radish, a *Drosophila* mutant deficient in consolidated memory

**Elisabeth Folkers, Peter Drain†, and William G. Quinn**

Department of Brain and Cognitive Sciences and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

*Communicated by Emilio Bizzí, May 10, 1993*

**ABSTRACT** We have characterized the behavior and genetics of the *Drosophila* mutant radish (*ras* gene). Initial learning of radish flies in two olfactory discrimination tests is high, but subsequent memory decays rapidly at both early and late times after training. Anesthesia-resistant memory (consolidated memory) is undetectable in radish flies 3 hr after training. The mutant shows normal locomotor activity and normal sensitivity to the odor cues and electric-shock reinforcement used in the learning tests. The radish gene maps within a 180-kb interval in the 11D-E region of the X chromosome.

Study of *Drosophila* mutants can relate defined genes to learning behavior. Several mutations have been isolated that severely reduce learning and short-term memory (1–5). Two of these, dune and rutabaga, directly affect metabolism of the second-messenger cAMP (3, 6–11). A role for cAMP in learning was specified by studies with its usual target enzyme, cAMP-dependent protein kinase A. Flies transformed with heat-shock-inducible transgenes that perturb protein kinase A activity show inducible disruptions in learning behavior (12). These findings in *Drosophila* complement results with the mollusc *Aplysia* (13), and together they have confirmed the role of the cAMP-signaling cascade in invertebrate learning and short-term memory.

Only a little is known about the molecular substrates of long-term memory. Here we describe the mutant radish, which seems relevant to this issue. Radish flies show high initial learning followed by a pronounced memory decay that continues several hours after training until memory becomes undetectable. Moreover, anesthesia-resistant memory (consolidated memory) is strongly reduced in radish. This phenotype is unlike that of other learning mutants isolated so far. Biochemical assays with the mutant (E.F. and K. W. Choi, unpublished results) indicate normal adenyl cyclase activity and protein phosphorylation patterns, which also suggests that the metabolic lesion in radish may be of a different type than in dune and rutabaga. Consequently, studies of radish may be informative on the mechanism of memory consolidation.

**MATERIALS AND METHODS**

**Stocks and Culture Conditions.** Wild-type *Drosophila melanogaster* were of the C-S strain. The radish stock was derived from C-S after chemical mutagenesis with ethylmethane sulfonate (14), followed by crosses to recover X-chromosome-linked mutations. Mutagenized lines were then screened for deficient learning performance measured 1–2 min after olfactory discrimination training in the Quinn, Harris, and Benzer measurement of learning and memory (QHB assay; ref. 15). Mutagenesis, crosses, and behavioral screening were by P. P. Szibor and W. G. Q.

All fly stocks were kept at 25°C, 45% relative humidity, on a 16:8 hr light/dark cycle. Flies were raised on standard cornmeal medium (16) in plastic bottles. Twenty-four hours before behavioral experiments, the flies were transferred to fresh glass bottles with cornmeal medium.

**Genetic Mapping.** For recombination mapping, homozygous radish females were mated to males with X chromosome-linked markers y, cv, v, f, car (17). F1 female offspring were crossed individually to FM7 males (17), yielding F2 males with parental and recombinant arrangements of the visible markers and radish. Single such F2 males were used to generate lines, whose members had identical X chromosomes, via crosses with the balancer FM7. These lines were tested for 1-hr memory in the QHB assay (15).

Deletion stocks (17, 18), used for finer genetic localization, are listed below, followed by their breakpoints in brackets. The stocks are: Df(1)l–L3 [9F10;10A7–8], Df(1)RA37 [10F1;10F9–10], Df(1)JA26 [11A1;11D-E], Df(1)C246 [11D-E;12A1–2], Df(1)HA29 [12A6–7;12D3], Df(1)KA9 [12E1;13A5], Df(1)ls2726 [13F1;14B1], Df(1)D7 [14C7-D1;14E3-F1], and Df(1)ID34 [14F1–2;14F6]. The proximal breakpoint of Df(1)NI05 [10F7;11D-E] was previously placed in 11D1 (17). Our molecular analysis indicates that it is between the proximal breakpoints of JA26 and of Df(1)sc206–14 [11D3-8]. All these stocks were from the National *Drosophila* stock center at Bloomington, IN, except for *sd*2726, *D7*, and *ID34*, which were from B. Ganetzky (University of Wisconsin, Madison, WI).

