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Conditioning of leg position in normal and mutant *Drosophila*

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ABSTRACT **Individual *Drosophila melanogaster*, with or without heads, can be trained to lift their legs to avoid electric shock. This behavior is similar to the operant conditioning previously demonstrated in intact and headless cockroaches. More than 90% of headless wild-type flies learned to our criterion. In contrast, three mutants (*dunce*, *cabbage*, and *turnip*), originally selected for failure to learn in an olfactory discrimination paradigm, tended to perform poorly in this new learning situation. The difference in learning behavior between normal and mutant flies is distinguishable in individuals and may be useful for mosaic analysis.**

Populations of *Drosophila* can be trained to avoid an odor by shocking the flies in its presence (1, 2), and single-gene mutants that fail to learn this olfactory task have been isolated (3, 4). We would like to find physiological or chemical processes that are altered in these mutants and thus gain clues about how normal flies learn. First, however, we need to know whether the mutations really interfere with learning *per se* or merely produce poor performances in our test by causing sensory defects or general debility. One of the mutants, *dunce*, has been carefully characterized and found to have normal olfactory acuity, motor coordination, and overall activity (3). Therefore, *dunce*'s poor performance is not due to gross peripheral or neurological derangement; however, more subtle behavioral abnormalities cannot be ruled out. The learning-deficient mutants *turnip* and *cabbage* (4) are under more suspicion in this respect because they show slight deficiencies in phototaxis, which is not a learned behavior. It would be advantageous to develop a variety of learning paradigms for *Drosophila*. Testing normal flies and the mutants in several learning tasks would help separate "shallow" mutants, with sensory deficits or deficiencies in a particular kind of learning, from mutants with more general learning disabilities.

One widely studied example of "simple" insect learning is the operant conditioning shown by the cockroach in Horridge's paradigm (5, 6). In this test the animal, with one leg free to move, is suspended over an electrolyte solution. If a voltage is applied so that the animal receives a shock when a wire on its leg extends into the solution, it often learns to keep the leg flexed. Decapitated cockroaches learn even more readily. Here we report that normal *Drosophila*, especially headless ones, learn very reliably to flex their legs in the Horridge paradigm. The flies can also learn to extend their legs to avoid shock. Three mutant fly stocks, originally selected for deficient olfactory discrimination learning, also do poorly in the leg-flexion and leg-extension tests.

MATERIALS AND METHODS

Fly Stocks and Culture Conditions. *Drosophila melanogaster* of the Canton-special (C-S) wild-type strain and three mutant derivatives were used. The X-linked, ethylmethane sulfonate-induced mutants *dunce*^{1} (3), *dunce*^{2} (7), *cabbage*^{PS264} (4), and *turnip*^{PS274} (4, 8) were all originally isolated (methods of ref. 3) because they showed consistently poor learning scores in an olfactory discrimination paradigm. The *dunce* gene has been mapped between salivary bands 3D1 and 3D3 on the X chromosome (7). Mutations in this gene abolish activity of a cyclic AMP phosphodiesterase (isozyme form II) (7). The *turnip* gene (4, 8) maps between the X-linked markers *forked* (56.7) and *carnation* (62.5) (unpublished); *cabbage* (4) has not yet been genetically mapped. The *dunce*^{1}, *cabbage*, and *turnip* mutations complement one another for learning ability (4), which indicates that they involve different genes. All flies were maintained at 25°C on a standard cornmeal medium (9) in half-pint milk bottles. Before testing, flies were transferred to new culture bottles and given at least 20 min to clean themselves. Approximately equal numbers of males and females were used for each genotype; no sex-related differences in performance were apparent.

Preparation for Testing. Our procedure was adapted from Horridge's method for cockroaches (5, 6). A fly to be tested was anesthetized with carbon dioxide and placed under a dissecting microscope. The fly was positioned, ventral side up, on a wax "lollipop," a 1-cm disc of tackiwax on a wooden stick (Fig. 1). The wings were spread laterally from the fly and secured firmly in the wax with Dumont no. 5 forceps so that the dorsal thorax was in light contact with the wax.

