortexed (75 sec at 1720 oscillations/min) and returned to the apparatus in time for the test odor pulse.

DARK REACTIVITY

To assay locomotor reactivity in response to mechanical agitation, flies were tested in the dark in the countercurrent apparatus described by Benzer (1967), at 25°C and 70% relative humidity as described by Boynton and Tully (1992). Usually 25–35 flies were used in a single run and never <23 or >37 flies. After the flies were loaded and allowed to acclimate to the apparatus for 60 sec, the apparatus was held vertically and rapped sharply several times on a padded benchtop to initiate the escape response of the flies and was then laid flat on the benchtop. The flies were allowed to run into the distal tubes for 30 sec for each of five cycles. Dark reactivity scores were calculated as simple averages, with each fly being given a score equal to the number of the tube reached at the end of the run; this ranged from 0 to 5.

STATISTICS

PIs are distributed normally (Tully and Gold 1993), so untransformed data were analyzed parametrically with the Macintosh software package JMP 3.1 (SAS Institute, Inc.). For multiple pairwise planned comparisons, an experimentwise error rate of $\alpha = 0.05$ was maintained; the critical *P* values for individual comparisons were adjusted accordingly (Sokal and Rohlf 1981; Audesirk and Audesirk 1989) and are listed for each experiment. All of these behavioral experiments were performed in a balanced fashion, with *N* = 2 PIs collected per day per group. Raw TTC scores from habituation experiments were near-normal (slightly leptokur-

totic) and were subjected to a one-way analysis of variance (ANOVA). Spontaneous recovery and dishabituation scores were dichotomous (1 or 0) and so were subjected to a log-likelihood analysis. For habituation experiments, equal numbers of individuals per genotype were tested each day. Dark reactivity scores follow a binomial distribution and thus were subjected to an arcsine square root transformation before being subjected to a one-way ANOVA. For dark reactivity experiments, equal numbers of groups of individuals per genotype were tested each day. In all experiments, the experimenter was blind to genotype.

LEARNING IN WILD-TYPE, MUTANT AND GENETIC VARIANTS CARRYING THE *Df(1)JA27* DEFICIENCY

PIs from four GENOTypes (*tur/tur*, *tur/JA27*, *tur⁺/JA27*, and *tur⁺/tur⁺*) were subjected to a one-way ANOVA with GENOTYPE ($F_{(3,18)} = 21.70$, $P < 0.001$) as the main effect (Fig. 1). The three planned comparisons were deemed significant if $P \leq 0.017$.

LEARNING IN WILD-TYPE FLIES AND RECOMBINANTS BETWEEN *y v f (tur⁺) car* AND *tur* FLIES

PIs from nine GENOTypes (Can-S, *y v f* #87, *y v f* #139, *y v f* #111, *y v f* #94, *y v f* #19, *y v f*

Figure 1: Deletion mapping of the original *tur* mutation. Wild-type (Can-S; *tur⁺*) and mutant (*tur*) flies were tested for associative learning as homozygotes and as heterozygotes with the X-chromosomal deficiency JA27. The significant difference between scores for *tur/JA27* and *tur⁺/JA27* show that the *tur* mutation maps to this deficiency interval. $n = 6$ for all genotypes except *tur/tur* ($n = 4$).

#64, *y v f* #138, and *y v f tur⁺ car* homozygotes) were subjected to a one-way ANOVA with GENO ($F_{(8,27)} = 278.34$, $P < 0.001$) as the main effect (Fig. 2a). Tukey-Kramer unplanned pairwise comparisons ($\alpha = 0.05$) were performed subsequently.

LEARNING IN WILD-TYPE FLIES AND HETEROZYGOUS *y v f tur* RECOMBINANT FLIES

PIs from eight GENOTypes (Can-S, *y v f* #87/Can-S, *y v f car* #4/*tur*, *y v f car* #48/*tur*, *y v f car* #5/*tur*, *y v f* #19/*tur*, *y v f* #57/*tur*, and *y v f* #87/*tur*) were subjected to a one-way ANOVA with GENO ($F_{(7,24)} = 35.21$, $P < 0.001$) as the main effect (Fig. 2b). Tukey-Kramer unplanned pairwise comparisons ($\alpha = 0.05$) were performed subsequently.

COMPARISONS OF WILD-TYPE, MUTANT, AND GENETIC VARIANTS CARRYING THE *Df(1)JA27* DEFICIENCY AND THE *y v f* MORPHOLOGICAL MARKERS

PKC activity Mean PKC specific activity values were calculated for five genotypes (Can-S, *y v f tur/y v f tur*, *y v f tur/y v f JA27*, *y v f tur⁺/y v f JA27*, and *y v f tur⁺/y v f tur⁺*) and subjected to a square-root transformation (Fig. 3). These transformed values then were subjected to a one-way ANOVA with GENOTYPE ($F_{(4,15)} = 5.33$, $P = 0.007$) as the main effect. The two planned comparisons were deemed significant if $P \leq 0.025$.

Learning PIs from five GENOTypes (Can-S, *y v f tur/y v f tur*, *y v f tur/y v f JA27*, *y v f tur⁺/y v f JA27*, and *y v f tur⁺/y v f tur⁺*) were subjected to a one-way ANOVA with GENOTYPE ($F_{(4,25)} = 16.72$, $P < 0.001$) as the main effect (Fig. 4). The four planned comparisons were deemed significant if $P \leq 0.013$.

