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Drosophila Learning: Behavior and Biochemistry

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INTRODUCTION

When an organism learns, when it assimilates new information about its surroundings and responds by altering some aspect of its behavior, what transient and permanent neuronal changes take place? Since a satisfactory answer to this question would be of fundamental importance, psychologists and biologists alike have rushed into analyses of the phenomenon.

Through the work of Pavlov, Thorndike, Skinner, and others, general ideas about learning have been catagorized; simplified procedures such

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as classical conditioning, instrumental conditioning, operant conditioning, and maze learning have been designed for testing various species to facilitate our understanding of more complex forms of learning. Use of these simpler procedures has allowed us to formalize some of the basic properties of learning and memory. Three of the most general properties are as follows. (1) Associative learning, measured as an increase in performance levels, is a result of the temporal pairing of stimulus/response with reinforcer; (2) performance levels will extinguish (wane) with time, if stimulus/response–reinforcer contingencies are eliminated; and (3) performance levels will be retained, given enough training, but retention of a recently formed stimulus/response–reinforcer association is susceptible to disruptive effects such as cold shock, electroconvulsive shock, and several forms of anesthesia. In addition to these global characteristics of learning, more subtle aspects have been described [e.g., blocking, overshadowing (see Mackintosh, 1974; Razran, 1971)].

Within the last decade, work in invertebrates has begun to reveal enticing details of biochemical and physiological events associated with simple forms of learning (see Quinn, 1983, for a review). Most notable of these is the work in *Aplysia* by Kandel and his colleagues on two forms of nonassociative learning, habituation and sensitization (Kandel *et al.*, 1981; Walters *et al.*, 1982). Habituation is a decrease in behavioral response due to the repeated presentation of an initially novel stimulus. Sensitization is an increase in responsiveness to a variety of stimuli as a result of the presentation of a strong or noxious stimulus (Walters *et al.*, 1982; Mackintosh, 1974). *Aplysia*'s defensive gill-withdrawal reflex is monosynaptic, and behavioral modification reflects changes in synaptic transmission efficacy at the synapse between sensory and motor neurons. Repeated stimulation of siphon sensory neurons causes habituation of this reflex's motor neurons (Pinsker *et al.*, 1970; Carew *et al.*, 1972) by decreasing the number of open Ca^{2+} channels in the sensory neuron's presynaptic terminal, which diminishes binding of vesicles to release sites, decreasing the probability of neurotransmitter release to the motor neuron's postsynaptic receptors (Castellucci *et al.*, 1970; Castellucci and Kandel, 1974; Klein and Kandel, 1978). Sensitization of the gill-withdrawal reflex, produced by shocking the head, increases synaptic transmission at the same synapse that is associated with habituation (Castellucci and Kandel, 1976). The sensitization is apparently mediated by neurons, stimulated by the shock, which release serotonin onto the sensory cell's (presynaptic) nerve ending. Serotonin is believed to activate an adenylate cyclase in the presynaptic terminal, which then stimulates cAMP synthesis (Kandel *et al.*, 1981; Klein and Kandel, 1978; Tomosky-Sykes, 1978; Bailey *et al.*, 1981). Inactivation of voltage-dependent K^+

channels, mediated by cAMP-dependent protein kinases, causes a decreased K^+ current that secondarily increases the influx of Ca^{2+} into the terminals, thereby facilitating transmitter release (Walters *et al.*, 1982; Klein and Kandel, 1980).

Recently, Kandel and coworkers have demonstrated associative learning in *Aplysia*, and they are proceeding to investigate the relationship between sensitization and classical aversive conditioning. The most promising results are with discriminative conditioning of the gill-withdrawal reflex (Carew, Hawkins, Abrams, and Kandel, unpublished). In this case, the US-paired input exhibits enhanced synaptic transmission, apparently because of K^+ inactivation. This result implicates the monoamine-activated cyclase system in associative conditioning, which is already known to function in nonassociative learning.

Currently, evidence consistent with Kandel's model is accumulating in *Drosophila* studies. Quinn, Byers, and others have characterized biochemical abnormalities in several single-gene mutant strains that cannot learn a shock-avoidance task as well as wild-type flies. To date, biochemical deficiencies have been identified in four of these mutants, and each deficiency involves a component of the monoamine-activated cyclase system (see below for details).

These biochemical findings are exciting. There are a myriad of molecular genetic techniques, unequaled in other higher organisms, that can be brought to bear on the problem in fruit flies. The entire *Drosophila* genome might be screened to identify every gene that affects learning. These genes might be cloned; studying the structure and function of their corresponding (wild-type) proteins might unravel the biochemical and physiological puzzles. Ultimately, the molecular biology of learning in *Drosophila* might serve as a model, the essence of which is generalizable across species, like the structure and function of DNA.

All of this admittedly grueling but potentially important work hinges on one question: Can *Drosophila* learn? Unfortunately, the majority of reports that have claimed to show learning in fruit flies (or other flies) is fraught with methodological or conceptual errors. My intent in these pages is not to review exhaustively the history of learning in flies. That job has been tackled by T. R. McGuire in this issue. Instead, I discuss some aspects of three studies by Platt *et al.* (1980), by Medioni and Vaysse (1975), and by Quinn *et al.* (1974), which do convince me that *Drosophila* can learn. The only drawback of each of these studies is that learning levels are modest at best.

The olfactory conditioned avoidance paradigm of Quinn *et al.* (1974) apparently is the most productive but also the most controversial *Drosophila* learning test. This procedure was used to isolate the learning

mutants mentioned above. Ironically, their study provides the least convincing behavioral evidence for learning among the three claims discussed below. The most notable weaknesses are that avoidance behavior of *individual* flies cannot be recorded and avoidance behavior is confounded with phototactic behavior. There are, however, more subtle aspects of this procedure that render it rapid and effective for accurately measuring, and then comparing, the average amount of associative conditioning in wild-type and mutant populations. I think it appropriate to devote some space to discuss these points further and to summarize the biochemical abnormalities associated with four mutant strains that cannot learn the olfactory avoidance task.

One final introductory remark should be made. After spending several days practicing the conditioning procedure of Quinn *et al.* (1974) as a new postdoctoral fellow in Quinn's laboratory, I was able to reproduce the learning levels reported in the original study. There were methodological shortcomings, but I was convinced, nonetheless, that *Drosophila* did learn to avoid olfactory cues and that they could learn even better, if their task was made simple enough. Near the end of this article, I describe my own olfactory conditioned avoidance procedure, which eliminates a number of confounding variables. The resulting learning levels are surprisingly high; nearly 95% of conditioned flies make a correct response.

Drosophila CAN LEARN

Drosophila Can Learn to Discriminate Tactile Cues

The study by Platt *et al.* (1980) included a 30-unit sequential T-choice-point "maze" to demonstrate learning of an instrumental response. Following a correct response to discriminative stimuli (presence or absence of paper in their Experiment 2) that were presented simultaneously at a horizontal choice point, individual flies from a strain bred for high negative geotaxis were "rewarded" with the opportunity to ascend a vertical alley, leading to another choice point. By consistently rewarding one substrate texture, the sequence of left and right turns could be randomized, allowing the experimenters to exclude turn biases and "correcting" behaviors as possible explanations for the flies' improved performances (cf. Hay, 1975; Bicker and Spatz, 1976). Essentially, substrate texture was the only consistent cue in the experimental (learning) group. Platt *et al.* (in their Experiment 1) were able to disregard odor trails from previous flies (Pluthero and Threlkeld, 1979) as a factor by reversing the choice-point arms after each individual fly's training session, so that the previously incorrect substrate cue was correct for the next fly. Under these conditions, no effect on choice behavior due to odor trails was detected.

