Studies on Learning and Cyclic AMP Phosphodiesterase of the *dunce* Mutant of *Drosophila melanogaster*.

Thesis by

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This thesis is dedicated to the memory
of James Olds, teacher and friend.

Acknowledgements

My first conception of this dissertation occurred on October 5, 1971. On that day I was a second year graduate student in the Department of the Psychological Foundations of Education at the University of Minnesota, entirely ignorant of genetics, and was unexpectedly treated to a clear and elegant lecture on the specification of protein structure in the triplet code of DNA. It seemed to me very likely that the new and rapidly advancing knowledge of molecular genetics could be turned to advantage in the study of behavior. Professor Val Woodward informed me that this conception was not novel, and urged me to pursue it as a student of Seymour Benzer; for this excellent advice I am very grateful. I also wish to thank Professors John Rhetts and Carol Deppe and Senior Research Associate Gary Miner of the University of Minnesota.

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Abstract

Normal Drosophila learn to avoid an odorant associated with electric shock. This dissertation describes the isolation of two X-linked mutants that fail to display this learning, in spite of being able to sense the odorants and electric shock. The mutants are proved to be alleles of one gene, and are named dunce^{1} and dunce^{2}. The finding that dunce^{2} females are sterile led to the discovery that the dunce gene was independently known in two other laboratories.

Kiger, Davis, and Golanty of the University of California at Davis find (one) that normal Drosophila have two forms (I and II) of soluble cyclic AMP phosphodiesterase, differing in molecular weight and other properties; (two) that a gene appearing to control form II is included within chromosomal bands 3D3 and 3D4; and (three) that females with homozygous deficiency of these two bands are sterile. Mohler of the University of Iowa isolated 225 X-linked female-sterile mutants. Among these Davis and Kiger found two that map within 3D3 and 3D4 and lack the form II of soluble cyclic AMP phosphodiesterase.

The two mutants of Mohler learn poorly. They do not complement dunce^{1} or dunce^{2} in learning nor dunce^{2} in fertility, and therefore are alleles of the dunce gene. Mapping of learning with genetic deficiencies and duplications places dunce^{1} and dunce^{2} within region 3D3 and 3D4. Females with homozygous deficiency of five adjacent bands, including these two, are viable and vigorous, but learn poorly and are sterile.

The soluble cyclic AMP phosphodiesterase II is inactive or absent in homogenates of dunce^{1} and dunce^{2} flies. In addition, cyclic AMP levels are elevated in the dunce mutants, as expected if cyclic AMP

phosphodiesterase activity is deficient.

It is concluded that the dunce gene of normal Drosophila has functions in olfactory learning performance, reproduction, and cyclic AMP metabolism. Recent work with other organisms has suggested that cyclic AMP may be intimately involved in mechanisms of learning; the dunce mutant should prove helpful in testing this.

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Chapter 1

Introduction

1. Some Learning Theory.

Genes and environment interact in the functioning of nervous systems. To memory, the environment presents conditional relationships that represent ways to satisfy wants and avoid hurts. The genes provide storage and recall mechanisms, but do not specify memory content.

Much of learning seems to occur by formation or strengthening of connections between situation and behavior (1-4). The formation and strengthening of connections is primarily under the control of certain biologically significant stimuli including food, water, heat, cold, pinches, bites, poisons, and sour or bitter tastes. These stimuli, termed reinforcers or rewards, not only elicit behavioral responses, but also elicit learning.

A reward simultaneously strengthens many connections. An animal, when rewarded or punished, may form learned connections of visual, olfactory, tactile, or auditory cues with almost any feature of its current behavior, including body position, limb movement, vocalization, etc. This suggests that the effects of a rewarding stimulus are in some form propagated widely through the brain.

The first major advance in the neurophysiology of learning was the discovery about 1954 that stimulation of certain brain sites via microelectrode can be rewarding. The first discovery, by Delgado and his colleagues, was of punishing brain stimulation (5). The interpretation of this was ambiguous, since it was well known that shocks to skin or peripheral nerves were also punishing. Soon afterwards, Olds and Milner

observed positive reward by brain stimulation (6). By 1962, learning with brain stimulation as positive reward had been observed in monkey, cat, dog, rat, dolphin, and goldfish (7-12). Olds demonstrated that the same electrode could reinforce learning to press the bar of a Skinner box and learning the route through a complex maze (13). These findings suggested the hypothesis that the electrodes were activating the nervous pathways by which natural rewards exert their effects. Despite intensive work in many laboratories, this hypothesis is not yet proven nor disproven (14,15).

Rewarding sites are widely distributed in the brain. Olds has estimated that perhaps one quarter of electrodes placed randomly in a rat's brain would deliver positive reinforcement when stimulated. The successful sites, however, are not randomly distributed, but lie along certain tracts and pathways. When the systems of neurons using noradrenaline, dopamine, and serotonin as neurotransmitters were discovered and traced by fluorescence histochemistry during the 1960's, it became apparent that their locations correspond closely to the distribution of reward sites. Their cell bodies are clustered in a few small regions of brainstem and midbrain and their axons travel in bundles to almost all brain regions (16,17). Neurons of this shape would be well suited to deliver information on the pleasurable or painful consequences of behavior to many or all of the brain locations generating behavior. Testing the hypothesis that these neurons are the final common pathways of natural reward has proven very difficult. Pharmacological and surgical experiments have produced abundant evidence that dopamine, noradrenaline, and serotonin are intimately involved in emotion, motivation, and reward, but their exact roles remain unclear (14,15). Since

the cell surface receptors for dopamine, noradrenaline, and serotonin are often found to be coupled to adenyl cyclases (see Chapter 6), cyclic AMP may serve as a signal at some step of the learning process.

Learning is termed associative when connections are formed between current stimuli and current behavior. One type of non-associative learning is sensitization, defined as "a prolonged enhancement in the behavioral response to one stimulus as a result of the presentation of another stimulus" (18). (It is non-associative because the sensitized reflex need not be active when the sensitizing stimulus occurs.) Sensitizing stimuli are often identical to the reinforcing stimuli of associative learning.

Kandel and his colleagues (18,19) have described a molecular mechanism mediating sensitization of the gill withdrawal reflex of the marine mollusc Aplysia californica. Sensitization of the reflex involves increased efficiency of synaptic transmission (more neurotransmitter released per nervous impulse) at a particular synapse in the abdominal ganglion, the sensitizing stimulus being carried by neurons from the head. These neurons probably release serotonin, and the presynaptic element of the sensitizing synapse seems to bear serotonin receptors that are associated with adenyl cyclase. The effect of a natural sensitizing stimulus can be mimicked by electrical stimulation of the connective from the head, extracellular application of serotonin to the abdominal ganglion, injection of cyclic AMP into the presynaptic cell of the synapse, or application to the ganglion of isobutyl methyl xanthine, an inhibitor of cyclic AMP phosphodiesterase. These findings suggest that synaptic efficiency can be altered in some cells by a pulse of intracellular cyclic AMP. A similar conclusion was reached by

Shimihara and Tauc (20) studying a different synapse of Aplysia, and was suggested with much less complete evidence for some vertebrate synapses (21,22). It will be interesting to learn whether similar mechanisms operate during associative learning.

2. Neurogenetics of Learning

Neurogenetics is the marriage of modern molecular genetics with neurobiology and psychology. In principle, neurogenetics involves characterization of normal behavior and neural function, isolation of mutants with altered function or behavior, and use of the mutants for further probing into the mechanisms of behavior. The pioneers of this approach are Max Delbrück, Seymour Benzer, Sideny Brenner, and Julius Adler. The subjects being explored with mutations at this moment include embryology and development of nervous systems (23-25), neurotransmitter metabolism (26,27), synaptic transmission (28), conduction of action potentials (29), sensory transduction (30-33), muscle function (34,35), motor coordination (36), stereotyped behavior patterns (37), and learning (38,39). Neurogenetics is progressing most rapidly in species for which genetic knowledge is well developed, especially the bacterium Escherichia coli and the protozoan Paramecium tetraurelia (both being considered as one-cell nervous systems), the nematode worm Caenorhabditis elegans, and the fruit fly Drosophila melanogaster. Among mammals, neurogenetics is most advanced for the human and the mouse, but in no mammalian species is there a convenient method for induction and selection of new mutations.

For neurogenetics of learning, the first problem was to find a species with good genetics and good associative learning. Before 1970,

there was no convincing evidence for associative learning in any fly and Dethier (40) was speculating that flies might have lost all learning capacity during their evolution. In 1971, Nelson (41) demonstrated classical conditioning of the proboscis extension reflex of the blowfly Phormia regina. In 1974, Quinn, Harris, and Benzer (42) demonstrated associative learning in Drosophila with a procedure suitable for selection of mutants with altered learning; their procedures are used for tests of learning throughout this thesis and will be carefully described in Chapter 2. Fukushi (43,44), Medioni and Vaysse (45), and Menne and Spatz (46) offered additional evidence of associative learning in flies, using various sensory modalities.

The reinforcers now known to be effective for learning in flies are sucrose (positive reward), and electric current, intense vibration, or quinine sulfate (negative rewards). The sensory cues that can be learned include odors, colors, and tastes.

As a quantitative index of learning performance, Quinn, Harris, and Benzer defined a learning index, Λ , as the fraction of flies avoiding the stimulus previously coupled with reinforcement minus the fraction avoiding the control stimulus. Positive values of Λ indicate learning (maximum = 1.0) and a zero score indicates no learning. They observed learning scores of $\Lambda = 0.33 \pm 0.02$ for olfactory avoidance learning and $\Lambda = 0.09 \pm 0.02$ for visual avoidance learning in Drosophila. The definition of Λ extends readily to other learning experiments with flies and the following scores can be computed from published data: $\Lambda = 0.28$ for classical conditioning with taste cues in Phormia (Nelson (41), figure 4); $\Lambda = 0.55$ for classical conditioning with visual cues in the housefly Musca domestica (Fukushi (44), figure 2); $\Lambda = 0.27$

for learned inhibition of proboscis extension in Drosophila (Medioni and Vaysse (45), figure 2); and $\Lambda = 0.15 \pm 0.02$ for visual avoidance learning in Drosophila (Menne and Spatz (46), figure 8).

These examples of learning in flies provided a starting point for a neurogenetic analysis of learning. The next step was to find genetically controlled differences in learning performance. In principle, learning performance might be genetically changed either by progressive selection of bright strains and dull strains, or by one-step mutation. McGuire and Hirsch (47), using the training procedures of Nelson with Phormia, selectively bred good learners and poor learners for 26 generations. The learning performance of the bright line increased during the first 10 generations of selection, then remained for 16 generations at a plateau corresponding to $\Lambda = 0.61$. The dull line reached zero learning performance within three generations of selection and did not change further. D. Fulker and his students at the Royal Bethlehem Hospital, Beckenham, Kent, England, performed a similar selection experiment (personal communication) using the olfactory learning procedures of Quinn, Harris, and Benzer. To ensure a heterogeneous starting population, they interbred nine wild-type strains (including Canton-S). They then selectively bred a bright line and a dull line with continuous selection for ten generations. The learning score of the bright line was consistently about 0.35 and the learning score of the dull line reached zero. These results indicate that learning performance is under genetic control. However the bright lines and dull lines probably differ in many genes, making further analysis very difficult.

Analysis would be simpler if the whole difference in learning

performance were a consequence of mutation of one gene. Using the procedures of Quinn, Harris, and Benzer, Dudai tested 34 known behavioral, biochemical, or morphological mutants of Drosophila to see whether any had strong effects on learning performance (48). In this test of learning, good phototaxis is necessary for successful learning performance (see page 12). In general poor learning in the mutants was correlated with poor phototaxis. Among those mutants with approximately normal phototaxis, some mutants learned normally and some had intermediate learning, but none were very poor at learning.

Since 1974, six new mutations affecting learning performance have been found in Drosophila. The first of these was dunce, which is the subject of the following chapters. The other five were isolated by Quinn, Szilber, and Booker at Princeton University. The mutant amnesiac has normal learning performance if tested immediately after training, but forgets abnormally quickly (39). The mutant turnip as a homozygote performs poorly when tested immediately after training. However, the turnip/+ heterozygote learns normally but forgets even more quickly than amnesiac (39). The mutant cabbage and two unnamed mutants perform poorly at all times after training and differ from dunce and each other in certain other ways still being characterized. Genetic mapping and complementation testing by Szilber and Quinn indicate that the six mutants define six independent genes on the X-chromosome (unpublished).

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Chapter 2

Isolation, Characterization, and Genetic Mapping of dunce[1].