Olfactory acuity PIs from five GENOTypes (Can-S, *y v f tur/y v f tur*, *y v f tur/y v f JA27*, *y v f tur⁺/y v f JA27*, and *y v f tur⁺/y v f tur⁺*) and two ODORS (MCH and OCT) were subjected to a two-way ANOVA with GENO ($F_{(4,30)} = 15.92$, $P < 0.001$) and ODOR ($F_{(1,30)} = 4.15$, $P = 0.05$) as main effects and GENO $\times$ ODOR ($F_{(4,30)} = 1.52$, $P = 0.22$) as an interaction term (Fig. 5). The two planned comparisons were deemed significant if $P \leq 0.025$.

Figure 2: Pavlovian conditioning of *tur* recombinants. (a) Homozygous *y v f car*⁺ recombinant lines were tested for associative learning. Scores of the recombinant lines ranged from wild-type to a level near that of the original *tur* line (cf. Fig. 1). The range of scores seen suggests the recovery of recombinants on either side of the *tur* mutation. As all the recombinants were *y v f*, the lower score of the parental *y v f car* line must have resulted from some locus at the proximal end of that X chromosome, probably *car* itself. *n* = 4 for all genotypes. (b) Lines with recombinant *y v f* chromosomes, some with and some without *car*, were tested for learning when heterozygous with the original *tur* chromosome. These results demonstrate that recombinant line *y v f* #87 carries the *tur* mutation, and that this is responsible for the defect in associative conditioning. In addition, *y v f* #87 was tested as a heterozygote with *Can-S*. *n* = 2 for all genotypes except *y v f* #87/*tur* (*n* = 4), *y v f* #87/*Can-S* (*n* = 8), and *Can-S* (*n* = 10).

Shock reactivity Pls from five GENOTYPES
(*Can-S*, *y v f tur*/*y v f tur*, *y v f tur*/*y v f JA27*, *y v*

Figure 3: Protein kinase C activity of outcrossed *tur* recombinants. The difference between the mean specific activity values for *y v f tur*/*y v f JA27* and *y v f tur*⁺/*y v f JA27* flies shows that the defect in PKC activity maps to the *tur* mutation. Values are presented as the mean $\pm$ S.E.M. *n* = 3 for all genotypes.

f tur⁺/*y v f JA27*, and *y v f tur*⁺/*y v f tur*⁺) were subjected to a one-way ANOVA with GENO ($F_{(4,31)} = 13.69$, $P < 0.001$) as the main effect (Fig. 6). The one planned comparison was deemed significant if $P \leq 0.05$.

Figure 4: Pavlovian conditioning of outcrossed *tur* recombinants. The difference between the learning scores of *y v f tur*/*y v f JA27* and *y v f tur*⁺/*y v f JA27* flies shows that the performance deficit in our Pavlovian conditioning assay maps to the *tur* mutation. Except for *Can-S*, all flies tested were female and were homozygous for the markers *y v f*. Scores are presented as the mean $\pm$ S.E.M. *n* = 4 for all genotypes.

Figure 5: Olfactory acuity tests of the odors used in Pavlovian conditioning. (a) avoidance of MCH; (b) avoidance of OCT. There were no significant differences among the scores of the genotypes tested. Each value is mean $\pm$ S.E.M. $n = 4$ for all genotypes.

Habituation Raw TTC scores from five GENOTypes (Can-S, *y v f tur/y v f tur*, *y v f tur/y v f JA27*, *y v f tur⁺/y v f JA27*, and *y v f tur⁺/y v f tur⁺*) were subjected to a one-way ANOVA with GENO ($F_{(4,425)} = 10.29$, $P < 0.001$) as the main effect (Fig. 7a). The two planned comparisons were deemed significant if $P \leq 0.025$.

Spontaneous recovery and dishabituation Dichotomous spontaneous recovery (SR) or dishabituation (DIS) scores from five GENOTypes

Figure 6: Test of shock reactivity. This test measures the flies' ability to sense and escape from the electric shock used in Pavlovian conditioning (Boynton and Tully 1992). The difference between the scores of *y v f tur/y v f JA27* and *y v f tur⁺/y v f JA27* flies shows that the defect in shock reactivity maps to the *tur* mutation. Each value is mean $\pm$ S.E.M. $n = 8$ for all genotypes except *y v f tur/JA27* ($n = 4$).

(Can-S, *y v f tur/y v f tur*, *y v f tur/y v f JA27*, *y v f tur⁺/y v f JA27*, and *y v f tur⁺/y v f tur⁺*) were subjected to a log likelihood analysis with GENOTYPE ($\chi^2_{(4)} = 9.759$, $P < 0.045$ for SR, and $\chi^2_{(4)} = 31.49$, $P < 0.001$ for DIS) as the main effect (Fig. 7b). The two planned comparisons for SR and DIS were deemed significant if $P \leq 0.025$.

Dark reactivity Transformed dark reactivity scores from five GENOTypes (Can-S, *y v f tur/y v f tur*, *y v f tur/y v f JA27*, *y v f tur⁺/y v f JA27*, and *y v f tur⁺/y v f tur⁺*) were subjected to a one-way ANOVA with GENOTYPE ($F_{(4,903)} = 182.85$, $P < 0.001$) as the main effect (Fig. 8). The four planned comparisons were deemed significant if $P \leq 0.013$.

