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Learning and Memory in *Drosophila*, Studied with Mutants

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Ten years ago, impressed with the power of genetics applied to straight molecular biology, we set out to study learning using single-gene mutations. We were attracted to this problem in part because of its humanistic interest and in part because the similarity of learning phenomenology across species suggested some simplicity and universality for the underlying mechanism. In retrospect, our approach seems very naive, and perhaps we deserved to flounder. Nevertheless, luck has been with us. Learning-deficient mutants were isolated, and several of them turned out to have well-defined biochemical lesions. These lesions tied in with a mechanistic model formulated to explain nonassociative learning in another organism, *Aplysia*. Behavioral and biochemical analyses of the mutants together with other work indicate that monoamine-activated cAMP responses are involved in several types of learning in different species.

Ten years ago, "we" was one of the authors and Seymour Benzer, who had pioneered the systematic study of *Drosophila* behavior patterns with single-gene mutations, and who each year had offered an A+ and a case of beer to any student who convincingly trained fruit flies. Several dry years passed. At length, some undirected experiments suggested to us that flies were particularly attentive to odors and that they were distressed after walking on electrified grids. We decided to use these cues and this reinforcement and to try to condition the flies differentially to olfactory cues, because such discriminative conditioning provides internal controls ensuring that the observed effect is associative learning. The basic training and testing procedure (Quinn et al. 1974) will be outlined here, because it was used to select mutants and because more recent fly-learning experiments tend to be variations on this procedure.

Training Populations and Selecting Mutants

About 40 flies are placed in an apparatus with tubes and trained by alternate exposure to two chemical odorants (denoted A and B), one of which is coupled to 90-V electric shock. They are then subjected to a simple test (Dudai et al. 1976) in which they are transported to a chosen point between tubes containing odors A and B, and the direction in which they run is observed. Odor concentrations are arranged so that naive flies distribute themselves fifty-fifty between the two odorants. If the flies were shocked during training in the presence of odor A, they now tend to run toward odor B (35–65%). On the other hand, if they were shocked in the presence of odor B, they now run toward odor A (65–35%). The relevant results of this type of experiment can be expressed as a simple number, i.e., the fraction of the population avoiding the shock-associated odor minus the fraction avoiding the control odor (averaged for groups of flies trained in opposite directions). This numerical training index, Λ, normally can vary between 1 (perfect learning) and 0 (no learning). In the fly-learning experiment outlined above, it would be 0.30. A typical set of ten real experiments gives $\Lambda = 0.34 \pm 0.03$.

The learning effect (differential odor avoidance based on pairing with shock) is easily demonstrated and easily quantified, because working with populations of flies gives instant statistics. However, in the original paradigm the learning effect was small; the difference in odor avoidance amounted to only one third of the population. We were unable to teach all of the flies all of the time. Recently, Jellies (1981) and also we have improved the procedure to obtain much stronger learning—Λ values of 90, corresponding to 95% correct odor choice by trained flies. The major procedural improvements are (1) eliminating disturbances to the flies, such as mechanical shaking, (2) presenting odor stimuli at carefully controlled concentrations in laminar air currents, and (3) sequestering flies in a chamber for classical conditioning, so that exposure to odorants and shock is made inevitable for several training cycles. Figure 1 shows the learning obtained with our best procedure.

Given a reliable "group" learning test, selecting mutants is simple in principle, although brutally tedious in practice. Male flies from an inbred wild-type stock (C-S) are mutagenized, and their progeny are mated in appropriate genetic crosses to produce many populations, with the flies in each population having identical, mutagenized X chromosomes (Lewis and Bacher 1968; Dudai et al. 1976). Most mutations do not seriously alter learning behavior, but if a relevant one is present, it will affect all of the flies of a given population. One selects learning mutants by training each population in the olfactory learning procedure described above and retaining those that show little learning, i.e., that give

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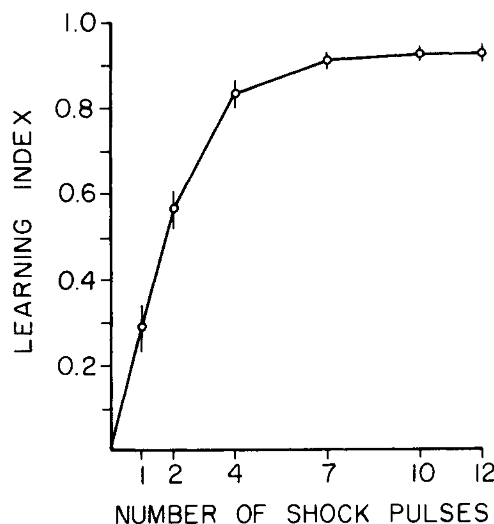