For deletion mapping, females with one of the deletions above and an X-chromosomal balancer [FM6, FM7, or C(1)RM y,f,(17)] were crossed to either radish or C-S males. Female offspring from this cross, heterozygous for the deletion and for either radish or radish*, were selected under CO2 anesthesia and tested 24 hr later either for 1-hr memory in the QHB assay or for 6-hr memory in the Tully–Quinn measurement of learning and memory (TQ assay; ref. 2).

**Cloning.** The DNA in the 11D-E region between the breakpoints of Df(1)NI05 and Df(1)C246 was cloned by chromosomal walking (19). We entered the region via an inversion, *In(1)sc206–14* (17), the breakpoints of which connected this region (11D3-8) with the previously cloned achaete-scute complex in 1B2-3 (20). We made a genomic library from the inversion stock (obtained from the Bloomington stock center) in Afix vector (Stratagene). We obtained a phage (Asc22, figure 1 in ref. 20) containing DNA of the 1B2-3 region from J. Modolell (Universidad Autónoma de Madrid, Madrid, Spain) via K. White, (Brandeis University, Waltham, MA) and from it isolated a 2.8-kb *HindIII* fragment near the breakpoint. Screening the inversion library (21) with this probe, we identified a clone with material in 11D-E by restriction analysis and confirmed this with *in situ* hybridization to C-S chromosomes (22). We used a 3.0-kb *HindIII* fragment from the clone to start the chromosomal walk.

Abbreviations: OCT, 3-octanol; MCH, 4-methylcyclohexanol; QHB assay, Quinn, Harris, and Benzer measurement of learning and memory; TQ assay, Tully and Quinn measurement of learning and memory.

†Present address: Department of Molecular and Cellular Physiology, Stanford University Medical School, Stanford, CA 94305.
To make a library for walking, we isolated DNA from C-S flies, partially digested it with Sau3A, selected fragments between 16 and 22 kb with a salt gradient, and cloned them into AfI vector. From the entry point we walked in both directions, isolating overlapping clones by plaque-hybridization screens. The continuity of the walk was verified after each step by chromosomal in situ hybridization. The endpoints of our walk were the relevant breakpoints of Df(1)N105 and Df(1)C246. We ascertained that we had crossed these breakpoints by hybridizing clones from the walk (i) to DNA blots prepared from flies heterozygous for the relevant deletions and (ii) to salivary chromosomes from larvae of the same genotype.

**Measuring Learning and Memory.** Olfactory discrimination learning and subsequent memory of flies were measured in two different olfactory learning paradigms. Apparatuses and experimental procedures are described in detail in ref. 15 (with modifications cited in ref. 23) and in ref. 1. Training in both procedures consisted of separate presentation of two chemical odors, 3-octanol (OCT) and 4-methylcyclohexanol (MCH). One of the two odors was negatively reinforced with electric shock; the other odor was an unreinforced control. A test for learning or memory consisted of exposing the flies to OCT and MCH, both without reinforcement, and measuring the fraction of the population that avoided each odor. Such tests were done between 2 min and 24 hr after training.

The second teaching method (the TQ assay) differs from the first in several respects. (i) Training follows classical conditioning models, in that the flies are unable to affect the timing and intensity of the stimuli they experience. (ii) The odors are presented in air currents. (iii) Training and testing take place in darkness and with minimal agitation of the flies. (iv) Testing consists of a direct choice between simultaneously presented odors. Flies, transported to a choice point between tubes with converging air currents containing the two odors, are given 2 min to choose an odor and enter the corresponding tube.

In both assays outlined above, a measurement of learning actually consisted of two experiments, with two naive groups of flies trained to avoid opposite odors of the pair (OCT and MCH). For such experiments the calculated score is the fraction of the population avoiding the shock-reinforced odor minus the fraction avoiding the unreinforced odor. Scores for both halves of a learning measurement were averaged to give a learning index, L.