This study included two important controls—randomization and reversal. Flies in the randomization group received the same amount of exposure to both discriminative cues and reinforcement as flies in the experimental group, but pairing of a texture cue with opportunity to ascend a vertical tube was randomized throughout the maze. Thus, the only difference between this group and the experimental group was that a texture cue was not temporally associated with reinforcement in the former. In the reversal group, substrate cues and reinforcement were paired, but the correct cue was switched during the second half of training trials. Flies in this group had to "forget" what they first learned and then relearn the new substrate cue–reinforcement contingency.

Both the experimental and the randomization groups showed an increase over trials in the number of correct responses, but a consistently higher average performance level was obtained by the experimental group (67.5 vs. 49% total correct responses). A rough estimate of the average amount of associative learning can be made by subtracting the average performance level of the randomization group, which reflects the combined effects of nonassociative factors, from that of the experimental group. By this method, an average of about 18.5% of correct responses in the experimental group can be attributed to associative learning. Results from the reversal control group provided evidence that performance in the maze was due neither to habituation nor to odor trails. "The dramatic increase in 'incorrect' responses in . . . [the reversal group] clearly demonstrates that the subjects were using substrate texture to aid in finding the vertical alley" (p. 309).

Furthermore, Platt *et al.* may have isolated the variable that distinguishes their reproducible results from the unsuccessful replications of past studies (Yeatman & Hirsch, 1971; also see McGuire, this issue). A significant difference between their procedure and those of Murphey (1967, 1973), who first used negative geotaxis as a reinforcer in a T maze, and Yeatman and Hirsch (1971) was the way that flies were handled between trials. Unlike the 30-unit multiple T maze used by Platt *et al.*, Murphey and Yeatman and Hirsch used a single T maze, transferring flies back to the start tube after every trial. Platt *et al.* showed that improvement in performance over trials could be eliminated by disrupting a fly's progress through a 14-unit maze after every two choice points. This was accomplished by aspirating the fly out of the previous unit's vertical alley and into the "start tube" of the next pair of choice points.

Although the instrumental discrimination learning experiment of Platt *et al.* has yet to be replicated by an independent laboratory, it is encouraging that they have produced similar effects (1) using a new maze that differed appreciably from the original maze in its construction and

in the speed with which flies ran through it, (2) using different texture cues, and (3) in a different laboratory (that of Hirsch) under different environmental conditions of light, humidity, and temperature.

Drosophila Can Learn to Suppress Their Tarsal Reflex

Medioni and Vaysse (1975a,b) tethered individual, hungry flies to stationary insect needles, allowing them to "walk" around the surface of a rotating kymograph drum. At specified intervals, the flies would walk across a piece of filter paper soaked in sucrose solution, the concentration of which was adjusted to 10 times each fly's initial threshold for proboscis extension. During training, if a fly extended its proboscis in response to sucrose stimulation of the front tarsi (defined by the authors as the tarsal reflex), it received a 0.5-mA shock (DC) from an electrifiable grid located beneath the mid and hind legs. Each fly was given 30 trials at 60 s intervals. The grid was washed every other trial to minimize odor cues, and the filter paper was remoistened with sucrose.

Learning in this procedure involves suppression of a response, which might also result from fatigue or from habituation to repeated sucrose presentation. To rule out these alternative explanations, a second control group was trained as above but with sucrose alone, without shock. Finally, Medioni and Vaysse also included an explicitly unpaired control group in their experiment, which they called a pseudoconditioning control. These flies were yoked to their conditioned counterparts, so that they received a similar shock (US) experience 12 s after walking across sucrose filter paper.

All three groups showed some increase in suppression of tarsal reflex responses as trials progressed. However, the mean percentages of total correct responses in the habituation and explicitly unpaired control groups (about 8 and 15%, respectively) were less than that of the conditioned group (36%). Medioni and Vaysse estimated the average amount of associative conditioning by subtracting group performance levels in a manner similar to that discussed above for Platt *et al.*, and they concluded that ". . . nearly two-thirds of the suppressed tarsal responses [in the conditioned group] resulted from the formation of an associative link . . ." (1975b, p. 5). However, this level of learning represents associative responses only on about 21% of the trials in a test. The authors also showed that conditioned suppression of the tarsal reflex could be extinguished by repeated presentation of sucrose alone.

Conditioned suppression of the tarsal reflex also was accomplished by Medioni *et al.* (1978), using quinine as the US instead of shock. More recently, Dejianne *et al.* (1984) have replicated these results and have

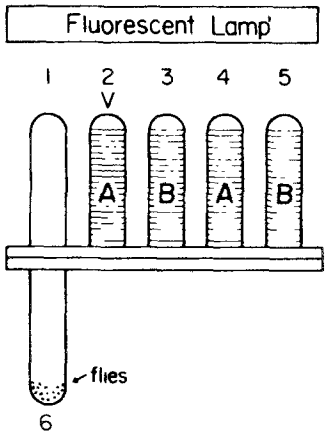

Fig. 1. The apparatus used by Quinn *et al.* consisted of a "start" tube that could slide past, or into register with, a row of five tubes similar to the start tube (polystyrene test tubes, 17 × 100 mm). Tube 1 was a "rest" tube with holes at the end. Tubes 2–5 contained electrifiable grids coated with odorants [1% (v/v) in ether]. Tubes 2 and 4 were coated with OCT, while tubes 3 and 5 were coated with MCH. During training, tubes 2 and 3 were used with shock applied to tube 2. Tubes 4 and 5 were used for the test trial in order to remove flies from any odor cues they may have left on the grids during training.

shown a stronger effect in fewer trials by presenting quinine after sucrose each trial, regardless of a proboscis extension response to sucrose. This classical conditioning procedure may represent a form of Pavlovian counterconditioning.

Drosophila Can Learn to Avoid Previously Shocked Odors

Unlike the previous two studies, Quinn *et al.* (1974) did not set out to condition flies individually. Instead, they ". . . sought to devise a paradigm suitable for mutant isolation, in which flies can be trained and tested *en masse*" (p. 708). The basic procedure involved exposing a group of about 40 flies alternately to two chemical odorants [usually 3-octanol (OCT) and 4-methylcyclohexanol (MCH)], the first of which was paired with a 90-V (AC) electric shock. Odor concentrations were chosen so that naive flies avoided each odor equally. After three training cycles, the flies were tested by exposing them sequentially to the two odors without shock.

To begin conditioning, flies were introduced into the start tube (see Fig. 1), holding the apparatus vertically and shaking the flies to the bottom. The start tube was shifted into register with the proper grid tube, and then the apparatus was laid horizontally in front of a fluorescent lamp. The flies ran from the start tube into the grid tube, induced by their positive phototactic responses. For each training cycle, flies were exposed sequentially to the rest tube (60 s), tube 2 (15 s), the rest tube (60 s), and tube 3 (15 s). During the test cycle, which followed training by 60 s, flies were tested in the same sequence but tubes 4 and 5 were used, instead

of 2 and 3, in order to remove any pheromone cues deposited by flies during training. The numbers of flies avoiding the grids were counted visually. So, to eliminate experimenter bias, the sequence of odors during testing was the reverse of that during training in about half of the experiments, and the odor in each tube was not known by the experimenter.

During training, flies that avoided the shock-associated odor were exposed less to it than to the control odor, possibly producing more habituation to the latter. To test for this effect, flies in some experiments were presented with a novel odor during the test cycle instead of the control odor. No differential habituation was indicated, however, because avoidance of the control odor was no greater than avoidance of a novel odor (naive flies were equally averse to both odors).