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Genetics

dunce, a mutant of *Drosophila* deficient in learning

(conditioned behavior/olfactory discrimination/behavior genetics)

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

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

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

MATERIALS AND METHODS

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

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

Before testing, males were separated from females in a nitrogen atmosphere, which immobilizes flies but does not affect subsequent learning (5). For training, about 40 flies were placed in the start tube and the apparatus was laid horizontally, with tubes 1 through 5 pointing at a horizontal 15-W daylight fluorescent lamp (Westinghouse F15T8/D). After the flies had explored the rest tube for about 1 min, the apparatus was held vertically and tapped sharply on a rubber mat to bring the flies to the bottom of the start tube. The holders were slid to set the start tube in register with tube 2 and the apparatus was replaced on the table. The flies, strongly phototactic, ran toward the light and were shocked in the presence of odorant A. After 30 sec, the flies were shaken back into the start tube, and the slide shifted so that the flies could run into the rest tube again for 30 sec. Next was a similar run for 30 sec into tube 3, containing odorant B but no shock, followed by a 30 sec rest. The complete sequence was repeated twice, thus totaling three training trials with each odorant.

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

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

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

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

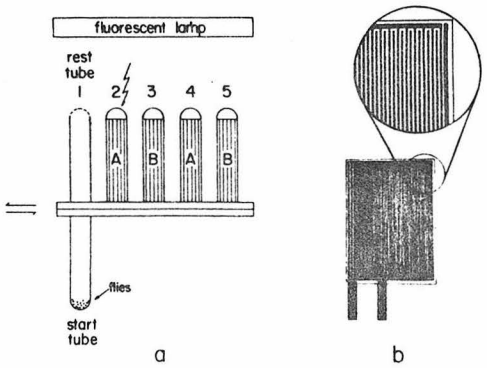

FIG. 1. Apparatus used for training and testing flies. (a) Two plastic blocks holding tubes slide on a dovetail joint, so that the start tube can be shifted into register with tubes 1 through 5. Tube 1, the rest tube, is perforated for ventilation. Tubes 2 through 5 contain grids with odorants A or B.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

RESULTS

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

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

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

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

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

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

*For a side view of the apparatus, see figure 5, page 16.

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

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

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

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

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

Other Tests on *dunce*. The external morphology of *dunce* flies is normal. The viability of *dunce* eggs, larvae, and pupae is normal, and the adults have a normal life span. The mutant has essentially normal phototaxis, geotaxis, flight, locomotor activity, and sexual courtship. In crowded culture bottles, *dunce* flies do seem to become somewhat weaker and more sluggish than normal. The electroretinogram (9) is normal. Synaptic

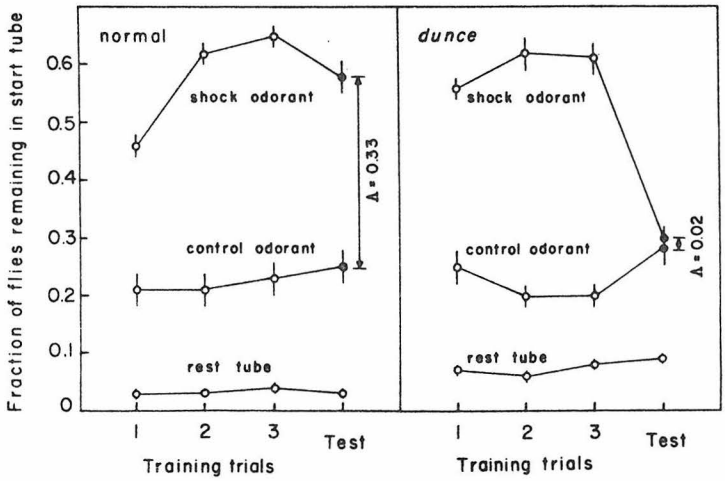

FIG. 2. Behavior of normal and *dunce* flies during training and testing. Fraction of flies in start tube after 15 sec is shown for the three training trials and for the test. Each point represents the mean ± SEM for 11 experiments; 0.5% 3-octanol and 0.5% 4-methylcyclohexanol served as odorants. The learning index, Λ, is the fraction of flies avoiding the shock-associated odorant during the test minus the fraction avoiding the control odorant during the test.

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

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

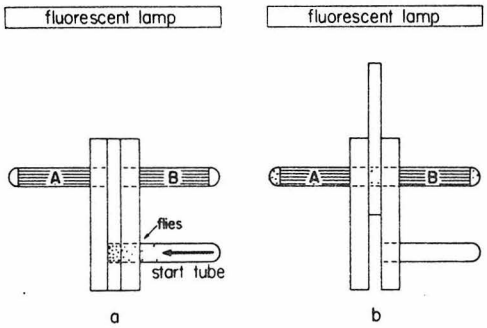

FIG. 3. Choice-chamber apparatus for testing the ability of flies to sense odorants. (a) The flies are shaken from the start tube into the sliding compartment. (b) The compartment is shifted into register with tubes containing odors A and B. After 30 sec, the compartment is shifted out of register and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

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

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

DISCUSSION

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

What could the defect in *dunce* be?

(*i*) Although the mutant has essentially normal phototaxis, can sense the odorants, and is deterred by the electric shock,

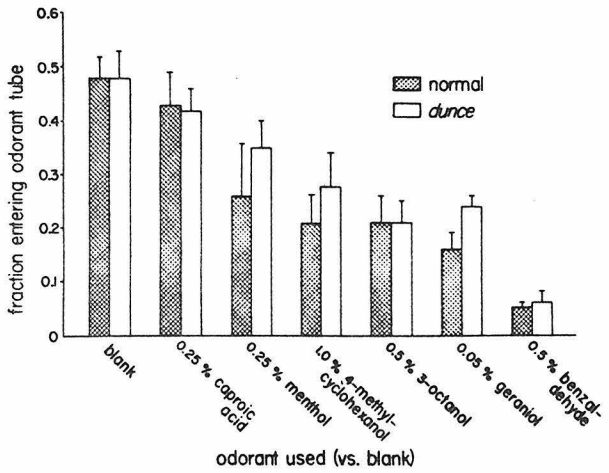

FIG. 4. Response of normal and *dunce* flies to various odorants in the choice-chamber apparatus of Fig. 3. Two-tenths milliliter of the specified odorant in ethanol was spread on one grid. The other grid was a blank with 0.2 ml of ethanol alone. Before use, the grids were dried for 5 min to evaporate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

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

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

Table 2. Mapping of the *dunce* mutation

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

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

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

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

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

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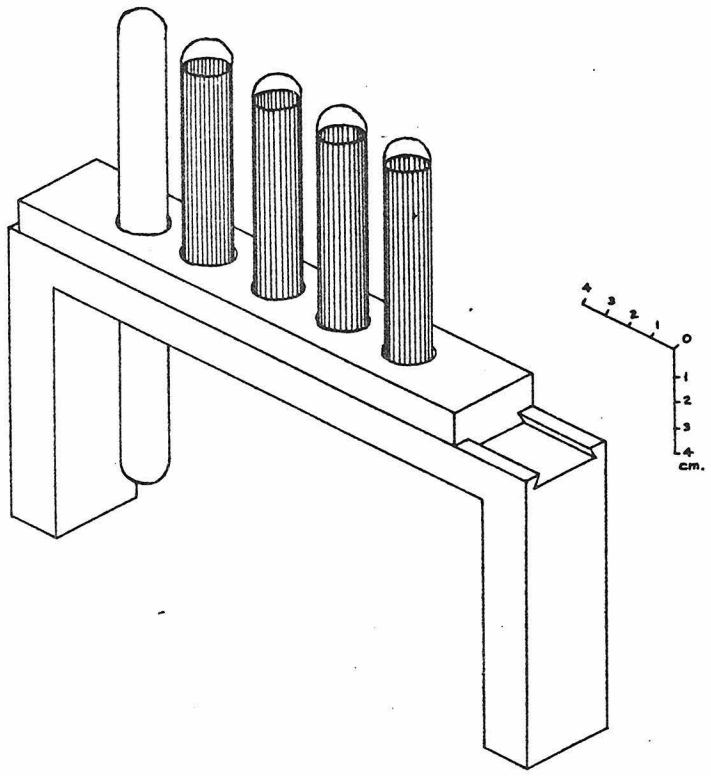

Figure 5. A side view of the apparatus of figure 1a.

Additional Information on *dunce*.

1. Temperature-sensitivity of normal and *dunce* learning.

Study of temperature-sensitive mutations of *dunce* could reveal the critical times at which the normal *dunce* gene is needed for successful learning. To determine the temperature range over which normal flies can learn successfully, and whether the phenotype of *dunce* is sensitive to temperature, normal and mutant flies were cultured at 17° or 25° and tested at various temperatures. The results are shown in Table 3. Normal flies cultured at either temperature learned successfully between 17° and 29°. At 34° the flies raised at 25° learned normally, but those raised at 17° had poor phototaxis and were not tested. At

Table 3. Effects of temperature on learning.

Temperature During Learning Test	Culture Temperature (until 1/4 to 2 hours before testing)	Learning Index (Λ)	
		Normal	dunce[1]
13°	17°	slow (phot.= 50%)*	slow (phot.= 30%)*
	25°	very slow (phot.= 2%)*	very slow (phot.= 3%)*
17°	17°	0.42 ±0.05 (n=3)	0.04 ±0.02 (n=3)
	25°	0.35 ±0.05 (n=3)	0.05 ±0.01 (n=3)
23°	17°	0.39 ±0.04 (n=4)	0.01 ±0.04 (n=4)
	25°	0.39 ±0.03 (n=6)	0.04 ±0.02 (n=4)
29°	17°	0.25 ±0.05 (n=3)	-0.02 ±0.04 (n=3)
	25°	0.29 ±0.06 (n=3)	0.05 ±0.04 (n=3)
34°	17°	slow (phot.= 70%)*	slow (phot.= 50%)*
	25°	0.29 ±0.06 (n=3)	0.05 ±0.02 (n=3)

*Learning was not tested unless phototaxis was greater than 70% of normal.

13°, all flies had phototaxis too weak to permit a reliable test of learning. The dunce mutant showed poor learning at all these temperatures with no sign of abnormal temperature-sensitivity.

2. The learning performance of individual flies.

The study of genetic mosaics can reveal the site of action of a mutant gene (1,2). The procedure requires that the mutant phenotype be accurately scored for each mosaic individual used. For the case of learning, this will require new techniques because the measures of learning so far described for Drosophila have involved testing of populations. If the behavior of a population is indicative of the behavior of the individuals in it, then it should be possible to reliably measure learning by repeated testing of individuals. I have attempted this.

The learning experiment developed for populations was modified for use with individual flies. A short grid was used for testing so that the lip of the grid where the fly made its choice to stop or proceed was clearly observable through the wall of the test tube and not partially obscured by the learning apparatus (see figure 5). The average learning of populations tested on the short grids was $\Lambda = 0.37 \pm 0.02$ (n=20), not significantly different from scores on the normal grids. The movements of each fly onto and off of the grid were timed with the help of a metronome beating once per second and recorded in a shorthand notation. A plug of sponge rubber was placed 2 cm from the mouth of the start tube, in order to shorten the distance to be run on each trial. Training trials in the electrified or control training grids (tubes 2 and 3 of figure 1 of this chapter) were 8 seconds

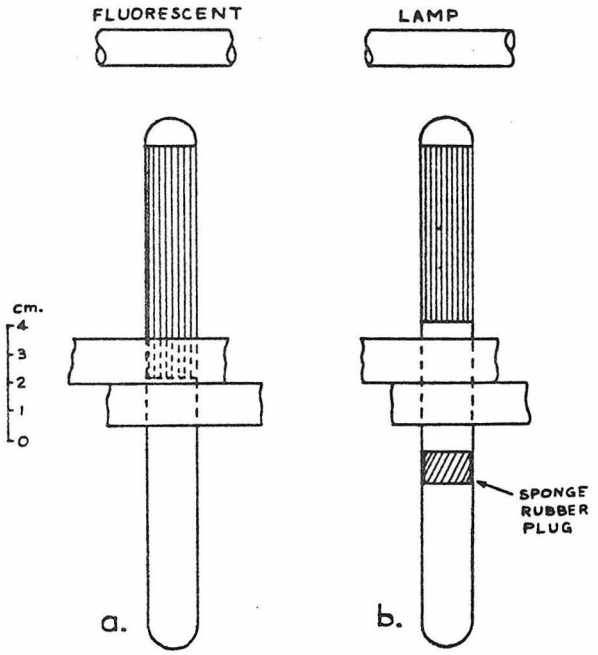

Figure 6. Comparison of the experimental arrangements for testing learning performance of populations (a) and individuals(b). In arrangement (b) the first few steps of the fly onto the grid are clearly visible, while in arrangement (a) they are partially obscured by the plexiglas frame.