Results

DELETION MAPPING

Previous work had demonstrated that the *tur* mutation was completely recessive to the wild-type allele in its effect on initial learning in our Pavlovian classical conditioning assay (Tully and Quinn 1985; see also Fig. 2b), which enabled us to

map the mutation using chromosomal deletions. In our initial attempt, we used a subline of the origi-

Figure 7: Olfactory habituation of the jump response. (a) Number of trials required to reach our criterion of not jumping in response to the odorant (5% benzaldehyde) for four successive trials (TTC = 4). Each value is mean $\pm$ S.E.M. $n = 96$ for all genotypes. (b) DIS (shaded bars) and SR (open bars) of the jump response after habituation. A test pulse of odorant was presented 2 min after each fly reached criterion; during this period the fly was either vortexed for 75 sec in its training chamber (dishabituation) or left undisturbed (spontaneous recovery). Each value is mean $\pm$ S.E.M. $n = 40$ (dishabituation) and $n = 49$ (spontaneous recovery) for all genotypes. The differences between the scores of *y v f tur/y v f JA27* and *y v f tur⁺/y v f JA27* flies for TTC and DIS show that the causes of these differences map to the *tur* mutation; there was no difference among spontaneous recovery scores for the genotypes tested.

Figure 8: Dark reactivity of *tur* flies. Each fly was given a score equivalent to the position of the tube it ended in (from 0 to 5). The difference between the scores of *y v f tur/y v f JA27* and *y v f tur⁺/y v f JA27* flies shows that the locomotor defect revealed by the dark reactivity assay maps to the *tur* mutation. Each value is mean $\pm$ S.E.M. Six groups of each genotype were tested, for individual flies $n = 174$ (*y v f tur/y v f tur*), $n = 178$ (*y v f tur/JA27*), $n = 187$ (*y v f tur⁺/JA27*), $n = 183$ (*y v f tur⁺/y v f tur⁺*), and $n = 186$ (Can-S).

nal *tur* mutant stock. Booker and Quinn (1981) referred to unpublished experiments that put *tur* between the markers *f* and *car* on the X chromosome. Accordingly, we mapped the *tur* mutation against several X-chromosomal deficiencies in this region (data not shown) as did Choi et al. (1991).

All *tur/Df* heterozygotes performed normally in the Pavlovian conditioning apparatus except for *tur/Df(1)JA27*, which gave marginal results (Choi et al. 1991). We therefore repeated the complementation experiment with this deficiency chromosome (Fig. 1). We suspected that the difference in PI between *tur⁺/Df(1)JA27* and *tur/Df(1)JA27* ($P = 0.002$) reflects what could truly be called a learning deficit, and that the slightly larger difference between *tur/Df(1)JA27* and the *tur/tur* homozygote ($P < 0.001$) was the result of some factor in the *tur* line's genetic background, which was complemented by the *Df(1)JA27* ("JA27") background. It is also worth noting that fewer *tur⁺/JA27* and *tur/JA27* flies eclosed than did their *tur⁺/FM7a* and *tur/FM7a* sisters, indicating the JA27 chromosome itself had some negative effect on viability. These *tur/FM7a* flies scored indistinguishably from *tur⁺/tur⁺* and *tur⁺/JA27* (data not

shown); as their autosomal genetic background is identical to that of their *tur*/*JA27* sisters, the different X chromosomes must be solely responsible for the difference in PIs between *tur*⁺/*JA27* and *tur*/*JA27*.

The *JA27* chromosome carries two deletions, one removing region 18A5;18D1-2 (Lindsley and Zimm 1992) and the other taking out (roughly) 20A4-5;20E-F (Miklos et al. 1986). Given the previous unpublished data (Booker and Quinn 1981) mapping the *tur* mutation between *f* (15F1-3) and *car* (18D1-2), *tur* must be in the 18A5;18D1-2 interval.

RECOMBINANT LINES

To remove any autosomal modifiers of *tur* that might have accumulated during stock maintenance, and to make the genetic background of the mutant more like that of the *tur*⁺ (Can-S) control strain, the original *tur* stock was outcrossed for at least five generations to a strain containing the X-chromosomal balancer *FM7a* and Can-S autosomes (cantonized *FM7a*). No *tur* males were seen after a few generations of outcrossing, indicating that there was an X-linked lethal mutation in the original mutant stock and that one or more autosomal suppressors of lethality were removed by cantonizing the line.

This lethal mutation was a likely cause of at least some of the performance difference between *tur*/*JA27* and the *tur*/*tur* homozygote described above. To remove any possible confounding effects of the lethal and its associated autosomal suppressor(s), recombinant lines were generated by crossing *tur* males to *y v f car* females, mating the heterozygous daughters to cantonized *FM7a* males, and using the F₂ females singly to establish independent lines by matings with cantonized *FM7a* males. (This approach allowed us to rescue any potential lethal recombinants.) Therefore, each recombinant X chromosome used in this work consisted of material from the distal end of the multiply-marked *y v f car* chromosome and the proximal portion of the original *tur* chromosome. (The marker *y* is at the distal end of the X chromosome, *car* at the proximal end.)

