dunce, a mutant of *Drosophila* deficient in learning

(conditioned behavior/olfactory discrimination/behavior genetics)

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ABSTRACT   Normal *Drosophila* learn to avoid an odorant associated with electric shock. An X-linked mutant, *dunce,* has been isolated that fails to display this learning, in spite of being able to sense the odorant and electric shock and showing essentially normal behavior in other respects.

The mechanism of learning presumably involves altered interactions among neurons, based on molecular events. Assuming that the biochemical apparatus responsible for plasticity is specified by the genes, one might isolate mutants deficient in learning. This could lead to a dissection of the learning process. In *Drosophila,* the method of mosaic analysis (1) could permit one to pinpoint the critical anatomical foci which, when altered by mutation, cause learning deficiency.

Quinn, Harris, and Benzer (2) demonstrated learning in *Drosophila* using an olfactory discrimination paradigm. Pseudoconditioning, habituation, and odor preference were excluded as explanations. We report here the isolation and characterization of a mutant that appears to have normal sensory and motor mechanisms, yet is unable to learn in this paradigm.

MATERIALS AND METHODS

Flies and Mutagenesis. Normal *D. melanogaster* of the Canton-Special (C-S) wild-type strain were maintained on cornmeal medium (3). Mutagenesis by ethylmethanesulfonate was according to Lewis and Bacher (4). Treated males were mated to virgin, attached-X females, so that each F_1 progeny male carried a treated X-chromosome received from his father. Each individual F_1 male was mated to attached-X females, producing a stock in which the males carried identical, potentially mutant, X-chromosomes. To screen for mutations affecting learning ability, we tested each such population of males.

The Learning Test. The paradigm was similar to that of Quinn *et al.* (2). The apparatus (Fig. 1a) consisted of two sliding Plexiglas holders accepting plastic test tubes (Falcon no. 2017, 17 × 100 mm). Tube 1 was a "rest tube" with about 15 holes at the far end, made with a hot needle. Tubes 2 through 5 contained copper grids etched on flexible epoxy backing (Fig. 1b), rolled up to fit inside the tubes with connecting tabs folded out. Odorants were spread over the grids in 0.2 ml of solution in absolute ethanol, allowing 5 min for evaporation. The shock voltage was 90 V ac (60 Hz). Between experiments, grids were cleaned in ethanol and the apparatus was washed with soap and water.

Before testing, males were separated from females in a nitrogen atmosphere, which immobilizes flies but does not affect subsequent learning (5). For training, about 40 flies were placed in the start tube and the apparatus was laid horizontally, with tubes 1 through 5 pointing at a horizontal 15-W daylight fluorescent lamp (Westinghouse F15T8/D). After the flies had explored the rest tube for about 1 min, the apparatus was held

vertically and tapped sharply on a rubber mat to bring the flies to the bottom of the start tube. The holders were slid to set the start tube in register with tube 2 and the apparatus was replaced on the table. The flies, strongly phototactic, ran toward the light and were shocked in the presence of odorant A. After 30 sec, the flies were shaken back into the start tube, and the slide shifted so that the flies could run into the rest tube again for 30 sec. Next was a similar run for 30 sec into tube 3, containing odorant B but no shock, followed by a 30 sec rest. The complete sequence was repeated twice, thus totaling three training trials with each odorant.

To test for learning, we then shifted the flies to tube 4, containing odorant A on a fresh grid (but with no voltage applied). After 15 sec, the number of flies in the start tube was counted. After a 30 sec rest, the flies were tested against tube 5, containing odorant B (fresh grid, no voltage), and the number of flies in the start tube at 15 sec was recorded. Selective avoidance of the shock-associated odorant indicated learning. To control for odor bias, we performed a reciprocal experiment with a fresh population of flies, in the same apparatus, with the voltage transferred from tube 2 to tube 3. For each half-experiment, the fraction of flies avoiding the shock-associated odorant minus the fraction of flies avoiding the control odorant was determined. The learning index Λ was defined as the average of the two values. $\Lambda = 1$ represents perfect learning; $\Lambda = 0$ indicates no learning.