In this report, the term "initial learning," indicates that flies are tested within 2 min after training; "memory" indicates that flies were tested at later times. For memory measurements with the TQ assay flies were kept in vials with food between training and testing.

**Measuring Cold-Anesthesia-Resistant Memory.** This was done with the TQ assay under the conditions of ref. 24. After training, flies were kept in vials with food at 25°C for 2 hr. They were then transferred into prechilled 25-ml plastic vials (Sarstedt; no. 58.490), and the vials were immersed in ice water (0°C). The flies stopped moving within 20 sec. After 2 min of cooling, the flies were transferred into new vials with food at 25°C. They started moving within 30 sec. The flies were kept in these vials for an additional 60 min until the test for memory.

**Measuring Sensory Acuity and Locomotor Ability.** The tendency of C-S and radish flies to avoid the odors OCT and MCH was assayed under nonlearning conditions, as described in ref. 12 modified from ref. 2. Avoidance of electric shock was also measured as in ref. 12.

The fast-phototactic response of radish and C-S flies (their tendency to run toward light when disturbed) was measured in two situations: (i) in the testing phase of the QHB assay (15); and (ii) in a countercurrent assay (25). In the latter case, the phototactic index \( \phi \) was inferred from the displacement of the mean of the population distribution from the starting tube, \( \bar{x} \); for five repeated countercurrent cycles, \( \phi = \bar{x}/5 \).

Statistics. Scores from behavioral tests are reported as mean ± SEM for \( n \) determinations. Error bars in all figures indicate SEMs. Student's \( t \) test (two-tailed unless otherwise specified) was used to assess behavioral differences between two stocks. The one-tailed Dunnett test (26) was used to compare the learning indices of multiple stocks with that of a reference group. The Dunnett test was run with the SAS statistical software package version 6.03 (27), on an 80386-microprocessor-based personal computer network.

**RESULTS**

The mutant radish was isolated by P. P. Sziber and W.G.Q. in a screen for X chromosome-linked ethylmethylene sulfonate-induced mutants that fail to learn an olfactory discrimination task (8, 15). Subsequent studies of the behavior of radish flies revealed good initial learning followed by rapid memory decay. This memory phenotype was retained after autosomes of the radish stock were replaced with wild-type C-S autosomes.

When tested in the olfactory-discrimination paradigm of ref. 15 (the QHB assay), radish flies display 70% of wild-type initial learning performance. Subsequent memory decays abnormally rapidly. Learning indices for radish decrease to undetectable levels within 60 min—a time at which C-S flies retain 52% of their initial learning performance (Fig. 1).

**Fig. 1.** Memory retention of mutant radish (óż) and of C-S wild-type (c) flies, measured in the QHB assay. The two stocks were assessed in parallel—i.e., the assays were run under the same conditions and in the same experimental series. Learning indices were measured immediately after training and at 1 hr represent average values from 12 determinations (i.e., \( n = 12 \)). For learning indices at other times, \( n = 6 \). Learning indices of radish flies are significantly lower than those of C-S flies at all time points shown (all \( P < 0.01 \), two-tailed \( t \) tests). Vertical error bars in this and subsequent figures indicate SEMs.
It seemed possible that the mutant's poor performance resulted not from intrinsic forgetfulness but from the particular conditions during training and testing. Therefore, we tested radish flies in another olfactory learning situation, the TQ assay (1). In this assay the defect in the radish mutant appears more specific to memory. Mutant radish flies show 88% of wild-type initial learning, and subsequent memory decays rapidly. Six hours after training, radish flies show no significant conditioning effect, whereas the learning index for C-S flies is still 0.33 (Fig. 2). Memory of radish remains undetectable 24 hr after training ($\Lambda = -0.01 \pm 0.03$; $n = 8$). The corresponding memory score of C-S flies trained and tested in parallel is $0.10 \pm 0.05$ ($n = 8$).