Figure 1. Learning acquisition curve for wild-type *Drosophila* in a new, classical conditioning paradigm. Populations of about 150 flies are trained by placing them in a cylindrical chamber lined with conductive grid material and blowing pulses of two odorants past them in air currents with or without concomitant electric shock. The odors are administered by placing little cups of pure odorant in the air current (500 ml/min) before the training chamber and letting the odors enter the air current by evaporation (evaporative surface = 50 mm^2 for 3-octanol and 80 mm^2 for 4-methylcyclohexanol). A training sequence consists of 60-sec exposure to 3-octanol plus shock, 30-sec rest, 60-sec exposure to 4-methylcyclohexanol without shock, and 30-sec rest. During the octanol exposure, the indicated number of electric shock pulses (each 1.25 sec, 60 V DC) are administered with a Grass stimulator. For testing, the flies are tapped into a sliding compartment and transferred to the center of a T-maze choice point (modified from Dudai et al. 1976). Here, flies encounter two air currents coming from opposite arms of the T maze, one with the shock-associated odorant 3-octanol, the other with the control odorant 4-methylcyclohexanol (concentrations and flow rates as above). Typically, 95% of the flies run to the control odorant. After 120 sec, the flies are trapped in the arms they have chosen, anesthetized, and counted. The training and testing procedure is repeated with new flies as above but with shock paired to 4-methylcyclohexanol, and a learning index is calculated as in the text. Each point and error bar represents mean ± S.E.M. for four experiments. Control experiments, in which flies were trained with shock alone, odor alone, or shock and odor explicitly unpaired in time, all gave learning indices of zero ± 0.02 (from T.P. Tully and W.G. Quinn, in prep.).

Λ values less than 0.05. Any such suspect population is analyzed in further behavioral experiments to be sure that the behavioral alteration in the mutants is genuinely interesting, not simply a defect in olfaction, locomotion, or general activity.

The first such mutant, isolated by D. Byers at Caltech, was *dunce* (Dudai et al. 1976). Five other mutations in the *dunce* gene have since been found. Three more mutations, *cabbage, turnip,* and *rutabaga*, all affecting different genes, were isolated at Princeton by P. Sziber (see Aceves-Piña and Quinn 1979; Quinn et al. 1979). A fourth mutant, *amnesiac*, isolated by Sziber, learned normally but forgot within 1 hour (Quinn et al. 1979), compared with 4–6 hours for normal flies (Dudai 1979). All of these mutants could sense electric shock and the odorants used to train them. All showed the normal tendency to migrate toward light, although *cabbage* and *turnip* ran more slowly than wild-type flies. Because of the breeding techniques used to isolate them, all the mutations were X-linked; *dunce, rutabaga,* and *amnesiac* have since been carefully mapped (see Duerr and Quinn 1982). *dunce, turnip, cabbage,* and *rutabaga* showed virtually no learning (Λ = 0.00–0.05) on the olfactory discrimination learning test (Aceves-Piña and Quinn 1979). It remained to be seen whether they were "shallow" learning mutations, affecting only the tasks for which they were isolated, or "deep" ones, which affected learning in a variety of situations using different cues, reinforcements, and responses. The answer seems to be that the mutations are deep, but not perfectly so.

Several learning tests were devised, mostly variations on the basic discriminative scheme above, with different cues or reinforcements. Wild-type *Drosophila* larvae turned out to be nearly as acute at olfactory learning (Λ = 0.26) as adult flies, and larvae of the learning mutants failed to learn (Aceves-Piña and Quinn 1979). Menne and Spatz (1977) at Freiburg developed an elegant, automated procedure and trained flies to discriminate between different colored lights, avoiding a color that was associated with severe mechanical shaking. Dudai and Bicker (1978) and Folkers (1982) at Freiburg tested the available learning mutants in this visual test and found that although they could eventually learn this task, it took them many more trials to do so. However, of all the tests developed so far, visual learning is the test on which the mutants' performance is the least altered.

Tempel et al. (1983) trained flies to discriminate between odors as above, but substituted reward for punishment. They found that hungry flies would specifically migrate *toward* odorants previously associated with the opportunity to feed on sucrose. The magnitude of the learning effect after training with sucrose (Λ = 0.36) was similar to that observed after training with electric shock (Λ = 0.34) if measured immediately. However, memory after reward persisted much longer—for days rather than 4–6 hours. Substituting reward for punishment also lengthened the memory span for *amnesiac* flies (3 hr vs. 1 hr). The most surprising results came with the *dunce* and *rutabaga* mutants, which had shown virtually no learning in the shock-avoidance test. Mutant *dunce* flies, tested with reward, learned normally but forgot within 1 hour. *rutabaga* flies learned fairly well (Λ = 0.16) but forgot even more rapidly (see Fig. 2) (Tempel et al. 1983). The simplest explanation for these findings is that *dunce* and *rutabaga* are actually memory mutants; they can learn even in shock-avoidance training, but their memory span under those conditions is so short and labile that it is virtually undetectable (see also Dudai 1980). This finding is considered more fully below.

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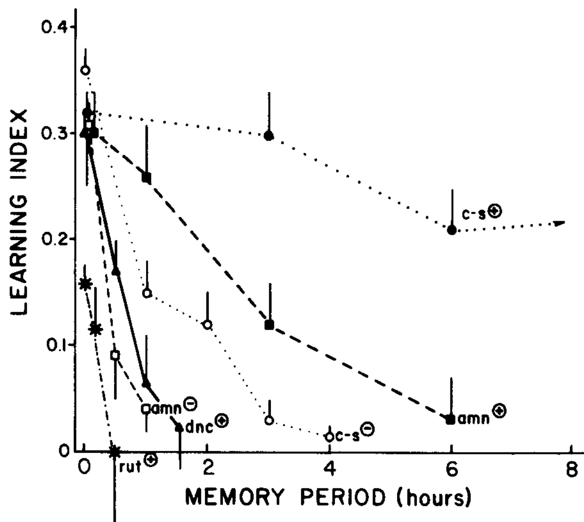