Behavioral Tests. The ability of flies to sense the odorants on the grids was measured in the choice-chamber apparatus of Fig. 3. A new group of flies was used for each test. Tubes 1 and 2 contained grids, one coated with 0.2 ml of an odorant in ethanol, the other being an ethanol blank. As in the learning experiments, the ethanol was allowed to evaporate beforehand. Each grid was used for two runs. About 60 flies were introduced into tube 0 and shaken into the sliding compartment. The apparatus was then laid horizontally with tubes 1 and 2 parallel to a fluorescent lamp (at 6 cm distance) to equalize the light intensity in both arms. The sliding compartment was then shifted into register with tubes 1 and 2. After 30 sec, the slide was shifted away and the number of flies trapped in each tube (and the small number remaining in the chamber) was counted.

Phototaxis and geotaxis were measured by countercurrent distribution (6). Flying ability was measured as described by Benzer (7). Spontaneous locomotor activity was measured by the total distance walked as a function of time in a long glass tube of 8 mm inside diameter (8). All learning and other behavioral experiments were carried out at 20–23°.

Chemicals. 3-Octanol, 4-methylcyclohexanol, and geraniol were from K&K Laboratories (Plainview, N.Y.). Benzaldehyde, amyl acetate, and menthol were from Mallinckrodt Chemical Works (St. Louis, Mo.). Caproic acid was from National Biochemicals Corp. (Cleveland, Ohio). Caproic acid was from National Biochemicals Corp. (Cleveland, Ohio), and stearic acid and quinine sulfate were from Matheson, Coleman and Bell (Norwood, Ohio).

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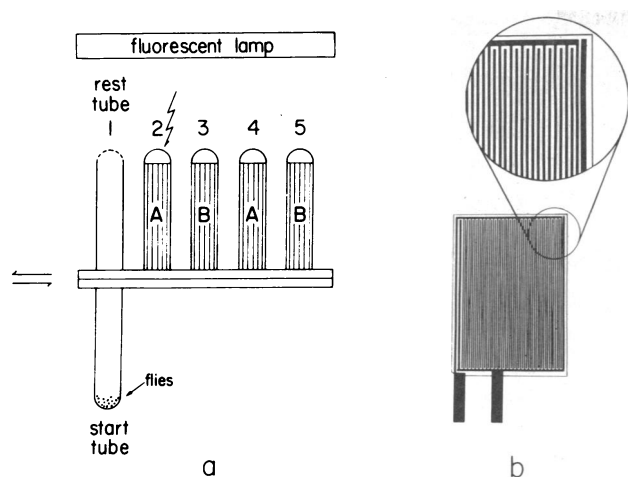*Proc. Natl. Acad. Sci. USA 73 (1976)*   1685

FIG. 1. Apparatus used for training and testing flies. (a) Two plastic blocks holding tubes slide on a dovetail joint, so that the start tube can be shifted into register with tubes 1 through 5. Tube 1, the rest tube, is perforated for ventilation. Tubes 2 through 5 contain grids with odorants A or B. Tubes 2 and 3 are used for training. The lightning bolt indicates voltage on the grid. Tubes 4 and 5 are for testing. (b) Printed-circuit grid for shocking flies. The bars of the grid are 0.5 or 1 mm wide and are separated by 0.5 mm. The grid is rolled up and inserted into a tube, which is plugged into the apparatus. Conductive tabs for applying voltage are bent around the tube rim to the outside.

RESULTS

Isolation of *dunce*. Normal flies, during training, avoid the shock-odorant combination, but show much smaller avoidance of the control odorant (Fig. 2). On testing, they still preferentially avoid the odorant that had been associated with shock, yielding a learning index Λ of 0.31 (SEM $\pm$ 0.02, $n = 18$).