The recombinants then were tested as homozygotes for learning (Fig. 2a). These lines produced a range of scores, with some lines giving values similar to wild type (*y v f* #19, *y v f* #64, and *y v f* #138; *P* values > 0.05) and others nearly 20 points lower (*y v f* #87, *y v f* #139, *y v f* #111,

and *y v f* #94; *P* values < 0.05), suggesting that we had recovered lines from recombination events on either side of the *tur* mutation. Mean PIs among these lines likely varied, in part, because of genetic background differences attributable to a founder effect (each recombinant line was established from a single female; Dura et al. 1993).

The difference in scores between *y v f car* and wild type (*P* < 0.05) suggested that something on the *y v f car* chromosome adversely affects learning. Mildly deleterious effects of body- and eye-color mutations on learning in this procedure have been observed previously (Tully and Gergen 1986; Drain et al. 1991; Tully and Gold 1993), as have effects of *car* on brain morphology (McCarthy and Nickla 1980).

To be certain that our low-scoring recombinants were not performing poorly because of whatever was responsible for the reduced performance of *y v f car* flies, we tested flies heterozygous for the original *tur* chromosome and recombinant chromosomes carrying *y v f* and either *car* or *car*⁺ (Fig. 2b). The parental *y v f car* recombinants performed as well as Can-S when heterozygous with the *tur* chromosome (*P* > 0.05), demonstrating that whatever defect was causing the poor performance of *y v f car* homozygotes is recessive. The poor performance of *y v f* #87/*tur* flies (Fig. 2b) demonstrated a failure of the *y v f* #87 chromosome to complement *tur*, and thus indicated that this recombinant bears the *tur* mutation. That the *y v f* #87/Can-S flies scored as well as the Can-S controls confirmed that the effect of *tur* is recessive in this assay.

For the remaining experiments described in this paper, we used two recombinant lines with high (*y v f* #64) and low (*y v f* #87) learning scores (Fig. 2a), after outcrossing them for at least five generations using the cantonized *FM7a* line (see Materials and Methods). Because these presumably represent crossover events on either side of the *tur* mutation, we will henceforth refer to these lines as *y v f tur*⁺ and as *y v f tur*, respectively. Hemizygous *y v f tur* males were viable after outcrossing, indicating that the lethal was distal to the *tur* locus and was absent from the recombinant chromosome. The use of an identically marked recombinant deficiency chromosome, *y v f JA27* (courtesy of K.-W. Choi, Baylor College of Medicine, Houston, Texas), allowed us to compare the results of the recombinant homozygotes together with those of the deficiency heterozygotes in subsequent experiments, because all genotypes

now carried and expressed the same morphological markers.

PKC ACTIVITY

We assayed each of five genotypes for PKC activity (Fig. 3). We observed a significant difference in membrane-associated PKC activity from head homogenates between $y\ v\ f\ tur^+/y\ v\ f\ JA27$ and $y\ v\ f\ tur/y\ v\ f\ JA27$ ($P = 0.040$). Our results thus confirmed the finding of Choi et al. (1991) that the reduction in PKC activity in this recombinant *tur* line maps to the *JA27* deficiency interval.

PAVLOVIAN CONDITIONING

The relevant comparison to assess any putative effect of *tur* on associative (Pavlovian) olfactory avoidance conditioning is between $y\ v\ f\ tur^+/y\ v\ f\ JA27$ and $y\ v\ f\ tur/y\ v\ f\ JA27$ (Fig. 4). This comparison revealed a significant difference of 23 points in conditioning scores ($P < 0.001$). There was no dominant effect of the *JA27* deficiency on performance in this assay, as revealed by comparing the average score of the genotypes with the deficiency with the average score of the corresponding homozygotes (+/Df and -/Df vs. +/+ and -/-, $P = 0.87$).

OLFACTION AND SHOCK REACTIVITY

Up to this point, our results were in complete agreement with those of Choi et al. (1991). To properly interpret performance deficits from our Pavlovian conditioning experiments (i.e., to ascribe the performance deficit specifically to associative learning and not to a task-relevant sensorimotor defect), however, it was necessary to verify that the genotypes being compared had no significant differences in their abilities to perceive and respond to the stimuli used for conditioning. For the study of recessive single-gene mutations, this approach is most valid only when comparing +/Df versus -/Df genotypes, which restricts contributions to any phenotypic differences to genetic loci within the region of the chromosomal deficiency.

The aversiveness of the two odorants MCH and OCT was tested by giving untrained flies a choice between each odor versus air (Fig. 5a,b). With this assay, no significant differences were detected between $y\ v\ f\ tur^+/y\ v\ f\ JA27$ versus $y\ v\ f\ tur/y\ v\ f\ JA27$ for either MCH ($P = 0.17$) or OCT ($P = 0.61$).

To assess possible differences in the perception of, and response to, electric shock, untrained flies were tested for their ability to escape from 60 V DC current delivered in one arm of the T maze (Fig. 6). Here we detected a significant reduction in avoidance by $y\ v\ f\ tur/y\ v\ f\ JA27$ compared with $y\ v\ f\ tur^+/y\ v\ f\ JA27$ ($P = 0.03$). The magnitude of this effect on percent avoidance during the shock reactivity assay (11%) is similar to the magnitude of the effect on the PI during Pavlovian conditioning (23 points), because the difference between two PIs is arithmetically equivalent to twice the difference between two percent avoidance scores (see Materials and Methods for equations).