Figure 2. Memory retention in normal and mutant flies after training with positive reinforcement or negative reinforcement. Solid lines and solid symbols denote training with sucrose; broken lines and open symbols denote training with electric shock reinforcement. (●,○) Wild-type C-S; (■,□) *amnesiac*; (▲) *dunce*; (*) *rutabaga*. The mutants *dunce* and *rutabaga* show virtually no learning after negative reinforcement with standard procedure (Dudai et al. 1976; Aceves-Piña et al. 1979). Here, trained with positive reinforcement, they do learn but show abnormally rapid forgetting. (Reprinted, with permission, from Tempel et al. 1983.)

Training Individual Flies

In all of the learning experiments above, flies were trained and tested en masse. This is a real experimental convenience. It circumvents the problem of variability in movement among genetically similar individuals and has enabled us to select the learning mutants. Nevertheless, there are situations in which one wants to know whether a given individual can or cannot learn. This is particularly true of work using genetic mosaics, where no two individuals are alike. For this, a way to train larger insects can be scaled down for *Drosophila*. If a tethered cockroach (Horridge 1962) is shocked every time it extends one leg, it rapidly comes to maintain that leg in a flexed position. This postural change is due to associative learning because it occurs only if shock is made contingent on leg position. Cockroaches show less extraneous movement and perform this task even better if their heads are removed. This is not completely surprising, because insects have about a third of their CNS in thoracic ganglia.

Horridge's training procedure (Horridge 1962) can be readily adapted to *Drosophila*, given sufficient dexterity in tying wires on fly legs (Booker and Quinn 1981). Wild-type flies learned about as well as cockroaches. The behavioral change was associative, and flies could also be trained to *extend* their legs, by shocking them when their legs were flexed. Perhaps because leg flexion is a simple task and training is intensely repetitive, wild-type flies, particularly headless ones, performed well: 92% learned to a set criterion keeping their legs flexed over 90% of the time in a given 10-minute period. The learning mutants did worse: only 20% of *turnip* flies, 25% of *dunce* flies, and 45% of *cabbage* flies met this criterion (Fig. 3). Scores on the leg-extension were comparable (Booker and Quinn 1981). Note, however, that some individuals of all genotypes did learn. As in other tests, the learning disabilities in the mutants appear to be relative, not absolute.

The behavioral separation between mutant and wild-type individuals, although imperfect with this test, was good enough for a start at mosaic mapping of the anatomical site(s) involved in learning. Booker (1982) made mosaics that had *dunce* and *dunce*/+ (essentially normal) tissue patches, which were made distinguishable with cuticular and enzyme markers that allowed identification of mutant tissue (see Kankel and Hall 1976). He tested these flies in the leg-flexion test and then sectioned them and scored the tissue distribution of mosaics that had passed and those that had failed the test. The imperfect reliability of the behavioral test and other complications severely lowered the resolution of the mosaic map, introducing an element of uncertainty in the exact fate-map assignment. Nevertheless, one tendency is clear in the data. The anatomical site that showed the closest correlation to learning lay anterior to the thorax (Fig. 4) in the region expected of neural tissue in the head (see Kankel and Hall 1976). Apparently, flies need normal brains to learn. This result, seemingly obvious, becomes paradoxical when one remembers that these were headless flies, with brain tissue destroyed before testing. If pressed, one can make several rationalizations. For example, mutant neurons from the head might make processes with functional synapses in the thoracic ganglion, processes that survive beheading and continue to function critically during the testing period. Any detailed understanding of what is going on will have to await a better behavioral assay.

Another instance of modifiable behavior, expressed in individuals, that may have relevance to the lives of flies in the wild is the courtship-depression effect of Siegel and Hall (1979). Male flies placed with sexually unreceptive females become, as it were, discouraged. For about 3 hours afterward, they show markedly less ardor in courtship, even with receptive virgin females. Some recent behavioral experiments (Tompkins et al. 1983) suggest that this change represents associative learning. Still, perhaps the best evidence for this is the fact that learning mutants show less courtship depression than wild-type flies and that the depression effect is present in *amnesiac* males, but lasts less than 1 hour (Siegel and Hall 1979).

In all of the fly-learning tests described above, the experimenters have gone to some pains to ensure that the behavioral changes were *associative*; i.e., that the changes occurred only if the animal had experienced two environmental stimuli close enough together in

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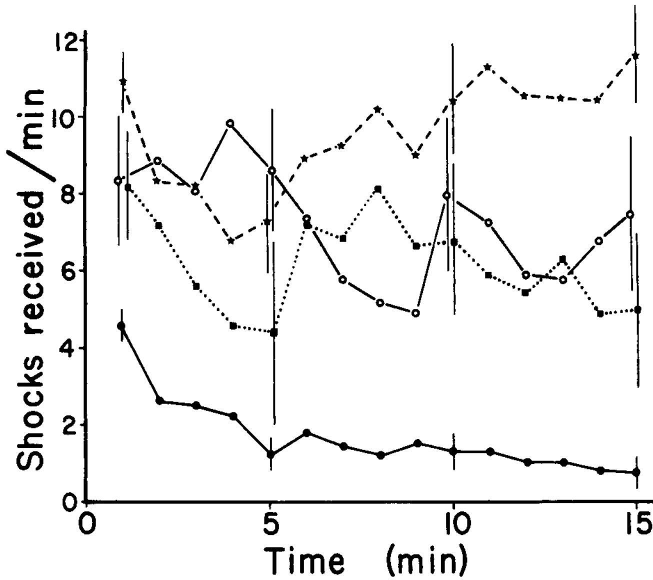

