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Classical conditioning and retention in normal and mutant *Drosophila melanogaster*

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Summary. By changing the conditioned discrimination paradigm of Quinn et al. (1974) from an instrumental procedure to a classical (Pavlovian) one, we have demonstrated strong learning in wild-type flies. About 150 flies were sequestered in a closed chamber and trained by explosing them sequentially to two odors in air currents. Flies received twelve electric shock pulses in the presence of the first odor (CS+) but not in the presence of the second odor (CS–). To test for conditioned avoidance responses, flies were transported to a T-maze choice point, between converging currents of the two odors. Typically, 95% of trained flies avoided the shock-associated odor (CS+).

Acquisition of learning was a function of the number of shock pulses received during CS+ presentation and was asymptotic within one training cycle. Conditioned avoidance increased with increasing shock intensity or odor concentration and was very resistant to extinction. Learning was best when CS+ presentations overlap shock (delay conditioning) and then decreased with increasing CS-US interstimulus intervals. Shocking flies immediately before CS+ presentation (backward conditioning) produced no learning. Nonassociative control procedures (CS Alone, US Alone and Explicitly Unpaired) produced slight decreases in avoidance responses, but these affected both odors equally and did not alter our associative learning index (Λ).

Memory in wild-type flies decayed gradually over the first seven hours after training and still was present 24 h later. The mutants *amnesiac, rutabaga* and *dunce* showed appreciable learning acquisition, but their memories decayed very rapidly during the first 30 min. After this, the rates of decay slowed sharply; conditioned avoidance still was measurable at least three hours after training.

Abbreviations: *OCT* 3-octanol; *MCH* 4-methylcyclohexanol; *C-S* Canton-Special; *CS* conditioned stimulus; *US* unconditioned stimulus

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Introduction

Drosophila can learn a variety of associative tasks (see McGuire 1984; Tully 1984 for reviews). Several studies have employed discriminative (differential) conditioning procedures, in which two stimulus cues are presented during training, but only one is paired temporally with reinforcement. Variations on this general discriminative conditioning procedure have been run successfully with pairs of odor cues, colored lights or substrate textures as the discriminanda, with shock, quinine or mechanical shaking as negative reinforcements, and with sucrose or the opportunity to run upwards (negative geotaxis) as positive reinforcements (Quinn et al. 1974; Menne and Spatz 1977; Platt et al. 1980; Tempel et al. 1983).

Flies also can learn to modulate reflex responses. When proboscis extensions to sucrose are followed by electric shock or by quinine applied to the tarsi, flies can learn to suppress their usual proboscis extension reflex (Medioni and Vaysse 1975; DeJianne et al. 1985). Even headless flies will learn to keep their legs retracted or extended to avoid shock (Booker and Quinn 1981; cf. Horridge 1962). Finally, associative learning has been implicated in experience-dependent components of *Drosophila* courtship (Siegel and Hall 1979; Tompkins et al. 1983).

Two laboratories have used the olfactory discrimination conditioning paradigm of Quinn et al.

(1974) to isolate single-gene mutations that affect associative learning (see Aceves-Pina et al. 1983 for a review). So far, recessive mutations in five X-linked genes have been found. Mutant *dunce, rutabaga, turnip* and *cabbage* flies cannot learn to discriminate olfactory cues (Dudai et al. 1976; Aceves-Pina and Quinn 1979). Mutant *amnesiac* flies can learn to discriminate odors normally, but they forget the odor-specific avoidance response much faster than wild-type flies (Quinn et al. 1979; Dudai 1983). These mutants perform poorly in several of the other learning tasks mentioned above, although their impairments are often quantitative rather than absolute (Dudai and Bicker 1978; Siegel and Hall 1979; Booker and Quinn 1981; Folkers 1982; Gailey et al. 1982, 1984). In particular, *dunce* and *rutabaga* flies can learn approach responses (Tempel et al. 1983) and avoidance responses (Dudai 1979, 1983) in some learning tests, but they learn less well and forget more quickly than *amnesiac* flies.

Interestingly, four of these mutations also are deficient in sensitization or habituation of the proboscis extension reflex (Duerr and Quinn 1982). These results, which suggest that associative and nonassociative learning may be mechanistically related, are supported by behavioral evidence in blowflies (Tully et al. 1982) and are consistent with direct biochemical and neurophysiological experiments on *Aplysia* (Carew et al. 1983; Hawkins et al. 1983; Kandel et al. 1983; Walters and Byrne 1983).

The original olfactory shock-avoidance paradigm of Quinn et al. (1974) gave reproducible results, and it allowed the isolation of mutant strains that could not learn, because flies could be tested *en masse*. However, the learning effect was weak; only about two thirds of trained flies avoided the shock-associated odor. We wondered whether imperfections in the conditioning procedure were responsible for this limited effect. We were encouraged by a report by Jellies (1981), who devised a classical (Pavlovian) conditioning procedure, using odors as cues and electric shock as reinforcement to elicit much stronger conditioned avoidance in wild-type *Drosophila*. By modifying several of Jellies' procedural details, by carefully controlling the odor concentrations, and by designing a new conditioning apparatus, we were able to demonstrate very strong learning levels in wild-type flies.

Below, we report results from experiments with wild-type flies that (1) characterize acquisition and extinction of classically conditioned olfactory avoidance responses, (2) measure the effects of odor concentration and shock intensity, (3) delineate the temporal constraints on odor-shock pairings, (4) assess the effects of nonassociative factors and (5) show retention of conditioned avoidance responses for at least 24 h. In addition, we have begun to study learning and memory in the mutants *amnesiac, rutabaga* and *dunce*.

Materials and methods

Flies. Drosophila melanogaster of the Canton-Special (C-S) wild-type strain and three X-linked, single-gene mutant derivatives were used in this study. The mutants *dunce*^{1} (Dudai et al. 1976) and *rutabaga*^{PS511} (Aceves-Pina and Quinn 1979) were isolated in the original olfactory shock-avoidance test (Quinn et al. 1974) because they produced low average learning indices ($\Lambda \leq 0.05$). Mutant *amnesiac*^{PS801} flies learned normally in that test but showed abbreviated memory retention (Quinn et al. 1979).

Homozygous stocks of these mutants apparently tend to accumulate genetic modifiers that cause average learning scores to increase slowly toward wild-type (P.P. Sziber, J.S. Duerr, R. Booker and WGQ, unpublished data). We tried to minimize this problem in three ways: (1) We maintained 12–24 sublines of each mutant stock, tested each subline before a series of experiments began, and kept the sublines with the behavioral phenotype of the original line, i.e., the poorest learners. (2) When necessary, we removed autosomal modifiers by replacing the autosomes of a mutant stock with those from the C-S wild-type strain, using a *y*;Pm/CyO;Sb/TM6 double balancer stock and a C(1)DX stock (hereafter called $yf\widehat{XX}$) that had been backcrossed repeatedly to C-S flies (Lindsley and Grell 1968). (3) In one series of critical memory experiments with *rutabaga* flies, we used females with fresh C-S autosomes and also with X-chromosomes originating from different sources. To breed these flies, we crossed *rutabaga* males (with C-S autosomes) to females heterozygous for the X-chromosomal balancer FM7a (with C-S autosomes) and the deficiency Df(1)KA9. This X-chromosome deficiency lacks chromomeres 12E1-13A5 and fails to complement the *rutabaga* mutation both behaviorally and biochemically (Livingstone et al. 1984). The *rut*/KA9 female progeny were apparently homozygous-null at the *rutabaga* locus (Livingstone et al. 1984), were heterozygous for any X-linked *rutabaga* modifiers, and had wild-type (C-S) autosomes.

All stocks were maintained at 25 °C, at 60% relative humidity and on a 16/8 h light/dark cycle with lights-on at 9:00 AM. They were raised on a standard cornmeal medium (Cline 1978) in half-pint milk bottles. Twelve to 24 h before an experiment, the flies were transferred to fresh food bottles without anesthesia. Training began between 10:00 AM and 8:00 PM the following day. In the memory retention experiments, testing was completed by 10:00 PM. Most flies were 24–48 h old when training began, but occasionally 48–72 h old flies were used. Within these ranges of age and training and testing times, no differences in conditioned avoidance were discernible. Sex of the flies did not affect learning scores, so males and females were trained and tested together.