A learning index was calculated as the fraction of flies avoiding the shocked odor (CS+) minus the fraction avoiding the unshocked (control) odor (CS−) during the test cycle. As a control for odor bias, the above procedure was repeated with another group of flies, but the previously shocked odor became the control odor, and vice versa. The learning index for a complete experiment (Λ), defined as the average of the two reciprocally trained groups, is 0.0, if flies do not learn and avoid each odor equally, or 1.0, if flies learn and all avoid the shocked odor but none avoids the control odor. The authors stated that such an ". . . experimental design rules out pseudoconditioning as an explanation for the results, since the second part of the experiment serves as a control for the first . . ." (p. 710).

Trained and tested this way, flies produced a mean learning index of 0.34 ± 0.02. The conditioned avoidance behavior could be extinguished when trained flies were repeatedly exposed to odor cues without shock. This decreased avoidance was not due to weaker odor concentrations, because the flies could be retrained using the same grids. Moreover, it did not result from decreased "alertness," because shocking flies in the absence of odors did not restore avoidance behavior. The authors also showed that, following extinction, the same group of flies could be retrained to avoid the original control odor (reversal training). In addition, when conditioned avoidance was not extinguished, flies would retain such behavior for at least an hour. If the usual training procedure was repeated four times at 2-h intervals, conditioned avoidance was present 24 h later ($\Lambda = 0.12 \pm 0.02$).

Since flies were tested in groups, another experiment was conducted to ". . . test whether the selective avoidance is a property of individuals or a collective 'stampede' effect" (p. 710). Separate groups of flies, one *yellow* and the other wild-type, were conditioned to avoid different odors (either OCT or MCH). But before the test cycle, the two groups were

mixed. The two learning indices that resulted from testing the mixed groups and scoring each phenotype separately were about 32% lower than those obtained by testing each phenotype separately. Thus, a moderate stampede effect was detected. "Nevertheless," Quinn *et al.* concluded, "the fact that the two types will separate indicates that the information for the proper choice resides in the individual flies" (p. 711).

One further experiment was done to decide if individuals in the wild-type (Canton-S) strain were heterogeneous for learning ability or if learning was ". . . due to a stochastic component in the behavior of all flies" (p. 711). The authors reasoned that if an "intelligent subset" of individuals existed in the population, it would be included in the subgroup avoiding the shock-associated odor during the original test cycle. Then if original avoiders and nonavoiders were tested again separately, the former would produce a higher learning index than the latter. When this hypothesis was tested by training flies, separating avoiders from nonavoiders, and then retraining and retesting each group 24 h later, the authors found no difference between performances of the subgroups [$\Lambda_{(\text{avoiders})} = 0.31 \pm 0.02$; $\Lambda_{(\text{nonavoiders})} = 0.34 \pm 0.05$]. Thus, they concluded ". . . the expression of learning is probabilistic in every fly. There is no evidence for an 'intelligent' subset of the population" (p. 711).

The olfactory conditioned avoidance experiment of Quinn *et al.* has been replicated by two independent laboratories. Hewitt *et al.* (1983) used the conditioned avoidance phenotype for a biometrical analysis and in a bidirectional selection experiment, and Savvateeva and Kamyshev (1981) conditioned wild-type (Canton-S) flies and temperature-sensitive mutants that were isolated for their effects on cyclic AMP metabolism. Hewitt *et al.* summarized their replication of the procedure by saying, ". . . the highly reliable [replicable] overall learning index, the appropriate pattern of extinction over trials, the absence of large interfering effects, and the demonstration of some degree of genetic control . . . support the original claims of Quinn *et al.* (1974)."

What Constitutes a Valid Claim for Learning?

Having delineated experimental details of the three learning claims above, we now can examine what makes them so convincing. Each of these studies has met two essential criteria: (1) at least a portion of performance levels resulted from the temporal pairing of stimulus/response with reinforcer; and (2) other properties characteristic of learning, such as acquisition, were demonstrated. Unlike Platt *et al.* and Medioni and Vaysse, an acquisition curve was not shown by Quinn *et al.*, although such data were recorded and acquisition was observed (W. G. Quinn,

personal communication). A similar acquisition curve for wild-type (Canton-S) flies was included by Dudai (1977). In addition to observing acquisition curves, Platt *et al.* and Quinn *et al.* further characterized the flies' discriminative behavior by showing reversal learning, which doubled as powerful proof that flies were attending to substrate cues and to olfactory cues, respectively. Medioni and Vaysse and Quinn *et al.* demonstrated extinction of their conditioned behaviors. Quinn *et al.* also were able to show retention of conditioned avoidance up to 24 h after training.

One of the most confounding problems in the history of attempts to demonstrate learning in flies is the occurrence of rather strong nonassociative effects such as sensitization or pseudoconditioning, defined here as increases in responsiveness over trials resulting from repeated exposure to the stimulus, or to the reinforcer, respectively (Nelson, 1971; Murphey, 1973; Tully and Hirsch, 1983). Accordingly, inclusion of randomization and explicitly unpaired control groups in the studies by Platt *et al.* and Medioni and Vaysse, respectively, is a significant advance in experimental design. Performance levels in each control group reflect the combined effects of sensitization, pseudoconditioning, habituation, etc. In both studies, the fact that performance levels were higher in the experimental groups, where stimulus/response and reinforcer were paired every trial, then performance levels in the control groups is primary evidence for the formation of associative responses in some individuals. It is worth noting, however, that neither of these procedures may be perfect controls. Some evidence exists that the chance pairing of stimulus/response and reinforcer in a randomization control may produce some associative responses, especially when stimulus/response–reinforcer pairings occur early in training (Benedict and Ayres, 1972). On the other hand, conditioned inhibition may occur during an explicitly unpaired control procedure, producing lower-than-expected performance levels (Rescorla, 1967).

No attempt was made by Quinn *et al.* (1974) to eliminate, or to assess the magnitude of, nonassociative effects on conditioned avoidance behavior. However, their experimental design allows the calculation of an *associative* learning index for a population, which is unbiased by nonassociative factors. During a conditioned discrimination procedure, the unshocked odor serves as an explicitly unpaired control (Rescorla, 1967), and avoidance of it reflects the combined and interactive effects of sensitization, pseudoconditioning, and native odor preference. These nonassociative effects are eliminated from the overall learning index arithmetically, by subtracting the fraction of flies avoiding the control odor (CS −) from the fraction of flies avoiding the shock-associated odor (CS +)

to obtain a learning index for each reciprocal experiment and, then, by averaging these two learning indices.

This computation is slightly more confusing than those subtraction methods discussed for Platt *et al.* or by Medioni and Vaysse, but the three methods are analogous. Each subtracts the average response level of a nonassociative control group from the average response level of a conditioned group; the difference reflects the average proportion of responses due only to associative effects. In no case can individuals showing a preponderance of associative responses be identified. The arithmetic used by Quinn *et al.* is as follows.

> Let $CS+_o$ and $CS+_m$ denote the fraction of flies avoiding OCT and MCH when each was previously paired with shock. Similarly, let $CS-_o$ and $CS-_m$ denote the fraction of flies avoiding OCT and MCH when each was not previously paired with shock. Then, the learning index from one experiment is
>
> $$\lambda_o = CS+_o - CS-_m$$
>
> and the learning index from the reciprocal experiment is
>
> $$\lambda_m = CS+_m - CS-_o.$$
>
> Finally, the overall learning index is
>
> $$\Lambda = \frac{\lambda_o + \lambda_m}{2} = \frac{(CS+_o - CS-_m) + (CS+_m - CS-_o)}{2},$$
>
> which can be rearranged to
>
> $$\Lambda = \frac{(CS+_o - CS-_o) + (CS+_m - CS-_m)}{2}$$
>
> or
>
> $$\Lambda = \frac{(CS+_o + CS+_m) - (CS-_o + CS-_m)}{2}.$$

Since the only difference between CS− and CS+, whether they are OCT or MCH, is the temporal association of CS+ with the US, any overall learning index significantly greater than zero is the result of associative conditioning.

