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Rescue of the learning defect in dunce, a *Drosophila* learning mutant, by an allele of rutabaga, a second learning mutant

(memory/cyclic AMP/adenylate cyclase)

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ABSTRACT **rutabaga[1] (*rut[1]*), a *Drosophila* learning mutant, has adenylate cyclase (EC 4.6.1.1) with reduced basal activity and the absence of calcium/calmodulin-stimulated activity. A second learning mutant, dunce, is defective in cyclic AMP degradation due to decreased or absent phosphodiesterase activity. These opposing biochemical defects allow *rut[1]* to partially suppress the female sterility caused by elevated cyclic AMP levels in dunce flies. Selection of mutations that suppress dunce sterility has led to the isolation of two rutabaga alleles. The alleles (*rut[2]* and *rut[3]*) decrease basal adenylate cyclase activity [Bellen, H. J., Gregory, B. K., Olsson, C. L. & Kiger, J. A. (1987) *Dev. Biol.* 121, 432–444] but, unlike the original rutabaga mutation, leave the calcium/calmodulin-stimulated activity intact. Behaviorally, the two alleles also differ from *rut[1]*. One of the mutations partially rescues the dunce learning defect, and flies bearing both alleles learn. Calcium responsiveness may thus be the crucial component of adenylate cyclase activity required for associative learning.**

Neurophysiology has long provided the basis for exploring the molecular mechanisms underlying complex behavior. Genetics complements this analysis with the introduction of single-gene mutations that provide a specific noninvasive method to induce biochemical changes. *Drosophila*, with its rich history of classical genetics and techniques available to introduce and express altered genes, is an ideal organism in which to apply the genetic approach to behavioral biochemistry. Happily, physiology and genetics have so far implicated the same biochemical pathways as underlying learning and memory.

Investigation of learning and memory in the sea snail *Aplysia* has resulted in a possible model of simple learning. Kandel *et al.* (1) propose that sensitization, a simple nonassociative form of behavioral plasticity, relies on presynaptic facilitation. A sensitizing stimulus causes a neurotransmitter, possibly serotonin, to be released onto the sensory nerve terminal, activating in turn adenylate cyclase and cyclic AMP-dependent protein kinase. The kinase then phosphorylates and closes K^+ channels, depolarizing the cell. Subsequent stimulation thus induces enhanced neurotransmitter release from the sensory neuron, producing behavioral sensitization.

Kandel *et al.* (1) have expanded their model to include associative learning. A second stimulus [conditioned stimulus (CS)] presented immediately before the sensitizing stimulus [unconditioned stimulus (US)] produces a greater response than either the CS or US alone. The additional biochemical change in the sensory neuron seems to be a consequence of neuronal activity, probably Ca^{2+} entry, immediately prior to neurotransmitter release onto the presynaptic terminal. Adenylate cyclase can produce cyclic AMP in response to both Ca^{2+} and monoamines and is, therefore, a likely substrate for the synergism between CS and US.

Several *Drosophila* mutations that affect learning and memory disturb the monoamine-activated adenylate cyclase cascade. Mutants at the Dopa decarboxylase (*Ddc*) locus cannot synthesize dopamine or serotonin (2) and they do not learn (ref. 3, but see ref. 4). dunce (*dnc*) flies have elevated cyclic AMP levels due to a defective structural gene for the cyclic AMP-dependent phosphodiesterase (5–8). dunce flies can learn but learn less well than wild-type controls and forget much more rapidly (9, 10). Since dunce and other "learning" mutants as well do learn in some olfactory as well as visual assays (11), they might be more accurately characterized as partial memory mutants. I retain the term "learn" here as a convenient shorthand.

One *Drosophila* mutation affects the key enzyme in cyclic AMP regulation: rutabaga[1] (*rut[1]*) flies lack the calcium/calmodulin-sensitive component of adenylate cyclase (12, 13) and show learning and memory defects comparable to those of dunce (10, 13). Since the dunce and rutabaga mutations influence cyclic AMP metabolism in opposite directions, the possibility arises that learning requires a certain range of cyclic AMP levels. On the other hand, the *Aplysia* model of associative learning requires a specific calcium-induced cyclic AMP stimulation. In *rut[1]* flies the absent calcium response, rather than the overall cyclic AMP decrease, may be the critical defect. Double-mutant dunce, *rut[1]* flies have cyclic AMP levels nearer to normal than either strain alone, but these flies still do not learn, casting further doubt on the importance of cyclic AMP levels alone (13). Although double-mutant flies do not learn, rutabaga does partially compensate for one dunce phenotype—female sterility. The isolation of suppressors of dunce sterility has produced mutations with various amounts of cyclase activity (14). I have used these alleles to assay the relative contribution of basal versus calcium-stimulated adenylate cyclase activity to learning.