Conditioning apparatus. We extensively modified the choice chamber apparatus of Dudai et al. (1976) so that (1) odor cues and electric shock reinforcement were made inescapable during training, (2) odor cues were presented in relatively high concentrations in streams of air and were kept uniform from experiment to experiment and particularly from training to testing, and (3) flies were disturbed as little as possible, especially during training. The apparatus (Fig. 1A) consisted of a training tube (Fig. 1B) with 95% of its inner surface electrifiable, a sliding

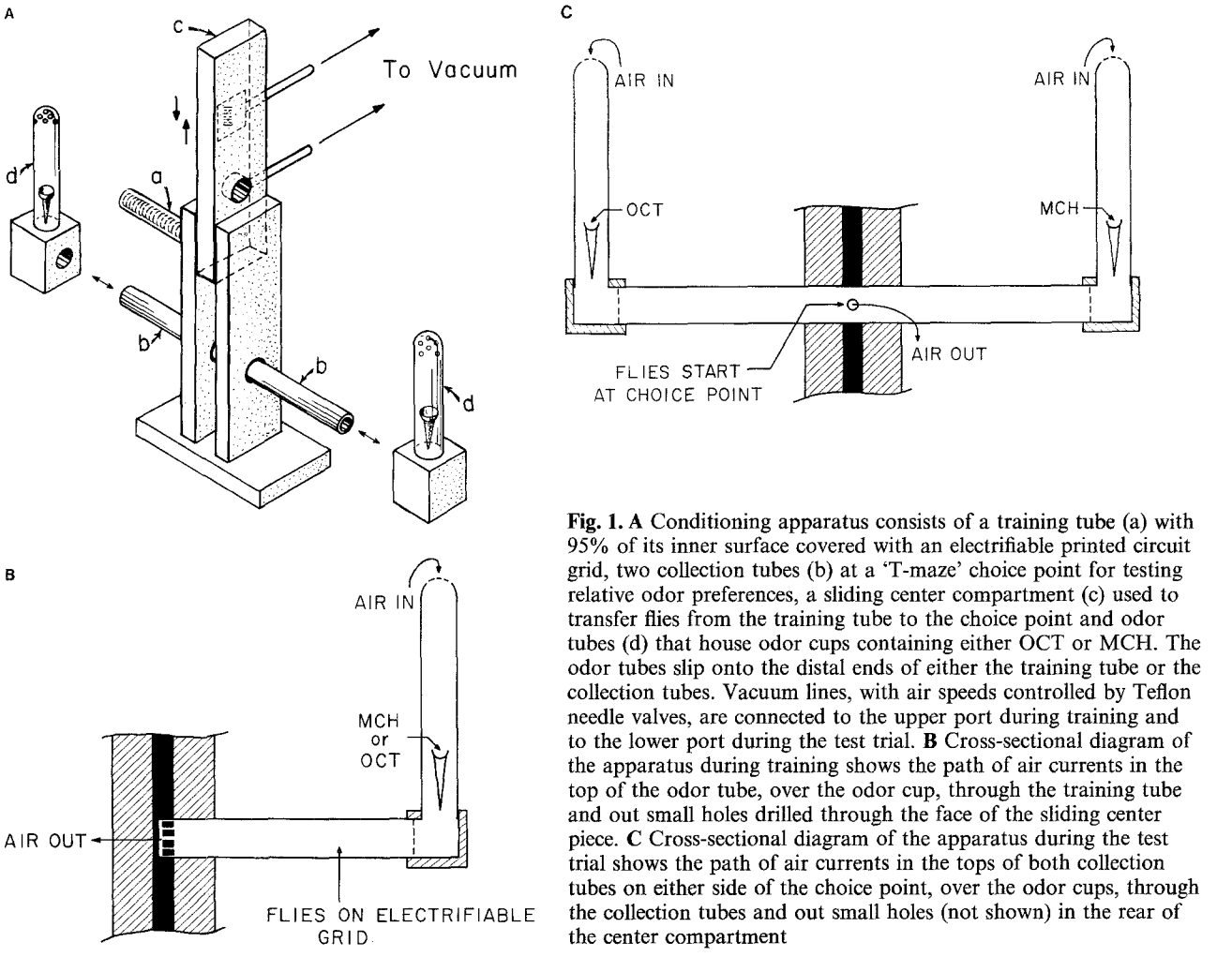

Fig. 1. A Conditioning apparatus consists of a training tube (a) with 95% of its inner surface covered with an electrifiable printed circuit grid, two collection tubes (b) at a 'T-maze' choice point for testing relative odor preferences, a sliding center compartment (c) used to transfer flies from the training tube to the choice point and odor tubes (d) that house odor cups containing either OCT or MCH. The odor tubes slip onto the distal ends of either the training tube or the collection tubes. Vacuum lines, with air speeds controlled by Teflon needle valves, are connected to the upper port during training and to the lower port during the test trial. **B** Cross-sectional diagram of the apparatus during training shows the path of air currents in the top of the odor tube, over the odor cup, through the training tube and out small holes drilled through the face of the sliding center piece. **C** Cross-sectional diagram of the apparatus during the test trial shows the path of air currents in the tops of both collection tubes on either side of the choice point, over the odor cups, through the collection tubes and out small holes (not shown) in the rear of the center compartment

center compartment to transfer flies after training, and a two-arm choice point (Fig. 1C) for testing relative odor avoidance responses. The training tube consisted of a polystyrene 17 × 100 mm test tube (Falcon plastics #2017) cut to a length of 81 mm from the top of the tube. A removable nylon mesh screen (#50) on the distal (sawed-off) end of the training tube prevented flies from escaping while allowing odorized air streams to enter. A 57 × 81 mm electrifiable grid, with the copper pattern printed on flexible epoxy backing (as described in Quinn et al. 1974), lined the inside surface of the training tube, and a separate circular grid also covered the nylon screen at the distal end of the chamber. Many 0.5 mm holes were drilled through the epoxy backing between the copper lanes of the circular grid to allow air to enter the training tube. Each arm (collection tube) of the choice point used for the test trial, consisted of a Falcon 17 × 100 mm polystyrene test tube with fifteen 0.5 mm holes melted through the bottom to allow air to enter the collection tubes and to exit at the center choice point.

The odorants were contained in cups about 10 mm deep, made by cutting off the bottoms of disposable borosilicate culture tubes (Fisher Scientific). These 'odor cups' were glued to the wide ends of micropipet tips (Sarstedt #70.760), appearing like little bird baths on pedestals. Once glued, the odor cups were dipped in Sigmacote (Sigma Chemicals) to coat the glass surfaces with hydrophobic film, air-dried overnight, then baked at 65 °C for 30 min. Before an experiment, the odor cups were filled with pure solutions of either 3-octanol (OCT) or 4-methylcyclohexanol (MCH). Each odor cup was housed in an 'odor tube', consisting of a 17 × 100 mm Falcon polystyrene test tube with sixteen 0.5 mm holes melted through the bottom, turned upside-down to cover the odor cup, and a lucite base, which supported the odor cup, anchored the cover tube and afforded passage for the odorized air into the collection tube (see Fig. 1B and C).

Suction for two conditioning apparatuses was generated with a rotor-style vacuum pump (Arthur H. Clark Co., Model #5KH3366 102FX), which produced a pulse-free stream of air. The air exhaust from the pump was piped out of the room. Latex tubing was connected to the vacuum pump at one end and was split into two pairs of inlets at the other end, using three Y-connectors. One inlet from each pair was connected to the upper port of a conditioning apparatus, the other was connected to the lower port (see Fig. 1A). Air speed was controlled separately at the four inlets with 4-mm Teflon needle valves (Fisher #14-630-7B). Using a flow meter, air speed was adjusted to 11 ml/s for the upper port and to 22 ml/s for the lower port of each pair of inlets.

During training, the appropriate inlet was connected to the upper port of the sliding center piece (Fig. 1A), drawing air in the top of an odor tube, over the odorant in the odor cup, through the training tube and out through thirty eight 0.5 mm holes in the face of the sliding center piece (Fig. 1B). During

testing, the other inlet was connected to the lower port of the sliding center piece, drawing air at 11 ml/s equally through each odor tube attached to its collection tube and out sixty 0.5 mm holes in the back side of the center chamber (Fig. 1C). Under these conditions of constant air speed, odor concentrations were adjusted by varying the diameters of the odor cups until naive flies distributed themselves 50:50 when given a choice between OCT and MCH. Unless stated otherwise, 10 mm and 8 mm (inside diamter) cups were used for MCH and OCT, respectively.

A blueprint of the apparatus and a set of directions for its use are available from T.T.

Conditioning procedure. At the start of a training cycle, about 150 flies were aspirated into the training tube, the nylon screen was placed over the distal end of the training tube, the vacuum hose was connected to the upper port of the sliding center piece, and a blank (empty) odor tube was slipped gently onto the end of the training tube, providing flies with a stream of relatively odorless air from the room. During this rest interval, the grid was connected to a Grass S44 stimulator, which was turned off for the moment but was set to deliver 1.25 s, 60 V square-wave pulses at 5-s intervals. After 90 s, the blank odor tube was replaced with an OCT odor tube, and the stimulator was switched on. Flies reacted visibly to a shock pulse either by freezing or by jumping. They appeared to recover and return to normal locomotor behavior during the subsequent 3.75-s interpulse interval. After 60 s, the stimulator was switched off and the OCT odor tube was replaced with the blank odor tube for 30 s, followed by the MCH odor tube for 60 s without shock and, finally, by the blank odor tube again for 30 s more. During training, we were careful not to shake or jar the flies; minimizing disturbances to the flies appeared to be necessary to obtain maximal learning scores.

Immediately following training, flies were transferred to the sliding center compartment by turning the conditioning apparatus on its side and gently tapping three times. Flies were retained there for 90 s, while the collection tubes were snapped into place at the choice point and the MCH and OCT odor tubes were slipped onto the distal ends of the collection tubes. Five seconds before the test trial began, the appropriate vacuum inlet was attached to the lower port of the sliding center piece, and then the center compartment was slid smoothly into register with the choice point. Flies had 120 s to disperse from the center compartment into the collection tubes. The sliding center compartment then was pulled up quickly, trapping the flies in the collection tubes they had chosen. Finally, flies in each collection tube were anesthetized and counted. Usually, 5 to 10 flies remained in the center compartment; these were counted visually.