HABITUATION

We also looked at the behavior of wild-type and mutant flies in habituation of the jump reflex to olfactory cues—a nonassociative learning task (Boynton and Tully 1992). Whereas flies jump and attempt to fly away upon the initial presentation of a noxious odor, this response wanes and eventually disappears with repeated presentations.

To verify that the effect of *tur* was recessive in this assay, we tested the heterozygous genotype. The TTC score of female $y\ v\ f\ tur^+/y\ v\ f\ tur$ flies was 3.5 ± 0.4 , the same as that of $y\ v\ f\ tur^+/y\ v\ f\ tur^+$ flies (3.6 ± 0.4 ; $P = 0.818$) and significantly different from that of mutant $y\ v\ f\ tur/y\ v\ f\ tur$ flies (1.9 ± 0.2 ; $P = 0.001$; $n = 103$ for all genotypes). These data show that the effect of the *tur* mutation on habituation is recessive to the wild-type allele. The dishabituation score of the heterozygote (0.67 ± 0.07) was not different from that of the $y\ v\ f\ tur^+$ homozygote (0.67 ± 0.07 ; $P = 1.00$) or that of the mutant (0.50 ± 0.07 ; $P = 0.092$; $n = 46$ for all genotypes). [Note that a significant effect of the mutation on dishabituation was seen in other experiments (see below).] SR scores were not significantly different between the heterozygote (0.12 ± 0.05) and either $y\ v\ f\ tur^+$ (0.08 ± 0.04 ; $P = 0.508$) or $y\ v\ f\ tur$ homozygous animals (0.04 ± 0.03 ; $P = 0.160$; $n = 51$ for all genotypes).

Mutant $y\ v\ f\ tur/y\ v\ f\ JA27$ flies reached our criterion of four successive no-jumps in 1.3 ± 0.1 trials (Fig. 7a) significantly more quickly than did $y\ v\ f\ tur^+/y\ v\ f\ JA27$ flies (2.5 ± 0.3 ; $P = 0.007$). Both of the recombinant chromosomes yielded lower scores in this assay in combination with the *JA27* deficiency chromosome than when homozygous, suggesting a dominant effect of the deficiency (+/Df and -/Df vs. +/+ and -/-, $P = 0.006$).

The same pattern was true for dishabituation (Fig. 7b): Significantly more *y v f tur⁺/y v f JA27* flies jumped in response to an odor pulse given after the fly was vortexed, compared with *y v f tur/y v f JA27* flies (0.38 ± 0.8 vs. 0.17 ± 0.6 ; $P = 0.049$). There was also a dominant effect of the *JA27* deficiency here: The average scores of the deficiency-bearing versus homozygous flies were significantly different ($P < 0.001$). All genotypes showed similar, low levels of (2-min) spontaneous recovery (Fig. 7b). The SR scores for *y v f tur⁺/y v f JA27* and *y v f tur/y v f JA27* flies (0.04 ± 0.3 vs. 0.00 ± 0.00 ; $P = 0.887$) were not significantly different and there was no apparent dominant effect of the *JA27* deficiency ($P = 0.169$). The *tur* mutation thus produced faster-than-normal habituation and lower levels of dishabituation in this assay.

DARK REACTIVITY

The defects displayed in our shock-reactivity test, faster-than-normal habituation, and abnormally low dishabituation were all consistent with *tur* flies having locomotor problems, so we next looked at dark reactivity (cf. Boynton and Tully 1992). In this test, flies are separated on the basis of locomotor activity in response to vigorous mechanical agitation, using the Benzer (1967) counter-current apparatus in the dark. Lines with normal activity levels distribute themselves throughout the apparatus, whereas sluggish flies tend to stay in the starting tube.

The *tur* mutation was semi-dominant in this assay, as the heterozygous *y v f tur⁺/y v f tur* animals (1.37 ± 0.08 ; $n = 240$) were significantly less reactive than the *y v f tur⁺/y v f tur⁺* homozygotes (2.47 ± 0.10 ; $n = 238$; $P < 0.001$) but significantly more reactive than the mutant *y v f tur/y v f tur* flies (0.17 ± 0.03 ; $n = 211$; $P < 0.001$). Thus, we still were able to deficiency-map the *tur* mutation in this assay.

As can be seen in Figure 8, *y v f tur/y v f JA27* flies scored much lower than *y v f tur⁺/y v f JA27* flies (0.52 ± 0.06 vs. 1.96 ± 0.08 ; $P < 0.001$). This difference showed that the locomotor reactivity defect associated with the *tur* mutation mapped to the same locus as the learning and PKC defects.

Discussion

Six *Drosophila* mutants from the first learning and memory screens of D. Byers and W.G. Quinn

have been described in the literature: *dnc* (Dudai et al. 1976), *rut* (Livingstone et al. 1984), *amnesiac* (*amn*) (Quinn et al. 1979), *radish* (*rad*) (Folkers et al. 1993), *cabbage* (*cab*) (Aceves-Piña and Quinn 1979), and *tur* (Quinn et al. 1979; Choi et al. 1991). Both *dnc* and *rut* have been characterized in some detail behaviorally, the genes cloned and sequenced, and their protein products identified (Chen et al. 1986; Qiu et al. 1991; Levin et al. 1992). Feany and Quinn (1995) reported the identification of a P-element insertion mutation that failed to complement *amn* behaviorally, and suggest that *amn* may define a neuropeptide gene. Although the *rad* gene product is not known, *rad* mutants have been shown to have normal olfaction, shock reactivity, and long-term associative memory (Folkers et al. 1993; Tully et al. 1994). Much less has been published about *tur* and *cab*. In the absence of the sort of genetic or biochemical handles that enabled the cloning and molecular characterization of *dnc* and *rut*, the roles of these “poor cousins” of learning and memory processes have been more difficult to pin down.