The fly was given 10 min to recover from anesthesia, then fed 0.1 M sucrose with a no. 00 artist's brush until it stopped drinking. A tungsten wire (25-μm diameter, no. 218, 0.98 mg/meter, tolerance 2, General Electric, Cleveland, OH) was threaded between the fly's thorax and the wax, then bowed up from the wax so it contacted the dorsal thorax in the notch between the scutellum and the postscutellum. It was fixed in this position by anchoring it in the wax with warm forceps at two sites about half a millimeter apart on either side of the thorax. All the legs except the right metathoracic leg were pressed into the wax with warm forceps to immobilize them (Fig. 1). A very thin tungsten wire (12.5 μm diameter, no. 218, 0.25 mg/m, tolerance 2, General Electric) was tied in a slipknot. One end of the wire was pulled very tight, and the other end was adjusted to give a compact knot with a slip noose about 0.5 mm in diameter. The noose was looped over the free metathoracic leg to the fourth or fifth tarsal segment, then tightened snugly around the cuticle by pulling carefully on the sliding wire ends with Dumont no. 5 forceps. One end of the wire was trimmed to about 2 cm and bent if necessary so that it formed an extension of the fly's leg. Ordinarily, the other end of the wire was cut about 0.1 mm from the knot with iridectomy scissors. For leg-extension learning, this second end was left long. In this case, it was bent to form a loose (12 cm) loop before being anchored in the tackiwax, then continued to a grounded contact.

For so-called "headless" flies, the brain was destroyed by pinching the head with hot forceps. Actually cutting off the head with a razor blade produced similar changes in behavior,

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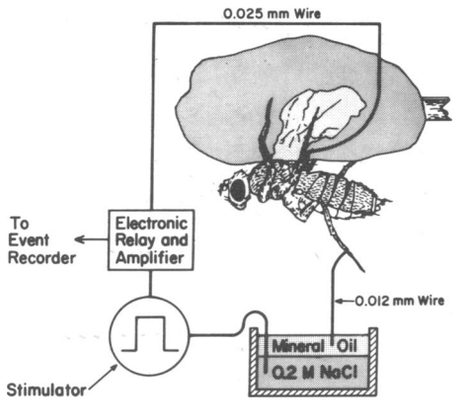

FIG. 1. A fly is fixed to a 1-cm wax lollipop by the wings, with five legs immobilized in the wax. A very thin wire is tied around the free metathoracic leg. The wax lollipop with fly is placed in a micromanipulator above a dish of saline solution covered with 4 mm of mineral oil. The wire tied to the fly's leg just touches the saline when the leg hangs down in the resting position. A second wire from the thorax of the fly runs through the outputs of a stimulator into the saline. When the wire from the fly's leg is in contact with the saline, the circuit is complete, and the fly receives shocks from the stimulator every 4 sec. An event recorder, connected via an electronic relay, records shocks experienced by the fly.

but such genuinely decapitated flies often deteriorated more quickly, even when the neck wound was sealed with petroleum jelly.

Testing the Flies. All tests were carried out in a dark, quiet room at 22°C and 40% relative humidity. A Narishige MM3 micromanipulator was positioned over a 100-mm Petri dish containing 0.2 M NaCl solution overlaid with 4 mm of paraffin oil to minimize surface tension effects. The lollipop was attached by the stick to the micromanipulator with the tackiwax disc horizontal, so that the fly (now rightside up) was on the bottom with the wire hanging straight down from its leg (Fig. 1). The fly was lowered until the wire penetrated the mineral oil and just made contact with the saline solution when the free leg hung in the rest position. The fly, positioned this way, could flex its leg (typically with its femur and tibia above its back) to break the contact between wire tip and saline solution. However, the wire tip always remained in the mineral oil; and the weight of the wire (0.05 mg), mineral oil viscosity, and surface tension combined to ensure that the wire always hung vertically down from the leg.