(instead of 30 seconds for populations). On the 3rd, 9th, and 15th training trials in each group of 20, the flies were forced to remain on the electrified grid for 5 seconds. Rest periods were 10 to 15 seconds (instead of 30 seconds for populations). Testing trials (in tubes 4 and 5 of figure 1) lasted up to 29 seconds depending upon the behavior of the fly. Preliminary tests showed that flies which reached the end of the tube adjacent to the lamp rarely left that region. Therefore if a fly reached the end on a test trial, it was immediately returned to the rest tube and recorded as having entered the grid. Testing

trials in which the fly did not reach the far end of the grid were ended when the fly had accumulated 15 not necessarily consecutive seconds on the grid (scored as entering) or off the grid (scored as avoidance). Pairs of training trials with interspersed rests were followed by pairs of testing trials with interspersed rests, for up to 20 testing trials of one fly. Some flies were then stored in culture vials for one to three days before a second identical sequence of 20 training trials and 20 testing trials. Flies were usually chosen at random for training, but were occasionally pre-selected for good phototaxis. Males and females were taken in alternating sequence.

To help rule out the possibility of experimenter bias, about half the flies were trained and tested with a sequence of testing odorants unknown to the experimenter. To accomplish this without the constant presence of an assistant, the testing tubes were labelled with differently-valued electrical resistors so that their identities could be recorded on a chart recorder in series with a battery. After each testing trial on which a fly had responded differently in the two testing tubes, the tube identities were recorded, then the tubes were removed from the apparatus, shuffled, and reinserted. The average result of these blind runs was not significantly different from the overall average. Control experiments were run with normal populations in each apparatus before and after its use with a single fly, and showed that the repeated handling of the testing tubes did not disturb the odorant distribution or alter the effectiveness of the apparatus.

In this way 29 normal individuals were tested 600 times, averaging 21 tests each. Collectively, they avoided the shock-associated odorant in 387 tests (65%) and the control odorant in 198 tests (33%); their

average score was equivalent to $\Lambda = 0.33 \pm 0.04$ (s.e.m.). Nineteen dunce individuals were tested 640 times, averaging 34 tests each. They avoided the shock-associated odorant on 359 of the tests (56%) and the control odorant on 328 (51%); their average score was equivalent to $\Lambda = 0.04 \pm 0.03$. These learning scores of individuals are very close to those seen with populations and seem to demonstrate that the capacity for learning is inherent in individual flies. The same conclusion was reached with different evidence by Quinn et al. (3).

The learning scores of individual flies ranged widely -- from 0.0 to +0.75 for groups of 20 trials for normal flies and from -0.35 to +0.35 for groups of 20 trials of dunce. (A negative score indicates that the fly avoided the odorant previously associated with shock less often than it avoided the control odorant.) Calculations suggest that reliably scoring 95% of the flies as normal or dunce 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 numbers of trials or to true individual differences.

Even during training, a substantial difference was seen between normal and dunce individuals. After a few shocks, the normal flies rarely stepped again onto the electrified grid; this was true also when the forced shocks were omitted. Even after several severe shocks, the dunce flies continued to venture occasionally onto the electrified grid, though less often than onto the control grid. The same phenomenon is apparent in figure 2 of this chapter, which shows an increase of avoidance of the electrified grid greater for populations of normal flies than for populations of dunce. It is unknown whether the increased

avoidance in normal flies is due to learning.

If repeated tests are to be used for mosaic mapping of dunce, it is important to know whether learning scores change with repeated training. The learning scores of the set of individual flies are plotted as a function of trial number in figure 7. Trials are collected into groups of five, because sample sizes are rather small and consequently the results for individual trials are variable. The normal flies performed at about the same level throughout the 40 trials. Even after 40 trials the performance of dunce flies did not improve.

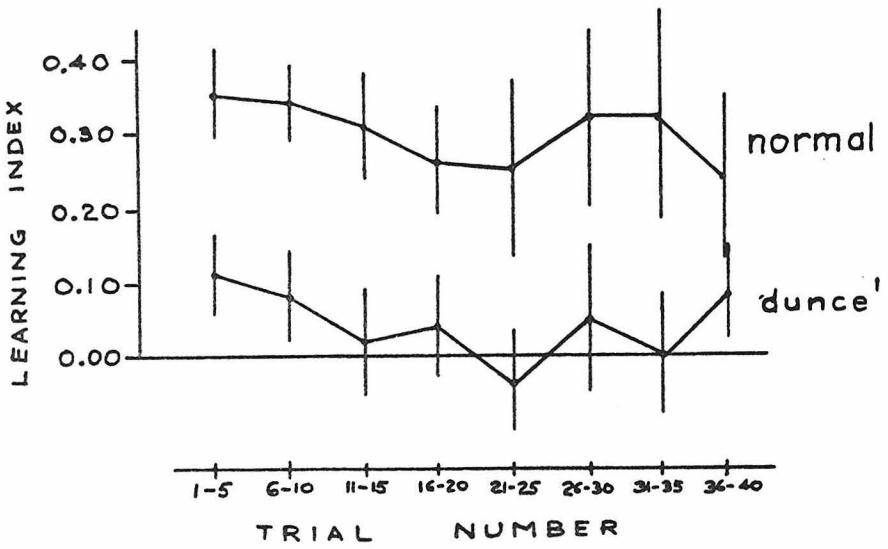

Figure 7. Learning performance of normal and dunce flies trained and tested individually. The apparatus and procedures regularly used with populations were modified as described in the text. Plotted are the mean scores (± s.e.m.) of 29 normal flies, 5 tested 40 times and 24 tested 4 to 29 times; and 19 dunce flies, 13 tested 40 times and 6 tested 20 times. The standard errors are larger in the later trials because fewer flies were tested.

To score enough mosaics for a blastoderm fate map using this method would not be practical, since about 20 hours of labor would be required

for each mosaic individual. Nonetheless, the results encourage me in the belief that study of dunce in mosaics is possible.

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Chapter 3

A Second Mutant Allele of *dunce*.

The *dunce* mutant described in Chapter 2 was discovered May 21, 1974 in the 38th mutant candidate line tested. I discovered a second mutant November 12, 1974 in the 276th line tested. All other mutants found which learned poorly also had gross motor or phototactic defects and were eliminated from further consideration. Screening continued at Cal Tech until June 1977, when about 1500 lines had been tested by Marlyn Woo, William Quinn, Yuh-Nung Jan, Elizabeth James, Yadin Dudai, and me.

The new mutant resembles *dunce* in learning performance and behavior. The evidence to be presented in Chapter 4 demonstrates that the new mutant and *dunce* do not complement each other and so must be alleles of the same functional gene. The new mutant is designated $dunce^{2}$ and the original *dunce* mutant is henceforth designated $dunce^{1}$. Dudai (1,2) has referred to $dunce^{1}$ as $dunce^{DB38}$, and to $dunce^{2}$ as $dunce^{DB276}$. In this chapter I describe the traits of $dunce^{1}$ and $dunce^{2}$.

Olfactory learning scores Λ in this and the following chapters were measured as described on page 12 with 4-methylcyclohexanol and 3-octanol as odorants. The learning scores and tests of fertility are reported as means $\pm$ one standard error of the mean.

1. Phenotypic similarities between $dunce^{1}$ and $dunce^{2}$.

The olfactory learning performance of normal and $dunce^{2}$ males and females is shown in Table 4, with $dunce^{1}$ included for comparison. The performance of $dunce^{2}$ is poor (Λ = 0.03 by males, 0.01 by females). No

Table 4. Learning performance of normal and *dunce* males and females.

Genotype	Learning Index (Λ)	
	Males	Females
normal	0.33 ±0.02 (n=8)	0.33 ±0.04 (n=8)
dunce^1	0.02 ±0.02 (n=8)	0.07 ±0.03 (n=8)
dunce^2	0.03 ±0.02 (n=8)	0.01 ±0.03 (n=8)

differences between the sexes are seen in normal or mutant learning. The *dunce*^{2} flies are of normal appearance, have the normal strong phototactic drive that is essential for the olfactory learning test, and have normal geotaxis and locomotor activity. With the choice-chamber apparatus of figure 3, page 14, Dudai tested the ability of *dunce*^{2} flies to detect and avoid odorants and found no abnormalities (2). In all these characteristics *dunce*^{2} resembles *dunce*^{1}.

The *dunce*^{1} and *dunce*^{2} flies share a curious trait rarely seen in the normal (Canton-S) strain from which the *dunce* mutants are derived. This is a tendency, while standing or running, to briefly spread the wings about 30° outward and upward. Flies with their wings spread seem to be as phototactic as the others, and as already stated, phototaxis of *dunce*^{1} and *dunce*^{2} is normal. A fly with outspread wings usually returns them to the normal resting position within 60 seconds. Therefore this trait is probably not due to gross muscular degeneration, as in some *wings up* mutants, but is a behavioral feature of some sort. Its significance is obscure.

2. Phenotypic differences between *dunce*^{1} and *dunce*^{2}.

Two differences between the mutants are observed. The first, a

substantial one, is that dunce^{2} homozygous females are almost fully sterile. The second difference, relatively minor, is that dunce^{2} individuals are less likely to survive to adulthood than dunce^{1} or normal individuals. Genetic mapping shown in figures 10 and 11 demonstrates that these five traits, dunce^{1} learning and fertility and dunce^{2} learning, fertility, and viability map together (± 3 map units) on the genetic map of the X-chromosome. But first the traits themselves must be described.

In early work, the dunce^{2} mutation was propagated solely in the male line by repeated mating with attached-X females. Females homozygous for the X-chromosome carrying dunce^{2} can be generated using the In(1)FM7a balancer chromosome but are so nearly sterile that a homozygous stock can not be established. Most dunce^{2} females produce no offspring but some produce a few. This observation prompted an examination of reproduction in normal and dunce^{1} flies, revealing that dunce^{1} females also have reduced fertility. This difference in dunce^{1} is comparable in size to the variability seen in normal reproduction and had previously escaped notice.

Infertility would result if the females simply were not fertilized. To test this, normal or mutant virgin females (45 of each genotype) were placed individually in observation chambers with one or two normal males. Most of the females participated in the normal rituals of Drosophila courtship, and 73% began copulation within 60 minutes; couplings lasted about 20 minutes, as is usual for Drosophila (3). No statistically significant differences in latency or duration of copulation were seen between normal, dunce^{1}, and dunce^{2} females. Some of the females which had copulated were cultured alone until after apparent exhaustion of

their supplies of stored sperm (within about 10 days). They were kept 15 days in food vials replaced at 5 day intervals. All their adult offspring were counted. Three females did not survive 15 days and their results were discarded. The results of the 67 survivors are plotted in figure 8, in which each point represents the total reproduction by one female after one fertilization. Normal females averaged 181 ± 9.1 offspring, dunce^{1} females 116 ± 11.6 (64%), and dunce^{2} females 1.6 ± 0.8 (1%). These differences are statistically significant; the statistical probability of equal fertility in dunce^{1} and normal females is $p<0.001$ (t test; one-tailed). The data of figure 8 may not represent the maximum fecundity after one fertilization, since later experiments suggested that reproduction was limited by the quantity of food available to the offspring. Nevertheless, it is clear that dunce^{1} and dunce^{2} females are less fecund than normal females, even when fertilized, and that the difference is not due to an overt defect in courtship or copulation.

To test whether dunce males are fertile, normal, dunce^{1}, and dunce^{2} males were mated in all combinations with normal, dunce^{1}, and dunce^{2} females. In each case, normal females were fertile, dunce^{1} females were intermediate, and dunce^{2} females were almost sterile. It appears that the three types of males are equally fertile.