Amnesiac is a previously reported mutation (2, 5) that predominantly affects memory (1, 4). For a direct comparison, we measured the learning and memory of radish and amnesiac flies in parallel experiments by using the TQ assay. During the first 4 hr after training, learning and memory scores of the two mutants are indistinguishable (Fig. 2). After this time the phenotypes differ. Memory of radish flies continues to drop to zero at 6 hr—whereas memory of amnesiac flies is stable. Memory scores of radish and amnesiac mutants differ significantly both at 6 hr ($P < 0.01$) and at 8 hr ($P < 0.01$) after training.

The fact that the memory of radish flies continues to decline for several hours after training suggested that the mutant might be deficient in a late memory process. One experimental definition of long-term memory is based on disruption studies (28). Immediately after training, memory is severely reduced by treatments such as concussion, electroconvulsive shock, or anesthesia. At later times, memory is consolidated into a form that survives these treatments. Consolidated memory has been found in wild-type Drosophila with experiments using cold-induced anesthesia (24, 29). The mutant amnesiac showed essentially wild-type levels of anesthesia-resistant memory in two different learning assays (4, 24). Here we applied the methodology to the radish mutant. We used the conditions of Tully et al. (24), anesthetizing the flies 2 hr after training and measuring memory 1 hr later. We confirmed earlier results, (figure 2 in ref. 24), including the observation of high levels of anesthesia-resistant memory in the amnesiac mutant. Memory of radish flies, measured under these conditions, is near zero (Fig. 3). In other words, the cold-induced anesthesia treatment, which has little effect on the memory of amnesiac flies, virtually abolishes memory performance in radish flies.

The high initial learning scores of radish flies indicate that this mutant can smell the odors and sense the electric shock used in the learning assays. Nevertheless, we directly measured the sensitivity of the mutant to these stimuli under nonlearning conditions (12). Naive flies were given a choice of entering either of two tubes with converging air currents, one current with an added odor (OCT or MCH), the other current of ambient air. With each odor the fraction of radish flies avoiding the tube with odor (measured after 2 min) is statistically indistinguishable from that of wild-type flies. Avoidance of OCT was as follows: $0.81 \pm 0.02$ ($n = 20$) for radish, $0.81 \pm 0.02$ ($n = 20$) for C-S flies. Avoidance of MCH was as follows: $0.81 \pm 0.01$ ($n = 10$) for radish, $0.83 \pm 0.01$ ($n = 10$) for C-S flies.

We used a similar method to measure the sensitivity of the mutant to electric shock (12). Given a choice between a tube with an electrified grid and a tube with a nonelectrified grid, $0.83 \pm 0.02$ ($n = 10$) of C-S wild-type flies avoided the tube with the shock. Here again radish flies (0.78 ± 0.01, $n = 10$) performed indistinguishably.

To assess the locomotor ability of the mutant we tested its fast-phototactic response in two situations. (i) During the testing phase of the QHB learning assay, we counted the fraction of the population $\phi$ that entered an illuminated tube within 15 sec in the intervals between runs to odor tubes (15). Under these circumstances, radish flies ($\phi = 0.88 \pm 0.02$; $n = 24$) were behaviorally indistinguishable from C-S flies ($\phi = 0.90 \pm 0.02$; $n = 24$). (ii) We measured the tendency of the flies to run repeatedly toward light, using the countercurrent assay of Benzer (25). The two stocks performed indistinguishably in this test as well: $\phi = 0.73 \pm 0.03$ ($n = 8$) for radish flies; $\phi = 0.80 \pm 0.02$ ($n = 8$) for C-S flies.

![Fig. 2. Memory retention of radish (●), amnesiac (□), and wild-type C-S (○) flies, measured in the TQ assay. The three genotypes were assayed in parallel. Learning indices for wild-type and radish flies represent averages from the same number of determinations ($n = 8$–13) at a given time. For amnesiac, $n = 4$–8. SEMs for immediate learning scores are smaller than the symbols. The learning indices of radish flies are significantly lower than those of C-S flies ($P < 0.01$) at all intervals shown. Learning indices of radish flies are significantly lower than those of amnesiac flies at 6 ($P < 0.01$) and 8 hr ($P < 0.01$).](image)