The wire contacting the fly's back was attached to one output of a Grass S44 stimulator. Another wire continued from the other stimulator output to the salt solution in the Petri dish, so the circuit from the stimulator through the fly was complete when the wire from the fly's leg touched the water (Fig. 1). The stimulator was set to deliver a shock consisting of a pair of pulses (10 msec long, 50 msec apart) once every 4 sec. These shock parameters were selected for the following reasons. To activate the recording circuit overall shock pulse had to be about 15 msec long; given this constraint the duration of each pulse was kept as short as possible to minimize injury to the fly. The pair of very short pulses activated the recorder and worked better than a single pulse; i.e., the flies learned better and showed less involuntary twitching. Performance also improved as the intershock interval was increased from 1 to 4 sec; it remained steady at longer intervals. Before training began, the stimulus voltage was adjusted just high enough to elicit a noticeable twitch reaction to a shock. In 85% of experiments reported here the stimulus voltage was 80–90 V, but stimuli in a few cases were as low as 60 or as high as 120 V. (The variability may be attributable to differences in resistance among individual flies in our apparatus; these values, measured from dorsal wire to leg wire, ranged between 10 and 30 MΩ.) Shocks of less than 40 V elicited no avoidance learning. In the last 60 experiments of this series, the shock was standardized at 90 V; this did not alter the results detectably.

After the fly was positioned and the voltage was adjusted, the stimulator was turned off and the fly was given a rest. The recorded experiment began 5 min later when the stimulator was turned back on. To record the fly's behavior, an electronic relay was placed in the circuit between stimulator and bath. The amplified relay output activated a moving-chart event recorder, which gave a graphic record of shocks experienced by the fly.

Yoked-Animal Controls. These experiments consisted of a 30-min yoked training session followed by a 60-min posttest. For each experiment two flies (designated P, positional, and R, randomly shocked) were prepared for training and set up in similar circuits, each with its own saline bath and stimulator as in Fig. 1. During the yoked-training phase, the P animal was situated over the bath as usual, with the wire from its leg extending slightly into the saline solution. The R animal was positioned with its leg wire deep in the saline bath so it could not break the circuit no matter how it positioned its leg. The relay output of the P circuit was connected to trigger the stimulator in the R circuit. In this situation both animals received shocks whenever the P animal extended its leg.

In the 5-min interval between yoked training and posttest, neither animal received shocks. The simulators were uncoupled and the R fly was repositioned above the saline bath so that it could break the circuit by flexing its leg. In the posttest, both animals were in identical, independent circuits. Thus the R animal started, and the P animal continued, in the standard situation for leg flexion training (Fig. 1).

Leg-Extension Learning. A fly was prepared as above, except that the wire on its foreleg was grounded. The fly was positioned as usual over the saline solution. When the wire dangling from its leg failed to make contact with the saline, the voltage between ground and saline (≈400 mV) activated an electronic trigger circuit, whose amplified output triggered the stimulator; in this mode the stimulator administered shocks every 4 sec as above. If the fly extended its leg, the saline was grounded, the stimulator was not activated, and the fly escaped shock.

Statistics. The Mann–Whitney *U* test was used for all estimates of differences in performance. To assess improvement in avoiding shock, we measured the change during the first 15 min of testing. The parameter used was the number of shocks received by the flies in minutes 1–3 of testing minus number of shocks received in minutes 13–15. To assess differences between mutant and normal flies in shock-avoidance performance, the parameter used was the number of shocks received by mutant flies in minutes 13–15 minus number of shocks received by normal flies in minutes 13–15.

RESULTS

Learning Performance. In most respects, learning behavior of normal *Drosophila* in the leg-flexion task parallels that of cockroaches (5, 6). Intact flies, even without electric shock, often kicked their legs spontaneously and in response to lights, drafts, or substrate vibration. Their performance in the leg-lifting paradigm was variable, and many individuals never appeared to learn. Nevertheless, when responses of flies were averaged, there was a significant tendency ($P \leq 0.05$) for intact wild-type flies to reposition their legs to avoid shock for increasing periods of time as the test proceeded (Fig. 2) and some further improvement in behavior for 60 min (data not shown).