Figure 3. Average learning performance of headless normal and mutant flies in the leg-lifting task. For each genotype, the average frequency of shocks received for all individuals tested is shown as a function of time. Low shock values indicate that the flies tend to hold their legs up, avoiding shocks. Error bars at 5, 10, and 15 min indicate S.E.M.; variations at other times are comparable. The larger error bars for the mutants are primarily due to smaller sample sizes; their variability in performance was only slightly higher than wild-type flies. (●) C-S wild-type (50 flies); (○) *dunce* (20 flies); (■) *cabbage* (20 flies); (*) *turnip* (20 flies). (Reprinted, with permission, from Booker and Quinn 1981.)

time to suggest that one stimulus predicted the other. Animals also show simpler, nonassociative forms of learning: habituation, a decrease in responsiveness to a stimulus presented repeatedly, and sensitization, an increase in general responsiveness after a strong or noxious stimulus. Habituation and sensitization may seem less interesting than associative learning, but they are much better understood—their underlying mechanisms have been worked out in considerable detail in *Aplysia* (for review, see Kandel and Schwartz 1982). We wondered whether the (largely unknown) mechanism for associative learning had points of similarity with the (largely known) mechanism for, say, sensitization, at least in depending on the same gene products. To test

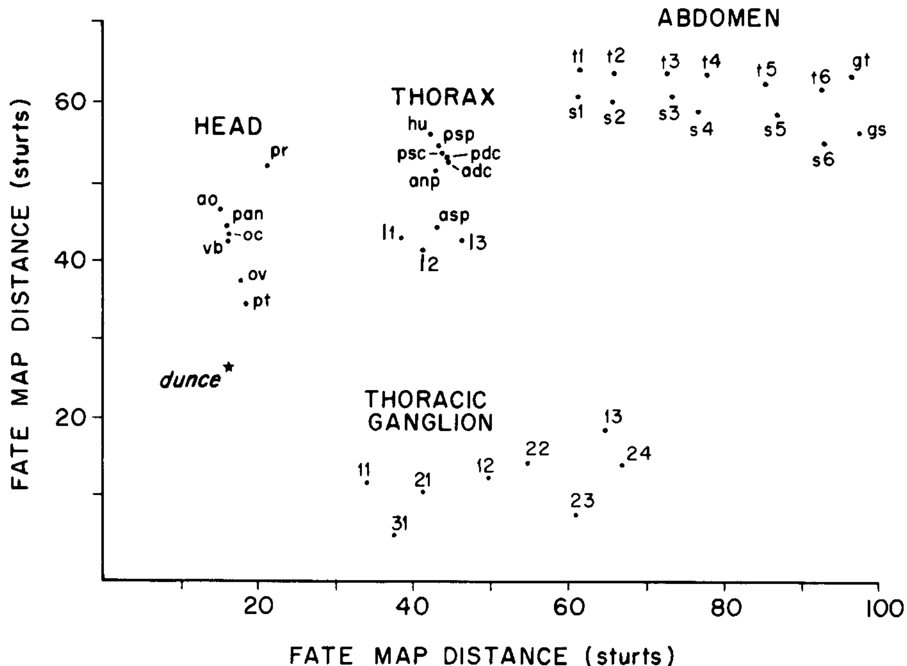

Figure 4. Fate map of *Drosophila* blastoderm showing location of cuticular landmarks for head, thorax, and abdomen, location of internal thoracic ganglion sites, and the site (behavioral focus) for leg-lifting learning behavior (Fig. 3) as influenced by the *dunce* gene. Analysis is from 126 appropriately marked mosaics, generated and scored as in Kankel and Hall (1976), and our nomenclature for landmarks follows theirs. Cuticular landmarks were scored using the marker, *yellow*. Thoracic ganglion sites were scored using histochemical staining for acid phosphatase enzymatic activity. The destruction of head tissue to improve behavior (see text) prevented direct scoring of cephalic ganglion sites. The fate map sites for landmarks and for the *dunce* leg-lifting focus were assigned by computer algorithms kindly provided by J. Flanagan (1976, 1977). The *dunce* focus lies in the region expected for cephalic ganglion sites (see Kankel and Hall 1976). However, this assigned location, although done objectively by computer, is an approximate one because of ambiguities in behavioral scoring (R. Booker and W.G. Quinn, in prep.).

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this possibility, Duerr examined habituation and sensitization in the proboscis extension reflex of *Drosophila*. As expected (see Dethier 1976), normal flies showed both forms of plasticity. More to the point, the associative learning mutants showed abnormalities: *dunce* and *turnip* had low habituation, and *dunce, rutabaga,* and *amnesiac* showed unusually brief sensitization (Duerr and Quinn 1982). The results suggested, albeit indirectly, that associative and nonassociative learning *do* have underlying mechanistic similarities, an idea strengthened by biochemical analysis of the mutants (see below) and, recently, by direct physiological analysis in *Aplysia* (Hawkins et al. 1983; Walters and Byrne 1983).