Some experiments were performed to explore the nature of the poor fertility of the females. Upon dissection, normal, dunce^{1}, and dunce^{2} females (15 of each) were found to possess ovaries of similar size and appearance. Each normal ovary contained about a dozen mature eggs; ovaries of dunce^{1} and dunce^{2} contained sometimes a dozen, but more usually four to ten mature eggs each. Ovaries of dunce^{2} females were

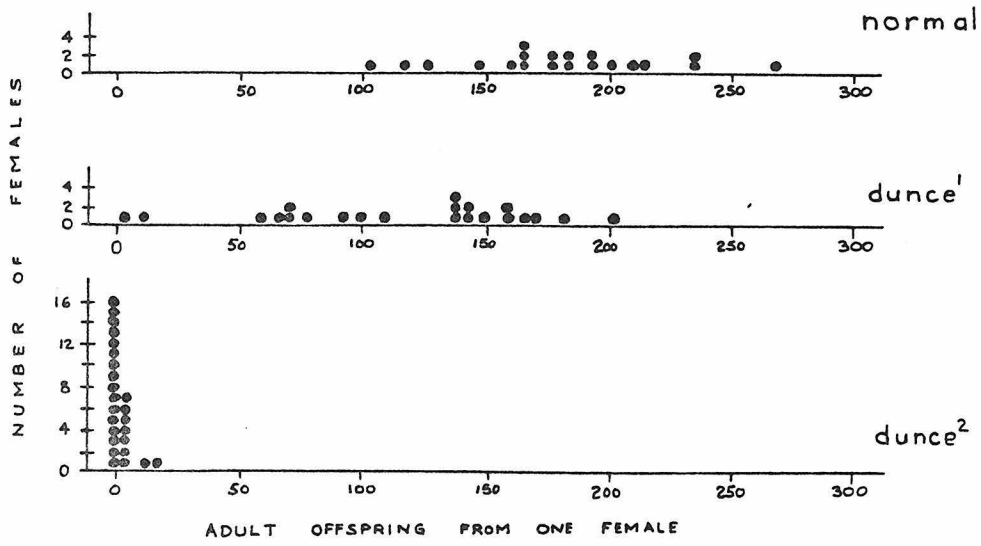

Figure 8. Reproduction by normal, dunce[1], and dunce[2] females fertilized once by a normal male. After fertilization the females were cultured alone until after apparent exhaustion of the supply of stored sperm, and all their adult offspring were counted.

as well stocked with mature eggs as ovaries of dunce[1]. When groups of 20 normal fertilized females were offered a yeast-covered agar-with-sucrose surface in a dark, humid, quiet chamber, they deposited eggs at the rate of about 50 per female per day. In the same conditions, after fertilization by normal males, dunce[1] females averaged 12 eggs per day (24%) and dunce[2] females 4 per day (8%). Thus dunce[1] and dunce[2] females produce and lay eggs, though more slowly than normal. Observation of 800 eggs laid by normal females showed that 97% hatched and 88% reached adulthood; those adults were 53% male. Of 800 eggs laid by dunce[1] females (fertilized by normal males), 76% hatched and 70% reached adulthood (49% male). Only 85 eggs of dunce[2] females (by normal males) were

obtained for observation; of these, 32% hatched and 5% (2 males and 2 females) reached adulthood. These observations do not give a simple picture of the dunce infertility. The mutant ovaries contain fewer mature eggs, fewer eggs are laid, fewer of the laid eggs hatch, and in the case of $dunce^{2}$ a smaller fraction of the newly-hatched larvae reach adulthood. It is as if the normal dunce gene product is used at more than one step of normal reproduction.

The experiments just described measured the viability of the offspring of dunce females fertilized by normal males; of these offspring the males were dunce and the females were heterozygous +/dunce. In the case of $dunce^{2}$, even the $+/dunce^{2}$ offspring were very poorly viable (5%). Separate experiments were conducted to measure the viability of dunce offspring of normal females. Virgin attached-X females were mated with normal, $dunce^{1}$, or $dunce^{2}$ males. Two classes of adult offspring appear from such crosses: females with the maternal attached-X chromosome and males with the paternal single X. Since the attached-X offspring should have constant viability, the relative viability of the male offspring in the different crosses may be compared using sex ratios. About 3000 adult progeny were counted from each cross. The viability of $dunce^{1}$ was 92(±4)% and $dunce^{2}$ 69(±4)% of normal (Canton-S), arbitrarily 100%. That is $dunce^{2}$ viability is reduced by about one-third, even when the mothers are genetically normal.

3. Genetic mapping of $dunce^{1}$ and $dunce^{2}$.

Once the viability and fertility phenotypes of the dunce stocks were characterized, it became necessary to test whether the additional phenotypes were consequences of the learning mutations or of indepen-

dent X-linked mutations. (Independent autosomal mutations would not have persisted in the stock with the breeding regimen used.) Figure 9 is a genetic map of the Drosophila X-chromosome showing the approximate location of $dunce^{1}$. If the mutation causing poor fertility in the $dunce^{1}$ stock is to the right of chocolate then recombinants between dunce and chocolate should have good learning and poor fertility or good fertility and poor learning. Even if an independent mutation were near dunce then some recombinants between yellow and chocolate might separate learning from fertility.

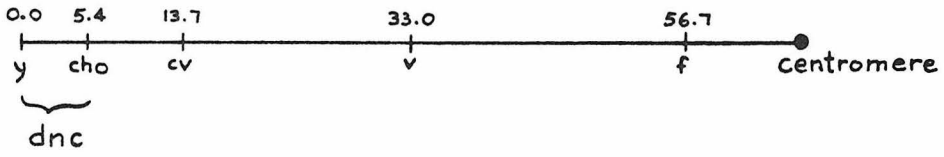

Figure 9. Genetic map of the X-chromosome of Drosophila, showing the loci of the mutants yellow(y), chocolate(cho), crossveinless(cv), vermilion(v), and forked(f). In Chapter 2 it was shown that $dunce^{1}$(dnc) maps between yellow and chocolate.

To generate the appropriate recombinants, $dunce^{1}$ males were mated with homozygous y cho cv v f females and the resulting heterozygous dnc^{1}/y cho cv v f females were mated with normal males. The male recombinant progeny (either y^{+} cho cv v f or y cho^{+} cv^{+} v^{+} f^{+}) were mated singly with attached-X females and the resulting stocks were cultured for three generations, yielding many X-isogenic male descendents of each original recombinant male. The learning ability of these males was tested in the usual way. In control experiments y cho cv v f males

had normal learning ($\Lambda = 0.30 \pm 0.02$; n=16), indicating that the marker genes used do not disturb learning ability. To generate females for tests of fertility, male descendents of the original recombinant males were mated with homozygous dunce^{1} females; the progeny would have normal or poor fertility, depending on whether the male carried dunce or dunce^{+} (because dunce infertility is recessive; Table 5 of Chapter 4). Fertility was measured by culturing individual females with three males for six days, then counting all adult offspring appearing within 17 days of culture at 25°; the results for females which did not survive six days were discarded. The learning performance and fertility of the recombinants are shown in figure 10a. The two traits appear to map together between yellow and chocolate.

For dunce^{2}, it was not known whether any of the phenotypes would map between yellow and chocolate. By crosses similar to those just described for dunce^{1}, a set of dunce^{2} recombinants with crossovers distributed along the X-chromosome was obtained. The fertility of recombinant/dunce^{2} heterozygous females was tested as with dunce^{1}, with one modification: the difference between normal fertility and dunce^{2} sterility is so great that the offspring of the fertile females were not counted as in the dunce^{1} crosses, but merely estimated in a glance. (In figure 10b the fertile females were arbitrarily assigned 50 offspring, a number not far from the true number.) The results demonstrated that dunce^{2} sterility also maps between yellow and chocolate. To test whether learning maps with sterility, the learning performance of some of the recombinants with crossovers between yellow and chocolate was measured. The correlation of learning with fertility is plotted in figure 10b. It appears that learning and fertility of dunce^{2} map to-

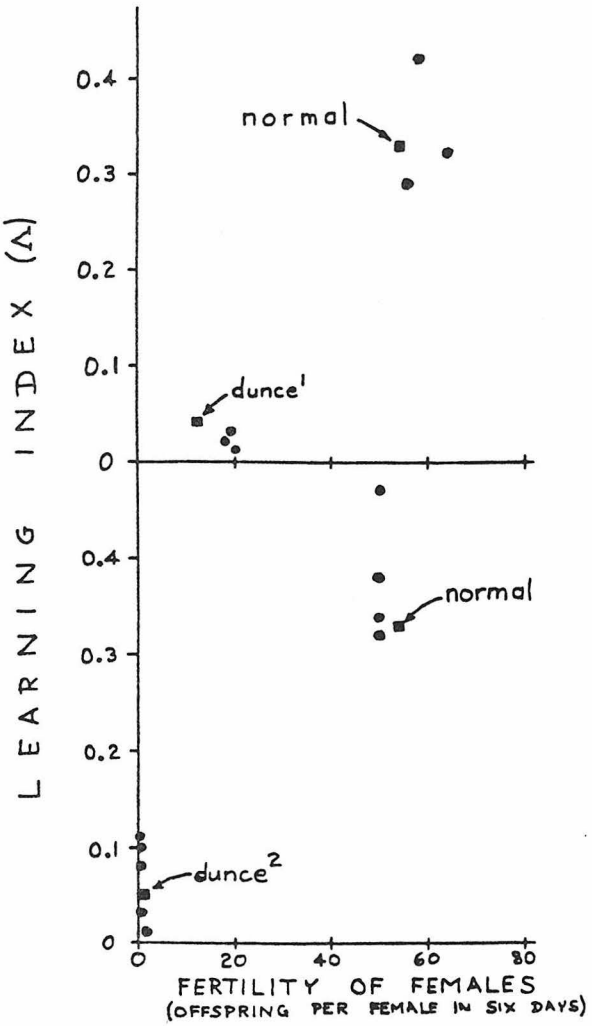

Figure 10. Mapping of learning relative to fertility in <u>dunce</u>[1] and <u>dunce</u>[2] recombinants. The squares (■) represent the parental types. Each circle (●) represents one recombinant with crossover between <u>yellow</u> and <u>chocolate</u>. The recombinant learning scores are the means of 3 to 6 tests and each fertility score is the mean of 17 to 20 females in (a) and 7 to 10 females in (b).

gether between yellow and chocolate. The viability of the same recombinants was tested by mating males from the recombinant lines with virgin attached-X females and recording the sex ratios among the progeny. The results, plotted in figure 11, show that the viability phenotype maps with the others. No attempt was made to map the wing posture trait described earlier in this chapter.

These results do not reveal any separation of $dunce^{1}$ and $dunce^{2}$ phenotypes but do not necessarily show that the poor learning and infertility are due to the same genes because the numbers of recombinants tested are very small, and independent but closely-linked mutations on the $dunce^{1}$ or $dunce^{2}$ chromosomes would not have been separated. A complementation test of learning and fertility in $dunce^{1}/dunce^{2}$ heterozygous females is described in the next chapter.

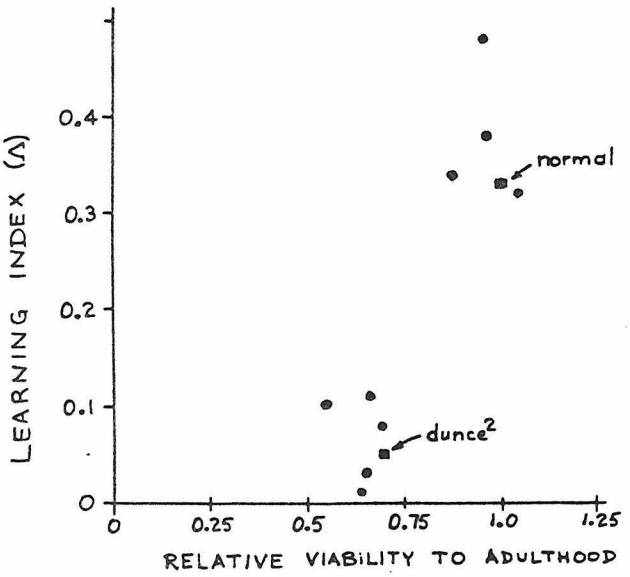

Figure 11. Mapping of learning relative to viability in the $dunce^{2}$ recombinants of figure 10b. Symbols are as in figure 10. Normal (Canton-S) viability is arbitrarily placed at 1.0.

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Chapter 4

Two Independent Discoveries of the *dunce* Gene

In 1978, knowledge of the function of the *dunce* gene was greatly advanced by the discovery that the same gene was independently known in two other ways by two other groups. Mohler of the University of Iowa found 225 X-linked female-sterile mutants, including two now known to be allelic with *dunce*. Kiger and Golanty of the University of California at Davis identified a small chromosomal region containing a gene which appears to control a cyclic AMP phosphodiesterase of *Drosophila*; *dunce* is that gene. This chapter presents the genetic and behavioral evidence for these conclusions, and Chapter 5 presents the biochemical data on cyclic AMP phosphodiesterase activity of $dunce^{1}$ and $dunce^{2}$.

1. The origin of the alleles $dunce^{M11}$ and $dunce^{M14}$.

About 1974, Kiger and Golanty set out to locate the genes specifying or regulating the cyclic AMP phosphodiesterase activity of *Drosophila*. They used segmental aneuploids (1), flies in which a chromosomal segment is duplicated or deficient. Since the amount of a gene product is often proportional to the "dose" of the gene, assay for a specific enzyme in the nearly complete set of *Drosophila* segmental aneuploids can usually reveal the location of a specific gene (2,3). Problems arise when two or more enzymes have the same activity, as appears to be the case for *Drosophila* cyclic AMP phosphodiesterase (see Chapter 5). Nonetheless, Kiger and Golanty (4) found a region controlling a large part of the activity and mapped it within bands 3D3 and 3D4 of the

X-chromosome. Females heterozygous for a certain pair of overlapping deletions were entirely deficient in five adjacent bands including these two. The females had no visible abnormalities, but most were sterile and the others produced only a few offspring each (5). The two papers (4,5) describing this work appeared in April 1977 and came to my attention in May 1978. The widely-suspected involvement of cyclic AMP in learning, the coincidence of poor female fertility, and the coincidence that 3D3 and 3D4, like *dunce*, are between *yellow* and *chocolate* on the genetic map immediately prompted the hypothesis that the gene affecting phosphodiesterase activity and the *dunce* gene could be identical. In further support of this hypothesis, I knew then that *dunce* (like 3D3 and 3D4) is outside the limits of the duplication *white*$^{+}$*Y* (see below).