The following criteria were chosen for a learning-deficient mutant: (*i*) during testing, the flies fail to avoid selectively the shock-associated odorant; (*ii*) the abnormal performance cannot be ascribed to sensory or motor defects. Approximately 500 mutagenized X-chromosome lines were tested, using 3-octanol and 4-methylcyclohexanol as odorants A and B. About 20 of these lines met the first criterion; of these, only one also satisfied the second. The strain was made homozygous, and named *dunce*‡ (*dnc*).

Behavior of *dunce* in the Learning Paradigm. Fig. 2 shows results of learning tests of the mutant strain compared with normal flies. During the three training trials, the mutant flies show normal phototaxis and are deterred by the charged grid. When subsequently tested, however, they do not selectively avoid the shock-associated odorant.

To determine whether the deficiency in performance of *dunce* is specific to the odorants used in screening, various other odorants were tested, including alcohols, acids, aldehydes, and esters. The results are shown in Table 1. All the combinations listed are effective learning cues for normal flies, but not for *dunce*.

Different reinforcements were tried. Normal flies showed learning for voltages ranging from 20 to 140 V; *dunce* showed very little. Quinine sulfate powder, dusted onto a grid, can substitute for shock as a negative reinforcement for normal flies (2) ($\Lambda = 0.21$, Table 1). The learning ability of *dunce* is deficient with this reinforcement ($\Lambda = 0.05$, Table 1).

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‡ Term of derision applied to followers of John Duns Scotus, theologian, d. 1308, regarded by 16th century humanists as an enemy of learning.

Table 1. Learning performance of normal and *dunce* flies

Odorants used	Learning index (Λ)	
	Normal flies	*dunce*
0.5% 4-Methylcyclo-hexanol versus 0.5% 3-octanol	0.31 ± 0.02 (18)	0.04 ± 0.02 (17)
0.25% 3-Octanol versus blank	0.28 ± 0.06 (4)	−0.02 ± 0.03 (4)
0.5% 3-Octanol versus blank	0.48 ± 0.02 (4)	0.10 ± 0.01 (4)
1.0% 4-Methylcyclo-hexanol versus blank	0.28 ± 0.03 (3)	0.08 ± 0.01 (3)
0.25% Menthol versus blank	0.24 ± 0.03 (4)	0.05 ± 0.04 (3)
0.05% Geraniol versus blank	0.24 ± 0.06 (4)	0.05 ± 0.02 (4)
0.5% 4-Methylcyclo-hexanol versus 1.0% stearic acid	0.29 ± 0.05 (3)	0.01 ± 0.02 (3)
0.25% Caproic acid versus blank	0.29 ± 0.05 (3)	0.04 ± 0.07 (3)
0.5% Benzaldehyde versus blank	0.28 ± 0.04 (3)	0.06 ± 0.04 (3)
0.1% Benzaldehyde versus 0.5% amyl acetate	0.30 ± 0.03 (4)	0.03 ± 0.08 (4)
0.5% 4-Methylcyclo-hexanol versus 0.5% 3-octanol, with quinine as negative reinforcement	0.21 ± 0.04 (3)	0.05 ± 0.04 (3)

Ethanol was used as blank. Values are mean ± SEM with the numbers of experiments in parentheses.

Ability of the Mutant to Sense the Odorants. There is a tendency of flies to avoid some of the odorants used. In the learning paradigm, this is largely overcome by the strong phototactic drive. The choice-chamber apparatus of Fig. 3 was designed to use this avoidance as a test of the ability of flies to sense odorants. The fraction of flies entering the odorant tube is plotted in Fig. 4 for various substances. It appears that *dunce* can sense the odorants.