Biochemical Lesions in Learning Mutants

The fly-learning project has involved molecular aspects lately. Several of the learning mutations turn out to produce well-defined metabolic defects once one knows where to look for them. The first to be understood in this way was *dunce.* Kiger and Golanty (1977, 1979) were interested for biochemical reasons in cyclic nucleotide metabolism and they decided to map the genes coding for the relevant enzymes (e.g., guanyl cyclase and cAMP-dependent protein kinase) as a prelude to further analysis. Techniques are available in *Drosophila* that allow one to do such mapping, even before mutants are isolated (for review, see O'Brien and MacIntyre 1978). In this indirect way, cAMP was found to be hydrolyzed by at least two phosphodiesterases in flies, and one phosphodiesterase enzyme, PdE II, was localized to a well-defined genetic region (Kiger and Golanty 1977, 1979).

D. Byers at Caltech noticed that the genetic locus for the *dunce* region mapped very near the published site for PdE II. He did genetic complementation tests, and then direct biochemical tests, in collaboration with R. Davis and J. Kiger, which showed that *dunce* flies had low levels of PdE II enzyme and high levels of cAMP (Byers et al. 1981). Continuing this line of work, Kauvar (1982) and Shotwell (1983) obtained good evidence that *dunce* is the structural gene for PdE II enzyme. Enzymatic activity correlates directly with the number of *dunce* gene copies present. Among the several different mutations isolated in this gene, one, *dunce*^{1}, alters the enzyme's K_m; another, *dunce*^{2}, increases the enzyme's thermolability measured in vitro.

A second learning mutation, *rutabaga*, alters another enzyme involved in cAMP metabolism: adenylate cyclase (Livingstone et al. 1982; M.S. Livingstone, unpubl.). This abnormality was first detected as an increase in K_m, i.e., a *decrease* in the enzyme's apparent affinity for its substrate, ATP, measured in crude extracts. Although some earlier work suggested cAMP metabolic abnormalities in several learning mutants (Uzzan and Dudai 1982), the mutant difference in *rutabaga* became reliable only when specific tissues were examined. At first, Livingstone could detect the mutant's abnormal kinetics only in tissue from the abdomen, with brain enzyme apparently normal (Livingstone et al. 1982). This seemed counterintuitive, since work with mosaics and common sense suggested that the changes underlying learning occur in the brain. New evidence by Livingstone has eased this difficulty. The *rutabaga* mutation also causes a threefold increase in the thermolability, measured in vitro, of adenylate cyclase from abdominal tissue. With this difference she was able to show a similar abnormality in 10–20% of enzyme from brain (Livingstone et al. 1982). Therefore, the most likely explanation for *rutabaga's* effect on learning is that it alters a form of adenylate cyclase that constitutes a minority of brain enzyme but that is critically important in some neurons and synapses involved in plasticity.

In the case of *rutabaga*, as with *dunce*, the mutant enzyme's thermolability measured in vitro and the altered K_m suggest that the mutation lies in the structural gene for the catalytic enzyme. Other findings that support this idea are that (1) adenylate cyclase enzymatic activity in crude extracts from heterozygotes has two distinct thermal decay rates and two K_m values (Livingstone et al. 1982) and (2) cyclase catalytic activity correlates with the number of gene copies of the *rutabaga*$^+$ gene (M.S. Livingstone, unpubl.). If the *rutabaga*$^+$ gene does code for an adenylate cyclase, then this conclusion, taken together with the finding that most brain cyclase activity is unaffected by the mutation, implies the presence of different isozyme species of catalytic cyclase. If true, it would be the first good evidence for multiple adenylate cyclases. In fact, experiments with mammalian cell hybrids suggest a different picture, indicating that one type of cyclase enzyme functions interchangeably with different hormone receptors. The findings with *rutabaga* suggest that the adenylate cyclase biochemistry may have hidden complexities.

At present, we cannot strictly rule out the possibility that the *rutabaga* mutation affects some tightly bound, stoichiometrically limiting regulator of adenylate cyclase rather than the catalytic subunit itself. We do have evidence that the GTP-binding stimulating subunit, N_S (Ross et al. 1978), is not involved. Cyclase activity in abdominal tissues continues to depend on the number of *rutabaga* gene copies even in the presence of forskolin (Seamon et al. 1981) or Mn^{++} ions, agents that circumvent this regulatory protein (Livingstone et al. 1982).