A "gedanken" experiment might clarify this point. Suppose that 20% of a group of naive flies avoid OCT and 20% avoid MCH, and suppose that pseudoconditioning (but not associative conditioning) exists after presenting shock alone to a group of flies in the training cycle of Quinn *et al.*—rest (60 s), shock (15 s), rest (60 s), and no shock (15 s). Then, the behavioral effect of pseudoconditioning can be measured during a test cycle—rest (60 s), OCT (15 s), rest (60 s), and MCH (15 s). One of two outcomes is possible. Either (1) an equal number of flies will avoid both odors, say 40%, or (2) more flies will avoid one odor than the other, say 60% avoid OCT and 40% avoid MCH. A learning index can be calculated

for this experiment by arbitrarily designating OCT as CS+ (the "shock-associated" odor) and MCH as CS− (the "control" odor) and then subtracting the fraction of flies avoiding MCH from the fraction of flies avoiding OCT. In 1, the learning index is 0.0, and in 2, it is 0.2. Next, the reciprocal experiment is done by training and testing another group of flies as above, but now MCH is designated CS+ and OCT is CS−. Pseudoconditioning should have the same effect on behavior in this reciprocal experiment, so the learning index for 1 is 0.0 and that for 2 is −0.2. Finally, when the two experiments are averaged, as in a complete conditioning experiment, the overall learning index is 0.0 for 1 and also 0.0 for 2. In either case, the effect of pseudoconditioning does not contribute to the magnitude of the overall learning index, even though the nonassociative effect exists.

We can construct similar gedanken experiments to test the effect of sensitization or the combined effects of sensitization, pseudoconditioning, and habituation, etc. In these cases, too, the magnitude of the overall learning index is unaffected by the presence of the nonassociative factors. In addition, any other factor that results in an odor bias will not influence the overall learning index. The difference between CS+ and CS−, averaged over reciprocal experiments, is a measure free of nonassociative effects and odor biases. I must emphasize, though, that nonassociative effects on conditioned avoidance behavior in individuals may, in fact, exist. Such effects just do not bias the overall learning index of the tested population.

Learning in groups of flies tested *en masse* has been described as ". . . a property of populations not of individuals" (McGuire and Hirsch, 1977; Menne and Spatz, 1977), because individual flies cannot be scored. In the Quinn *et al.* procedure, indistinguishable individual flies are exposed, as a group, to a shock-associated odor (CS+) and then, independently, to a control odor (CS−). It is not known whether a given fly avoided both odors, neither odor, or one but not the other. Furthermore, since flies are tested only once for their avoidances of CS+ and CS−, individual probabilities to avoid each odor (number of avoidance responses in N repeated test trials) cannot be estimated.

In spite of these procedural limitations, several observations indicate that *individual* flies learn to associate odor with shock in the Quinn *et al.* procedure. (1) Individual flies smell the odors. Individual flies receive shock. And since more flies avoid an odor previously paired with shock than an odor that has not been paired with shock (naive groups of flies avoid each equally), at least some flies must have learned, to some degree, the temporal contingency between odor and shock. This deduction is a fact rooted in statistical theory; it is not an article of faith. (2) Results

from the Quinn *et al.* experiment on "stampede" effect suggest that flies act independently during the test trial. (3) Byers (1980) confirmed this by training and testing individual flies using a procedure similar to that of Quinn *et al.* The learning index ($\Lambda = 0.33 \pm 0.04$) that resulted from combining these individual scores did not differ from the learning index ($\Lambda = 0.37 \pm 0.02$) that resulted from groups of flies tested *en masse*.

Although Byers (1980) showed that individually trained wild-type flies, on average, could be conditioned, he was not able to detect reliably any individual differences (IDs). Individual learning indices (based on 20-trial tests) ranged from 0.00 to 0.75. Byers suggested ". . . that reliable scoring of 95% of the flies as normal [learner] or dunce [nonlearner] would require about 200 test trials of each. Since no fly has been tested more than 40 times, it is not possible to determine directly from these data whether the observed individual variability is due to the small number of trials or to true individual differences" (p. 21). Byers' conclusion agrees with that of Quinn *et al.*, based on results from their test–retest experiment (see above).

At first glance, the occurrence of minimal or no IDs for conditioned avoidance in a wild-type population may seem counterintuitive, since the Canton-S strain most likely is genotypically heterogeneous (see McGuire and Hirsch, 1977). However, there is evidence from a study on rat maze learning that IDs in conditioned behavior attenuate during acquisition as performance levels reach an asymptote. Tryon (1931) observed that IDs diminished as training progressed. Individuals differed mainly in the rate at which they learned to navigate the maze. However, most were capable of similar performance levels given enough practice. Such may be the case for shock-avoidance conditioning in the wild-type, Canton-S strain. In more general terms, even if genotypic heterogeneity is known to exist in a population, it is still an empirical question whether phenotypic heterogeneity exists for any behavioral measure.

Surprisingly, Hirsch (1979) claims that Byers ". . . presents *no* convincing evidence of individual learning, because Byers has explicitly reported 'it is not possible to determine directly from these data . . . true individual differences' (p. 21). Byers remained unconvinced that his own evidence had demonstrated individual learning!" McGuire and Hirsch (1977) similarly question the Quinn *et al.* learning claim, partly because Quinn *et al.* were unable to detect an "intelligent subset" (individual differences) in their test–retest experiment.

Whether individuals can learn and whether individual differences (IDs) in learning exist are two unrelated questions. Perhaps two conceptual examples may clarify this. Suppose we ask a group of college professors, "What equals 2 + 2?" Most likely, all of them will say, "4." If

so, no IDs will exist. Can we then conclude that this "behavior" is not learned? More specifically, suppose we shock groups of, or individual, flies in the presence of one odor but not another (both odors are equally aversive to naive flies). Then we give the flies one or more choices between the two odors, neither of which is shock associated in the test trial, and every fly avoids the previously shocked odor every trial. Again, no IDs in choice behavior will exist. Can we conclude that this conditioned behavior is not learned? No. The two criteria for learning above are necessary and sufficient.

This is not to say that reliably measured IDs are undesirable or unimportant. A wide variety of experiments from mosaic analysis to bidirectional selection can be used to analyze further a learning phenotype, if, and only if, IDs can be detected reliably. To this end, Zawistowski (1983), in Hirsch's laboratory, has developed a conditioned discrimination procedure for blow flies, which permits the reliable measurement of IDS in associative conditioning.

There is a historical precedent for Hirsch's interpretation of the Quinn *et al.* test–retest results. McGuire and Hirsch (1977) quote Yeatman and Hirsch (1971), who were discussing an analysis of preimaginal conditioning in *Drosophila* (see McGuire, this issue, for background on preimaginal conditioning). "Manning (1967) has made the valuable suggestion . . . (which is) the test necessary to distinguish between the alternative interpretations of habituation and conditioning. Such a test would involve running for a second trial those flies choosing the odour on the first trial. A conditioning interpretation predicts that those flies initially choosing an odour would choose it again on the second trial. If only habituation . . . were involved, the choice on the second trial might be random" (p. 5193). In Manning's study, rearing *Drosophila* larvae on medium containing geraniol changed adult flies' preference: when groups of flies were given a choice between geraniol and air, 12.1% of adults preferred geraniol when reared on normal medium, while 46.7% of adults preferred geraniol when reared on geraniol medium. Manning's test–retest experiment showed that retesting geraniol-reared flies that chose correctly on the first trial still yielded a 50–50 distribution between geraniol and air. He concluded that habituation could not be excluded as a possible explanation for preimaginal conditioning.