The finding in region 3D3 and 3D4 of a gene affecting fertility and a gene affecting cyclic AMP phosphodiesterase does not prove that the phosphodiesterase and fertility genes are one. The relationship between the traits could be examined by assaying the enzyme levels in female-sterile point mutants within 3D3 and 3D4. For this purpose, Davis and Kiger (unpublished) obtained some of the 225 X-linked female-sterile mutants isolated by Mohler (6,7). An allelic pair of these mutants proved to map at 3D3 or 3D4 and to have abnormal cyclic AMP phosphodiesterase activity. It thus seemed very likely that the infertility and altered enzyme activity were phenotypic traits of one gene.

I then obtained the two mutants of Mohler to test their learning and their ability to complement *dunce*^{1} and *dunce*^{2}. The two mutants were originally named 11-761 and 14-756. With knowledge of our results (in Table 5) Mohler renamed them *dunce*^{M11} and *dunce*^{M14}, respectively.

Both were induced by mutagenesis with ethylmethanesulfonate(EMS) on X-chromosomes bearing the recessive mutations yellow(y), crossveinless (cv), vermillion(v), and forked(f). Before learning was tested, they and a y cv v f strain from Mohler were crossed for two generations with strains derived from the normal Canton-S strain, in order to reduce possible genetic variation due to chromosomes other than the X. Viability of Mohler's mutants was measured in crosses to attached-X females as described in Chapter 3; the viability of y cv v f $dunce^{M11}$ is 39(±7)% and the viability of y cv v f $dunce^{M14}$ is 56(±12)% relative to y cv v f controls.

2. Complementation test of $dunce^{1}$, $dunce^{2}$, $dunce^{M11}$, and $dunce^{M14}$.

If the female-sterile mutants of Mohler are alleles of dunce, then they should learn poorly when homozygous, and should also show poor learning and fertility when heterozygous with $dunce^{1}$ or $dunce^{2}$. On the other hand, if Mohler's mutations are in a gene other than dunce, then the heterozygotes with $dunce^{1}$ or $dunce^{2}$ should be fertile.

The olfactory learning scores and fertility statistics of normal (abbreviated as +) and mutant flies, +/mutant heterozygotes, and some combinations of one mutant with another are presented in Table 5. The learning score of normal flies is $\Lambda = 0.33$; y cv v f flies do less well ($\Lambda = 0.23$). The mutants $dunce^{1}$, $dunce^{2}$, $dunce^{M11}$, and $dunce^{M14}$ all show poor learning ($\Lambda = 0.04$, 0.05, 0.01, -0.01, respectively).

Phototaxis by y cv v f, y cv v f $dunce^{M11}$, and y cv v f $dunce^{M14}$ flies is poor, respectively 68%, 65%, and 62% of normal. Such large reductions of phototaxis interfere with the measurement of learning (8) and may account for the low score of y cv v f. However the approximate

equality of the reductions in phototaxis shows that little of the change is due to the dunce mutation itself.

The +/dunce heterozygotes do not learn as well as +/+ homozygotes (Table 5) and thus dunce is incompletely recessive in learning (i.e. one normal gene is insufficient for normal learning). The learning scores of flies heterozygous for any two different dunce mutations are low (Λ = 0.07, 0.06, 0.06 for the three different heterozygous genotypes tested). This is consistent with allelism but inconclusive because two different incompletely recessive mutants, even if not allelic, might add their effects. That ambiguity was resolved by the tests of fertility of the heterozygotes.

Table 5. Complementation test of $dunce^1$, $dunce^2$, $dunce^{M11}$, and $dunce^{M14}$.

Genotype	Learning Index (Λ)[a]	Fertility of Females (adult offspring per female in six days)[b]		
+ (Canton-S)	0.33 ±0.01	(n=463)	54 ±2.5	(n=36) (100%)
y cv v f	0.23 ±0.04	(n=6)	58 ±10	(n=10)
$dunce^1$	0.04 ±0.01	(n=88)	12 ±1.8	(n=36) (22%)
$dunce^2$	0.05 ±0.01	(n=47)	0.9 ±0.39	(n=32) (1.6%)
$dunce^{M11}$	0.01 ±0.02	(n=6)	0.06 ±0.06	(n=64) (0.1%)
$dunce^{M14}$	-0.01 ±0.03	(n=6)	0.0	(n=20)
$dunce^1/dunce^2$	0.07 ±0.02	(n=6)	24 ±2.1	(n=35) (44%)
dnc^1/y cv v f dnc^{M14}	0.06 ±0.03	(n=4)	not tested	
dnc^2/y cv v f dnc^{M11}	0.06 ±0.02	(n=10)	2.4 ±0.96	(n=39) (4.4%)
+/$dunce^1$	0.26 ±0.02	(n=9)	60 ±2.7	(n=36)
+/$dunce^2$	0.25 ±0.05	(n=9)	62 ±2.7	(n=35)
+/y cv v f $dunce^{M11}$	0.19 ±0.03	(n=7)	46 ±3.0	(n=44)
+/y cv v f $dunce^{M14}$	0.21 ±0.01	(n=4)	not tested	

a. mean ± s.e.m. of n tests of learning.
b. mean ± s.e.m. of n females.

Fertility was measured by culturing single females with three normal males each for six days, then counting all adult offspring appearing within 17 days of culture at 25°; the results for females not surviving six days were discarded. Reproduction by normal females was 54 offspring per female (Table 5), an arbitrary score limited by the quantity of food (5 ml.) available to the progeny. Under the same conditions, $dunce^{1}$ females averaged 12 offspring and the other mutants one or fewer each. Infertility of the dunce mutants is recessive; that is one normal gene is sufficient for normal fertility. Therefore the poor fertility of $dunce^{1}/dunce^{2}$ and $dunce^{2}/dunce^{M11}$ females provides good evidence that $dunce^{1}$, $dunce^{2}$, and $dunce^{M11}$ are alleles of one gene.

3. Mapping of dunce with genetic deficiencies and duplications.

The finding of allelism, taken with the finding of Davis and Kiger that $dunce^{M11}$ and $dunce^{M14}$ map at 3D3 or 3D4, leads to two definite predictions: $dunce^{1}$ and $dunce^{2}$ should map within region 3D3 and 3D4, and deletions of 3D3 and 3D4 should act like mutant alleles of dunce and cause poor learning.

In the upper part of figure 12 are illustrated some of the deficiencies and duplications used by Kiger and Golanty. The lower part of the figure shows a portion of the X-chromosome between yellow and chocolate (see figure 9) drawn by Beerman (9) from the giant chromosomes of the larval salivary gland, with bands numbered according to the system of Bridges (10). Bridges counted 1012 bands on the X; the 25 bands shown in figure 12 thus represent about 2.5% of the cytological length of the X-chromosome. The locations of the genes white(w), Notch(N), and diminuitive(dm) are indicated.

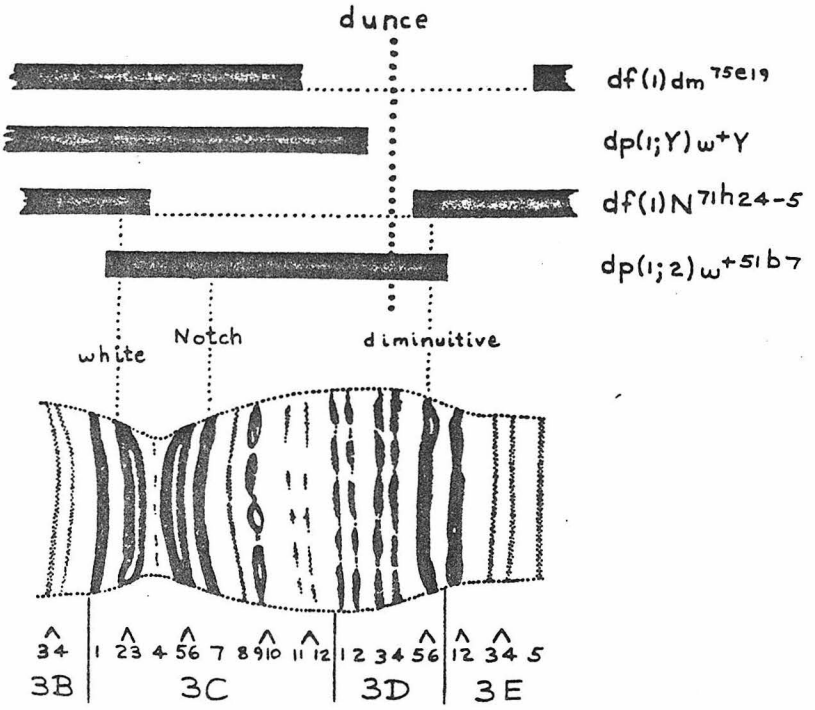

Figure 12. Mapping of dunce with genetic deficiencies(df) and duplications(dp). The location of dunce is based on the genetic evidence of Tables 6-9. The infertility and cyclic AMP phosphodiesterase traits of dunce were first mapped within 3D3 and 3D4 by Kiger and Golanty (4,5). The rearrangement breakpoints were located by Lefevre (11) and are shown in the upper part of the figure; the presence of chromosomal material in a deficiency or duplication chromosome is indicated by a dark horizontal band and the absence of it by a gap. The drawing of a portion of the salivary gland X-chromosome in the lower part of the figure is reproduced from Beerman (9). The region shown is approximately 2.5% of the length of the salivary gland X-chromosome.

The cytological analysis of deficiency(df) and duplication(dp) breakpoints used in the mapping of dunce is by G. Lefevre (11) who suggests that breakpoint assignments be considered as having a possible error of one band in either direction (personal communication). Deficiency(1)diminuitive^{75e19*} is a deletion of bands 3C12 through 3E4; deficiency(1)Notch$^{71h24-5}$ is a deletion of bands 3C4 through 3D4; duplication(1;Y)white^{+}Y is a Y chromosome with X-chromosomal bands 2D2 through 3D2 or 3D3 attached to it; and duplication(1;2)white^{+51b7} is a second chromosome with X-chromosomal bands 3C2 through 3D6 inserted at region 52F. Before learning was tested, these strains were crossed for two generations with C(1)DX(attached-X) or In(1)FM7a strains carrying Canton-S autosomes in order to reduce possible genetic variation due to chromosomes other than the X.

For mapping of dunce, the deficiency or duplication strains were crossed with normal or dunce strains or with each other. Not all possible combinations were tested, but the results obtained do lead to an unambiguous conclusion. Complete results of all combinations tested are included in Tables 6, 7, 8, and 9. Learning was measured as previously described. Fertility was measured as for the complementation test of Table 5, with one modification. When a sterile female was cultured in the standard medium previously used, the bacteria, yeast, and absence of larvae caused the food to crack and become treacherous so that up to half of the females were trapped and dead within a few days. Larvae rendered the food safer and few fertile females did not survive six days. Normal virgin females were also likely to become

* 75e19 and other numbers of this format represent dates in Drosophila mutant names, in this case 19 May 1975.

trapped and therefore the problem did not seem to be due to any special weakness of the mutants. To counteract the problem, a diluted culture medium was used. It dried and cracked more slowly, and most sterile females survived six days. However, the quantity of food available to the offspring severely limited the maximum reproduction of the fertile females, usually to 10 to 40 progeny after six days of egg-laying. For every mutant except $\underline{dunce}^{1}$, the resolution of this test was sufficient for unambiguous classification. The females that died during the six days were excluded from the summaries of results; their inclusion would have altered the quantitive scores slightly, but not the conclusions drawn.

The results of the mapping can be simply considered in the order of the breakpoints along the chromosome. Table 6 shows the results for $\underline{df(1)diminuitive}^{75el9}$. The $\underline{+}$/deficiency heterozygotes had good learning ($\Lambda = 0.26$), comparable to $\underline{+}/\underline{dunce}$ heterozygotes. The $\underline{dunce}^{2}$/deficiency flies learned poorly ($\Lambda = 0.02$). The $\underline{+}$/deficiency females were fertile, though rather weakly, and the $\underline{dunce}^{M11}$/deficiency and $\underline{dunce}^{2}$/deficiency

Table 6. Mapping of $\underline{dunce}$ vs. $\underline{df(1)diminuitive}^{75el9}$.