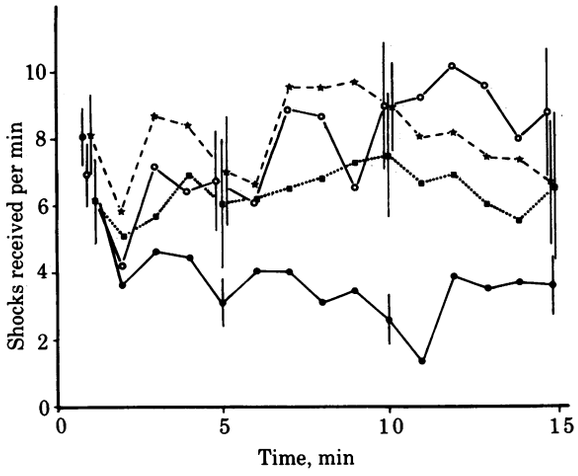

FIG. 2. Average learning performance of intact flies (normal and mutant) in the leg-lifting task. For each genotype, the average frequency of shocks received for all individuals tested is shown as a function of time. A fly can receive a maximum of 15 shocks per minute; low shock values indicate that the flies tend to hold their legs up. Error bars at 1, 5, 10, and 15 min show SEM. Variations among flies at other times are comparable; they are omitted here for clarity. ●, C-S (20 flies); ○, *dunce* (10 flies); ■, *cabbage* (10 flies); ★, *turnip* (10 flies).

In contrast, the mutants showed no general improvement over time. Their average performance tended to deteriorate somewhat during the test, perhaps because of the effect of many repetitive shocks (Fig. 2). Nevertheless, a few individuals of each mutant type did learn. In these instances, their behavior appeared similar to that of normal flies.

"Headless" flies (i.e., with cerebral ganglia destroyed) gave much more consistent behavior. When observed visually they showed little of the spontaneous leg motion characteristic of intact individuals. "Headless" wild-type flies learned more decisively than intact flies [P (headless learning = intact learning) ≤ 0.05], perhaps because they were less susceptible to "distraction" from sensory stimuli to the head. Many individuals learned almost immediately, keeping their legs flexed after three to five shocks (Fig. 3). "Headless" mutant flies did less well than "headless" normals even in the first minute, and they showed no improvement. All three mutants differed significantly ($P \leq 0.01$) from normal flies.

Fig. 4 shows the average rate of leg movement for mutant and normal flies during these tests. None of the mutants seemed hyperactive or lethargic relative to C-S flies. The mutants also showed normal activity at later times (data not shown).

In order to have an objective basis for comparing learning performance of individual flies, we chose an arbitrary criterion; a fly was considered to have learned if it received 10 or fewer shocks during the 10-min interval from 15 to 25 min after the start of training. Learning curves for individual "headless" flies are shown in Fig. 5, and Table 1 summarizes individual performances. It is evident that more normal flies (92%) than mutant flies (25–45%) learn to criterion.

With *dunce* flies there is good evidence that the deficiencies in olfactory learning (3) and leg-flexion learning result from the same genetic lesion, because flies carrying an independently arisen mutation in the gene [$dunce^2$ (2, 7)] also do poorly in both tests. A similar argument holds for the *turnip* mutation, which continues to disrupt both types of learning in a recombinant in which the autosomes plus about 90% of the X chromosomes have been replaced by new material. For these mutants, both deficits map to a defined genetic region. The *cabbage* mutation has not yet been mapped, but in this case we know that poor

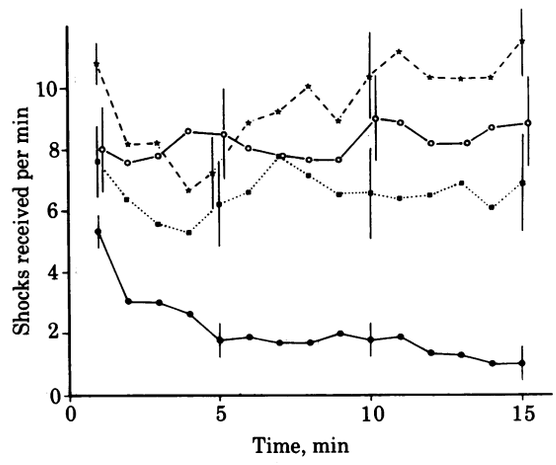

FIG. 3. Average learning performance of "headless" flies (normal and mutant) in the leg-lifting task. Axes and error bars are as in Fig. 2. The larger error bars for the mutants are primarily due to smaller sample sizes; their variability in performance was only slightly higher than that of C-S flies. ●, C-S (50 flies); ○, *dunce* (20 flies); ■, *cabbage* (20 flies); ★, *turnip* (20 flies).

performance in both learning tasks is X-linked (unpublished data).