Training and testing were repeated with a new group of naive flies as above, except that MCH was presented first and was paired with shock during training. Accordingly, the shock-associated odor alternated between OCT and MCH when several successive experiments were run. During the test trial, OCT and MCH always were placed on the left and right collection tubes, respectively. This procedure, together with the alternation of shock-paired odors in successive runs, was designed to minimize any side-bias from pheromone cues in the collection tubes.

Flies were trained and tested at 22°C and 50% relative humidity. A red darkroom lamp (Testrite Instrument Co., Inc., Model 3A with a standard 6 watt photolab bulb and with filter removed) was placed 15 cm behind and 34 cm above the apparatus. The lamp was aimed so that light rays were centered on the choice point and were perpendicular to the collection tubes, allowing equal amounts of light to fall on each. Dark-adapted flies appeared to orient slightly toward the dim red light source. Nevertheless, their phototactic drive seemed very weak; naive flies appeared relatively attentive to odor cues and were induced by anemotaxis to walk upwind from the choice point into the collection tubes.

Preliminary observations showed that flies ran more quickly and learned better with tubes and grids previously occupied by other flies. Therefore, new collection tubes and training tubes with new grids were 'aged' by keeping naive flies in them overnight, and were used repeatedly without washing between experiments.

This basic conditioning procedure was varied several ways to measure some properties of conditioned avoidance behavior and to assess nonassociative effects on learning. These variations are described in the Results section.

Learning index. As in Quinn et al. (1974), a learning index (Λ) was calculated as the fraction of flies avoiding the shock-associated odor (CS+) minus the fraction of flies avoiding the unshocked control odor (CS–), averaged over two groups of flies – one shocked in the presence of OCT, the other shocked in the presence of MCH. Because both odors were presented simultaneously to flies at the choice point during a test trial, flies were considered to have avoided an odor when they ran into the opposite collection tube. If all flies failed to learn, then the index would be 0; if they all avoided the shock-associated odor (perfect learning), the index would be 1.

Statistics. Numerical confidence limits and error bars indicate standard errors of the mean. Sample sizes (N) for experiments using the learning index indicate the number of complete experiments run (Λs), in which one group of about 150 flies had OCT paired with shock and another group had MCH paired with shock. Sample sizes for experiments using percent avoidance indicate the numbers of groups of 150 flies tested. Statistical significances of the differences between two means were assessed with Student's *t*-test. Comparisons among three or more means were assessed with 1-way or 2-way analyses of variance (ANOVAs) as described in Sokal and Rohlf (1969).

Results

Acquisition

To try to measure learning acquisition, we trained different groups of flies, repeating the training cycle described in Methods (60 s of electric shock paired with one odor, 30 s of 'rest' with no odor, 60 s of a second odor with no shock, 30 s rest) one, two, three or five times before giving them a choice between OCT and MCH in a test trial (without reinforcement). Training cycles after the first began immediately after the last 30-s rest interval of the previous cycle. The learning index we use here, as in previous papers, is the fraction of flies that avoid the shock-paired odor (CS+) minus the fraction of flies that avoid the control odor (CS–), averaged for two groups of flies – one trained to avoid OCT, the other trained to avoid MCH. The mean learning index after one training cycle was 0.89 ± 0.01 ($N = 5$). For a learning index of 0.89, typically 93% of flies avoided the CS+, 4% avoided the CS– and

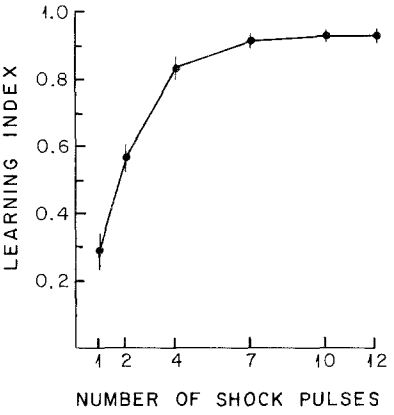

Fig. 2. Learning acquisition as a function of the number of shock pulses. Different groups of flies received the indicated numbers of 1.25-s shock pulses during a single training cycle. Each point represents 8–10 experiments (As)

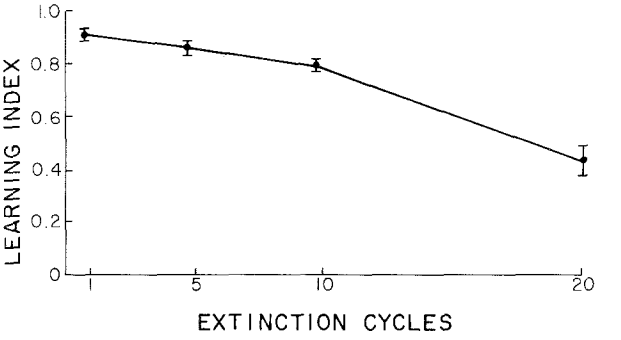

Fig. 3. Extinction of conditioned avoidance. Different groups of flies received the indicated numbers of extinction cycles after one training cycle (12 pulses of 60 V DC shock). Each point represents four experiments

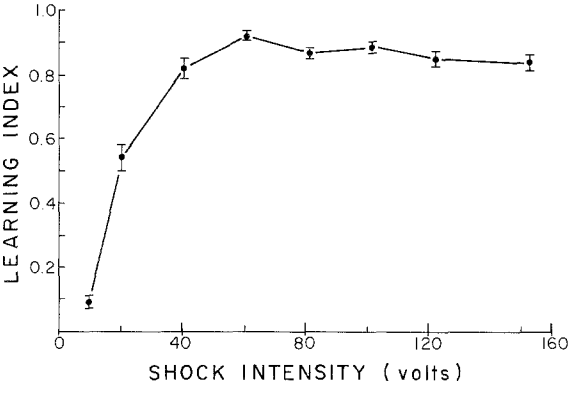

Fig. 4. Learning as a function of shock intensity. Different groups of flies received 12 1.25-s pulses of DC shock at the indicated voltage during a single training cycle. Each point represents 8–14 experiments

3% remained in the center compartment after the 120-s test trial.

Learning indices were 0.89 ± 0.01 after one training cycle, 0.90 ± 0.02 ($N = 5$) after two cycles, 0.88 ± 0.01 ($N = 5$) after three cycles and 0.90 ± 0.01 ($N = 9$) after five cycles. Thus, conditioned avoidance was maximal after one training cycle. Additional training cycles up to four produced neither higher performance levels nor any signs of fatigue by the flies.

Since the performance levels of flies were maximal after one 60-s exposure to an odor paired with shock pulses, we thought that acquisition of conditioned avoidance might be a function of the number of shock pulses that flies received during the first training cycle. To test this idea, different groups of flies were given from one to twelve 1.25-s shock pulses during a single training cycle. (The stimulator was set to deliver the appropriate number of pulses per minute, and the onset of the first pulse occurred randomly within the period of time equal to the interpulse interval.) In Fig. 2, mean learning indices are plotted as a function of the number of shock pulses, showing acquisition of conditioned avoidance. Learning levels rise quickly to an asymptote within 7–10 shock pulses. Apparently, individual shock pulses are acting as acquisition *trials* during a single 60-s exposure to the odor cue (CS+).

Extinction

To measure extinction, flies were conditioned for one training cycle. Then, different groups received various numbers of extinction cycles, beginning immediately after the last 30-s rest interval of the training cycle. These extinction cycles were identical to the usual training cycle, except shock was not presented with either odor. Figure 3 shows the mean learning index as a function of the number of extinction cycles. After one training cycle, 20 extinction cycles were necessary for conditioned avoidance responses to attenuate to 50% of maximum levels.

Effects of shock intensity and odor concentration

Next, we exposed different groups of flies to a range of shock intensities or odor concentrations, while holding all the other training and testing conditions constant. Figure 4 shows mean learning indices as a function of shock intensity. Conditioned avoidance levels are low at 10 V and rise quickly to a maximum near 60 V. There is a slow decline in conditioned avoidance levels from 80 to 150 V, which may reflect some disruption or damage to the flies by stronger shock.

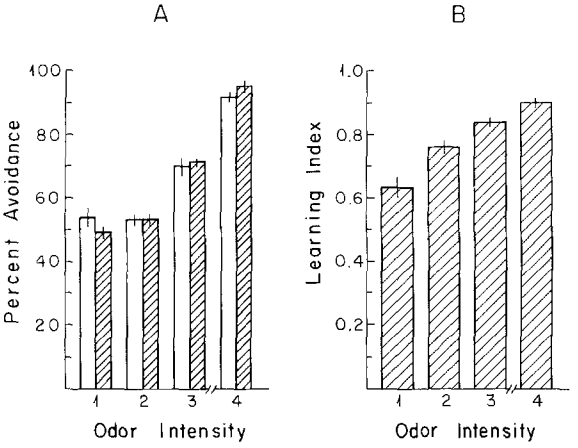

Fig. 5 A, B. Learning as a function of odor avoidance levels of naive flies. **A** Percent avoidance of OCT (open bars) and MCH (hatched bars) by naive flies in choice between one odor vs. fresh air measured at three odor concentrations in Groups 1–3. Inside diameters of OCT and MCH odor cups were 0.78 and 1.04, 1.56 and 2.08, and 8.00 and 10.00 mm for Groups 1–3, respectively. MCH odor cup sizes were chosen arbitrarily; OCT odor cup sizes then were chosen so that naive flies distributed themselves 50–50 in a choice between OCT and MCH. Group 4 used the same odor cup size as Group 3, but flies in Group 4 were tested in summer (July) instead of spring (April) for Groups 1–3. Percent avoidances increase with increasing odor concentration and also appear to be higher in the summer than in the spring. Each point represents six groups of about 150 flies. **B** Mean learning scores increase with increasing percent avoidances. Each point represents 6 experiments