Genotype	Learning Index (Λ)	Fertility of Females (adult offspring per female in six days)
$\frac{+}{df(1)dm^{75el9}}$	0.26 ±0.03 (n=5)	14 ±1.1 (n=20)
$\frac{y\ cv\ v\ f\ dnc^{M11}}{df(1)dm^{75el9}}$	not tested	0.3 ±0.26 (n=23)
$\frac{dnc^{2}}{df(1)dm^{75el9}}$	0.02 ±0.02 (n=5)	2.6 ±1.0 (n=28)

females were almost sterile. The dunce/deficiency flies would learn poorly if the deficiency uncovered the dunce gene or if the deficiency stock carried one or more dominant mutations elsewhere that disturb learning. The good learning score of the +/deficiency heterozygotes seems to rule out the hypothetical dominant mutations and therefore dunce very probably maps within region 3C12 to 3E4.

Table 7 shows the results with dp(1;y)white^{+}Y(w^{+}Y). The +/w^{+}Y males had normal learning (Λ = 0.34) and dunce^{1}/w^{+}Y males learned poorly (Λ = 0.05). Through the sequence of crosses diagrammed in figure 13, it was possible to recover a few females carrying the w^{+}Y. The dunce^{2}/dunce^{2}/w^{+}Y and dunce^{M11}/dunce^{M11}/w^{+}Y females were sterile and control FM7a/dunce^{M11}/w^{+}Y females were fertile. Thus the white^{+}Y duplication appears not to include the dunce gene, which must therefore map to the left of 2D2 (outside figure 12) or to the right of 3D2.

Table 8 shows the results for df(1)Notch$^{71h24-5}$. The +/deficiency females had a learning score lower than is usual for dunce heterozygotes (Λ = 0.18), but well above typical scores of dunce homozygotes. The dunce^{1}/deficiency and dunce^{2}/deficiency flies learned poorly (Λ = 0.05 and 0.02, respectively). The +/deficiency females were fertile and the dunce^{2}/deficiency females were sterile. The interpretation of learning scores given for df(1)diminuitive^{75e19} applies here also and therefore the dunce gene very probably maps within the boundaries of the Notch$^{71h24-5}$ deletion.

Since the dunce gene is deleted in df(1)diminuitive^{75e19} and df(1)Notch$^{71h24-5}$, the females heterozygous for the two deficiencies should express the most extreme possible dunce phenotype. These females were generated by the cross illustrated in figure 14, and appeared

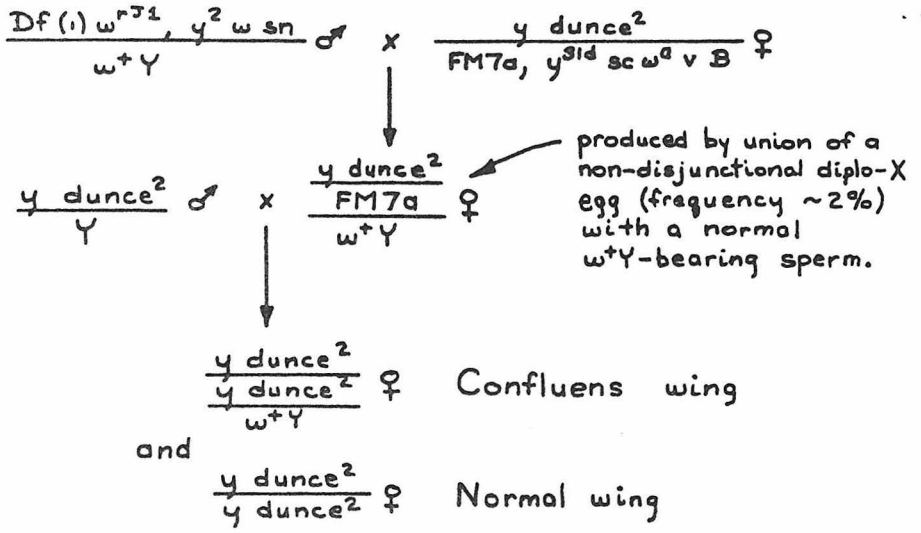

Figure 13. Sequence of matings producing homozygous dunce females carrying the white$^+$Y(w^+Y) duplication. The fertility of these females is listed in Table 7. Other classes of progeny are not shown. FM7a is a multiply-inverted X-chromosome used here because it increases the frequency of primary non-disjunction, suppresses recombination, and carries the dominant mutation Bar(B). The Confluens wing phenotype is due to duplication of the Notch locus at 3C7. Mutant phenotypes are described in the Appendix.

Table 7. Mapping of dunce vs. dp(1;Y)white$^+$Y.

Genotype	Learning Index (Λ)	Fertility of Females (adult offspring per female in six days)
$\frac{+}{w^+Y}$	0.34 +0.07 (n=4)	(male)
$\frac{dnc^1}{w^+Y}$	0.05 +0.02 (n=5)	(male)
$\frac{FM7a}{y\ cv\ v\ f\ dnc^{M11}} / w^+Y$	not tested	23 ± 6.2 (n=8)
$\frac{y\ cv\ v\ f\ dnc^{M11}}{y\ cv\ v\ f\ dnc^{M11}} / w^+Y$	not tested	0.0 (n=9)
$\frac{y\ dnc^2}{y\ dnc^2} / w^+Y$	not tested	0.0 (n=6)

Table 8. Mapping of *dunce* vs. *df(1)Notch*$^{71h24-5}$.

Genotype	Learning Index (Λ)	Fertility of Females (adult offspring per female in six days)
$\frac{+}{df(1)N^{71h24-5}}$	0.18 ±0.04 (n=5)	22 ± 2.6 (n=8)
$\frac{dnc^1}{df(1)N^{71h24-5}}$	0.05 ±0.01 (n=4)	not tested
$\frac{dnc^2}{df(1)N^{71h24-5}}$	0.02 ±0.02 (n=6)	0.0 (n=50)

among the progeny about half as frequently as their normal sisters. They were detected because they express the dominant phenotype of notched wings, due to heterozygous deficiency of the *Notch* locus at 3C7, but they are otherwise of normal appearance. They are vigorous, apparently healthy, and have good phototaxis (85% of normal). As shown in the top row of Table 9, they learn poorly (Λ = 0.03), and all so far examined by me have been entirely sterile.* From the same cross (figure 14) were obtained females heterozygous for the same two deficiency X-chromosomes, but carrying the *dp(1;2)white*$^{+51b7}$ second chromosome and thus having one dose of bands 3D3 and 3D4. They have a learning score typical of *dunce* heterozygotes (Λ = 0.26) and are fertile (Table 9). The same duplication restores learning and fertility to *dunce*^{2}/*df(1)N*$^{71h24-5}$ females (Table 9). Therefore this duplication carries a normal allele of the *dunce* gene.

* Kiger (5) reported that 8 of 20 females of this genotype produced some offspring, but more recently all observed by him also have been sterile (personal communication).

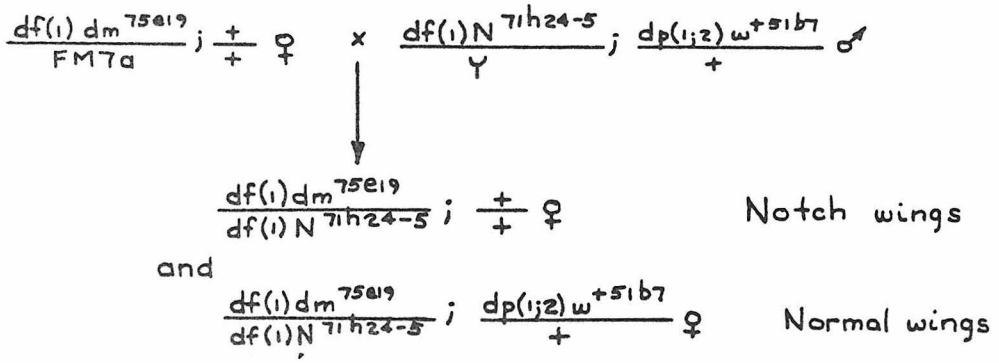

Figure 14. Scheme of the cross producing females with homozygous deficiency of the five bands from 3C12 to 3D4 inclusive. The deficiencies and duplications are illustrated in figure 12. The Notch wings are due to heterozygous deficiency of the Notch locus at 3C7.

Table 9. Behavior of flies with homozygous deficiency of the dunce locus and mapping of dunce vs. dp(1;2)white^{+51b7}.

Genotype	Learning Index (Λ)	Fertility of Females (adult offspring per female in six days)
$\frac{df(1)dm^{75e19}}{df(1)N^{71h24-5}}$; $\frac{+}{+}$	0.03 ±0.03 (n=6)	0.0 (n=30)
$\frac{df(1)dm^{75e19}}{df(1)N^{71h24-5}}$; $\frac{dp(1;2)w^{+51b7}}{+}$	0.26 ±0.03 (n=6)	23 ±1.2 (n=49)
$\frac{dnc^{2}}{df(1)N^{71h24-5}}$; $\frac{+}{+}$	0.02 ±0.02 (n=6)	0.0 (n=50)
$\frac{dnc^{2}}{df(1)N^{71h24-5}}$; $\frac{dp(1;2)w^{+51b7}}{+}$	0.27 ±0.07 (n=3)	18 ±1.9 (n=10)

Taken together, the results with df(1)diminuitive^{75e19}, df(1)Notch$^{71h24-5}$, dp(1;Y)white^{+}Y, and dp(1;2)white^{+51b7} indicate that the dunce gene maps within region 3D3 and 3D4.

There exists a rearrangement that separates 3D3 from 3D4. It is deficiency(1)Notch^{64j15}, which is a deletion of bands 3C4 through 3D3 (Lefevre, 11). Kiger and Golanty (12) found that the cyclic AMP phosphodiesterase activity of df(1)Notch^{64j15}/df(1)diminuitive^{75e19} females was intermediate (62%) between the normal level in +/+ females (100%) and the reduced levels in df(1)Notch$^{71h24-5}$/df(1)diminuitive^{75e19} females (29%). Kiger (5) recorded that df(1)Notch^{64j15}/ df(1)diminuitive^{75e19} females had a curious type of intermediate fertility; 80% of the individual females were normally fertile and 20% were sterile. Among the control females no individuals were sterile. I have observed the same sort of mixed fertility in df(1)Notch^{64j15}/ df(1)diminuitive^{75e19} females and also found that they learn poorly. All these findings might suggest that df(1)Notch^{64j15} has a dunce gene with partial function but are equally well-explained on the hypothesis that the strains of df(1)Notch^{64j15} used were genetically contaminated with one of the other deficiencies. This second hypothesis is simple to test, but I have not yet had the opportunity and Kiger apparently did not consider it.

The mutant diminuitive has been mapped to band 3D5 (13) and therefore must be close to dunce. Because female-sterility is among the traits of diminuitive, one must consider the possibility that dunce and diminuitive are allelic mutants of one gene. If this were so then heterozygous dunce^{1}/diminuitive and dunce^{2}/diminuitive females should be sterile and learn poorly. The data of Table 10 indicate that

dunce^{1}/*diminuitive* and *dunce*^{2}/*diminuitive* females are as fertile and proficient at learning as *dunce*/+ females; that is, the *dunce* and *diminuitive* mutants complement each other. Moreover, *diminuitive* mutant males have normal learning ($\Lambda = 0.42$). The successful complementation and good learning by the *diminuitive* mutants are strong evidence that *dunce* and *diminuitive* are independent genes.

Table 10. Complementation test of *dunce*^{1} and *dunce*^{2} with *diminuitive*.

Genotype	Learning Index (Λ)	Fertility of Females (adult offspring per female in six days)
+/+	0.33 ±0.04 (n=8)[a]	54 ±2.5 (n=36)[b]
+/dm	0.35 ±0.04 (n=3)	52 ±17 (n=10)
dnc^{1}/+	0.26 ±0.02 (n=9)[b]	60 ±2.7 (n=36)[b]
dnc^{1}/dm	0.23 ±0.02 (n=3)	62 ±10 (n=10)
dnc^{2}/+	0.25 ±0.05 (n=9)[b]	62 ±2.7 (n=35)[b]
dnc^{2}/dm	0.22 ±0.01 (n=3)	75 ±3 (n=10)
dm/dm	not tested	0.0 (n=35)
dm/Y	0.42 (n=2)	(male)

a. from Table 4. b. from Table 5.

In the strictest sense, the experimental findings described in this chapter do not prove that the poor learning and infertility are consequences of the same mutations since one might still imagine that a gene required for learning and an independent gene required for fertility lie adjacent in the chromosome, and that *dunce*^{1}, *dunce*^{2}, *dunce*^{M11}, and *dunce*^{M14} are all double mutants or small deletions that eliminate both. The argument against the possibility is its extreme improbability; I conclude that *dunce* is very probably one gene with a role in olfactory learning performance and a role in reproduction.

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Chapter 5

A Chemical Phenotype of the *dunce* Mutants.

Cyclic AMP phosphodiesterases catalyze hydrolysis of the phosphate ring of cyclic AMP (cyclic 3',5'-adenosine monophosphate) yielding 5'-AMP. This chapter presents the evidence that cyclic AMP phosphodiesterase activity is abnormal in the *dunce* mutants. The functions of cyclic AMP and cyclic AMP phosphodiesterase will be considered in Chapter 6.