![Fig. 3. Consolidated memory (i.e., memory resistant to cooling-induced anesthesia) in radish, amnesiac, and wild-type C-S Drosophila. Flies were trained as in the TQ assay. Two hours later they were cooled to 0°C for 2 min, a treatment that induced brief anesthesia. Memory was tested an hour later. Unperturbed 3-hr memory for these three genotypes was measured in parallel. Memory scores of anesthetized radish flies ($n = 8$) differ significantly from those of anesthetized C-S flies ($n = 12$, $P < 0.001$) and amnesiac ($n = 12$, $P < 0.01$) flies. Memory scores of anesthetized amnesiac flies are statistically indistinguishable from those of anesthetized C-S flies. (Memory scores of anesthetized and unanesthetized amnesiac flies are also indistinguishable.)](image)
We detected no abnormality in radish flies with respect to morphology, development, or viability during our handling and inspection of the flies.

The radish mutation has a semidominant effect on memory; in other words the forgetfulness of heterozygous radish/C-S flies is intermediate between the wild-type and the homozygous mutant. When measured 1 hr after training in the QHB assay, the learning index of heterozygous radish/+ female flies is 0.09 ± 0.02 (n = 20) versus 0.19 ± 0.02 (n = 12; P < 0.01; one-tailed t test) for C-S/C-S and 0.01 ± 0.02 (n = 12; P < 0.01) for radish/radish female flies. One-hour memory scores of C-S (0.19 ± 0.04; n = 6) and radish (−0.02 ± 0.03; n = 10) males are indistinguishable from those of their female counterparts. Memory in the TQ assay, measured 6 hr after training, also appears lower in radish/C-S than in C-S/C-S flies (see Fig. 6).

radish was isolated as an X chromosome-linked mutation. Recombination mapping (Fig. 4) places radish between the visible X-linked markers vermilion and forked. Analysis with deletions further localized the mutation within this region. Flies heterozygous for various deletions and radish were tested for 1-hr memory in the QHB assay (Fig. 5). Two of the deletion stocks tested had memory scores significantly lower than radish/C-S. These two deletions, Df(1)JA26 and Df(1)C246, overlap in the chromosomal region 11D-E.

![Graph](image)

**Fig. 4.** Genetic recombination mapping of the memory phenotype of the radish mutant. Heterozygous flies of the indicated classes were tested 1 hr after training in the QHB assay. Memory scores of radish and C-S flies were assayed in parallel. All recombinant classes that are both v+ and f+ have low memory scores. Conversely, all classes that are both v− and f− have high memory scores. These results indicate that the radish gene maps between the vermilion and forked genes. Different lines of the two recombinant classes with crossovers between vermilion and forked genes (+ + + f car and y cv v + +) have disparate memory scores. (Average scores for these subclasses are shown separately.) This disparity supports the inference that the radish gene lies in this interval. Memory scores of recombinants were compared with those of C-S (n = 100) and radish (n = 12) flies with one-tailed Dunnett tests. Statistical comparisons are presented below. L, Learning indices significantly lower than that of C-S wild-type (P < 0.05) and statistically indistinguishable from that of radish flies; H, learning indices significantly higher than that of radish flies (P < 0.05) and statistically indistinguishable from that of C-S flies. Each line within a recombinant class was tested an equal number of times, and scores were averaged. Classes or subclasses are as follows: + + + v + + (L, n = 12, 6 lines); + + + v + c ar (L, n = 12, 3 lines); + + + f c ar (L, n = 12, 2 lines); + + + f car** (H, n = 12, 2 lines); + + v f c ar (H, n = 10, 5 lines); + cv v f c ar (H, n = 4, 1 line); y cv v f c ar (H, n = 6, 2 lines); y cv v + + ** (H, n = 6, 1 line); y cv v + + (H, n = 6, 1 line); y cv v + + (L, n = 6, 1 line); y v + + + (L, n = 12, 6 lines); y + + + + (L, n = 6, 3 lines). We obtained no recombinants of the class (y cv v f +).