Recently, Choi et al. reported on the biochemical and behavioral characterization of *tur* (1991) and showed that PKC activity and performance in two olfactory learning assays were defective in *tur* mutants and that these defects mapped to the same region of the X chromosome. This led them to conclude that their data suggested a causal link between reduced PKC activity and defective associative learning. Although results from similar experiments presented here were in agreement with those of Choi et al., we went further with control experiments to show that the poor performance of *tur* cannot, in fact, be ascribed to defects in learning.

Upon thoroughly outcrossing representative recombinant *tur* lines and using Choi's recombinant *y v f JA27* chromosome (to facilitate comparisons by generating all genotypes with the same markers) our initial behavioral results were clear: All genotypes with the markers *y*, *v*, and *f* scored lower than Canton-S in the Pavlovian conditioning assay because of either some allele(s) in the marked region of the X chromosomes of the mutants or a specific, pleiotropic effect of one or more of the markers, and that *y v f tur* genotypes scored significantly lower than did *y v f tur⁺* genotypes (Fig. 4). This latter difference is the result of the *tur* mutation.

Concluding that a performance deficit in the Pavlovian assay is a bona fide result of a defect in

associative learning requires the demonstration that the mutants being tested are normal for any sensorimotor behaviors that subserve their odor-avoidance responses in the learning test. In our associative conditioning procedure, the flies must be able to perceive the two odors used, as well as the electric shock, and to respond to these by showing avoidance responses in the T maze. If a mutant cannot sense or respond normally to either of the conditioning odors, or if it cannot sense or respond normally to the electric shock used as negative reinforcement, one cannot conclude that associative learning per se is defective.

In the controls for shock reactivity, *y v f tur/y v f JA27* flies were significantly poorer at escaping the shock than were the *y v f tur⁺/y v f JA27* flies (Fig. 6). Thus, despite the facts that the *y v f tur* lines showed large reductions in PKC activity and in learning scores, and both of these defects mapped to the 18A5;18D1-2 region of the chromosome, the results of our control experiments effectively prevent us from saying that these flies are defective in associative learning.

A similar conclusion can be drawn from the olfactory habituation experiments (Fig. 7). The low-scoring *tur* recombinant, *y v f tur/y v f JA27*, reached our criterion of four successive no-jumps in response to a pulse of benzaldehyde more quickly than did the high-scoring line (*y v f tur⁺/y v f JA27*). This would be a most perplexing result if taken at face value, because it would suggest that the mutants learned faster than the controls, whereas other associative learning mutants habituate more slowly than wild type (T. Tully and S. Koss, unpubl.). Another explanation, though, was that the mutants simply tired more quickly, or were inherently more sluggish than the control lines. This interpretation was supported by the dishabituation results: In response to vortexing, previously habituated wild-type and *y v f tur⁺/y v f JA27* flies again jumped in response to a pulse of benzaldehyde. The *y v f tur/y v f JA27* line showed a reduced tendency to jump after such dishabituation (Fig. 7b).

A behavioral component common to our olfactory associative learning, shock reactivity, and habituation assays is locomotion. Thus we examined dark reactivity in these lines (Boynton and Tully 1992). Flies were given a chance to run in complete darkness (to avoid any interference from phototactic effects) in a countercurrent apparatus in response to mechanical shock. Their responsiveness was quantified as a function of the number of

times they reached the distal half of the training apparatus. Can-S and the *y v f tur⁺* line were very similar in their distributions. The apparent lethargy of the *y v f tur* lines in this test was striking (Fig. 8). This sluggishness was completely recessive to *y v f tur⁺* as well as to the balancer chromosome *FM7a* (data not shown). Thus the locomotor defect seen in these tests comapped to the *JA27* deficiency interval, just like the biochemical and other performance deficits.

Taken together, this profile of associative and nonassociative behavioral defects was similar to that of heteroallelic *latheo* (*lat*) flies (see below; Boynton and Tully 1992). Also like the *tur* flies, *Appl* (amyloid precursor protein-like) mutants are significantly poorer than wild-type controls at escaping electric shock (Luo et al. 1992). Although not tested for dark reactivity, *Appl* flies were shown instead to be defective in fast phototaxis (essentially the same assay, but using a fluorescent light source to attract the flies toward the distal tubes; Luo et al. 1992).

There is a clear conclusion to be drawn from our experiments: the *y v f tur* recombinant genotypes performed more poorly in our learning tasks than did their *y v f tur⁺* "siblings," but there is a drastic reduction in reactive locomotor activity that maps to the *tur* region. We believe this behavioral sluggishness affects shock reactivity, Pavlovian conditioning, and habituation. As a result, we conclude that performance, but not necessarily learning, is impaired by the *tur* mutation. The reduction in PKC activity that comapped to this region suggests not that PKC is necessary for associative learning in flies, but instead that PKC may be necessary for normal escape responses in flies.