MATERIALS AND METHODS

Fly Stocks. Wild-type flies were the Canton-Special (Canton-S) wild-type strain. *rut[1]* was originally isolated as a learning mutant, induced by ethyl methanesulfonate (EMS) mutagenesis in a Canton-S stock. *rut[2]* and *rut[3]* were induced by EMS mutagenesis and selected as suppressors of dunce sterility in a *y dnc[m14] ec f* stock (14); these mutants were kindly provided by J. A. Kiger, Jr. (University of California, Davis). The marker *f* was lost in the course of the mutagenesis. *y cv rut[2]* and *y cv rut[3]* stocks were prepared by recombination with *y cv v f car*. Presence of *rut[2]* and *rut[3]* was confirmed by suppression of dunce sterility. The double-mutant *y dnc[m14] ec rut[1]* chromosome was constructed by recombination of the parental dunce chromosomes with *rut[1]*.

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Abbreviations: CS or US, conditioned or unconditioned stimulus, respectively; EMS, ethyl methanesulfonate.

The presence of *rut*^{1} was confirmed biochemically and by suppression of dunce sterility.

Adenylate Cyclase Assays. Ten heads or abdomens from female or male flies were homogenized by hand in a glass–glass homogenizer. Membranes were prepared by centrifugation at 178,000 × *g* for 10 min. Adenylate cyclase was assayed by the method of Salomon (15). Free calcium was varied by using a 1 mM EGTA/$CaCl_2$ buffer (16). All comparisons are made in the same experiment, but each result represents data from at least three separate experiments. Protein was assayed using the method of Bradford (17). Adenylate cyclase activity in female and male heads and abdomens is qualitatively the same, but the calcium-sensitive component comprises a much greater proportion of total activity in abdomens (13). Abdomens are therefore assayed whenever possible. Unfortunately, the calcium-buffering system functions unreliably in assays of female abdomens, producing variations in the peak calcium sensitivity, so heads are used in the complementation analyses.

Learning. Learning ability was measured in an olfactory discrimination task with electric shock reinforcement, exactly as described by Quinn *et al.* (18). The numerical index of learning performance is defined as the fraction of flies avoiding the shock-associated odor minus the fraction of flies avoiding the nonreinforced control odor, averaged for reciprocal halves of the experiment. Results are reported as means ± SEM.

RESULTS

Bellen *et al.* (14) isolated two rutabaga alleles, *rut*^{2} and *rut*^{3}, and showed that they decrease adenylate cyclase activity approximately 30 and 10%, respectively. Since the original rutabaga mutation, *rut*^{1}, is remarkable for a complete absence of measurable calcium/calmodulin-sensitive adenylate cyclase activity (12, 13), I assayed mutants *rut*^{2} and *rut*^{3} for their cyclase response at various calcium concentrations. Like adenylate cyclase activity from most other organisms, *Drosophila* cyclase showed a biphasic response to calcium. Low (1×10^{-7} M) concentrations of calcium activated the enzyme, whereas higher concentrations inhibited it (Figs. 1 and 2). Hemizygous *rut*^{2} and *rut*^{3} flies showed essentially wild-type activation and inhibition, despite the decrease in basal activity (Fig. 1). The *rut*^{1} mutation defines two components of adenylate cyclase activity in *Drosophila* homogenates: calcium sensitive and calcium insensitive (12, 13, 19). *rut*^{1} removes the enzyme activity that responds to the divalent cation (12, 13, 19). *rut*^{2} and *rut*^{3} appeared to be hypomorphs that produce less of this calcium-sensitive adenylate cyclase. Fig. 1 shows that although the size of the calcium activation peak decreased in *rut*^{2} flies, the fold stimulation as a proportion of calcium-sensitive activity remaining was nearly constant. The decrease merely reflects that calcium-insensitive adenylate cyclase comprised a greater proportion of total enzyme activity in *rut*^{2} homogenates than in wild-type preparations, whereas the constant fold stimulation indicates that the calcium/calmodulin-sensitive activity remaining in *rut*^{2} flies had wild-type activation. These results are consistent with selective elimination of calcium-sensitive adenylate cyclase activity rather than loss of calcium/calmodulin regulation of the enzyme. Complementation tests also support this interpretation. The original rutabaga allele, *rut*^{1}, failed to complement the basal reductions of mutations *rut*^{2} and *rut*^{3} (ref. 14; Fig. 2), but calcium stimulation in *rut*^{2}/*rut*^{1} and *rut*^{3}/*rut*^{1} heterozygotes remained robust.