It appears that cyclic AMP phosphodiesterases have been studied only in relatively impure preparations, and to my knowledge cyclic AMP phosphodiesterase has not been highly purified from any organism. The cyclic AMP phosphodiesterase activity of tissue homogenates is usually found partly in a rapidly sedimenting particulate form and partly in a soluble form (1,2). Fractionation of the soluble activity on sucrose density gradients often separates multiple forms of activity with apparent molecular weights ranging from 130,000 d. to 30,000 d. (3-5). Even a single cell type, cultured cloned astroglioma cells, can yield multiple forms (6). The forms of cyclic AMP phosphodiesterase can differ in activity toward cyclic GMP, and in sensitivity to activation by an ubiquitous calcium binding protein of 10-20,000 d. which is similar in its properties to troponin-C of skeletal muscle (7,8). The relationships between the forms are unknown and it is clear that much remains to be learned about these enzymes.

1. Cyclic AMP phosphodiesterase of *Drosophila*.

Our present knowledge of cyclic AMP phosphodiesterase in Drosophila is due to the work of Kiger, Golanty, and Davis. Much of their work is recent and unpublished.

A crude homogenate of adult Drosophila contains cyclic AMP phosphodiesterase activity (9). After centrifugation of the homogenate at 100,000 x g for 60 minutes, about 70% of the initial activity remains dissolved in the supernatant and additional activity can be recovered from the sedimented pellet of nuclei, mitochondria, and cellular membranes (unpublished). Fractionation of the soluble activity on a sucrose density gradient separates two prominent forms of activity (10), designated form I and form II, as shown for normal females in the solid curve of figure 15. The apparent molecular weights are 96,000 d. for form I and 38,000 d. for form II (10). According to the studies of Davis (unpublished) on separated forms, form I hydrolyzes cyclic AMP ($K_m \sim 4\mu M$) and cyclic GMP ($K_m \sim 4\mu M$) and form II hydrolyzes cyclic AMP ($K_m \sim 2\mu M$) but is ineffective on cyclic GMP.

Davis finds that either form remains pure when recentrifuged and does not regenerate the other (unpublished); this suggests, but does not prove, that the occurrence of two forms is not simply an artefact due to aggregation. Whether the two forms share subunits is unknown. The apparent relative abundance of the two forms is sensitive to changes in the ionic composition of the solutions used during purification (10); the relative abundance in living flies is not known.

Homogenates of $\underline{dunce}^{M11}$ and $\underline{dunce}^{M14}$ flies have approximately half the normal level of cyclic AMP phosphodiesterase activity. Fractionation of the soluble activity of $\underline{dunce}^{M11}$ and $\underline{dunce}^{M14}$ in sucrose density gradients reveals that form II is absent from the gradients and

and form I is approximately normal (Davis, unpublished).

The conclusion of Kiger and Golanty (10) that form II is absent from flies with homozygous deficiency of band 3D4 is unconvincing and indirect, involving differential activation by added magnesium ion and inactivation by heat. The heat inactivation curves are variable and include unexpected and unexplained plateaus that make interpretation difficult. The magnesium activation curves (which run from 10^{-7}M to 10^{-1}M Mg^{++}) are compromised by the lack of chelating buffers at low Mg^{++} concentrations. Since the Mg^{++} concentration in living tissues is usually several millimolar (11,12), and since the homogenates were assayed at concentrations of 1 fly (with volume $\sim$ 1µl.) per ml. of homogenate (10), the magnesium activation experiments probably never actually extended below about 0.5×10^{-5}M Mg^{++}. On the one published sucrose density gradient for deficiency flies (10), the peaks of activity range from 5000 to 6000 counts per minute in a background of 4000 counts per minute and the results for the deficiency flies in the fractions corresponding with form II are so variable that a reliable conclusion on the activity of form II can not be reached. To my knowledge, careful direct measurements of the activity of form II in deficiency flies have not been made.

2. Cyclic AMP phosphodiesterase of dunce.

Since $dunce^{M11}$ and $dunce^{M14}$ lack form II on sucrose gradients (see above), and since $dunce^{1}$ and $dunce^{2}$ are alleles of $dunce^{M11}$ and $dunce^{M14}$ (Chapter 4), one may predict that $dunce^{1}$ and $dunce^{2}$ should be missing form II on sucrose gradients. R. Davis, Kiger, and I jointly tested this prediction in Kiger's laboratory at Davis, California.

Groups of 40 adult females were homogenized at 0° in 1.0 ml of 40 mM tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM $CaCl_2$, and 2 mM 2-mercaptoethanol; the same buffer was used in all succeeding steps. The homogenates were centrifuged at 105,000 x g for 60 minutes, then 0.2 ml. portions of the supernatants were layered on 4.6 ml. 5 to 20% sucrose density gradients and centrifuged at 165,000 x g for 18 hours. The gradients were divided into 0.18 ml. fractions and 0.08 ml. of each fraction was mixed with 0.02 ml. of 0.5 mM tritium-labelled cyclic AMP (New England Nuclear), incubated at 30° for 30 minutes, then heated at 90° for 2 minutes to stop the reaction. To each fraction was added 0.1 ml. of diluted venom (Sigma V-7000; 1 mg./ml.) from the Western Diamondback Rattlesnake, Crotalus atrox; this venom has a nucleotidase that converts 5'-AMP to adenosine but has no cyclic AMP phosphodiesterase. The cyclic AMP (and 30% of the adenosine) in each fraction was adsorbed with 1 ml. of Dowex-1 ion exchange resin (33% in 40% methanol: 60% water) and sedimented in a clinical centrifuge. The radioactive adenosine remaining in the supernatant of each fraction was counted by liquid scintillation spectrophotometry. Blank controls were treated identically except that no fly homogenate was added; the radioactivity in the blanks (4100 counts per minute) was subtracted from each fraction. The peak fractions contained about 15,000 counts per minute before correction. The cyclic AMP phosphodiesterase activity of each fraction was computed and corrected for the 30% underestimation of adenosine. Taking dilution factors into account, the cyclic AMP phosphodiesterase activity summed across the gradient fractions was equal to about half of the activity initially applied to each gradient.

The results are illustrated in figure 15. The Canton-S females

show the two forms of cyclic AMP phosphodiesterase of normal Drosophila. On the dunce^{1} and dunce^{2} gradients form I is normal and form II is nearly absent; the shoulders of activity at the position of form II may represent residual activities of about 10% or may be a third phosphodiesterase form.

In the experiments of figure 15, cyclic AMP phosphodiesterases were assayed at 10^{-4} M cyclic AMP, a concentration 25 to 50 times greater than the K_m's of the enzymes. Kauvar and Shotwell of this laboratory at Pasadena have repeated the experiments of figure 15 with adult Canton-S and dunce^{2} males and females, using 2×10^{-6} M cyclic AMP and with certain other modifications. They observed gradient patterns qualitatively similar to those for Canton-S and dunce^{2} in figure 15 (personal communication).

3. Cyclic AMP of dunce.

Since cyclic AMP phosphodiesterase hydrolyzes cyclic AMP, one might expect that the total amounts of cyclic AMP in the dunce mutants would be abnormally high. Davis and Kiger measured cyclic AMP concentration in normal flies and in flies with deficiency or duplication of region 3D3 and 3D4. In flies with homozygous deficiency of 3D3 and 3D4, cyclic AMP concentrations were increased several-fold over normal. Concentrations were slightly greater than normal in flies with heterozygous deficiency of 3D3 and 3D4 and were slightly lower than normal in flies with duplications of 3D3 and 3D4 (13).

Cyclic AMP levels of the dunce mutants have recently been assayed by the same methods. Cyclic AMP was measured by radioimmunoassay (from New England Nuclear) in which Drosophila cyclic AMP competed with

iodine^{125}-labelled cyclic AMP for antibody binding sites. Groups of 4-6 adults were homogenized at room temperature within 2 seconds after immersion in 0.1 M HCl (pH 1.1). The homogenates were heated for 2 minutes in a bath of boiling water, allowed to cool, then cleared by centrifugation at 27,000 x g for 15 minutes. The supernatant was made 0.05 M in sodium acetate, neutralized to pH 6.2 with sodium hydroxide, and recentrifuged. The supernatant was diluted with 0.05 M sodium acetate pH 6.2, and frozen at -80° until needed for the radioimmunoassay. Recoveries were estimated by adding a small amount of tritium-labelled cyclic AMP to the homogenates and were typically 95 to 100%. There are at least two possible sources of systematic error in these measurements: (1) any cyclic AMP tightly bound to protein in a way stable to heat and acid would not have been detected and (2) the tissue cyclic AMP levels may have altered during homogenization. These possibilities are uncontrolled in all the methods currently used for assay of cyclic AMP and their quantitative importance is unknown (14).

The results with Drosophila are presented in Table 11. From normal adults we were able to extract 1.3 picomoles of cyclic AMP per mg. live weight. From $dunce^{1}$ and $dunce^{2}$ we obtained 1.6 times as much and from $dunce^{M11}$ and $dunce^{M14}$ approximately 6 times as much. This is direct and independent evidence of altered cyclic AMP metabolism in the dunce mutants.

The normal cyclic AMP level, 1.3 pmole per mg. live weight, is equivalent to 1.7 μM when averaged over all intracellular and extracellular water, or 8.7 pmoles per mg. protein (since adult Drosophila are by weight 75% water and 15% protein; H.D. Mitchell, personal communication). In a previous measurement of cyclic AMP in Drosophila

adults, de Reggi and Cailla (15) found 7 pmoles per mg. protein. Our estimate for normal adults agrees closely with theirs and is within the range of concentrations recorded for other whole insects (16,17), and for various insect (18-25) and vertebrate tissues (14).

The evidence in this chapter demonstrates that metabolism of cyclic AMP is abnormal in the dunce mutants. It suggests, but does not prove, that the dunce locus may be the structural gene of cyclic AMP phosphodiesterase II.

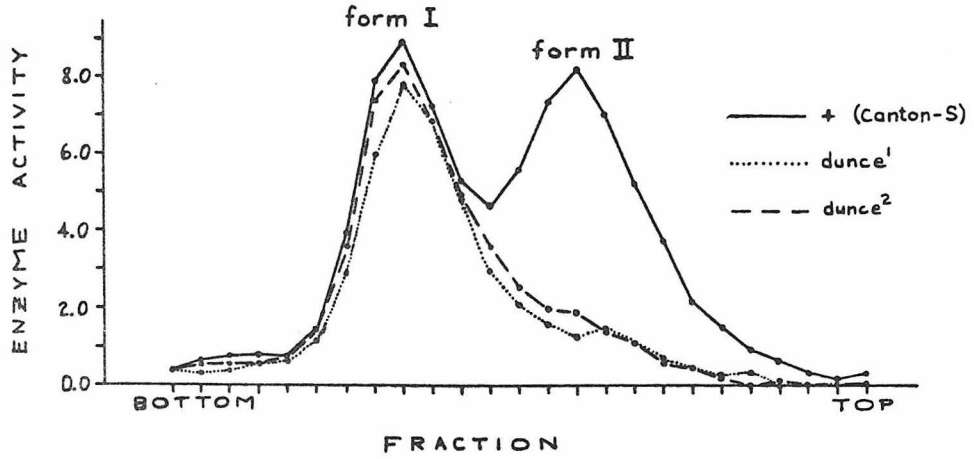

Figure 15. Sucrose density gradient fractionation of soluble cyclic AMP phosphodiesterase activity of normal, dunce[1], and dunce[2] flies. Units of enzyme activity are picomoles of cyclic AMP hydrolyzed per minute per mg. live weight.

Table 11. Cyclic AMP in normal and dunce flies.

Genotype	Cyclic AMP in adult whole fly[a] (pmoles per mg. live weight)	
	Males	Females
+ (Canton-S)	1.3 ±0.1 (n=4)	1.4 ±0.1 (n=6)
y cv v f	1.1 ±0.1 (n=6)	1.2 ±0.1 (n=6)
dunce[1]	2.3 ±0.1 (n=6)	1.9 ±0.2 (n=6)
dunce[2]	2.4 ±0.1 (n=6)	2.1 ±0.1 (n=5)
y cv v f dunce[M11]	5.9 ±1.0 (n=5)	6.7 ±0.5 (n=4)
y cv v f dunce[M14]	6.8 +0.7 (n=6)	8.2 ±0.8 (n=4)

a. Mean ± s.e.m. of n groups of 4-6 flies each.
Data of R. Davis (Byers, Davis & Kiger; manuscript in preparation).

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Chapter 6

Discussion and Conclusion

1. On the cellular function of cyclic AMP

Cyclic AMP was first discovered in 1958 as an intermediating agent in the activation of liver phosphorylase by the hormones epinephrine and glucagon (1). Since then it has been found in the cells of all animals, in fungi, and in bacteria. It appears to be a key regulatory agent in a wide variety of biological processes.