Differences in genetic background complicated the interpretation of our early behavioral data (Figs. 1 and 2a). The original *tur* X chromosome contained a lethal mutation distal to *tur* that was suppressed by one or more autosomal modifiers, as shown by (1) the eventual absence of male progeny when *tur* flies were outcrossed and (2) the viability of *y v f tur* male flies, which indicated that this lethal was distal to the *tur* mutation. The *JA27* chromosome initially was maintained over the *FM7a* balancer chromosome without outcrossing, allowing genetic background differences to accumulate over generations. The fact that fewer *+/JA27* and *tur/JA27* flies eclosed than did their *+/FM7a* and *tur/FM7a* sisters from the crosses that generated the flies tested in Figure 1 (data not shown) suggests that the *JA27* chromosome has a

negative effect on viability, making the accumulation of modifiers in the stock background likely. In addition, the background of the source stock for the marker chromosome *y v f car* was unknown. (Note that any nonisogenic population will have a variety of alleles present for most loci. "Accumulation of modifiers" refers to the phenomenon whereby selection pressure will act over generations to increase the representation of those alleles in the background that ameliorate a phenotypic defect. It does not refer directly to the accumulation of spontaneously arising mutations, which should be quite rare.)

Keeping these background differences in mind and knowing that such differences can easily affect performance in our olfactory conditioning assay (Gailey et al. 1991; Boynton and Tully 1992; Dura et al. 1993) makes it easier to understand our initial behavioral results. It is likely that the original *tur* homozygotes did poorly in the associative learning assay compared with *tur*/JA27 heterozygotes because 50% of the genetic background was different in the latter strain. The difference in the scores of *tur*⁺/JA27 and *tur*/JA27 is what reflects the effect of the *tur* mutation. It is worth pointing out that the same pattern of scores, *tur*⁺/*tur*⁺ = *tur*⁺/JA27 > *tur*/JA27 > *tur*/*tur*, was seen by Choi et al. (1991) using a *y v f tur* stock that was the same as ours, but their version of the JA27 chromosome did not carry *y*, *v*, and *f*.

Outcrossing with our cantonized FM7a stock diluted the representation of the autosomal suppressors of the distal lethal mutation in the original *tur* stock and recombination using the *y v f car* marker chromosome separated this lethal from *tur*. With the exception of the region of the various unique crossover points between *f* and *car*, the X chromosomes of the recombinant *tur* lines were identical, and the autosomes were only moderately cantonized initially. Note that each line derived from a single *y v f car*⁺/FM7a female with 25% of its autosomal material coming from the original *tur* stock, 25% from *y v f car*, and 50% from Can-S. Therefore the scores in Figure 2a can be compared among the recombinant lines and, to some extent, with that of Can-S.

Although one might expect the learning scores of the recombinant lines to fall cleanly into two classes, with those carrying the *tur* mutation scoring low and their "siblings" carrying the wild-type *tur*⁺ gene scoring high, we found that the different recombinants gave a range of learning scores. This continuum probably reflected the incomplete

equilibration of genetic background in these lines (recall that each line was tested soon after it was established, before outcrossing to a common background). We presume that it was similarly the removal of the restricted genetic background in the original *tur* flies that accounted for the apparent increase in *tur* scores between Figures 1 and 2a.

Choi et al. (1991) divided the seven *tur* recombinants shown in Figure 2a into two groups on the basis of their in vitro PKC activity. Using the same associative conditioning test as that described here, they went on to average the behavioral scores of the low and high PKC groups and used these two averages to conclude that the behavioral defect mapped to the same region as the biochemical deficiency. Because each recombinant line represents an independent crossover event, however, it was incorrect to pool and average these data. Apparently, the a priori assumption that PKC activity levels in these lines would correlate with their behavioral scores misled the authors, causing them to ignore the fact that in their hands recombinant #111 produced high learning scores despite having reduced PKC activity. This discrepancy in fact suggests a genetic separation of the two phenotypes, a conclusion directly at odds with the one they reached.

Our data, collected soon after the recombination experiments were done, showed that recombinant #111 in fact scored low in the Pavlovian conditioning test (Fig. 2a). Choi et al. (1991) collected their learning data at least 3 years after the recombinants were made homozygous. This interval allowed many generations for the recombinant lines to accumulate modifiers, which might have ameliorated the effects of *tur* on olfactory learning, shock acuity, and/or locomotion and thereby improved scores in the learning test. Thus, our data show that recombinant #111 rightfully should be considered behaviorally mutant, and the conclusion of Choi et al. that the biochemical and behavioral deficits comapped was correct, despite their mistaken analysis. Because of their lack of controls for sensorimotor behaviors, however, the authors nevertheless incorrectly ascribed the poor performance of their *tur* lines to a learning defect.

The effects of the *tur* mutation have been noted in a variety of learning paradigms, including larval learning (Aceves-Piña and Quinn 1979), leg-lift conditioning (Booker and Quinn 1981), habituation and sensitization to a sucrose stimulus (Duerr and Quinn 1982), and courtship conditioning (Gailey et al. 1982, 1984), as well as associative condi-

tioning with positive (Tempel et al. 1983) and negative (Quinn et al. 1979; Folkers 1982; Tully and Quinn 1985) reinforcement. All of these experiments (with the possible exception of Booker and Quinn 1981) used *tur* flies that had not been outcrossed to the control (Can-S) background, and therefore presumably bore the X-linked lethal, autosomal suppressors, and any other loci affecting performance that might have accumulated during maintenance of the stock. All of these experiments also required the flies to move during training and testing, but no independent quantitative tests of sensorimotor responses were included in these studies. In view of the locomotor defect of *tur*, this is particularly worrisome in the operant conditioning experiments of Quinn et al. (1979), Booker and Quinn (1982), and Tempel et al. (1983), because here reinforcement itself is contingent on the movement of the animals.