Yoked Control Experiments. Initially it seemed possible that the increased leg-lifting behavior observed during an experiment was not learned but was merely stronger muscle contraction induced by cumulative electric shock. The fact that *dunce*, *cabbage*, and *turnip* mutants show little increase in leg lifting in this situation makes such an explanation unlikely. Nevertheless, to settle the issue for *Drosophila* we repeated Horridge's yoked-animal control (5, 6). Two "headless" flies were prepared for training. The first (designated positional, P) was set up for training as usual. During the initial yoked-training session the second, control fly (designated radom-shock, R) was set up to receive a shock every time the P fly did. Thus the P animal received shocks whenever it extended its leg; the R animal experienced the same pattern of shocks without regard to its leg position. In this situation, if leg contraction is an artifact of shock, R and P animals should perform identically. On the

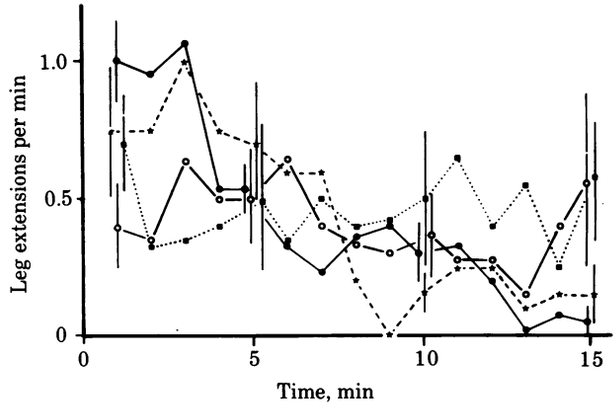

FIG. 4. Leg-movement activity of "headless" normal and mutant flies. The data come from the same experiments as Fig. 3. Leg extension by an individual was indicated by a shock to the fly occurring after an interval greater than 4 sec in which no shocks were received. Some leg extensions are missed by this method of recording, but separate control experiments show that such missed events are rare. ●, C-S (50 flies); ○, *dunce* (20 flies); ■, *cabbage* (20 flies); ★, *turnip* (20 flies). C-S flies tested without employing shock (monitored as with leg-extension learning) showed about a third of the activity of the flies shown here.

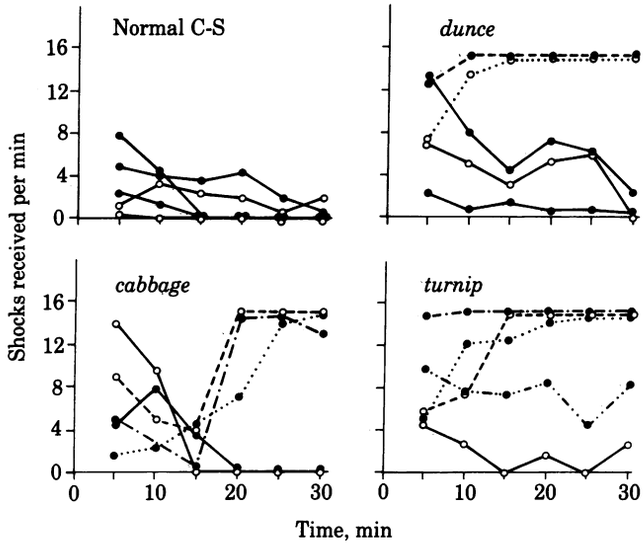

FIG. 5. Individual learning curves for five "headless" flies of each genotype (selected from the tested populations with the random-number function of a pocket calculator). A tested fly could receive a maximum of 15 shocks per minute. There was a tendency for the performance of a fly to deteriorate rapidly unless it learned to avoid shocks in the first few minutes. A solid curve indicates that the fly learned to criterion within 60 min; a broken curve indicates it did not.

other hand, if the observed behavior represents genuine learning, the randomly shocked animal should show no tendency to lift its leg during the yoked training session and should behave like a naive animal in the standard training situation during a posttest.