A major role of cyclic AMP in animals is as a "second messenger" or intracellular effector of hormones and neurotransmitters that do not themselves enter the cell. Embedded in the cell membrane of most or all animal cells are hormone receptors that are coupled with membrane-bound adenyl cyclase in such a way that attachment of a specific hormone outside the cell stimulates synthesis of cyclic AMP inside the cell. The hormones and neurotransmitters having specific receptors coupled with adenyl cyclase include epinephrine, norepinephrine, serotonin, dopamine, histamine, octopamine, glucagon, adrenocorticotrophic hormone (ACTH), antidiuretic hormone (ADH or vasopressin), and several others (2-4). Some of these neurotransmitters are known to have other cell surface receptors not coupled with adenyl cyclase (4,5).

Different cells respond in different ways to an increase of intracellular cyclic AMP concentration. Liver cells increase the rate of synthesis of glucose from stored starch; fat cells increase the rate of breakdown of triglycerides to free fatty acids; thyroid cells release thyroid hormone; etc. (2). The evidence summarized in Chapter 1 suggested that in some neurons the efficiency of synaptic transmission changes

in response to an increase of internal cyclic AMP. As a signal, cyclic AMP appears to have no more inherent meaning than do the sodium ions that enter a cell during an action potential.

Greengard (6) and others have argued that the role of cyclic AMP is to activate one or more protein kinases which, by phosphorylating proteins, lead to changes in cellular physiology. Rasmussen and Goodman (7) have summarized the evidence that changes in cellular cyclic AMP concentration often lead to changes in intracellular calcium concentration.

2. The metabolism of cyclic AMP.

Only two metabolic reactions of cyclic AMP have been discovered: the synthesis from ATP by adenyl cyclase and the hydrolysis by cyclic AMP phosphodiesterase. In all its other reactions with cellular proteins, cyclic AMP appears to remain chemically unchanged. The structure of cyclic AMP and the cyclic AMP phosphodiesterase reaction are illustrated in figure 16.

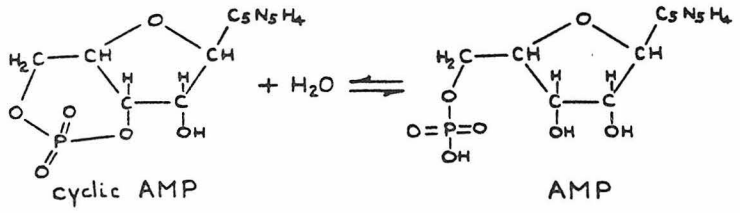

Figure 16. The reaction catalyzed by cyclic AMP phosphodiesterase.

Table 12. The metabolism of cyclic AMP.

Reaction	Standard Free Energy[a]	Ref.
Formation of cyclic AMP		
(1) $ATP + H_2O = PP_i +$ cyclic AMP	ΔG^o = +1.6 kcal/mole	8.
(2) $PP_i + H_2O = 2\ P_i$	ΔG^o = -6.8 kcal/mole	9.
$ATP + 2\ H_2O = 2\ P_i +$ cyclic AMP	ΔG^o = -5.2 kcal/mole	
Hydrolysis of cyclic AMP		
(3) cyclic AMP + H_2O = AMP	ΔG^o = -11.9 kcal/mole	8.

Reaction (1) is catalyzed by adenyl cyclase.
Reaction (2) is catalyzed by pyrophosphatase.
Reaction (3) is catalyzed by cyclic AMP phosphodiesterase.

a. At 25°, pH 7.3.

The free energies of synthesis and hydrolysis of cyclic AMP have been measured and are shown in Table 12. Using these values and assuming a temperature of 25°, a pH of 7.3, and physiological concentrations of other metabolites (adenosine triphosphate (ATP), 2 mM; adenosine monophosphate (AMP), 0.1 mM; orthophosphate (P_i), 1 mM; and pyrophosphate (PP_i), 10^{-11}M assuming pyrophosphate is in equilibrium with orthophosphate; 10,11), one may calculate that if the adenyl cyclase were active, and the phosphodiesterase inactive, then the concentration of cyclic AMP would tend toward a very high level, until essentially all of the ATP was converted to cyclic AMP. If only the cyclic AMP phosphodiesterase were active, then the concentration of cyclic AMP would eventually reach equilibrium at 10^{-13}M. The concentration of cyclic AMP inside cells is not known with accuracy, but is believed to be in

the vicinity of 10^{-6} or 10^{-7}M. Therefore both adenyl cyclase and cyclic AMP phosphodiesterase appear to be active in determining cyclic AMP concentration. Any change in the relative activity of the two enzymes would be reflected in a change of cyclic AMP concentration.

Some cells, including certain bacterial strains (12) and the red blood cells of birds (13), contain adenyl cyclase and cyclic AMP but no cyclic AMP phosphodiesterase. These cells appear to eliminate cyclic AMP by excretion. Excretion of cyclic AMP may also be important in other cases, since rats excrete more cyclic AMP per day than they contain at any one moment (14).

The observations of Hayaishi, Greengard, and Colowick (8) in Table 12 indicate that the phosphate ring of cyclic AMP includes a high-energy phosphate bond; the standard free energy of hydrolysis of the phosphate ring is greater than the standard free energy of hydrolysis of either of the two high-energy phosphate bonds of ATP.

From these considerations of the metabolism of cyclic AMP, it appears that a main function of cyclic AMP phosphodiesterase is to maintain a low background concentration of cyclic AMP against which hormonally-induced signals can be detected. In principle, it seems possible that hormones could alter cyclic AMP concentration by direct action on the cyclic AMP phosphodiesterase; to my knowledge, no mechanism of this sort has been demonstrated for any extracellular hormone or neurotransmitter. However, since some phosphodiesterases are regulated by calcium ion concentration, hormones or physiological states that alter calcium concentrations could indirectly control phosphodiesterase activity and hence cyclic AMP levels in some cells.

3. On the role of the normal dunce gene in the behavior of Drosophila.

The dunce mutants display two traits that are of major interest in the neurogenetics of learning; they perform poorly at olfactory learning and they lack one form of soluble cyclic AMP phosphodiesterase activity. Normal learning performance must involve numerous biochemical and physiological steps; an important question is whether dunce has a lesion specifically in a molecular mechanism of memory or in a process auxiliary to learning. A second important question is whether the poor learning in dunce is a consequence of aberrant cyclic AMP metabolism.

In Chapter 2 it was speculated that dunce flies might learn using a sensory modality other than olfaction. Dudai and Bicker (15) tested the learning of dunce in the visual learning paradigm of Menne and Spatz (16), using colors as cues and strong vibration as punishment. They observed that $dunce^{1}$ and $dunce^{2}$ learn almost as well as the Canton-S normal strain ($\Lambda = 0.12 \pm 0.02$ for Canton-S, $\Lambda = 0.09 \pm 0.01$ for $dunce^{1}$, and $\Lambda = 0.10 \pm 0.02$ for $dunce^{2}$). This seems to indicate that the dunce gene product is not universally required for learning in Drosophila. However, it is possible the two types of learning might depend on similar biochemical mechanisms but use different enzymes for similar functions. In particular, it is conceivable that the cellular changes that underlie visual learning occur by a cyclic AMP-dependent mechanism but in cells that contain only the form I cyclic AMP phosphodiesterase.

Another experiment addressing the question of which step of the pathway of learning is defective in dunce was carried out by Dudai (17). He showed that dunce flies are able to form an unstable olfactory memory, which decays more quickly than normal memory and which, unlike normal memory, is completely erased by exposure of the flies to a different

odor. In all of the learning tests which led to the isolation of dunce and which were used to characterize the mutant, the experimental design involved exposure to an intervening different odorant B between the last training exposure (odorant A + shock) and the first test exposure (odorant A). Dudai found that dunce flies exposed to odorant A + shock, then no odorant, then odorant A without shock specifically avoided the odorant A; dunce flies exposed for equal periods to odorant A + shock, then odorant B, then odorant A did not specifically avoid odorant A, in accord with all the previous observations on dunce. This observation is additional evidence that dunce flies can detect odorants (see Chapter 2) and suggests that dunce flies can form a transient association between odorant and shock, but cannot consolidate this into a stable memory.

To further explore the role of cyclic AMP in learning in Drosophila, several experimental approaches appear to be feasible. Each of the other five mutants in olfactory learning performance, described in Chapter 1, can be tested for defects in cyclic AMP metabolism. Conversely, if one could isolate mutants with aberrant levels of the other enzymes related to cyclic AMP, one could test their olfactory learning performance. A way of constructing such mutant strains would be to select mutant cells in tissue culture using the methods that have yielded mammalian tissue culture cells with mutations of cyclic AMP metabolism (18). The nucleus of such a mutant cell would then be injected into the polar plasm of a Drosophila egg, and the progeny of the adult which developed from that egg should include individuals bearing the mutation. In addition, one would like to know whether mutant flies with deficient learning performance in other sensory modalities are also defective in cyclic AMP metabolism, but because such mutants have not been identified, these studies must wait.

It would be of interest to test drugs which modify cyclic AMP metabolism for their effects on learning performance of normal flies, and for their ability to rectify the dunce lesions. Several inhibitors of cyclic AMP phosphodiesterases are known, including caffeine and isobutylmethylxanthine. Dudai (unpublished) found that feeding either of these drugs to normal flies caused a severe decrement in learning ability. This suggests that the ambient level of cyclic AMP is important during learning; therefore a developmental abnormality need not be invoked for the dunce mutants, although the possibility of such an abnormality cannot be excluded. The converse experiment of feeding dunce flies a drug which lowers cyclic AMP levels is not immediately feasible because suitable drugs are not known. The critical time of action of the dunce gene for learning could be explored with a temperature-sensitive allele of dunce, which could permit transient inactivation of the dunce gene product during development and during the course of memory storage and recall.

Recent work has revealed the major pathways for conduction of olfactory information within the insect brain (19-23). As the olfactory system becomes better known, it will be interesting to determine the distribution and level of activity of each form of phosphodiesterase in the different regions. This knowledge might lead to specific hypotheses on the location of modifiable synapses in insects that could be tested in further genetic, anatomical, and electrophysiological experiments. To study the distribution of phosphodiesterases will require purification of the two enzyme forms and preparation of specific antibodies that can be fluorescently labelled and used as selective staining reagents on tissue sections.

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Appendix

List of Mutants Used.

All are on the X-chromosome.

Map Position	Name		Phenotype
0.0	y	(yellow)	Cuticle color yellow. Hairs and bristles brown with yellow tips. (Normal cuticle is grey; normal hairs and bristles are black.) Recessive.
	y^2, y^{31d}		Alleles of y. Cuticle color yellow; hairs and bristles black. y^{31d}/y is like y^{31d}; $y^{31d}/+$ is like +. Thus y^{31d} is recessive to + but dominant to y. y^2 is also recessive to +, but dominant to y.
0.0	sc	(scute)	Certain bristles lost or reduced. Recessive.
1.5	w	(white)	Eye color pure white; ocelli colorless. (Normal eye is red, ocelli pink.) Recessive.
	w^a	($white^{apricot}$)	Allele of w. Eye color light yellow-orange. Recessive.
3.0	N	(Notch)	Homozygous N is lethal. In heterozygous N/+, the wing is incised at the tip.
	Co	(Confluens)	Wing veins irregularly thickened, especially toward the tip. The Co phenotype results from duplication of the Notch locus.
3.9	dm	(diminuitive)	Body small; bristles small and slender; homozygous females are sterile. Recessive.
5.4	cho	(chocolate)	Eye color brown. Recessive.
13.7	cv	(crossveinless)	Crossveins of wing absent. Recessive.
21.0	sn	(singed)	Bristles twisted and shortened. Some sn alleles are recessive female-steriles (sn^1, sn^5, etc.); with other alleles homozygous females are fertile (sn^2, sn^3, sn^4, etc.).

Map Position	Name		Phenotype
33.0	v	(vermilion)	Eye color bright scarlet. Eye color of cho v double mutant is light yellow-orange. Recessive.
56.7	f	(forked)	Bristles shortened, gnarled, or bent. Recessive.
57.0	B	(Bar)	Eye of B/B female or B male is a narrow vertical bar. Eye of B/+ female is kidney-shaped with an anterior nick. (Normal eye has a circular outline.) Dominant.

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The map position of dm was revised by G. Lefevre (personal communication).

The data in the following table show that the mutations y, dm, cho, cv, v, f, and B do not adversely affect learning performance.

Genotype	Learning Index (Λ)	
y cho cv v f	0.30 ±0.02 (n=16)	phototaxis approximately normal.
y B (y/B^{Sy} males)	0.29 ±0.04 (n=5)	
dm	0.42 (n=2)	