There is also evidence that N_i, the recently discovered inhibitory GTP-binding regulatory subunit (Cooper et al. 1979; Jakobs et al. 1983), is still functional in *rutabaga.* Mutant and wild-type extracts show similar inhibition at high GTP concentrations in $MnCl_2$ (M.S. Livingstone, unpubl.). The most likely possibility at present is that *rutabaga* affects the ability of the cyclase catalytic subunit to interact with calmodulin. Extracts from mutant heads and abdomens are relatively deficient in the normally observed activation by calcium (Fig. 5). Also consistent with this picture is the temperature sensitivity of mutant extracts; such ther-

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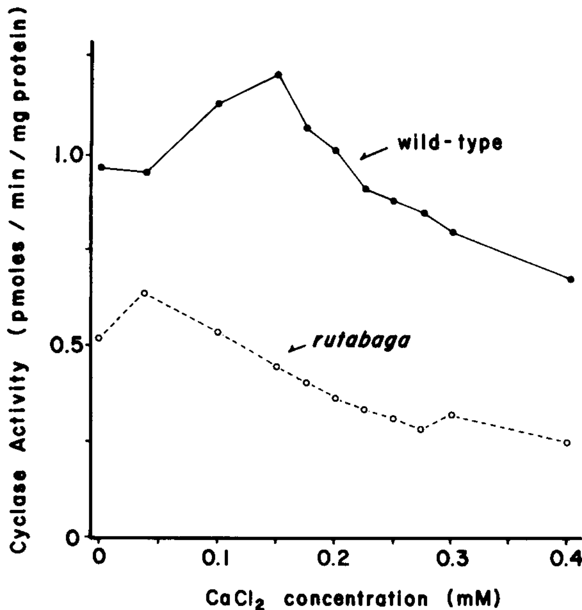

Figure 5. Calcium activation of adenylate cyclase from wild-type and *rutabaga* abdomens. Thirty *Drosophila* abdomens of a given genotype were homogenized in 1.2 ml of 0.1 mM Tris-acetate buffer, pH 7.6, containing 0.01 mM dithiothreitol and 0.4 mM EGTA. Of the low-speed supernatant, 40 μl was removed and added to 40 μl of assay cocktail (see Table 1 in Solomon 1979). Cyclase assays were run exactly following Solomon's procedure, except that the reaction solution contained 0.2 mM EGTA (M.S. Livingstone et al., in prep.).

molability is characteristic of cyclases which have been artificially stripped of calmodulin (Brostrom et al. 1978; Salter et al. 1981). In fact, direct experiments (M.S. Livingstone, unpubl.) indicate a relative inability of *rutabaga* cyclase to interact with exogenous calmodulin.

A deficiency in adenylate cyclase calcium activation, observed in a learning mutant, ties in very nicely with a plausible mechanism proposed to endow the *Aplysia* sensitization response with associative properties (Hawkins et al. 1983; Walter and Byrne 1983).

The *dunce* and *rutabaga* mutations both affect cAMP metabolism, but in opposite ways. The *dunce* mutations, which decrease phosphodiesterase activity, should increase overall cellular cAMP concentrations in the fly. The *rutabaga* mutation, which makes adenylate cyclase less active, should *decrease* cAMP levels. Direct measurement of cAMP levels in flies (Byers et al. 1981; M.S. Livingstone and P.P. Sziber, unpubl.) confirms these predictions. Why then do both mutations interfere with learning? Our working model is this: cAMP concentrations appear to be "buffered" in healthy cells, and a small change in concentration can be sufficient to produce a maximal cellular response (see Rasmussen et al. 1972; Nathanson 1977). A large perturbation of cAMP levels in either direction to beyond its normal range should disrupt the signaling properties of the system. (To take an analogy from neurophysiology, either a transmitter or its antagonist, applied in excess, will disrupt the signaling properties of neurons.) The *dunce* and *rutabaga* mutants may well induce such perturbations.

In vertebrate and invertebrate brains, adenylate cyclase is most frequently coupled to receptors for monoamine transmitters (for review, see Bloom 1976; Nathanson 1977). If cAMP is involved in learning, then probably one or more monoamine transmitters also are. In synaptic systems where the role of monoamines has been carefully studied, these transmitters often have a modulatory role; i.e., they act as accessory transmitters, increasing or decreasing the efficacy of synaptic signaling via another, principal neurotransmitter (for review, see Kupfermann 1981). This ties in well with ideas of learning as a change in the strength of connections in neural circuits. Moreover, the neuroanatomy of monoamine cells in vertebrates is consistent with a role in learning; the widely dispersed "sprinkler system" arrangements of monoaminergic systems seem particularly suited to alter the attentiveness or the retentiveness of large areas of the brain at once. Pharmacological studies in vertebrates, although intrinsically limited by drug side effects, cumulatively argue for a role of monoamines in learning, particularly for a dopaminergic role in positive reinforcement (see Wise 1978). Finally, in *Aplysia*, one monoamine, serotonin, is known to function in nonassociative learning (sensitization). For these reasons, many of us were eager to examine the fly learning mutants for abnormalities in monoamine transmitter synthesis. Livingstone did so, using high-performance liquid chromatography (HPLC) and electrochemistry (see Kissinger et al. 1981), and found all the learning mutants to be disappointingly normal (M.S. Livingstone, unpubl.). However, she and Tempel, examining the large published library of *Drosophila* stocks, found existing mutations (Wright et al. 1981) with which to examine the system. So far, the most relevant of these are a group of mutations, including temperature-sensitive mutations and deficiencies, that lie in the structural gene, *Ddc*, for dopa decarboxylase (Wright et al. 1981). This enzyme is very abundant in peripheral tissue, where it is vitally necessary for cuticle hardening. Wright (1977), who isolated the mutants, and also Dewhurst et al. (1972) had already adduced evidence for its presence in brain tissue. Livingstone and Tempel decided to try *Ddc* mutants in learning studies. First, they circumvented the usual lethal effect of the mutations by raising temperature-sensitive flies at permissive temperature (18°C) through development, eclosion, and cuticle hardening. Then they shifted the flies to restrictive temperature (29°C) for 3 days, which caused dopa decarboxylase activity to fall to undetectable levels but caused no loss of viability or overt behavioral abnormalities. Using radioactive precursors, they measured transmitter synthesis in isolated fly brains or in brain homogenates and found that *Ddc* mutants, in contrast to normal flies, were unable to carry out two relevant decarboxylation reactions: from L-dopa to dopamine and from 5-hydroxytryptophan to serotonin (Livingstone 1981; Livingstone and Tempel 1983). Synthesis of the third im-