In order to find out whether the poor performance of *dunce* could be due to a phototactic drive so strong as to override any learned avoidance, flies trained in the standard paradigm were tested in the choice-chamber apparatus, using the control odorant in one tube and the shock-associated odorant in the other, the light intensity being equal in both tubes. Normal flies selectively avoid the shock-associated odorant; *dunce* flies again show poor learning. In this experiment, the learning index is defined as the fraction of flies present in the control odorant tube minus the fraction in the shock-associated odorant tube at 30 sec. The values obtained were 0.33 (SEM $\pm$ 0.03, $n = 7$) for normal flies and 0.13 (SEM $\pm$ 0.05, $n = 7$) for *dunce*.

Other Tests on *dunce*. The external morphology of *dunce* flies is normal. The viability of *dunce* eggs, larvae, and pupae is normal, and the adults have a normal life span. The mutant has essentially normal phototaxis, geotaxis, flight, locomotor activity, and sexual courtship. In crowded culture bottles, *dunce* flies do seem to become somewhat weaker and more sluggish than normal. The electroretinogram (9) is normal. Synaptic
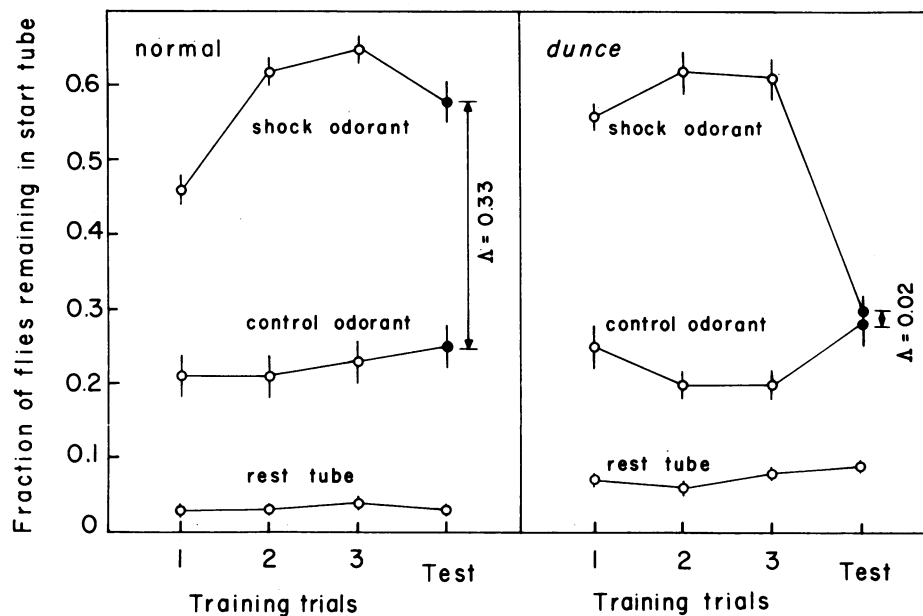
FIG. 2. Behavior of normal and *dunce* flies during training and testing. Fraction of flies in start tube after 15 sec is shown for the three training trials and for the test. Each point represents the mean ± SEM for 11 experiments; 0.5% 3-octanol and 0.5% 4-methylcyclohexanol served as odorants. The learning index, Λ, is the fraction of flies avoiding the shock-associated odorant during the test minus the fraction avoiding the control odorant during the test.

transmission at the neuromuscular junction in the larva, including facilitation (10), is normal.