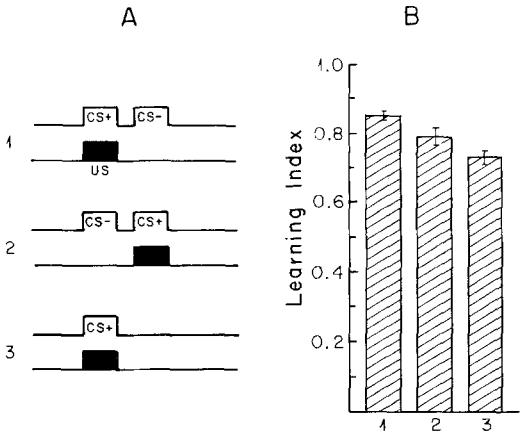

Fig. 6 A, B. Variations in training procedure. **A** Stimulus schedules for three different conditioning procedures. Group 1 is the standard discriminative classical conditioning procedure. Group 2 is discriminative classical conditioning where the second odor is shocked instead of the first. In group 3, only one odor is presented during training. **B** Mean learning indices after training with the indicated procedures. Each point represents 6 experiments

Conditioned avoidance also increases with odor concentration. Although we could not measure the actual concentration of airborne odors in our apparatus, we could vary odor concentrations by changing the surface area of exposed odorant in our odor cups, while keeping air flow constant. The 'effective concentration' of an odor can be measured behaviorally as the percentage of naive flies that avoid the odor in a choice against pure air (an absolute avoidance test trial). Figure 5 A shows the mean percent avoidance by groups of flies exposed to increasing concentrations of odors (OCT or MCH) in a choice against air. Figure 5 B shows that mean learning indices varied with odor concentration in Groups 1–3.

During the course of this study, we noticed that wild-type flies showed somewhat higher learning scores in the summer (May–September) than in the winter (October–April). We thought this seasonal variation might be due to differences in the flies' sensitivity to OCT and MCH. Accordingly, we tested a group of flies in July using the same odor cup sizes as Group 3 (tested in April). The mean percent avoidance for the July flies was higher than that for the April flies (Fig. 5 A; $t_{[10]}=7.793$, $P<0.001$ for OCT and $t_{[10]}=17.645$, $P<0.001$ for MCH). Figure 5 B shows that the July flies (Group 4) also learned better than the April flies ($t_{[10]}=3.957$, $P<0.001$). Thus, the strength of conditioned avoidance seems to vary with absolute avoidance levels of naive flies, whether differences in these levels arise from changes in odor concentration or from changes in olfactory acuity by the flies. [Since completing these experiments, we have discovered that a malfunctioning humidifier in the testing room allowed relative humidity levels to drop very low during the winter months. Recent experiments have indicated that 'summertime' performance levels can be achieved year-round as long as relative humidity levels are 50–60%.]

Variations in the training procedure

In our standard conditioning procedure, shock was paired with the first odor presented during training. We wanted to see if shocking the second odor rather than the first, or presenting only one odor, during training produced different learning scores. Stimulus schedules for these variations in our standard conditioning procedure are diagrammed in Fig. 6 A. The resulting mean learning indices are shown in Fig. 6 B. Both variations in conditioning procedure diminished performance levels somewhat, when compared to our standard procedure (Group 1). Shocking the second odor (Group 2, Fig. 6 A) instead of the first (Group 1) reduced

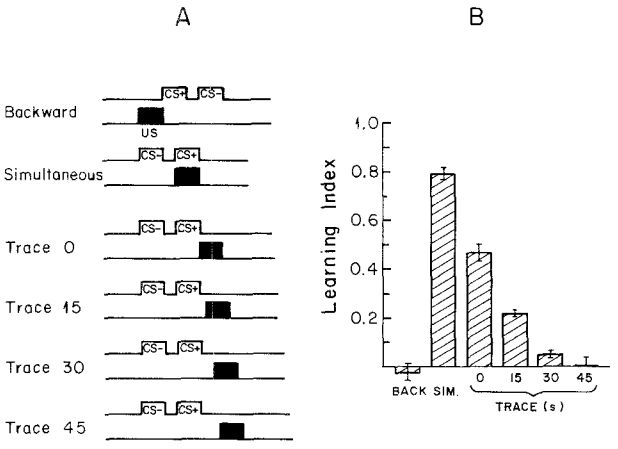

Fig. 7 A, B. Temporal specificity of CS-US pairings. **A** Stimulus schedules for backward conditioning, simultaneous conditioning and four trace conditioning intervals. Odor presentations (CS+ or CS−) lasted for 60 s; the length of the baselines indicates the amount of time flies spent in the training tube. **B** Mean learning indices for these CS-US pairings indicate that the US must *follow* CS presentations closely in order to produce strong learning, but they suggest the persistence of a CS trace. Each point represents 6–8 experiments

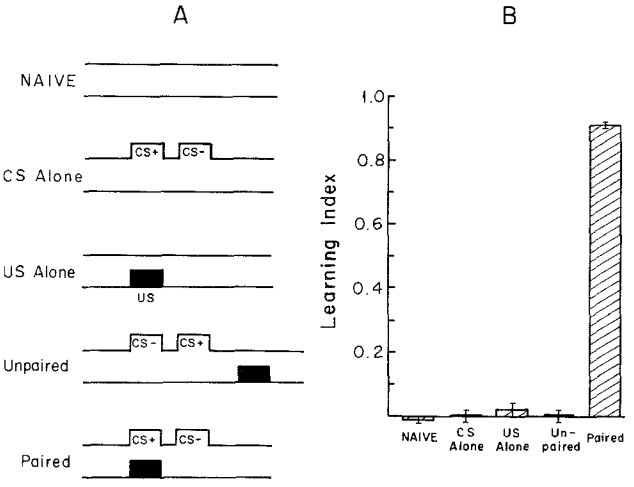

Fig. 8 A, B. Effect of nonassociative factors on the learning index. **A** Stimulus schedules for a naive group, three nonassociative control groups and a discriminative classical conditioning group. Odor presentations (CS+ or CS−) lasted 60 s. **B** Comparison of mean learning scores among these 5 groups shows that the learning index measures only associative learning. Learning indices for the CS Alone group were calculated by arbitrarily designating first odor as CS+. Learning indices for US Alone and Naive groups were calculated by designating two successive groups of flies as reciprocal experiments. Each point represents 5–16 experiments

learning scores by 7% ($t_{[10]}=2.54$, $0.02<P<0.05$). Using only one odor during training (Group 3) reduced learning scores by 14% ($t_{[10]}=4.46$, $P<0.001$).

We also tried reversal training. Groups of flies were given two standard training cycles, instead of one. During the first cycle OCT (or MCH) was paired with shock, while MCH (or OCT) was paired with shock during the second cycle. Then, these flies were tested with the usual choice between OCT and MCH. In spite of what they learned during the first training cycle, flies avoided the odor more recently paired with shock, producing a mean learning index of 0.26 ± 0.03 ($N=8$).

Temporal specificity of odor-shock presentations

In our standard classical conditioning procedure, shock and one odor presentation overlap temporally (delay conditioning). We decided to investigate CS-US intervals more closely. Figure 7 A shows the stimulus schedules for (1) a backward conditioning procedure, where the shock (US) ended before the first odor (CS+) was presented, (2) a discriminative classical conditioning procedure and (3) four trace-conditioning procedures, where shock (US) onset occurred 0, 15, 30 or 45 s after the second odor presentation (CS+) ended. In each group, the temporal relationship between the two odors was identical.

As Fig. 7B indicates, the delay conditioning procedure produced the highest mean learning index; backward conditioning produced no associative learning; and trace conditioning became less effective as the time interval increased between CS+ offset and US onset. It is possible that learning in our trace conditioning groups did not result from a true physiological trace of CS+ but simply from residual odor remaining in the training tube or on the flies. We consider this to be unlikely, however, because fresh air cleared the training tube every 1.54 s after an odor tube was removed.

Nonassociative controls

Figure 8A shows the stimulus schedules during training for flies receiving the discriminative classical conditioning procedure (Paired) and for four nonassociative control procedures, where (1) neither odors nor shock were presented (Naive), (2) the odors were presented alone, without shock (CS Alone), (3) shock was presented alone, without the odors (US Alone) and (4) shock presentation was separated in time from both odor presentations (Unpaired). In all other respects, conditions during

training and testing were identical in the classical conditioning and the nonassociative control groups (see Methods).

Our results (Fig. 8B) indicate that the mean learning index produced by the discriminative classical conditioning procedure results solely from associative learning. Presentations of the odors alone, shock alone, or the odors and shock explicitly unpaired yield mean learning indices near zero, while the conditioning (Paired) procedure produces a mean learning index of 0.89 ± 0.01. In addition, naive groups of flies, given a test trial without previous exposure to either OCT or MCH, give a mean learning index near zero.