Another deletion, Df(1)N105, extends into this region of overlap. Memory of Df(1)N105/radish flies appears low like radish, but it is not significantly lower than that of radish/C-S. This lack of statistical significance might be attributable to the relatively small difference in 1-hr memory scores between heterozygous and homozygous mutant flies observed in the QHB assay. Accordingly we measured the memory of the relevant deletion heterozygotes in the TQ assay, a regimen that elicits a greater difference in memory scores between homozygous and heterozygous radish flies. In this assay, the deletion uncovers the radish mutation—Df(1)N105/radish heterozygotes perform indistinguishably from homozygous radish flies and significantly worse than radish/C-S (P < 0.01). The learning indices of Df(1)JA26/radish and Df(1)C246/radish are also significantly lower than that of radish/C-S (P < 0.01; P < 0.01) in this assay (Fig. 6).

To initiate molecular studies of the radish gene, we cloned the DNA from this overlap region by chromosomal walking. We entered it via a breakpoint of the inversion sc260-H and walked in both directions with overlapping λ phages a total of 180 kb from the proximal breakpoint of Df(1)N105 to the distal breakpoint of Df(1)C246. The radish mutation is thus localized within 180 kb of cloned DNA.

**DISCUSSION**

Mutant radish flies show high initial learning followed by several hours of rapid memory decay. The mutant is also strikingly deficient in anesthesia-resistant memory. Its normal sensory and motor capability, as well as its good initial
Fig. 6. Deletion mapping of the memory of radish using the TQ assay. Flies with the indicated deletions were tested 6 hr after training. Black bars indicate memory scores for Df/radish heterozygotes (n = 5–8); white bars indicate memory scores for Df/C-S heterozygotes (n = 6–9). The control groups (radish/radish, n = 7; radish/C-S, n = 7; and C-S/C-S, n = 10) were assayed in parallel experiments. The learning indices of Df(1)C246/radish, Df(1)JA26/radish, and Df(1)NI05/radish are all significantly lower than that of radish/C-S (P < 0.05, one-tailed Dunnett test) and are indistinguishable from radish/radish.

learning, suggests that the mutation selectively lesions a process required for memory. The radish mutation is uncovered by the deletions Df(1)C246, Df(1)NI05, and Df(1)JA26, which overlap in a 180-kb interval in the 11D-E region of the X chromosome. This localization strongly suggests that the forgetfulness of radish results from an alteration in a single gene.

The genetic locus of radish is different from other mutations known to affect learning (2, 5, 10, 23, 30). The behavioral phenotype of radish also differs from that of previously studied learning mutants. Unlike dunc, rutabaga, and turnip, radish flies show high initial learning in both the olfactory discrimination tests used here (1–4). radish differs from latheo mutation (5) and from the transgenic flies with protein kinase A alterations (12) in its more pronounced effect on memory.

The mutation most similar to radish is amnesiac (1, 4). In the TQ assay, the memory decay curves of radish and amnesiac are indistinguishable until 4 hr after training. Thereafter the curves diverge; the score of radish flies falls to zero within 6 hr, whereas the memory of amnesiac flies is stable at this time. Reinforcing this difference in memory at late times is a clear disparity between the two mutants in anesthesia-resistant (consolidated) memory measured 3 hr after training. Levels of consolidated memory in amnesiac flies are statistically indistinguishable from wild-type values. In contrast, such memory is nearly undetectable in radish flies. This disparity strongly suggests that the two mutations affect different components of memory.

This combination of high initial learning, continuing memory decay, and absence of consolidated memory is unique among Drosophila mutants isolated so far. It suggests that molecular information on the radish* gene product will provide insights into long-lasting memory in Drosophila, particularly into the mechanism of consolidation.

We thank Herrmann Steller for his critical help with both the research and the manuscript. Patricia Sziber isolated the mutant, and Greg Solomon and Kwang-Wook Choi helped in its behavioral and biochemical characterization. Ho-Wei Hsu ran the Dunnett statistical tests. We thank Juan Model, Kalpna White, and Barry Ganetzky for clones and stocks. This work was supported by Grant BNS-9021698 from the National Science Foundation and was aided by a program grant from the Markley Foundation. E.F. was a fellow of the Deutsche Forschungsgemeinschaft.