Given their biochemistry, I wondered if *rut*^{2} and *rut*^{3} flies would learn. The two alleles were isolated as sterility suppressors of the dunce allele *dnc*^{M14}, which had to be removed by recombination before the behavioral assay could be performed. Since genetic background does influence learning

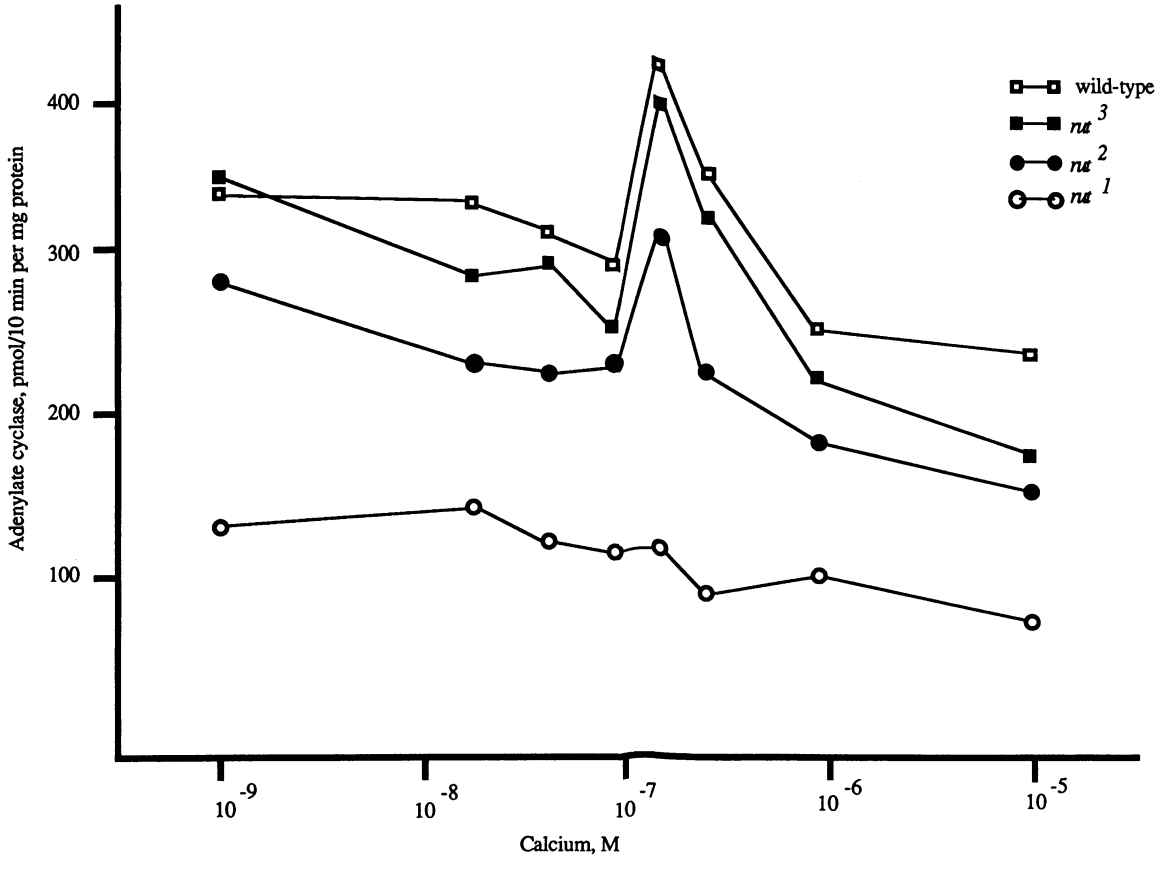

FIG. 1. Adenylate cyclase activity in membranes from abdomens of male wild-type, *rut*^{1}, *rut*^{2}, or *rut*^{3} flies.