We realized that nonassociative effects from our control procedures still could exist, in some cases, even though learning indices were zero. The test trial used in the standard conditioning procedure was designed to measure *relative* avoidance responses of flies by presenting OCT and MCH simultaneously. Such a test trial only can detect a differential change in avoidance of the two odors. Nonassociative factors could affect the flies' avoidance of both odors equally, producing no change in relative avoidance of both odors and resulting in a learning index near zero. Consequently, we modified the usual test trial to measure effects of nonassociative factors on the flies' (absolute) avoidance of OCT and MCH separately. Instead of testing all flies with a choice between OCT and MCH, we tested one group with a choice between OCT and Air and another with a choice between MCH and Air. This change in the test trial necessitated some modifications in the stimulus schedules during training (Fig. 9A), chiefly because flies were able to learn to avoid our relatively pure 'Air' as if it were a distinct odor cue (unpublished data).

Figure 9B shows the mean percent avoidance of flies for OCT or MCH vs Air after each of the training procedures. A 2-way analysis of variance (ANOVA), with ODOR and TRAINING group as main effects, indicates that (1) OCT and MCH produced different effects on avoidance responses ($F_{[1,70]} = 7.197$, $P < 0.01$), (2) the various training procedures produced different effects on avoidance responses ($F_{[4,70]} = 17.719$, $P < 0.001$) and (3) ODOR and TRAINING group interacted ($F_{[4,70]} = 3.234$, $0.01 < P < 0.025$). Avoidance levels in the nonassociative control groups were higher for MCH vs Air than for OCT vs Air, whereas the opposite was true in the conditioning (Paired) group. This may indicate that OCT was a more salient CS than MCH. Student-Newman-Keuls a posteriori tests ($\alpha = 0.05$) from separate 1-way

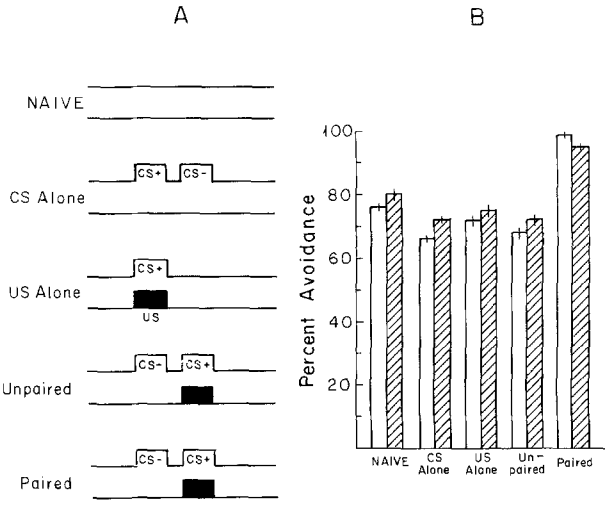

Fig. 9 A, B. Effect of nonassociative factors on single-odor avoidance. **A** Stimulus schedules for a naive group, three nonassociative control groups and a discriminative classical conditioning group. Odor presentations (CS+ or CS−) lasted for 60 s. Stimulus schedules for the US Alone and Unpaired are modified from those in Fig. 8A, primarily to take into account the fact that flies could learn to discriminate fresh air from OCT or MCH when they were shocked in its presence (unpublished data). To circumvent this complication, we paired shock with one odor (CS+) during training and then tested flies with a choice between fresh air and the other odor, which was CS− in the Unpaired group and a novel odor in the US Alone group. Flies in the Unpaired and Paired groups received identical training schedules. However, the test trial for the Unpaired group was a choice between CS− and Air, whereas that for the Paired group was a choice between CS+ and Air. The test trial for the CS Alone group was a choice between CS+ and Air for half of the complete experiments (N=4), between CS− and Air for the other half. These two CS Alone subgroups did not differ in their avoidance levels during the test trial ($t = 0.523$, $P > 0.5$), so their scores were combined here. **B** Percent avoidance of OCT vs Air (open bars) and MCH vs. Air (hatched bars) for each of the 5 groups. Compared to the percent avoidance levels of naive flies, each of the nonassociative control procedures produced a response decrement, whereas the Paired procedure caused an increase in percent avoidance levels. Each point represents 8 experiments

ANOVAs indicate that (1) mean percent avoidance responses of OCT from the US Alone, Naive and Paired groups differed from each other and from the CS Alone and Unpaired groups, which did not differ from each other and (2) mean percent avoidance responses of MCH from the Naive and Paired groups differed from each other and from the US Alone, CS Alone and Unpaired groups, which did not differ from each other.

On average, 79% of naive flies avoided the odors. Training with odors alone or with the odors and shock unpaired produced a 10% decrease in the flies' odor avoidance, whereas training with the shock alone procedure produced a 5% decrease. In

contrast to these control-procedure effects, conditioning flies by pairing shock with one odor increased avoidance by 18%, to 97%.

The response decrement produced by presentations of CS Alone may explain the smaller learning index in our one-odor conditioning experiment (Fig. 6, Group 3). Presenting only one odor (without shock) during training should result in a response decrement to that odor but not to a second, novel odor. This effect would produce an odor bias when the odor used during training and the novel odor were presented simultaneously during a test trial – fewer flies would avoid the odor used in training than the novel odor. If such an odor bias acted additively with conditioned avoidance during a one-odor conditioning experiment, the effect would be to produce slightly less avoidance of the shock-paired odor and a lower learning index compared to the usual two-odor conditioning experiment, in which flies are exposed to both odors that subsequently are used during a test trial.

The response decrement produced by presentations of US Alone was unexpected. Ordinarily, such a procedure causes behavioral sensitization (see Mackintosh 1974). We can suggest three possible explanations for our data: (1) Our US Alone procedure, in fact, did not produce any behavioral sensitization. (2) Behavioral sensitization may have occurred in our experiment, but its effect was to cause flies to be agitated or 'confused' during the test trial, which interfered with making the usual directed choice toward fresh air and away from the odor. (3) The observed response decrement may have resulted from generalized habituation, since the odor used during training (to keep 'Air' novel, see above) and the odor used during testing were both alcohols.

Pavlov (1927), Rescorla (1967, 1969) and others have shown that an explicitly unpaired control procedure can produce conditioned inhibition of the response normally elicited during excitatory conditioning. Since most conditioning procedures use CSs that initially elicit little or no behavioral responses, conditioned inhibition usually has to be measured by indirect methods such as retardation-of-acquisition and summation (see Rescorla 1968). However, in our conditioning procedure, naive avoidance responses to the CSs are greater than zero (Fig. 9B). If conditioned inhibition was present in the Unpaired group, it should act to produce lower avoidance levels in the Unpaired group than in the CS Alone group. Accordingly, our results suggest that neither the unpaired training procedure nor the discriminative classical conditioning procedure produced measurable conditioned inhibition.

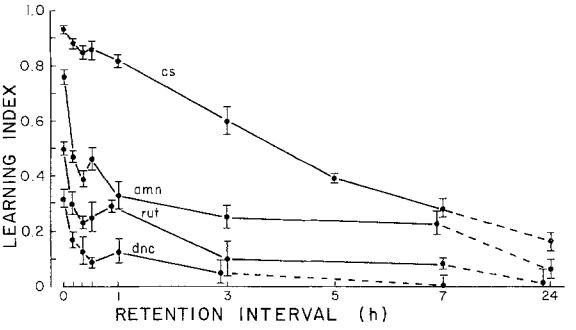

Fig. 10. Memory retention in normal and mutant flies. Different groups of wild-type (C-S) or mutant flies were tested at 0, 1/6, 1/3, 1/2, 1, 3, 5, 7 and 24 h retention intervals after a single training cycle (see text). Each point represents 4 experiments

Memory retention

After characterizing some properties of learning in wild-type flies and after verifying that the learning index is a relatively pure measure of associative learning, we wanted to see how long conditioned avoidance behavior was remembered by normal flies and by the mutants *amnesiac*, *rutabaga* and *dunce*. We gave flies one training cycle of the standard conditioning procedure (see Methods), after which we transferred them to a vial containing their usual food medium, where they remained undisturbed in the dark at 22 °C during the retention interval. About two min before the test trial began, we aspirated the flies from the food vial, via the training chamber, directly into the sliding center compartment. They remained there for 90 s, while the collection tubes were snapped in place at the choice point and the odor tubes were attached to the collection tubes. The flies then received their usual 120-s choice between OCT and MCH.

Figure 10 shows mean learning indices measured at various retention intervals for wild-type, *amnesiac*, *rutabaga* and *dunce* flies. These data suggest three conclusions: (1) The learning mutants *rutabaga* and *dunce*, like the memory mutant *amnesiac*, are capable of moderate levels of associative learning. (2) Retention curves for all three of these mutants look similar. They differ quantitatively rather than qualitatively. (3) In contrast to the wild-type retention curve, the mutant curves appear to be composed of two components. During the first half hour after training, retention in the mutants attenuated nearly

three times faster than in wild-type flies. Afterwards, retention decay rates slow dramatically in the mutants.

Over time, these mutant strains sometimes accumulate genetic modifiers, which compensate in some way for the primary mutation and ameliorate the learning deficit (see Methods). It seemed possible, therefore, that the two phases of each mutant retention curve reflected the additive effects of two different phenotypes in the population – a majority of flies with a mutant phenotype, forgetting entirely during the first hour, and a minority of flies with a 'modified' phenotype forgetting much more slowly.