Our experiments gave the latter outcome, indicative of learning. During the yoked training, the R flies, visually observed, showed no systematic tendency to contract their legs. (Because of the yoked circuit arrangement during this experiment, we could not monitor leg position electronically.) In the posttest situation, R flies performed much worse than P flies ($P < 0.01$). In fact they were even slower to flex their legs than naive animals (Fig. 6), perhaps because they had learned earlier that altered leg position was of no avail in shock avoidance. Note that these animals, unlike naive, "headless" C-S flies, received as many shocks as the mutants did in the first minute of training. During yoked training, 19 of 20 P animals learned to criterion. (Throughout this period the R animals were observed to keep their legs extended most of the time.) During the posttest, the same 19 P animals reached criterion again, whereas only 13 of the 20 R animals attained this level.

Table 1. Learning performance of individual flies

Conditions	Genotype	Total flies tested	Flies learning to criterion	
			No.	%
Leg-lifting test, "headless flies"	C-S	50	46	92
	dunce	20	7	35
	cabbage	20	9	45
	turnip	20	4	20
Leg-extension test, "headless flies"	C-S	20	15	75
	dunce	10	5	50
	cabbage	10	4	40
	turnip	20	5	25

A fly learned to our criterion if it received fewer than 10 shocks in the interval between 15 and 25 min after the start of testing (maximum possible shocks = 150).

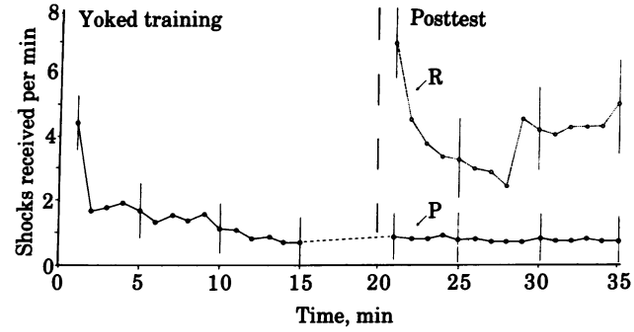

FIG. 6. Average learning performance in yoked control experiments. "Headless" C-S flies were used. ●, Positional flies (P; 20 individuals), trained as usual in both pretest and posttest. ○, Randomly shocked control flies (R; 20 individuals) each shocked at the same times as its P fly irrespective of its own leg position in the pretest; each was then trained positionally as usual in the posttest. Axes and error bars are as in Fig. 2. The R flies showed learning in the posttest, albeit less than naive flies did.

Leg-Extension Learning. If the induced leg-lifting represents genuine learning, the flies should also learn to extend their legs to avoid shock. [Leg-extension training has been tried on cockroaches, with mixed success (10, 11)]. To test this prediction, "headless" flies were prepared and set up as in the leg-flexion test, but the circuitry was modified so that a fly being tested received shocks when the wire from its leg *failed* to make contact with the water. In this situation, 15 of 20 normal flies learned to our criterion (Table 1). Learning by the mutants (Fig. 7, Table 1), was significantly inferior: P (*dunce*) < 0.01; P (*cabbage*) < 0.01; P (*turnip*) < 0.01. This is an important control for the mutants. Their failure to learn the leg-extension task indicates that their poor performance in the leg-lifting test is not due simply to muscular weakness or general debility, because downward leg extension should require less strength.

DISCUSSION

Normal C-S *Drosophila*, like cockroaches and locusts, can learn to alter the leg's position to avoid repeated electric shocks. The induced leg-flexion behavior is not merely an artifact of electric shock; an association between limb position and shock is essential.