Such a conclusion is not generalizable to the Quinn *et al.* test–retest experiment. In Manning's study, it is only because the alleged preimaginal conditioning changed choice behavior to essentially a 50–50 distribution that the habituation hypothesis was tested at all. If conditioning had produced an 80% preference for geraniol, for instance, habituation would not have been a plausible alternative explanation (and a 50–50 distribution

would not be expected in the retest). This precisely is the case in the Quinn *et al.* experiment. Shocking flies in the presence of one odor but not another changed the proportion of flies avoiding the two odors from equal avoidance to about 65–35, yielding a learning index of 0.33 ± 0.02. Habituation cannot account for these results. Equal habituation to both odors would yield a learning index of 0.00, and Quinn *et al.* did not detect differential habituation (see above).

There also are procedural differences between the Manning and the Quinn *et al.* test–retest experiments that make direct comparison of their results inappropriate. Manning retested flies soon after the first test. In contrast, Quinn *et al.* waited 24 h before retesting, which was beyond the retention period. To further eliminate residual memory effects before retesting, half of the flies were *retrained* to avoid the original shocked odor, and the other half were *retrained* to avoid the original control odor.

SINGLE-GENE MUTANTS

Single-Gene Learning Mutants Have Been Isolated

Once they were convinced that flies tested *en masse* could learn to associate olfactory cues with shock, Quinn, Byers, and others set out to isolate X-linked, single-gene mutations that disrupt normal learning. Individual male flies from a wild-type strain (Canton-S) were chemically mutagenized to produce an average of one mutation per chromosome (Lewis and Bacher, 1968). Their progeny then were mated in specific genetic crosses with special "balancer" strains to raise many populations, each derived from a single male, in which the X-chromosomes were identical within and among individual flies. Finally, a group of flies from each population was trained and tested in the conditioning procedure of Quinn *et al.* If the learning index of any population was less than 0.05, as compared to 0.33 for the parental, wild-type strain, the potential mutant strain was retained for further behavioral analysis. Learning mutants were those low-scoring strains that did not display serious abnormalities in other behaviors such as phototaxis, olfaction, locomotion, general activity, and reaction to shock.

The first such mutant strain, isolated by D. Byers at Caltech, was *dunce* (Dudai *et al.*, 1976). Since then, five other alleles of the *dunce* gene have been found (see Kauvar, 1982). At Princeton, P. Sziber isolated three additional mutant strains deficient in learning, *rutabaga, cabbage*, and *turnip* (see Aceves-Pina and Quinn, 1979; Quinn *et al.*, 1979), and a memory mutant, *amnesiac*, that learned normally but forgot four times faster than wild type (Quinn *et al.*, 1979). The mutant loci associated with *dunce, rutabaga*, and *amnesiac* have been mapped on the X chromosome,

while localization of the X-linked *turnip* and *cabbage* mutations is currently in progress. Mapping a mutant locus also greatly reduces the chance that the observed difference in the phenotypes of wild-type and mutant strains is due to the effects of two or more genes. Finally, the *Ddc* (dopa decarboxylase deficient) mutation, isolated by Wright (1977) in a screen for mutations affecting cuticle hardening in newly eclosed flies and located on the second chromosome, was found by Livingstone and Tempel (1983) to be a fifth single-gene mutation disrupting learning.

Flies from each of these mutant strains could sense electric shock and the odors used to train them. They also displayed normal phototaxis, but *cabbage* and *turnip* locomoted more slowly than wild-type flies. Subsequently, *turnip* flies were found to have abnormal morphology of nerves and muscles in the head and in the larval nervous system (see Hall, 1982). Thus, for this mutant, deficient learning may be a secondary consequence of the mutation(s). Until *turnip* is mapped, we do not know if only a single gene is involved. It also should be mentioned that learning levels of the four X-linked mutant strains tend to drift toward wild-type levels over generations, when these mutations are kept homozygous. Outcrossing a "drifted" mutant strain to the wild-type strain, which randomizes the genetic background, can lead to a return of the mutant phenotype. Apparently, selection pressure acts against less fit mutant phenotypes, causing an accumulation of modifiers in the genetic background. Since most of these mutant alleles are recessive (*turnip* is not entirely so), maintaining these genes in a heterozygous condition with a balancer chromosome prevents, or at least slows, the buildup of modifiers in the mutant strains.

These learning mutants perform poorly on a variety of tasks, many of which are variations of the conditioned discrimination procedure of Quinn *et al.* (1974). Wild-type *Drosophila* larvae avoid olfactory cues previously associated with shock ($\Lambda = 0.26$) nearly as well as adult flies. Moreover, larvae of the learning mutants failed to show avoidance behavior (Aceves-Pina and Quinn 1979). Tempel *et al.* (1983) also trained flies to discriminate between odors but substituted sucrose reward for shock punishment. Hungry flies that were originally averse to the odors would migrate *toward* an odor previously paired with sucrose. The average learning index after training with sucrose ($\Lambda = 0.36$) was similar to that from the shock avoidance procedure ($\Lambda = 0.34$). Retention of the sucrose-approach task, on the other hand, lasted for days, compared to hours in the shock-avoidance task. Surprisingly, *dunce* and *rutabaga* mutants acquired sucrose-approach behavior fairly well ($\Lambda = 0.30$ for *dunce* and $\Lambda = 0.16$ for *rutabaga*). However, this avoidance behavior disappeared in *dunce* flies within an hour, and even more quickly in *rutabaga* flies. These results suggest that *dunce* and *rutabaga*, in fact,

may be memory mutants like *amnesiac*. In the shock avoidance task, their memories may be so labile that they are virtually undetectable (also see Dudai 1979, 1981, 1983). In contrast to *amnesiac, dunce*, and *rutabaga, Ddc* mutants could not acquire approach behavior in the sucrose-reward task. More is said about *Ddc* flies below.

Menne and Spatz (1977) developed a procedure that trained flies to discriminate between different colored lights when one was paired with severe mechanical shaking. Their conditioning index, Δ, is similar to the Quinn *et al.* learning index, Λ. Folkers (1982) has tested the Canton-S wild-type strain and *dunce, rutabaga, turnip*, and *amnesiac* flies with the visual discrimination procedure (also see Dudai and Bicker 1978). She found that wild-type flies were capable of an average conditioning index (Δ) of 0.28 only after 24 training cycles, as opposed to a learning index of 0.33 after three cycles in the Quinn *et al.* experiment. *Dunce* (Δ = 0.16), *rutabaga* (Δ = 0.14), *turnip* (Δ = 0.18), and *amnesiac* (Δ = 0.16) flies each displayed some avoidance behavior after 24 training cycles. Interestingly, all of the mutants retained as much avoidance behavior as wild-type flies after a 2-h interval (Δ = 0.10). *Amnesiac* flies neither showed normal levels of avoidance behavior nor retained less than wild-type flies. These results from the visual discrimination procedure are most dissonant with other tasks.