We tested this hypothesis by comparing 3-h retention scores among (1) the original *rutabaga* stock, (2) a *rutabaga* stock with freshly replaced C-S (wild-type) autosomes and (3) females (with C-S autosomes) heterozygous for *rut* and Df(1)KA9, a small deficiency that uncovers the *rut* gene (Livingstone et al. 1984, see Methods). If the original *rutabaga* stock contained modifiers on the autosomes or on X-chromosomes that produced some phenotypically wild-type flies, one would expect the mean learning index after a 3-h retention interval for this stock to be higher than those indices from the other two *rut* stocks. In fact, the three 3-h retention scores did not differ significantly among these three *rut* stocks ($\Lambda = 0.10 \pm 0.06$, 0.10 ± 0.02 and 0.10 ± 0.03, respectively, for stocks (1), (2) and (3) above).

Wild-type and mutant strain differences in learning

While the main experiments of this study were proceeding, we tested several other wild-type and mutant stocks for learning. We did these experiments offhand, at various times during the year and sometimes with less-than-optimal performance of the conditioning apparatus. Therefore, we always tested wild-type C-S flies along with flies from the stocks of interest. Mean learning scores for 15 of these stocks are listed in the first column of Table 1, with scores of C-S flies tested the same day in the second column.

The 15 stocks listed in Table 1 are arranged into four sets to point out four findings:

(1) We measured large differences in mean learning scores among 'wild-type' laboratory strains of *Drosophila*. Of the stocks tested, C-S flies learned the best and Berlin flies learned the worst ($\Lambda = 0.52 \pm 0.08$). These results underline the importance of controlling for genetic background before comparing mutant and wild-type stocks from different origins. They also are congruent with earlier reports of variability among different wild-type stocks in other learning tests (Dudai 1977; Dudai and Bicker 1978).

Table 1. Comparison of learning scores among several wild-type and mutant strains (column 1), along with learning scores for C-S flies trained on the same day (column 2). The Texas strain was bred from a mixture of 12 Texas-inbred strains, while the T × B strain originated from a cross between the Texas and Berlin strains (Vargo 1984)

Strain	Learning index	C-S Learning index
C-S	0.90 ± 0.01	–
Texas	0.76 ± 0.04	0.90 ± 0.02
T × B	0.69 ± 0.11	0.87 ± 0.03
Oregon-R	0.55 ± 0.05	0.89 ± 0.02
Berlin	0.52 ± 0.08	0.90 ± 0.02
yf$\widehat{XX}$ (C-S)	0.78 ± 0.02	0.77 ± 0.03
FM7a/FM7a (C-S)	0.83 ± 0.04	0.89 ± 0.02
FM7a/C-S	0.90 ± 0.06	0.93 ± 0.01
rut/*rut* (C-S)	0.41 ± 0.01	0.87 ± 0.03
KA9/*rut* (C-S)	0.45 ± 0.03	0.87 ± 0.03
FM7a/*rut* (C-S)	0.88 ± 0.03	0.87 ± 0.03
tur/*tur*	0.60 ± 0.02	0.93 ± 0.01
tur/C-S	0.87 ± 0.04	0.91 ± 0.02
dnc^{M11}/dnc^{M11}	0.34 ± 0.04	0.84 ± 0.02
dnc^{M11}, *rut*/dnc^{M11}, *rut*	0.16 ± 0.03	0.84 ± 0.02
sbl	0.45 ± 0.01	0.82 ± 0.01

(2) Our *yf*$\widehat{XX}$ and the FM7a X-chromosome balancer stocks (with C-S autosomes) learned about as well as C-S flies. Evidently, the morphological markers on these chromosomes – *white* (eye), *Bar* (eye), *yellow* (body) and *forked* (bristle) – do not interfere much with normal learning. This is fortunate, because it gives us genetically convenient marked chromosomes with which to do crosses. The robustness of this particular learning test with respect to extrinsic chromosome rearrangements and to fairly severe alterations of eye and cuticular pigmentation and morphology stems, we would guess, from the fact that our conditioning procedure does not require visually oriented behavior, fast locomotion or particular coordination on the part of flies.

(3) The behavior of individual flies tested *en masse* is not influenced by others in the group (cf. Byers 1980; Jellies 1981; Tempel et al. 1983). We produced Df(1)KA9/*rut* and FM7a/*rut* flies from a cross between Df(1)KA9/FM7a females and *rut* males (see above) and trained and tested them together. After completion of an experiment, Df(1)KA9/*rut* and FM7a/*rut* flies were anesthetized and separated on the basis of morphological markers, and separate learning indices were calculated. The mean learning indices

of Df(1)KA9/*rut* and FM7a/*rut* flies tested together did not differ from those of *rut*/*rut* and C-S flies, respectively, both of which were tested in separate experiments but on the same day. This result also serves as a control for experimenter bias, since the experimenter could not distinguish Df(1)KA9/*rut* flies from FM7a/*rut* flies until after the experiment was over.

(4) Other learning mutants, though less extensively studied, also perform poorly in the classical conditioning test. Flies homozygous for the *turnip* learning mutation can learn about as well as *rutabaga* flies, while *tur*/C-S heterozygotes learn normally. Thus, the *turnip* allele acts as a recessive mutation for our learning phenotype. In contrast, the *turnip* allele seems to act as a dominant mutation for memory. Even though the mean learning score after a 30-min retention interval for *tur*/*tur* flies ($\Lambda = 0.40 \pm 0.03$) is different from that for *tur*/C-S flies ($\Lambda = 0.68 \pm 0.06$), the memories of both types of flies decay the same amount (0.20), whereas the memory of C-S/C-S flies decays much less (0.07). A second, more severe *dnc* allele (dnc^{M11}) has a learning deficit similar to dnc^{1} (see Fig. 10). More interestingly, the double learning mutant dnc^{M11}, *rut*, which has roughly compensating biochemical deficiencies and near-normal cAMP levels (Livingstone et al. 1984), learns much more poorly than either single mutant.

Discussion

Following Jellies' lead, we changed the conditioned discrimination paradigm of Quinn et al. (1974) from an instrumental procedure to a classical (Pavlovian) one. During training, two strongly, but equally, aversive odors were presented sequentially to a group of flies; the first odor was paired with shock (CS+), the second one was not (CS–). The flies were confined in the training tube, unable to escape the odor presentations or the electric shock regardless of their behavioral responses to either. Afterwards, we placed the flies in another situation, where they were given a choice between CS+ and CS– presented simultaneously without reinforcement. During this test trial, about 95% of the flies avoided the CS+ by walking toward the CS–.

A priori, there seems no reason for the flies to carry their passive training experience over to an active test situation – to orient and locomote so as to avoid the previously reinforced odor. Nevertheless, they do this very well, much more reliably than in previous fly learning tests. Moreover, the conditioned avoidance is difficult to extinguish and persists for a day. Perhaps our training procedure induces a reflexive, species-specific defense (escape) response – negative anemotaxis in the presence of noxious odors (see Mackintosh 1974). In any case, the associative strength of the resulting response in *Drosophila* now is as strong as those produced by similar training procedures in other invertebrates such as *Aplysia* or *Limax*, or even bees (Carew et al. 1983; Gelperin 1983; Menzel and Bitterman 1983).

It is difficult to say, even in retrospect, exactly why our particular combination of procedural details produced strong learning in *Drosophila*. However, three features of the classical conditioning paradigm probably contributed to our results: (1) Because the training situation was classical, in that shock reinforcement was not contingent on the flies' behavioral responses. (2) Our training and testing procedures allowed us to use odor concentrations much higher than in earlier studies. (3) We minimized distracting stimuli to the flies by training and testing them in dim red light without shaking or jarring. Flies appeared calmer and more attentive to odor cues.

Nonassociative factors often have complicated attempts to estimate the proportion of performance levels due to genuine associative learning in dipteran conditioning experiments (see McGuire 1984 for a review). Behavioral sensitization has proven particularly troublesome in excitatory conditioning experiments because it can induce the same increase in responsiveness as does associative learning. Discriminative (differential) conditioning is a way to alleviate this problem, because response levels to the CS– can provide a measure of the effects of behavioral sensitization (Rescorla 1967; Gilbert and Sutherland 1969; Mackintosh 1974; Carew et al. 1983). Quinn et al. (1974) used a discriminative conditioning procedure on *Drosophila* and substracted the mean level of response to CS– from the mean level of response to CS+, which, they assumed, would yield an index of associative learning unbiased by nonassociative effects. Furthermore, by averaging the difference scores from two groups of flies, one with OCT as CS+ and the other with MCH as CS+, they reasoned that the learning index would be unaffected by odor bias.

The fact that the discriminative conditioning procedure used in the present study was classical – with shock contingent on an odor presentation rather than on the behavioral response to that odor – allowed us to test Quinn et al.'s assumptions empirically. We trained flies with nonassociative procedures that are standard controls for classical conditioning experiments (CS Alone, US Alone and Explicitly Unpaired) and then gave flies a choice between OCT and MCH during a test trial.

The resulting learning index was zero for each nonassociative control procedure (Fig. 8B), verifying that the learning index is a measure of associative learning free from nonassociative effects. However, because OCT and MCH were presented simultaneously during the test trials, these data did not resolve whether nonassociative effects on odor avoidances actually were produced. If nonassociative factors affected avoidance responses to each odor equally, then the relative avoidance of OCT vs MCH would remain unchanged after training and the learning index would be zero. Accordingly, we trained flies in similar nonassociative procedures (see Fig. 8A and 9A), but then gave different groups of flies a choice between OCT vs Air or MCH vs Air during the test trial. In this manner, we could assess whether the nonassociative control procedures affected the flies' avoidance responses to OCT and MCH separately. In each case, the avoidance responses to OCT and MCH descreased the same amount. In contrast, avoidance responses to both OCT and MCH (when they were CS+) were enhanced after training with our classical conditioning procedure (Fig. 9B).