To test for a possible difference in the electrical conductivity of the adult cuticle which could affect sensitivity to shock, we made measurements of the current passing through single flies stepping across the grid lines, using an applied dc voltage in series with an oscilloscope as detector. Both normal and *dunce* gave similar deflections, corresponding to a leg-to-leg resistance of the order of 10^9 ohms. The effect of prolonged shock on the flies was assessed by measuring their subsequent phototaxis. Flies were forced into a tube containing a grid and shocked with 90 V ac for 60 sec. Fifteen seconds later, they were tested for phototaxis by countercurrent distribution. Both normal and *dunce* flies showed similarly reduced phototaxis, the probability of response per trial being decreased from 0.9 to 0.6. After about 10 min, the phototaxis of both strains recovered to normal.
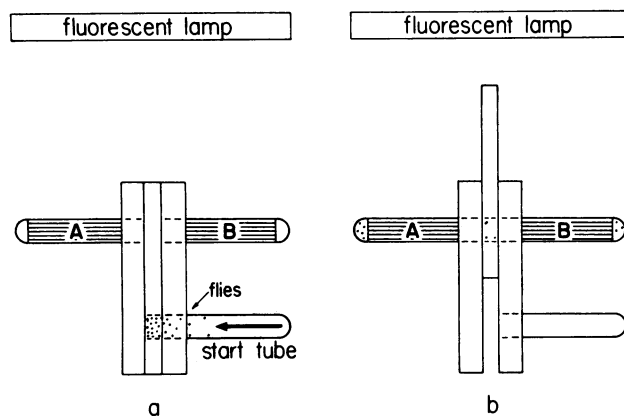

FIG. 3. Choice-chamber apparatus for testing the ability of flies to sense odorants. (a) The flies are shaken from the start tube into the sliding compartment. (b) The compartment is shifted into register with tubes containing odors A and B. After 30 sec, the compartment is shifted out of register and the numbers of flies trapped in each tube and in the central compartment are counted.

Genetics. The X-linked *dunce* mutation is incompletely recessive. Heterozygous *dnc*/ + females have a Λ of 0.23 (SEM ± 0.02, $n = 5$) in comparison to 0.31 (SEM ± 0.02, $n = 18$) for +/+ flies. Hemizygous males and homozygous females are equally deficient in learning; $\Lambda = 0.01$ (SEM ± 0.02, $n = 4$) for males and 0.05 (SEM ± 0.06, $n = 4$) for females.

To map the mutation, *dunce* males were mated to females homozygous for the following markers: *yellow* (*y*), *chocolate* (*cho*), *crossveinless* (*cv*), *vermilion* (*v*), *forked* (*f*), and a normal allele of *yellow* (*y+*) located near the centromere (11). The heterozygous F_1 females were then crossed to normal males, yielding males whose X-chromosomes had an opportunity to undergo recombination. The F_2 males included various recombinants for the morphological markers and *dnc*. These males could not be tested for the presence of *dnc* because some of the marker genes affect the activity of flies, interfering with learning performance. To overcome this difficulty, recombinant males were individually mated to *dnc*/*dnc* females to produce female progeny heterozygous for the recessive morphological markers, which do not affect learning ability when heterozygous. These flies were either homozygous or heterozygous for *dnc*, depending upon whether the *dnc* gene was present in the F_2 male. Testing these females reveals whether *dnc* was present in the F_2 male, since *dnc*/*dnc* females learn much more poorly than *dnc*/ +. The results are shown in Table 2 and indicate that the *dnc* gene is between *y* and *cho*.

DISCUSSION

During training, normal flies perceive the light, respond to it phototactically, sense the odorant and the electric shock, and integrate the sensory inputs. An association occurs between shock and odorant so that, in later testing, the odorant acts as a cue for avoidance. Poor performance in this paradigm could result from defects in any of these steps.

What could the defect in *dunce* be?