What about those experiments that saw no evidence of a motor defect? In the habituation and sensitization experiments of Duerr and Quinn (1982), the only movement required of the flies was proboscis extension. Their results showed that the *tur* flies were defective in habituation but normal for sensitization. The authors reported “no obvious differences” between Can-S and *tur* flies in viability, excitability, or acuity. In addition, in the courtship conditioning experiments of Gailey et al. (1982, 1984), *tur* flies courted at least as actively as the wild-type controls, which would not be expected of animals with a significant locomotor defect.

Two explanations present themselves. One is that the motor activity required in these assays may be unaffected by the defect we observed. It seems possible that the reactive locomotion that we looked at might be functionally distinct from motor patterns subserving feeding or courting behaviors. Our dark reactivity assay is in all likelihood not an appropriate control for behavioral tests such as proboscis extension and courtship, but because it is more task-relevant for the Pavlovian olfactory learning assay, it was the appropriate control for our experiments. From this perspective, it seems crucial to measure task-relevant sensorimotor responses during proboscis extension and courtship to make sense of those results.

Another explanation is that modifiers ameliorating adverse effects of the *tur* mutation may have accumulated in the original line as posited above, and, because the mutant was not outcrossed to equilibrate its genetic background with that of the

wild type, such modifiers could have affected performance in the proboscis extension and courtship assays. Thus, failure to outcross stocks before quantifying effects of single-gene mutations necessarily will call into question conclusions concerning learning.

What do our results mean for future work? The bottom line appears to be that a small but significant decrease in shock reactivity (~10%, or 20 PI points) is likely to reflect underlying locomotor problems. This has been the case not only for *tur*, but for *lat* (Boynton and Tully 1992; see below) and *Appl* (Luo et al. 1992) mutants as well. Investigating a phenotype as complex as learning has always been fraught with difficulty (see, e.g., Ricker et al. 1986); with the rapid development of the field of behavioral analysis in *Drosophila* and mice, it is particularly important now to establish basic criteria for determining what constitutes appropriate and sufficient evidence for discriminating true learning defects from other behavioral problems.

Where can we go from here with *tur*? Unless the locomotor defect can somehow be dissociated from *tur*, it will remain impossible to conclude that *tur* is a learning mutant in assays such as those described here, which rely on the flies showing learning through motor responses. One way to accomplish this dissociation hinges on the inference that the *tur* mutation may be amorphic (PKC activity levels and learning scores are identical for *y v f tur* homozygotes and *y v f tur/y v f JA27*; Figs. 3 and 4; Choi et al. 1991). The effect of the mutation on task-relevant sensorimotor function may be parallel to those observed for severe alleles of the *Drosophila* learning mutant *lat* (Boynton and Tully 1992). The *lat*^{P1} mutation was created by P-element mutagenesis and had a specific effect on associative learning. Whereas *lat*^{P1} was hypomorphic and had no effect on sensorimotor function when homozygous, flies heteroallelic for *lat*^{P1} and more severe (null) alleles showed reduced shock reactivity and olfactory acuity. The *lat*^{P1}/null animals were subsequently shown to be severely defective in the dark reactivity assay. Thus, in the case of *lat*, it appears that somewhat reduced levels of gene function can result in a “pure” learning defect and a further reduction causes more general defects in sensorimotor function (see Boynton and Tully 1992). This suggests that isolation of a hypomorphic allele of *tur* may allow us to assay learning without the confounding sensorimotor defects.

One intriguing early observation suggests an-

other approach. In the first published description of *tur*, it was asserted that although the putative effect of *tur* on associative learning was recessive, the mutation had a dominant effect on retention: Heterozygous flies forgot what they had learned much more quickly than did the wild-type controls. This effect was also seen in the larval learning experiments of Aceves-Piña and Quinn (1979) and in the associative learning task of Tully and Quinn (1985). Examining the performance of *tur/tur*⁺ heterozygotes using the recombinant *tur* lines already in hand, this time with complete control experiments, may reveal an effect of *tur* on memory with task-relevant sensorimotor function being normal.

A different strategy would be to address the question of PKC's behavioral effects directly. One approach would be to selectively mutagenize the known structural genes for PKC (Rosenthal et al. 1987; Schaeffer et al. 1989), using a targeted P-element mutagenesis strategy (Ballinger and Benzer 1989; Kaiser and Goodwin 1990; Sentry and Kaiser 1992; S.F. Goodwin, M. DelVecchio, C. Hoggel, T. Tully, and K. Kaiser, in prep.). Alternatively, inhibitors of other protein kinases have been used to show the importance of these kinases in *Drosophila* learning and memory (Drain et al. 1991; Griffith et al. 1993). Similarly, peptide inhibitors of PKC could be expressed in transgenic flies and the behavior of these animals assayed. Although tampering with Tur protein function might be expected to affect locomotor activity (on the basis of the results presented here), perhaps this reflects the absence of function during development of the mutant, and acute intervention might not have such severe effects. New experiments such as these may yet resolve the role of *tur* in learning and memory.

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