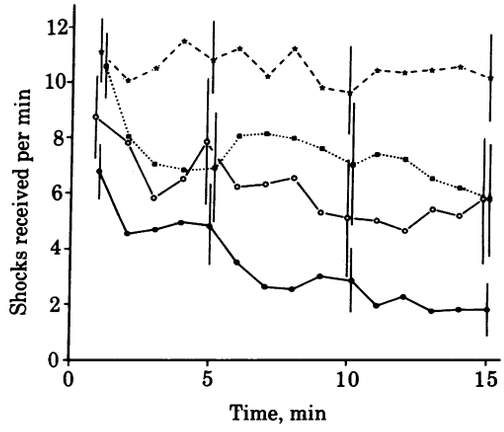

FIG. 7. Average learning performance of "headless" normal and mutant flies in leg-*lowering* test. Axes and error bars are as in Fig. 2. ●, C-S (20 flies); ○, *dunce* (10 flies); ■, *cabbage* (10 flies); ★, *turnip* (20 flies).

In the past we have trained *Drosophila* "en masse" (1, 3, 4, 8). Other groups (12–15) have tested *Drosophila* individually. All this previous work indicated that, at any given time, some flies in any population learned, while the others either failed to learn or failed to show their knowledge by "appropriate" performance. With the present paradigm, some normal flies still failed to show learning, but the proportion was low (8%). We can train nearly all of the flies all of the time, probably because the task is a relatively simple one, requiring no sensory discrimination, and because training is intensely repetitive (900 possible shocks per hr).

Our salient result is that individuals of three mutant types—*dunce*, *cabbage*, and *turnip*, all originally selected for absence of conditioned behavior in an entirely different task—tend to do poorly in leg-flexion learning as well. None of the mutant types, on average, showed improvement in shock avoidance as the test proceeded. With headless individuals, the mutants received more shocks than normal flies even during the first minute of training, so there was concern that their poor performance might have resulted from abnormalities unrelated to learning—e.g., hyperactivity, lethargy, deficient proprioception, or general weakness. Such artifactual explanations seem unlikely for two reasons. (*i*) All the mutants showed poor learning of leg extension as well as flexion. Thus they did not fail the flexion test merely because they were too weak to hold up their legs. (*ii*) Mutant and normal flies showed the same activity levels throughout the leg-flexion test (Fig. 4). The mutants did not appear abnormally excitable or sluggish.

For two of the mutants (*dunce* and *turnip*) there is good evidence that, in each case, a single genetic lesion alters learning in both olfactory and leg-lifting paradigms. Because the mutations interfere with different types of conditioning that use different sensory input and motor output channels, they probably affect the fly's nervous system. However, *dunce* flies can apparently learn in a third test, involving discrimination of visual cues (16, 17). Moreover, it should be noted that none of the mutations completely blocks learning in any test. Populations of *dunce*, *cabbage*, and *turnip* all have low, positive learning scores on the original olfactory paradigm. In the leg-flexion test, some mutant individuals appear to learn well. Evidently the mutants, like normal flies, learn better when the task is made easier.

What regions of the *Drosophila* nervous system mediate conditioned behavior? It is likely that different areas are involved in learning different tasks. Olfactory learning apparently occurs in the head. Work with larger flies and honeybees (18, 19) implicates supraesophageal brain structures (antennal lobes and mushroom bodies) in olfactory discrimination and learning. Moreover, severed heads of larger flies can be classically conditioned with odor cues (20). On the other hand, the thoracic ganglion must be sufficient for leg-position learning, because our headless flies can do it. The *dunce*, *turnip*, and *cabbage* mutations affect both types of learning. They may do this by altering some chemical or physiological process in cells distributed throughout the nervous system.

To further localize the neural areas involved in learning, we will need more sophisticated tools than hot forceps and a razor blade. One of the advantages of *Drosophila* for behavioral genetic analysis is the potential for mosaic mapping. Given a mutation that alters behavior, this technique allows one to determine the anatomical focus for the affected gene—the critical cell or group of cells whose genotype determines the behavior in question (21–23). Up till now mosaic mapping has not been applicable to learning—it requires that mutant behavior be reliably distinguishable from normal on the level of individuals (mosaics), and only about a third of the flies learned in any previous paradigm. The flies' more reliable performance in the leg-flexion test improves this outlook.

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