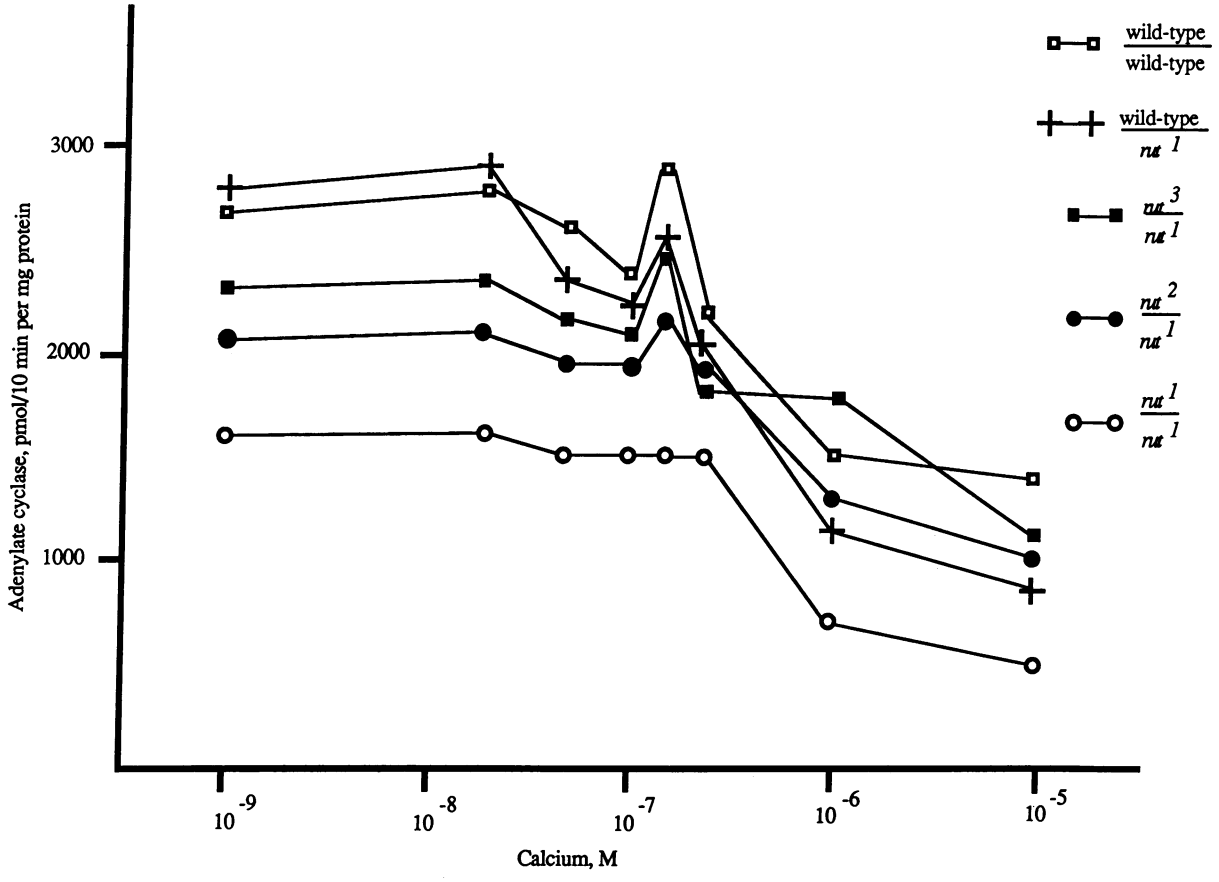

FIG. 2. Adenylate cyclase activity in membranes from heads of female wild-type, rut^1, wild-type/rut^1, rut^2/rut^1, and rut^3/rut^1 flies. Activity in heads and abdomens is qualitatively similar.

(10, 20), the chromosome used in the recombination, *y cv v f car*, was assayed as a control. These flies learned as well as wild-type flies (Table 1). Both rut^2 and rut^3 learned (Table 1). Learning in rut^3 flies was not distinguishable from controls. rut^2 flies learned about half as well as wild-type controls. Learning scores obtained for the *y cv* rut^2 stock were significantly different from wild-type scores or rut^1 values, suggesting that the rut^2 biochemical lesion results in a behavioral defect. The decrease was not, however, dramatic. Although the control chromosome learning values were not significantly different from Canton-S controls, an effect of genetic background could not be dismissed. Since the learning phenotypes of both rut^1 and rut^2 were recessive (ref. 13; Table 1), complementation analysis should reflect an additive effect of the two alleles. Unexpectedly, the data in Table 1 shows that rut^1 did complement the behavioral defect seen in rut^2 flies. The rescue was doubly surprising as rut^1 failed to complement the biochemical defect (ref. 14; Fig. 2).