The cockroach leg-position learning procedure of Horridge (1962) has been adapted to *Drosophila* (Booker and Quinn 1981). The conditioned behavior was associative, and individual flies could be trained either to extend their legs or to flex their legs, by shocking them appropriately. Wild-type flies, particularly headless ones, performed either task well. About 92% performed to a criterion of keeping their legs flexed (or extended) over 90% of the time during a 10-min period. The learning mutants did not perform as well. Only 20% of *turnip* flies, 25% of *dunce* flies, and 45% of *cabbage* flies met the criterion. Note, however, that some flies from each mutant strain reached criterion. Prhaps these individuals were those with more normalizing modifiers in their genetic background, or perhaps poor performers had a certain probability to reach criterion by chance (see Murphey, 1967).

The courtship-depression phenomenon of Siegel and Hall (1979) (see Siegel *et al.*, this issue) may be an instance of learning in a fitness-related behavior. Male flies exposed for a few minutes to sexually unreceptive females show markedly less courtship behavior for about 3 h afterwards, even in the presence of receptive virgin females. Behavior of the learning mutants supports the idea that courtship depression is conditioned. *dunce, cabbage, rutabaga*, and *turnip* males all showed less courtship depression than wild-type males, and the depression effect seen imme-

diately after "training" in *amnesiac* flies lasted only 15–20 min, instead of the usual 2–3 h (also see Gailey *et al.*, 1982; Hall, 1982). Recent behavioral experiments (Tompkins *et al.* 1983) suggest that this courtship-depression effect may be a form of associative conditioning.

Kyriacou and Hall (1984) also found that prior exposure to rhythmic pulse song, a component of male wing vibration during courtship (Kyriacou and Hall 1980), enhances subsequent receptivity to mating in females. This enhanced receptivity to mating was retained nearly 5 min. In contrast to normal flies, *amnesiac* females lost their enhanced receptivity within 1 min after prestimulation, and no enhancement at all was seen in *dunce* or *rutabaga* females.

In a formal sense, the enhanced female receptivity following acoustic stimulation described by Kyriacou and Hall can be considered a form of sensitization. Their observation that enhanced receptivity attenuates or is absent in the learning mutants corroborates a previous study by Duerr and Quinn (1982), which reports abnormal sensitization and habituation of the proboscis extension response in *dunce, rutabaga, amnesiac*, and *turnip* flies (also see Vargo and Hirsch 1982). This implied correlation between associative and nonassociative learning is not perfect though, because Duerr and Quinn found that sensitization is normal in *turnip*, one of the more severely learning-impaired strains. Nevertheless, the facts that three of four learning-impaired strains were deficient in sensitization and that all four did not habituate normally suggest that the two forms of learning may share mechanistic components—a conclusion also suggested from blow fly work (Tully *et al.*, 1982) and confirmed by direct evidence from *Aplysia* (Hawkins *et al.*, 1983; Walters and Byrne, 1983).

Deficiencies in the Monoamine-Activated Cyclase System Exist in Four Learning Mutants

The first mutant to be understood biochemically is *dunce*. Kiger and Golanty (1977, 1979) were interested in cyclic nucleotide metabolism and decided to map the genes coding for the relevant enzymes as a prelude to further analysis. Techniques are available in *Drosophila* that allow such mapping, even before mutants are isolated (see O'Brien and MacIntyre 1978). In this way, one of two forms of phosphodiesterase (PdE II) was localized to a well-defined genetic region on the X chromosome.

Byers *et al.* (1981) noticed that the genetic locus associated with *dunce* mutants mapped very near the published site for PdE II. Genetic complementation tests confirmed their suspicion that *dunce* and PdE II mapped to the same locus. In addition, biochemical tests on *dunce* flies

showed that they had low levels of PdE II and high levels of cAMP. Continuing this work, Kauvar (1982) and Shotwell (1983) obtained good evidence that *dunce* is the structural gene for PdE II. The *dunce*^{1} allele alters the enzyme's K_m, and *dunce*^{2} increases its (*in vitro*) thermolability. Combined, these data were the first hard evidence for the involvement of cyclic nucleotides in *Drosophila* learning.

A second learning mutant, *rutabaga*, alters another enzyme in this pathway—adenylate cyclase (Livingstone *et al.*, 1982; M. Livingstone, unpublished data; see also Aceves-Pina *et al.*, 1983, for more details). Gene dosage experiments suggest that the mutation lies in the structural gene for adenylate cyclase (Livingstone *et al.*, 1984). Whereas the *rutabaga* mutation caused a threefold increase in the thermolability of adenylate cyclase from abdominal tissue, a similar abnormality existed in only 10 to 20% of enzyme from brain tissue. The most likely explanation for *rutabaga*'s effect on learning is that it alters one form of adenylate cyclase that constitutes a minority of brain enzyme but that is critically important in some neurons and synapses involved in plasticity.

At present, the possibility cannot be strictly ruled out that the *rutabaga* mutation affects a regulator of adenylate cyclase rather than the catalytic subunit itself. There is evidence that neither the GTP-binding stimulating subunit, N_s (Ross *et al.*, 1978), nor the GTP-binding inhibitory subunit, N_i (Cooper *et al.*, 1979; Jakobs *et al.*, 1983), is affected in *rutabaga*. The most recent results so far indicate that *rutabaga*'s catalytic subunit is relatively unable to interact with calmodulin (Aceves-Pina *et al.*, 1983; Livingstone *et al.*, 1984).

In vertebrate and invertebrate brains, adenylate cyclase is most frequently coupled to receptors for monoamine transmitters (see Bloom, 1976; Nathanson, 1977). In synaptic systems where monoamines have been carefully studied, these transmitters often have a modulatory role. They act as accessory transmitters, increasing or decreasing the efficacy of synaptic release of another primary neurotransmitter (see Kupfermann, 1981). In *Aplysia*, one modulatory monoamine, serotonin, is known to function in nonassociative learning.

With this in mind, Livingstone and Tempel (1983) examined the large published library of *Drosophila* mutant stocks and found an existing temperature-sensitive mutation, *Ddc*^{ts1} [dopa decarboxylase deficient (Wright 1977)], that could not synthesize dopamine or serotonin from their precursors at restrictive temperatures (also see Livingstone, 1981; Dewhurst *et al.*, 1972). Synthesis of the third important monoamine transmitter, octopamine, was virtually normal in *Ddc* mutants, but the decarboxylation of its precursor was partially blocked by another mutation, *per*^{0}, which abolishes circadian rhythms (Konopka and Benzer 1971). They went on

to show that per^0 mutants learned as well as wild-type flies in the olfactory conditioned discrimination test, and they retained the conditioned behavior long afterwards. Temperature-sensitive Ddc^{ts1} mutants, on the other hand, learned reasonably well ($\Lambda = 0.12$) when raised at permissive temperatures but not when raised at restrictive temperature [$\Lambda = 0.00$ (Tempel *et al.*, 1984)]. Further experiments suggested the *Ddc* lesions decrease learning with no measurable effect on memory (Tempel and Quinn, 1982; Tempel *et al.*, 1983). This is in contrast to *rutabaga, amnesiac*, and *dunce* mutants, which seem to have attenuated memory spans.

In many cases, the binding affinity of neurotransmitter receptors is high enough and specific enough to be detected in biochemical filter assays using crude membrane extracts (cf. Pert and Snyder, 1973). Confirming previous reports (Dudai and Zvi, 1983, 1984), R. Smith and W. G. Quinn (unpublished data) have found specific, high-affinity serotonin receptor binding in wild-type flies. The serotonin receptor in *turnip* mutants, however, shows a dramatic decrease in the highest-affinity binding.