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portant monoamine transmitter, octopamine, was virtually normal in *Ddc* mutants, but the decarboxylation step here was partially blocked (Livingstone and Tempel 1983) by another mutation, per^0, which abolishes circadian rhythms (Konopka and Benzer 1971).

With this partial dissection of monoamine transmitter synthesis in hand, Tempel and Livingstone set out to measure learning in the relevant mutants. They found that per^0 mutants learned as well as normal flies in olfactory discrimination tests, and they remembered as long afterward, with either electric shock or sucrose as reinforcement. Severely affected *Ddc* mutants, on the other hand, showed no detectable learning with either reinforcement. Their behavior in other respects was nearly normal. Temperature-sensitive Ddc^{ts1} mutants learned reasonably well ($\Lambda = 0.12$), provided they had been raised at permissive temperature (18°C) but not ($\Lambda = 0.00$) if raised at 29°C. Tempel and Livingstone then constructed a number of *Ddc* stocks, with different combinations of alleles, raised at different temperatures, that gave a range of dopa decarboxylase enzyme activity levels from wild type to undetectable. In all stocks, the flies' learning performance in both olfactory tests correlated well with the dopa decarboxylase activity remaining (Tempel and Livingstone 1981). This correlation seems surprising, because the effects of synthetic enzyme levels on transmitter pools, transmitter secreted, and so on to overt behavior are unknown and extremely indirect. Still, we are left with robust phenomenology. With *Ddc* mutants the fact that one can modulate the severity of the behavioral defect has made it possible to measure what aspect of learning is affected. Tempel constructed stocks (e.g., $Ddc^{ts1}/+$ raised at 29°C) with a *partial* enzymatic block and tested them behaviorally. Although, as expected, their ability to learn was somewhat reduced ($\Lambda = 0.22$), their memory span was normal, after both positively and negatively reinforced training (Tempel and Quinn 1982). *Ddc* lesions appear to decrease learning with no measurable affect on memory. This is in contrast to *rutabaga* and particularly to *amnesiac* and *dunce*, mutations that, in the most sensitive tests, appear to abbreviate memory retention without affecting acquisition.

Biochemical studies with *Ddc* indicated that it blocked synthesis of two neurotransmitters, dopamine and serotonin. Which one is more critically involved with learning? We may be able to answer this question if we can ameliorate the mutant's learning deficit by feeding it precursors to one or the other transmitter, by analogy with L-dopa therapy for parkinsonian patients. Recent results suggest another way to identify the guilty transmitter. Neurotransmitters produce their effect by binding receptors on postsynaptic membranes. In many cases the binding affinity of receptors for transmitters is high enough and specific enough to be detectable in filter assays using crude membrane fractions from brain homogenates. This type of work is most dramatically exemplified by the identification of opiate receptors in mammalian brain (Pert and Snyder 1973). We are currently investigating the serotonin-binding activity of membrane fractions from wild-type and mutant heads (R.F. Smith and W.G. Quinn, unpubl.). Confirming previous reports (Dudai and Zvi 1982), we find specific, high-affinity binding in normal flies with at least two classes of serotonin-binding sites, shown in our case by the nonlinearity of Scatchard plots (Fig. 6). A more exciting result is that *turnip* membrane extacts show a severe reduction in binding affinity. The dissociation constant of the tightest binding component is raised from about 0.06 nM in wild-type membrane preparations to about 0.30 nM in *turnip* membrane preparations (Fig. 6). The observed difference in the Scatchard plots is consistent with the possibility that the *turnip* mutation eliminates the highest-affinity serotonin-binding component, without much affecting lower-affinity components. We need to do more work on this. Nevertheless, the results of Smith provide an opportunity to understand another learning mutant.