Our classical conditioning procedure elicits long-lasting memory retention in wild-type flies. With equivalent training, *amnesiac, rutabaga* and *dunce* all show moderate initial learning and relatively rapid memory decay during the subsequent 30-min retention interval. These results are congruent with those of Tempel et al. (1983), who used an operant, positively reinforced training procedure. A new finding is that memories in the mutants do not decay completely during the first hour after training. Instead, their memory decay rates slow considerably, and some retention still can be measured at least three hours later (Fig. 10). Preliminary experiments on memory consolidation in wild-type flies (Block and Tully, unpublished) have indicated that a cold-shock insensitive phase of memory begins to appear within 15 min after training (cf. Quinn and Dudai 1976; Dudai 1977; Tempel et al. 1983). The time-course of this consolidation process coincides nicely with the change in memory decay rates in the mutants. Thus, the *amnesiac, rutabaga* and *dunce* mutations affect acquisition somewhat and seem to affect an early memory phase, while leaving a later memory phase substantially intact. This idea is consistent with results from savings experiments with *amnesiac* (Quinn et al. 1979).

Our results are broadly consistent with a more mechanistic distinction between short- and long-term memory, based on evidence from *Aplysia*. If one of these animals is sensitized by tail shock, the decay of its enhanced gill-withdrawal responsiveness exactly parallels the decay of cAMP levels in the relevant sensory neurons for the subsequent 30 min or so. In this case, the chemical concomitant for retention of sensitization simply may *be* elevated cAMP in the cells. On the other hand, enhanced behavioral sensitization persists for hours or days after training, long after cAMP levels have returned to baseline (Castellucci et al. 1982; Schwartz et al. 1983). Another biochemical change must underlie this longer-term memory – perhaps an alteration in gene expression or cytoskeletal organization brought about by the acute, transient rise in cyclic nucleotide levels. So, for this (nonassociative) task in *Aplysia* there is an early phase of memory, lasting about 30 min, which seems to be related directly to cAMP, and a later phase, about which little is known.

Our retention data for the mutants suggest that such a mechanistic distinction between short- and long-term memory may hold for associative learning in *Drosophila*. We know that *dunce* and *rutabaga* have biochemical defects in cAMP metabolism (see below). Accordingly, if classical conditioning induces an increase in cAMP levels in the appropriate cells, then plausibly the kinetics of this transient increase in cAMP might be abnormal in *rutabaga* and *dunce*, as well as *amnesiac*, manifesting itself behaviorally as a rapid short-term memory loss. If the mechanism of long-term memory is different, then consolidation and long-term retention should be unaffected in the mutants, as they appear to be.

Kandel et al. (1983) have proposed a detailed biochemical model of classical conditioning based on their work in *Aplysia*. In their model, the relevant biochemical events all occur in the presynaptic terminals made by sensory neurons onto motorneurons of the gill-withdrawal reflex pathway. The US – strong shock to the tail – acts through a neural circuit to cause release of serotonin (or a neurotransmitter agonist) into the extracellular space around the sensory nerve terminal. This leads, by conventional receptor binding and G_s protein stimulation, to activation of adenylate cyclase. The consequent rise in cAMP produces, via kinase activation, protein phosphorylation and potassium channel inactivation, enhanced transmitter release from the sensory neuron terminal, which is a physiological change consistent with the behavioral change (Camardo et al. 1983; Kandel et al. 1983; Schwartz et al. 1983).

So far, this cascade of events, produced by the US (tail shock), is the same as that outlined earlier by Kandel and coworkers for behavioral sensitiza-

tion. New features of the biochemical model have been added to explain how CS-US pairings in classical conditioning interact to produce stronger behavioral responses than those produced by presentations of US alone, which cause sensitization. Physiological experiments (Hawkins et al. 1983; Walters and Byrne 1983) have indicated that the molecular signal for the CS (weak shock to the mantle) must be some consequence of action potentials in the sensory neuron – probably calcium influx. However, CS stimulation does not activate the adenylate cyclase system by itself. Instead, in the model, CS stimulation leads to a rise in intracellular calcium, which can amplify the US-induced cyclase response, provided CS presentations occur slightly before US presentations. The model uses a known biochemical entity for the point of convergence of CS-US interaction – a species of adenylate cyclase, found in vertebrates (Brostrom et al. 1977) and invertebrates (Dudai 1984; Livingstone et al. 1984), that is activated by calcium/calmodulin. Inclusion of this type of cyclase activation in Kandel's model can explain why paired CS-US presentations give stronger physiological and behavioral responses than presentations of CS and US unpaired or of US alone.

Biochemical abnormalities in four *Drosophila* mutants affect components of the adenylate cyclase system involved in Kandel's model of classical conditioning in *Aplysia*. Flies carrying a mutation in the dopa decarboxylase (Ddc) gene do not synthesize the neurotransmitters dopamine or serotonin (Dewhurst et al. 1972; Wright 1977; Livingstone and Tempel 1983), and they do not learn (Tempel et al. 1984). Preliminary work by K.W. Choi and R.F. Smith (unpublished) suggests that mutant *turnip* flies may have altered G_s protein. The *rutabaga* mutation affects a particular species of adenylate cyclase that is activated by calcium/calmodulin. Genetic and biochemical experiments suggest that the mutation lies either in the structural gene for adenylate cyclase or in some closely bound, stoichiometrically limiting protein (Livingstone et al. 1984). In vitro enzyme assays showed no detectable effect of calcium levels on *rutabaga's* adenylate cyclase activity (Dudai 1984; Livingstone et al. 1984), suggesting that the *rutabaga* allele may be an amorph with complete loss of gene function. Finally, the *dunce* mutations (there are five known alleles) affect phosphodiesterase II, one of two enzymes in fruit flies that catabolizes cAMP (Byers et al. 1981). The *dunce* locus most likely is the structural gene for this phosphodiesterase enzyme (Kiger and Golanty 1979; Byers et al. 1981; Kauvar 1982; Shotwell 1983).

Three of these four mutations were isolated independently in a screen for abnormal learning behavior, and all four affect different steps in the monoamine-activated adenylate cyclase pathway. This seems more than coincidental and suggests that the cAMP cell-signalling system may be involved with associative learning not only in *Aplysia* but also in *Drosophila*, an evolutionarily distant invertebrate. On the other hand, three behavioral results with *dunce* and *rutabaga* from the present classical conditioning procedure, suggest that some behavioral properties of these *Drosophila* mutants are not accounted for in the biochemical model of associative learning in *Aplysia*, as currently formulated:

(1) *Dunce* mutations decrease or eliminate phosphodiesterase II activity, leading to higher overall cAMP levels in mutant flies (Byers et al. 1981). The *rutabaga* mutation decreases or eliminates an adenylate cyclase activity, producing lower overall cAMP in mutant flies. In spite of these opposing effects on cAMP metabolism, both mutations induce similar abnormalities on learning and memory retention (Fig. 10, cf. Dudai 1983; Tempel et al. 1983).

(2) Given these opposing biochemical effects in *dunce* and *rutabaga* mutants, one might expect the mutations to compensate for one another. In some respects they do. The double mutant $dunce^{M11}$, *rutabaga* has near-normal cAMP levels in whole flies, and the *rutabaga* mutation may alleviate, to some extent, the female sterility associated with $dunce^{M11}$ (Livingstone et al. 1984). Nevertheless, the *dunce, rutabaga* double mutant learns much more poorly than either of the single mutants (Table 1).

(3) Biochemical measurements and genetic experiments suggest that the *rutabaga* mutation causes a severe or complete loss of detectable calcium/calmodulin-stimulated adenylate cyclase activity (Dudai 1984; Livingstone et al. 1984). As currently modeled in *Aplysia*, the biochemical mechanism for associative learning strictly depends on this species of adenylate cyclase. Accordingly, if this model held for *Drosophila*, then *rutabaga* flies should show little or no learning. In fact, *rutabaga* mutants can learn fairly well (Fig. 10, cf. Dudai 1983; Tempel et al. 1983). Thus, either the current model of associative learning in *Aplysia* is insufficient to explain *Drosophila* learning or some stimulation of *rutabaga's* adenylate cyclase by calcium/calmodulin occurs in vivo.

This is where fruit fly learning and biochemistry stands at the moment, with some strong results and some contradictions. We still are struck by the fact that different experimental approaches have im-

plicated a monoamine-activated cyclase pathway as central to associative learning and memory in both *Aplysia* and *Drosophila*. We also are encouraged by the concreteness of the current biochemical model. We find it easier to refine the model, revise it or even reject it than to work without biochemical building blocks. However, work on the *Drosophila* learning mutants indicates that the receptor-coupled adenylate cyclase system is complex; we only have begun to understand its details. We hope that continued work with the learning mutants, along with other work, will indicate where to look for novel properties in the system.