High-affinity ligand binding in cyclase-coupled systems is not a property of the receptor protein alone. Typically, the receptor interacts with a GTP-binding regulatory protein, N_s (Rodbell, 1980; Farfel *et al.*, 1981). Pursuing this, K. W. Choi, in Quinn's laboratory, has demonstrated a lower GTP affinity for a GTP-binding membrane protein in *turnip* flies (unpublished data). This observation suggests that the reduced high-affinity receptor binding in *turnip* may not be specific to serotonin. R. Smith has confirmed this by showing that octopamine high-affinity receptor binding also is reduced in *turnip* (unpublished data). Currently, then, the primary lesion associated with the *turnip* mutation appears to be in a GTP-binding protein, which functions as a regulatory subunit of the adenylate cyclase membrane complex. Further work is being done to corroborate these results.

The fact that all known biochemistry of the learning mutants seems to be involved in some aspect of the monoamine-activated adenylate cyclase pathway is striking. These disruptions are consistent with a plausible mechanism proposed to endow the *Aplysia* sensitization response with associative properties (see Hawkins *et al.*, 1983; Walters and Byrne, 1983). Castellucci *et al.* (1982) have suggested that the chemical change corresponding to a short-term memory may be simply an increase in cAMP concentration in relevant neurons.

A CLASSICAL CONDITIONING PROCEDURE PRODUCES STRONG OLFACTORY DISCRIMINATION

A number of observations indicate that several factors might be compromising potential learning levels in the Quinn *et al.* procedure. In an

early study, Dudai (1977) found a correlation between the learning index and a "nonphototaxis" index among several wild-type and mutant strains. This nonphototaxis index probably measured other behavioral effects such as locomotor activity, "startle-escape" response, etc., along with phototaxis, further confounding nonphototactic behavior with the learning index. Flies that did not run toward light fast enough would not reach the odor-coated shock grid. These and other data led Dudai to suspect that a learning index near 0.40 ". . . could merely reflect the limitations of the paradigms used" (p. 86).

In an effort to ". . . primarily remove phototaxis as a variable," Jellies (1981) confined a group of flies inside a chamber with 80% of its inner surfaces covered with an electrifiable grid. Pulses of airborne odors (either cyclohexanol or octanol) were blown through the training chamber, while flies were shocked in the presence of one odor but not the other. After training, flies were transferred to a separate T-maze choice chamber, in which both odors were presented simultaneously to flies located at the choice point. Airborne odors entered the ends of the arms of the maze and exited at the choice point, and flies moved upwind toward the odor they disliked the least. In this manner, Jellies obtained a learning index of 0.76 after three training cycles.

When I first tried the Quinn *et al.* shock-avoidance procedure, I was impressed and perplexed by two additional facts. First, the phototactic responses of wild-type flies, enhanced from banging the apparatus, were strong enough to "propel" virtually all of them into the grid tube of the shock-associated odor during the first training cycle. Even more striking was the flies' behavior the second time they were exposed to the shock-associated odor. At least 80% of them ran up to the edge of the grid but did not step onto it. Their "one-trial learning" was impressive. But what were the effects of the second and third training trials? Most certainly the flies smelled the odors without being shocked. Were these subsequent exposures to the shock-associated odor actually extinction trials for most flies? It was a disturbing possibility. The solution was to make sure that all flies always were shocked in the presence of the appropriate odor. In other words, a classical conditioning procedure, like that of Jellies, was indicated.

From a behavior-genetic perspective, too, the use of a classical conditioning procedure seemed like the correct approach. Quinn *et al.* (1974), as well as Platt *et al.* (1980) and Medioni and Vaysse (1975a), used an instrumental conditioning procedure. Presentation of the US was contingent on an inappropriate response to the conditioned stimulus. Such a procedure is not ideal because individuals or strains that may differ genotypically also may differ consequently in their self-induced exposure to

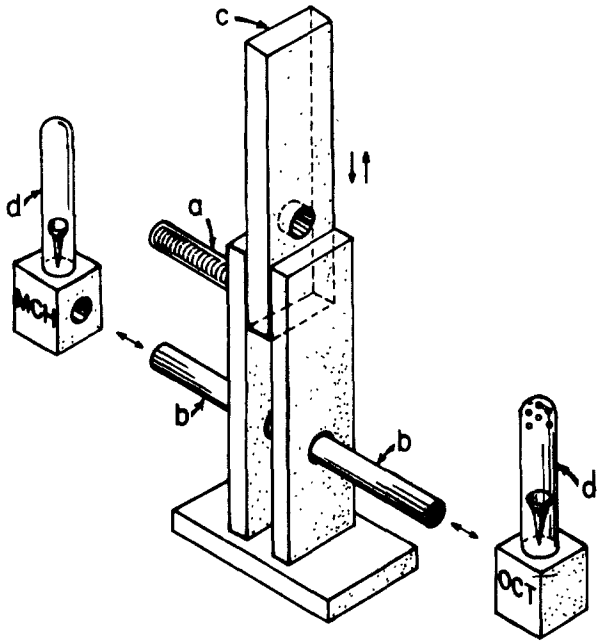

Fig. 2. The modified choice chamber apparatus consisted of a training tube (a), two simultaneous choice-point collection tubes (b), a sliding center compartment that transferred flies from the training tube to the choice point (c), and odor tubes that housed odor cups (8-mm OD for OCT and 12-mm OD for MCH) containing the odorants, mounted on micropipet tips (d). The plastic test tubes and shock grid were similar to those used by Quinn *et al.* (1974). Tubing, connected to a vacuum pump, was attached to openings in the center compartment (not shown). Air was drawn through holes in the tops of the odor tubes, into the training or collection tubes, and then out the center compartment. Flow rates were adjusted to 40 liters/h in each tube.

the US during training. It follows, then, that observed individual or strain differences may result from such "environmental" differences, from genotypic differences, or from interaction between the two. Classical conditioning procedures, on the other hand, tend to minimize environmental differences during training, as all subjects receive the same amount of exposure to both the CS and US. Thus, observed individual or strain differences can be attributed to underlying genotypic differences with more confidence.

I designed an apparatus functionally similar to Jellies' but that would not take excessive time or be too disruptive when transferring flies to the choice point. The apparatus, in which 95% of the inner surface of the training tube was electrifiable (see Fig. 2), was a modification of an odor choice chamber first used by Dudai *et al.* (1976). Pure odorant solutions

(OCT or MCH) were contained in "odor cups" (cut-off glass test tubes) glued to the tops of micropipet tips, which themselves were housed inside the odor tubes. Odor concentrations were adjusted by keeping airflow through the odor tubes constant (40 liters/h) and varying the diameters of the odor cups so that (1) 90% of naive flies avoided each odor vs. air and (2) naive flies distributed themselves 50–50 when exposed to OCT vs. MCH. The nature of shock reinforcement differed from Jellies' experiment. During presentation of the appropriate odor, 1.25-s pulses of 60-V DC shock were administered. Under such conditions, flies reacted to a shock pulse and recovered from it during the interpulse interval. Finally, flies were trained and tested under red light (15-W photographic safelight) at 22°C and 50% relative humidity. The arms of the choice point were perpendicular to the light source, allowing equal amounts of light to fall on either collection tube. The dim red light emitted a broad band of red wavelengths; dark-adapted flies seemed to orient slightly toward the light source. More importantly, flies under these light conditions moved more slowly than when under incandescent white light, appearing more attentive to odor cues.

To begin a training cycle, about 150 flies were aspirated into the training tube, and the grid was connected to a Grass S44 stimulator, set to deliver 60-V DC pulses. A blank "odor" tube was gently slipped onto the end of the training tube, providing flies with relatively odorless air for 90 s. Then the blank was replaced by a tube with odor A (either OCT or MCH), and the stimulator was switched on. Flies received 1.25-s pulses every 5 s for 60 s (a total of 12 pulses). Afterwards, the current was switched off and the odor A tube was replaced with the blank for 30 s, followed by the odor B tube (either MCH or OCT) for 60 s without shock. Finally, the odor B tube was replaced with the blank for 30 s more. When done carefully, this training procedure disturbed the flies minimally. In no circumstances were the flies shaken or jarred.