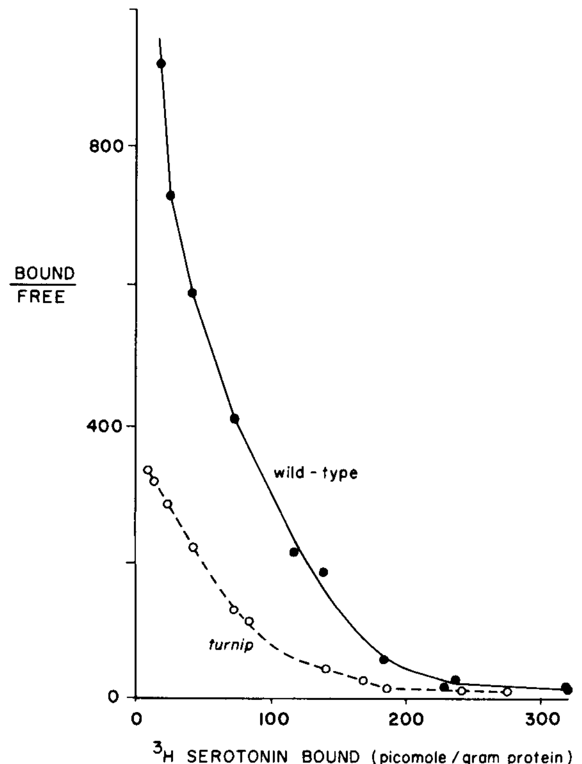

Figure 6. Scatchard plot of serotonin binding to membrane fractions from wild-type and *turnip* heads. Tissue preparation was similar to that of Gitschier et al. (1980), except that fresh tissue was used and the homogenate was preincubated for 20 min at 25°C prior to the final centrifugation to remove endogenous serotonin. The final membrane fraction was resuspended in 50 mM Tris-HCl buffer (pH 7.7) with 5 mM $MgCl_2$ at a final concentration of 19 mg heads/assay (2 ml total volume). Specific binding was assayed by incubating tissue for 60 min at 25°C with various concentrations of [^{3}H]serotonin from 0.02 nM to 36 nM in the presence or absence of 10 μM unlabeled serotonin. Samples were then rapidly filtered and counted. Ordinate units (bound/free) are picomoles/g protein/nM. (●) Wild-type; (○) *turnip*. The simplest interpretation of the plot is that *turnip* membranes lack a high-affinity binding component that is present in wild type.

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They also dovetail nicely with the defects in other *Drosophila* mutants, which all affect sequential steps in one biochemical signaling pathway.

Since four of the learning mutations affect important transmitters or metabolites, it is reasonable to ask why the mutants are not dead, let alone severely affected in nonlearning behavior. For *dunce, rutabaga,* and *turnip*, the answer may be that the metabolic affects are small, affecting only one isozyme in the case of *dunce*, subtly altering an enzyme's affinity for its substrate without abolishing activity in the cases of *turnip* and *rutabaga*. This excuse does not hold in *Ddc*; here, the metabolic lesion is drastic. We can only surmise that in *Drosophila* the monoamine systems are almost purely modulatory and do not disrupt vital circuits, an idea consistent with the small amounts of the relevant enzymes present in the fly's brain (Dewhurst et al. 1972).

CONCLUSIONS

This is where fly-learning genetics stands at the moment. Single-gene changes can interfere with learning in several tests, using olfactory, taste, visual, or proprioceptive cues, with positive or negative reinforcement, while leaving other behaviors substantially unaltered. Behavioral tests with the mutants have suggested experience-dependent components in behavioral patterns, such as courtship, which had appeared to be hard-wired. Work with mutants has provided evidence that associative and nonassociative learning are mechanistically related, a conclusion now confirmed by direct evidence from another system (Hawkins et al. 1983; Walters and Byrne 1983).

Because genes often specify enzymes, one can, with mutants and luck, jump directly from a behavior to a molecule. Work with the mutants directly implicates monoamines and cAMP as central to associative and nonassociative learning. The finding that *dunce* is a phosphodiesterase mutant was the first hard evidence for a role for cyclic nucleotides in associative learning. Behavioral examination of the mutants suggests a more detailed picture. Two mutations, *dunce* and *rutabaga*, which affect the kinetics of cAMP metabolism, appear to affect short-term memory decay rates. A third mutation, *Ddc*, which blocks a step before the cAMP response, primarily affects learning acquisition. These results tie in nicely with the suggestion of Castellucci et al. (1982) that the chemical change corresponding to a short-term memory may be simply an increase in cAMP concentration in the relevant neurons.

Could the mechanism underlying learning be so similar to a mere hormone response? This seems implausibly simple. On the other hand, deep problems in science often have simple answers, once the smoke has cleared. Learning may derive its phenomenological richness from the elaborate geometry of circuits in the brain, with the underlying mechanism kept simple. Nonassociative learning (in its short-term form) might be like a hormone response. Short-term associative learning could be nearly this simple, with enough metabolic embroidery to make the system responsive to a second stimulus, along the lines suggested by recent work in *Aplysia* (Hawkins et al. 1983; Walters and Byrne 1983). Things may be more complex, but they do not have to be.

The repeated links to *Aplysia* illustrate a limitation as well as a strength of the work with mutants. Single-gene mutations can provide well-defined perturbations of living systems. Work with them, properly interpreted, can lead directly from an animal's behavior to its molecular heart of hearts. Nevertheless, the search for the salient biochemical lesion in a given mutant is a needle-in-a-haystack enterprise unless there are clues on where to look. Such clues often come from other, nongenetic work. Since the search for mutant abnormalities then becomes highly directed, there is a good chance that someone will call important the first promising-looking difference found. Fortunately for the work at hand, there is good evidence that in *dunce, rutabaga* and *Ddc* mutations cause their primary lesions in the enzymes they are supposed to. At present, *turnip* seems half understood. Mutations like *amnesiac*, which may be relevant to a different problem of long-term memory storage, are presently not understood at all.

ACKNOWLEDGMENTS

Our work discussed here has been supported by National Institutes of Health grant GM-25578 to W.G.Q. and by equipment and assistance from the Whitehall Foundation at Princeton. We thank Ralph Greenspan for help with the manuscript.

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