Double-mutant dnc^{m14},rut^2 flies learned (Table 2). The learning scores obtained average only about one-half of wild-type but remained significantly higher than any other dunce or dunce, suppressor learning index. It seems unlikely that background differences are responsible for the partial rescue. Unlike flies with the *y cv* rut^2 and *y cv* rut^3 chromosomes, no recombination was necessary to assay the double mutants, as both rut^2 and rut^3 were induced directly on the dnc^{m14} chromosome during an EMS mutagenesis. Both the parental strain and the double mutant dnc^{m14},rut^3 were available for comparison, and neither displayed any learning (Table 2).

Table 1. Learning in mutant and wild-type flies

Genotype	Learning index	*n*
CS/CS	0.35 ± 0.02	12
rut^1/rut^1	0.04 ± 0.04	6
y cv v f car/y cv v f car	0.35 ± 0.03	4
y cv rut^2*/y cv* rut^2	0.19 ± 0.02*	18
y cv rut^3*/y cv* rut^3	0.35 ± 0.02	4
rut^1*/y cv* rut^2	0.36 ± 0.03	7
rut^1*/y cv* rut^3	0.38 ± 0.03	5

Learning index is presented as mean ± SEM. *n*, Number of trials. *Statistically significant difference ($P < 0.01$, one-way analysis of variance with supplementary Newman–Keuls test).

DISCUSSION

Two suppressors of dunce sterility have been biochemically and behaviorally characterized. Bellen *et al.* (14) induced these two mutants on a dnc^{m14} chromosome by using EMS as the mutagen. Based on consistent reductions in adenylate cyclase activity, complementation analysis of enzymatic activity and of ability to suppress dunce sterility (14), and meiotic mapping (H. J. Bellen and J. A. Kiger, Jr., personal communication), the two mutations were designated rut^2 and rut^3. I follow their terminology but acknowledge that adenylate cyclase activity depends on a number of proteins and

Table 2. Learning in dunce and dunce,rutabaga flies

Genotype	Learning index	*n*
dnc^{m14}/dnc^{m14}	0.01 ± 0.02	4
dnc^{m14},rut^1/dnc^{m14},rut^1	0.00 ± 0.03	3
dnc^{m14},rut^2/dnc^{m14},rut^2	0.17 ± 0.02*	15
dnc^{m14},rut^3/dnc^{m14},rut^3	−0.02 ± 0.04	4

Learning index is presented as mean ± SEM. *n*, Number of trials. *Statistically significant difference ($P < 0.01$, one-way analysis of variance with supplementary Newman–Keuls test).

that the two suppressors could actually be mutations in tightly linked loci encoding stoichiometrically limiting modulatory factors.

dunce flies carrying the rut^2 mutation learn. Double-mutant flies perform about half as well as wild-type flies in this paradigm. Any learning in dunce, rutabaga flies is surprising since dnc^{m11},rut^1 and dnc^{m14},rut^1 flies do not learn (ref. 13; Table 2). dnc^{m11},rut^1 flies perform even more poorly than either mutant alone in an associative learning paradigm (10). Since the dunce and rutabaga mutations influence biochemically opposing enzyme activities (phosphodiesterase and adenylate cyclase), one might expect that the two mutations would complement each other when present in the same fly. Indeed, dnc^{m11},rut^1 flies have near normal cyclic AMP levels, and rut^1 does partially suppress dnc^{m11} sterility (13). The inferior behavioral performance of the double mutant has thus been puzzling (10). The *Aplysia* model provides a possible answer: associative learning may require a synergistic response between the neurotransmitter released to the US and the result of neuronal activity triggered by the CS. If, as postulated, calcium influx mediates the CS signal and adenylate cyclase provides the molecular convergence point for the two signals, then rut^1 flies would lack the machinery for associative learning even if basal cyclic AMP levels were completely compensated by a dunce mutation. Learning in dnc^{m14},rut^2 flies but not dnc^{m14},rut^1 flies supports this model. rut^2 flies show a significant decrease in basal adenylate cyclase activity (30%) but have normal levels of calcium/calmodulin stimulation. It is therefore possible that this allele decreases adenylate cyclase activity enough to compensate for the elevated cyclic AMP levels in dnc^{m14} flies without abolishing the calcium/calmodulin stimulation required for associative learning.