Immediately following one training cycle, flies were readied for the test trial by gently tapping them into the sliding center compartment, where they remained for 60 s while the MCH and OCT odor tubes were slipped onto the ends of the collection tubes. The test trial began by sliding the center compartment into register with the choice point. Flies were allowed to disperse into the collection tubes for 120 s. Then the sliding center compartment was pulled up, trapping flies in the collection tubes. Finally, flies in each collection tube were etherized and counted. Usually, 5 to 10 flies remained in the center compartment, and only they were counted while unetherized.

In the reciprocal half of a complete experiment, another group of naive flies was trained and tested as above, except that shock was paired

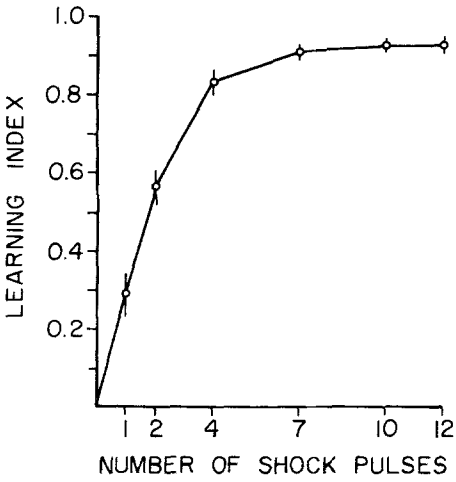

Fig. 3. Acquisition of the conditioned response is a function of the number of 1.25-s shock pulses (60 V DC). Each point on the graph represents the mean learning index ± SEM for at least eight complete experiments.

with the other odor during training. As by Quinn *et al.* (1974), a learning index (Λ) was calculated as the fraction of flies avoiding the shock-associated odor (they were in the collection tube attached to the control odor) minus the fraction of flies avoiding the control odor (they were in the other collection tube), averaged for the two halves of the experiment.

Figure 3 shows the mean learning index (± SEM) of wild-type (C-S) flies as a function of the number of shock pulses during one training cycle. Clearly, shock pulses acted as training trials. Conditioned avoidance levels were asymptotic after 10 shock pulses, producing an index of 0.91 ± 0.01, which indicates that 95% of flies avoided the shock-associated odor. Twelve pulses during a 60-s exposure to odor A was the maximum number attempted, because shorter interpulse intervals (3.75 s) seemed not to allow flies enough time to recuperate from shock. As many as five additional training cycles did not improve performance levels.

Resistance to extinction was very strong, even after one training cycle. Conditioned behavior of flies still produced a learning index of 0.44 ± 0.05 after 20 extinction cycles (one extinction cycle was a training cycle without shock). Flies also could be retrained immediately to avoid the original control odor, producing a learning index of 0.33 ± 0.02 after one retraining cycle. If flies were left undisturbed after one training cycle, they retained conditioned behavior for at least 24 h (Λ = 0.15 ± 0.03).

We have seen how the calculation of a learning index cancels the effects of nonassociative factors. Nevertheless, there is no substitute for empirical validation. Accordingly, I designed three control experiments.

(1) A sensitization control procedure exposed flies to the odors without any shock for one "training" cycle. (2) A pseudoconditioning control procedure shocked flies without exposing them to either odor. The blank odor tube was used in place of odor A and odor B tubes during the training cycle. (3) An explicitly unpaired control procedure exposed flies to both odors and to shock without temporally pairing shock with either odor. The unpaired control procedure presented odor A (60 s), blank (30 s), shock alone (60 s), blank (30 s), odor B (60 s), and blank (30 s). After all three nonassociative conditioning procedures, flies were tested immediately for their odor avoidance at the choice point in the usual manner. The average learning indices from the sensitization, pseudoconditioning, and explicitly unpaired control experiments were -0.02 ± 0.03, 0.01 ± 0.04, and 0.01 ± 0.01, respectively. No nonassociative effects of any kind biased the overall learning indices. Therefore, the learning index from the usual conditioning procedure was entirely the result of associative conditioning.

Two studies, using choice chambers similar to the one used here, have not detected "stampede" effects during the test trial, when flies were tested *en masse*. In an experiment similar in design to the one by Quinn *et al.* (1974), Jellies (1981) trained brown-eyed (*bw*) and wild-type flies to avoid different odors and then mixed these two phenotypically distinguishable groups of flies before the test trial. The odor preferences of each genotype in the mixed group were no different from those when the genotypes were tested separately. Tempel *et al.* (1983) trained small groups of flies using the sucrose-approach procedure. The flies then were tested one by one in an odor choice chamber for their odor preferences. The learning index ($\Lambda = 0.37 \pm 0.06$) resulting from flies tested individually did not differ from the learning index ($\Lambda = 0.36 \pm 0.04$) resulting from flies tested as a group. Apparently, individuals in a group behave independently during odor preference tests in these choice chambers.

Initial studies with the learning mutants show that *dunce, turnip, rutabaga*, and *amnesiac* are capable of moderate learning using the new conditioning procedure, but their memory spans are much shorter than that of wild-type flies. However, since the mutant flies' memories last for at least 3 h, memory consolidation experiments (see Quinn and Dudai, 1976; Tempel *et al.*, 1983) with the mutants are now possible. The classical conditioning procedure also is being used in mapping studies and to screen for new memory mutants.

Conditioned behavior produced by this new method is robust enough to permit investigations of additional learning characteristics. Of these, stimulus generalization (Mackintosh, 1974, pp. 484–542), overshadowing, and blocking (Kamin, 1968, 1969; Miles and Jenkins, 1973) seem partic-

ularly interesting. Such studies might uncover the components of stimuli to which flies pay attention.

Because this new conditioning procedure produces learning indices near 1.0 with small standard deviations in wild-type populations, small changes in learning ability caused by the administration of pharmacological agents may be detected. Many agonists and antagonists exist (in vertebrates) that affect specific components of the monoamine-activated cyclase system. Byers and Gustafsson (1984), for instance, have tested 41 compounds *in vitro* for their potential inhibiting effects on cAMP phosphodiesterases from *Drosophila*. Now such studies can be tried *in vivo*.

Finally, since conditioned avoidance is strong ($\Lambda = 0.91 \pm 0.01$) and since it does not extinguish rapidly ($\Lambda = 0.83 \pm 0.02$ after 10 extinction trials), the use of repeated test trials may separate reliably normal flies from mutant nonlearners, permitting a mosaic analysis to identify the foci of abnormal olfactory shock avoidance conditioning in mutant phenotypes.

CONCLUSION

Drosophila can learn. In fact, these flies can be conditioned to avoid an odor previously coupled to shock with astounding rigor. Historically, conditioned choice behavior in bees served as a model learning system in insects (see Erber, 1975). But now, fruit flies, so resistant to conditioning in the past, appear to be as "intelligent." With the genetic and molecular biological techniques available today (cf. Rubin and Spradling, 1982; Fujita *et al.*, 1982; Bender *et al.*, 1983), progress toward understanding the mechanisms of learning and memory in *Drosophila* should accelerate.

Biochemical analyses on the existing learning mutants already have begun to unravel the biological puzzles of learning. The discovery that mutations disrupting learning also affect components of the monoamine-activated adenylate cyclase system does not allow yet a grand synthesis of the molecular biology of learning. Nevertheless, the apparent success of the single-gene mutant approach has permanently altered the course of behavior genetic research using *Drosophila*.

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