(*i*) Although the mutant has essentially normal phototaxis, can sense the odorants, and is deterred by the electric shock,
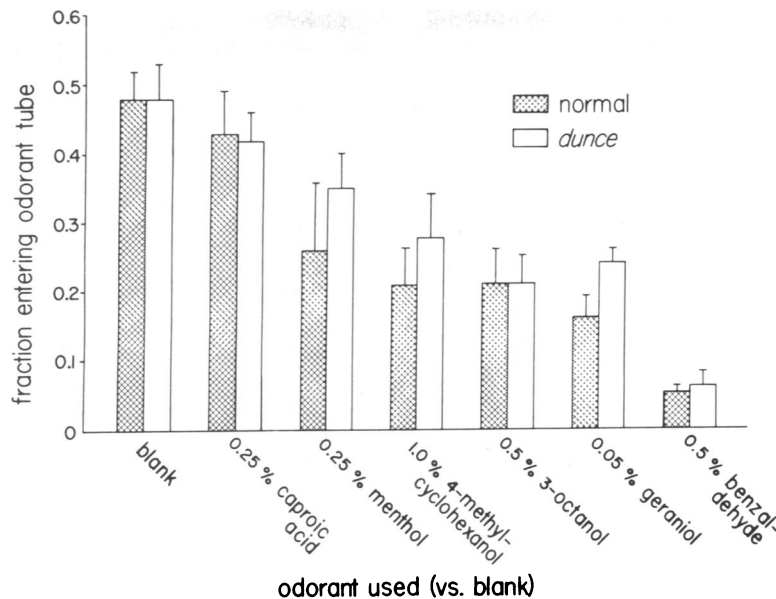
FIG. 4. Response of normal and *dunce* flies to various odorants in the choice-chamber apparatus of Fig. 3. Two-tenths milliliter of the specified odorant in ethanol was spread on one grid. The other grid was a blank with 0.2 ml of ethanol alone. Before use, the grids were dried for 5 min to evaporate the ethanol. Each column represents the average (± SEM) of 10 experiments, with 50–70 flies per experiment. A small number of flies remain in the central compartment, so blank vs. blank is slightly less than 50%.

there may exist some subtle sensory or motor process which is defective in *dunce* that becomes important under the conditions of the paradigm.

(*ii*) The defect could be in certain pathways connecting sensory input and motor output. The fact that *dunce* is able to avoid the odorants under conditions where learning is not required shows that some sensory-motor connections are intact. Nevertheless, others might be needed during learning. Using other sensory modalities, the mutant flies might learn.

Table 2. Mapping of the *dunce* mutation

Class	Learning index (Λ)
Parental chromosomes	
dunce	0.05 ± 0.03
$y\ cho\ cv\ v\ f\ y^+$	0.32 ± 0.03
Recombinant chromosomes	
$cv\ v\ f\ y^+$	0.10 ± 0.03
$v\ f\ y^+$	0.11 ± 0.02
$f\ y^+$	0.09 ± 0.02
y	$\begin{cases} 0.07 \pm 0.01\ (n = 6) \\ 0.26 \pm 0.03\ (n = 4) \end{cases}$
$y\ cho$	0.20 ± 0.02
$y\ cho\ cv$	0.25 ± 0.04
$y\ cho\ cv\ v$	0.20 ± 0.01
$y\ cho\ cv\ v\ f$	0.28 ± 0.03

Learning performance of the parental types and each recombinant class. Each chromosome was tested over *dunce* in heterozygous females to eliminate effects of markers. For recombinant types, each Λ is the average for 9–11 independently arising recombinants. For the parental types, the Λ represents the average of 9–11 determinations. The recombinant classes y^+ and *cho cv v f* y^+ are indistinguishable from one of the parental types with the markers used. The y recombinants could be clearly divided into two groups, each with Λ typical of one of the parental types. The results place the presumptive location of *dnc* between y and *cho;* i.e., between map positions 0.0 and 5.4 at the left tip of the X-chromosome.

(*iii*) The *dunce* mutation may interfere with accessory neural processes necessary for learning. For example, defects in arousal and motivation may interfere with information storage and retrieval (12), even though the machinery for learning is intact.

(*iv*) The *dunce* mutation may disrupt a molecular mechanism underlying neural plasticity.

Several molecular mechanisms have been implicated in learning. Some involve neurotransmitter metabolism (12) or protein synthesis (13). Biochemical measurements on *dunce* and normal flies might reveal possible defects in such mechanisms. Pharmacological treatments, if successful in reversing the mutant phenotype, could indicate the nature of the lesion. Preliminary experiments have not yet yielded any such clues. It is possible that the defect is confined to a very small anatomical region. If so, mosaic analysis should focus attention on the crucial region. Isolation of additional mutants might reveal various steps in the process of learning.

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