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References

Aceves-Pina EO, Quinn WG (1979) Learning in normal and mutant *Drosophila* larvae. Science 206:93–95

Aceves-Pina EO, Booker R, Duerr JS, Livingstone MS, Quinn WG, Smith RF, Sziber PP, Tempel BL, Tully TP (1983) Learning and memory in *Drosophila*, studied with mutants. Cold Spring Harbor Symp Quant Biol 48:831–840

Booker R, Quinn WG (1981) Conditioning of leg position in normal and mutant *Drosophila*. Proc Natl Acad Sci USA 78:3940–3944

Brostrom CO, Brostrom MA, Wolff DJ (1977) Calcium-dependent adenylate cyclase from rat cerebral cortex. J Biol Chem 252:5677–5685

Byers D (1980) Studies on learning and cyclic AMP phosphodiesterase of the *dunce* mutant of *Drosophila melanogaster*. Doctoral Dissertation. California Institute of Technology, Pasedina

Byers D, Davis RL, Kiger JA (1981) Defect in cyclic AMP phosphodiesterase due to the *dunce* mutation of learning in *Drosophila melanogaster*. Nature 289:79–81

Camardo JS, Shuster MS, Siegelbaum SA, Kandel ER (1983) Modulation of a specific K^+ channel in sensory neurons of *Aplysia* by serotonin and cyclic AMP dependent protein phosphorylation. Cold Spring Harbor Symp Quant Biol 48:213–220

Carew TJ, Hawkins, RD, Kandel ER (1983) Differential classical conditioning of a defensive withdrawal reflex in *Aplysia californica*. Science 219:397–400

Castellucci VF, Nairn AC, Greengard P, Schwartz JH, Kandel ER (1982) Inhibitor of adenosine 3':5-monophosphate-dependent protein kinase blocks presynaptic facilitation in *Aplysia*. J Neurosci 2:1673

Cline TW (1978) Two closely linked mutations in *Drosophila melanogaster* that are lethal to opposite sexes and interact with daughterless. Genetics 90:683–698

DeJianne D, McGuire TR, Pruzan-Hotchkiss A (1985) Pavlovian counterconditioning of proboscis extension in *Drosophila melanogaster*. J Comp Psychol 99:74–80

Dewhurst SA, Croker SG, Ikeda K, McCaman RE (1972) Metabolism of biogenic amines in *Drosophila* nervous tissue. Comp Biochem Physiol 43B:975–981

Dudai Y (1977) Properties of learning and memory in *Drosophila melanogaster*. J Comp Physiol 114:69–89

Dudai Y (1979) Behavioral plasticity in a *Drosophila* mutant, *dunce*^{DB276}. J Comp Physiol 130:271–275

Dudai Y (1983) Mutations affect storage and use of memory differentially in *Drosophila*. Proc Natl Acad Sci USA 80:5445–5448

Dudai Y (1984) A calcium-stimulated subpopulation of adenylate cyclase is a putative component of a memory apparatus in *Drosophila*. Neurosc Abstr 10:269

Dudai Y, Bicker (1978) Comparison of visual and olfactory learning in *Drosophila*. Naturwissenschaften 65:494–495

Dudai Y, Jan YN, Byers D, Quinn WG, Benzer S (1976) *dunce*, a mutant of *Drosophila* deficient in learning. Proc Natl Acad Sci USA 73:1684–1688

Duerr JS, Quinn WG (1982) Three *Drosophila* mutations which block associative learning also affect habituation and sensitization. Proc Natl Acad Sci USA 79:3646–3650

Folkers E (1982) Visual learning and memory of *Drosophila melanogaster* wild-type C-S and the mutants *dunce amnesiac turnip* and *rutabaga*. J Insect Physiol 28:535–539

Gailey DA, Jackson FR, Siegel RW (1982) Male courtship in *Drosophila*: The conditioned response to immature males and its genetic control. Genetics 102:771–782

Gailey DA, Jackson FR, Siegel RW (1984) Conditioning mutations in *Drosophila melanogaster* affect an experience-dependent behavioral modification in courting males. Genetics 106:613–625

Gelperin A (1983) Neuroethological studies of associative learning in feeding control systems. In: Huber F, Markl H (eds) Neuroethology and behavioral physiology. Springer, Berlin Heidelberg New York Tokyo, pp 189–205

Gilbert RM, Sutherland NS (1969) Animal discrimination learning. Academic Press, New York

Hawkins RD, Abrams SW, Carew TJ, Kandel ER (1983) A cellular mechanism of classical conditioning in *Aplysia*: activity-dependent enhancement of presynaptic facilitation. Science 219:400–405

Horridge GA (1962) Learning of leg position by headless insects. Nature 193:697–698

Jellies JA (1981) Associative olfactory conditioning in *Drosophila melanogaster* and memory retention through metamorphosis. Masters Thesis. Illinois State University, Normal

Kandel ER, Abrams T, Bernier L, Carew TJ, Hawkins RD, Schwartz JH (1983) Classical conditioning and sensitization share aspects of the same molecular cascade in *Aplysia*. Cold Spring Harbor Symp Quant Biol 48:821–830

Kauvar LM (1982) Defective cyclic adenosine 3'5' monophosphate phosphodiesterase in the *Drosophila* memory mutant *dunce*. J Neurosci 3:1347–1358

Kiger JA, Golanty E (1979) A genetically distinct form of cyclic AMP phosphodiesterase associated with chromomere 3D4 in *Drosophila melanogaster*. Genetics 91:521–535

Lindsley DL, Grell EH (1968) Genetic variations of *Drosophila melanogaster*. Carnegie Inst Washington. Publ 627

Livingstone MS, Tempel BL (1983) Genetic dissection of monoamine transmitter synthesis in *Drosophila*. Nature 303:67–70

Livingstone MS, Sziber PP, Quinn WG (1984) Loss of calcium/calmodulin responsiveness in adenylate cyclase of *rutabaga*, a *Drosophila* learning mutant. Cell 37:205–215

Mackintosh NJ (1974) The psychology of animal learning. Academic Press, New York

McGuire TR (1984) Learning in three species of Diptera: the blow fly *Phormia regina*, the fruit fly *Drosophila melanogaster* and the house fly *Musca domestica*. Behav Genet 14:479–526

Medioni J, Vaysse G (1975) Suppression conditionnelle d'un réflexe chez la Drosophile (*Drosophila melanogaster*): acquisition et extinction. CR Acad Sci (Paris) 169:1386–1391

Menne D, Spatz HC (1977) Colour vision in *Drosophila melanogaster*. J Comp Physiol 114:301–312

Menzel R, Bitterman ME (1983) Learning by honeybees in an unnatural situation. In: Huber F, Markl H (eds) Neuroethology and behavioral physiology. Springer, Berlin Heidelberg New York Tokyo, pp 206–215

Pavlov IP (1927) Conditioned Reflexes. Oxford University Press, Oxford

Platt SA, Holliday M, Drudge OW (1980) Discrimination learning of an instrumental response in individual *Drosophila melanogaster*. J Exp Psychol: Animal behavior processes 6:301–311

Quinn WG, Dudai Y (1976) Memory phases in *Drosophila*. Nature 262:576–577

Quinn WG, Harris WA, Benzer S (1974) Conditioned behavior in *Drosophila melanogaster*. Proc Natl Acad Sci USA 71:708–712

Quinn WG, Sziber PP, Booker R (1979) The *Drosophila* memory mutant *amnesiac*. Nature 277:212–214

Rescorla RA (1967) Pavlovian conditioning and its proper control procedures. Psychol Rev 74:71–80

Rescorla RA (1969) Pavlovian conditioned inhibition. Psychol Bull 72:77–94

Schwartz JH, Bernier L, Castellucci VF, Palazzolo M, Saitoh T, Stapleton A, Kandel ER (1983) What molecular steps determine the time course of the memory for short-term sensitization in *Aplysia*? Cold Spring Harbor Symp Quant Biol 48:811–819

Siegel RW, Hall JC (1979) Conditioned responses in courtship behavior of normal and mutant *Drosophila*. Proc Natl Acad Sci USA 76:3430–3434

Shotwell SL (1983) Cyclic adenosine 3'5'monophosphate phosphodiesterase and its role in learning in *Drosophila*. J Neurosci 3:739–747

Sokal RR, Rohlf FJ (1969) Biometry. Freeman, San Francisco

Tempel BL, Bonini N, Dawson DR, Quinn WG (1983) Reward learning in normal and mutant *Drosophila*. Proc Natl Acad Sci USA 80:1482–1486

Tempel BL, Livingstone MS, Quinn WG (1984) Mutations in the dopa decarboxylase gene affect learning in *Drosophila*. Proc Natl Acad Sci USA 81:3577–3581

Tompkins L, Siegel RW, Gailey DA, Hall JC (1983) Conditioned courtship in *Drosophila* and its mediation by association of chemical cues. Behav Genet 13:565–578

Tully T (1984) *Drosophila* learning: behavior and biochemistry. Behav Genet 14:527–557

Tully T, Zawistowski S, Hirsch J (1982) Behavior-genetic analysis of *Phormia regina*: III. A phenotypic correlation between the central excitatory state (CES) and conditioning remains in replicated F_2 generations of hybrid crosses. Behav Genet 12:181–191

Vargo MA (1984) The behavior-genetic analysis of *Drosophila melanogaster* using the central excitatory state. Doctoral Dissertation, University of Illinois, Champaign

Walters ET, Byrne JH (1983) Associative conditioning of single sensory neurons suggest a cellular mechanism for learning. Science 219:405–408

Wright TRF (1977) The genetics of dopa decarboxylase and methyl dopa sensitivity in *Drosophila melanogaster*. Am Zool 17:707–721