Although this demonstration of the interaction of dunce and rutabaga alleles on behavioral and biochemical levels provides further evidence that both mutations affect learning by altering the same molecular cascade in the same cells, it leaves an important question unanswered: Why do rut^1 flies display any learning in other olfactory and visual paradigms (10, 21, 22)? Perhaps additional biochemical processes can partially compensate for the absence of calcium/calmodulin cyclase activity. rut^1 cyclase may have some calcium/calmodulin stimulated activity *in vivo*. Separate second messenger systems might be involved in flies (4). The present result can neither support nor refute these possibilities but merely emphasizes the importance of calcium/calmodulin-stimulated cyclase activity to learning as measured by the olfactory avoidance paradigm used here.

The fact that flies bearing either rutabaga mutation rut^2 or rut^3 learn despite deficiencies in total enzyme activity also highlights the importance of the calcium-stimulated component of adenylate cyclase activity to the learning measured here. rut^2 does learn less well than wild type, suggesting that a 30% reduction in basal adenylate cyclase activity may produce a large enough reduction in the concentration of cyclic AMP to influence the molecular learning apparatus. A decrease in the total amount of adenylate cyclase competent to respond to calcium/calmodulin activation would also lead to a decrease in peak cyclic AMP concentrations during calcium entry (22). Thus, a defect in the mediation of the calcium signal could also explain the learning deficiency. Alternatively, the decrement in learning scores may be due to an alteration of genetic background that was introduced during the recombination and is unrelated to the biochemical deficiency. Support for this position is provided by the complementation data. rut^2/rut^1 heterozygotes learn as well as wild-type flies (Table 1). Since previous genetic evidence indicates that adenylate cyclase activity is proportional to the number of copies of the gene (13) (i.e., that rutabaga is a dosage-sensitive locus) and since rut^1 fails to complement rut^2 biochemically (ref. 14; Fig. 2), rut^1 should fail to complement any learning defect that resulted from a deficiency in adenylate cyclase activity. The data in Table 1 show, however, that rut^1 does complement rut^2 behaviorally. Given this inconsistency and the weakness of the learning defect in rut^2 flies, performance in other learning paradigms would be important to clarify the extent of the behavioral disability. It is comforting that the more severe biochemical defect, as measured *in vitro*, produces the behavioral defect (Fig. 1 and Table 1). In addition, rut^2 is the more effective suppressor of dunce sterility (14).

Biochemical analysis of the two rutabaga alleles, rut^2 and rut^3, supports the hypothesis that the locus encodes a calcium-sensitive adenylate cyclase catalytic subunit. Like rut^1, both mutations decrease adenylate cyclase activity in a dosage-dependent manner (ref. 14; Fig. 2). In contrast to the original mutation, neither rut^2 nor rut^3 decreases adenylate cyclase activity as much as rut^1, and both leave the calcium/calmodulin-sensitive cyclase activity intact. As seen most clearly in flies bearing the rut^2 mutation (Fig. 1), the total amount of calcium stimulation decreases in proportion to the reduction in basal enzyme activity, but the fold response remains relatively unaffected. The decrease in total stimulation reflects the fact that in homogenates of rut^2 flies the calcium-insensitive component of adenylate cyclase activity represents a greater proportion of total enzyme activity. The conservation of fold stimulation demonstrates that the remaining calcium/calmodulin-sensitive adenylate cyclase exhibits a wild-type response to stimulation. If, as seems likely, rut^2 and rut^3 represent mutations in the rutabaga locus, these data provide evidence that the two characteristics of the original rutabaga allele, decrease in basal activity and ablation of calcium/calmodulin activation, are separable. It is possible to remove a portion of the cyclase activity responding to calcium while sparing the ligand sensitivity of the remaining calcium-sensitive activity. The genetic segregation offered here has some implications for the nature of the rutabaga gene product. Previous biochemical and genetic analysis suggested that the rutabaga locus encodes either a stoichiometrically limiting cofactor conferring calcium/calmodulin sensitivity or an adenylate cyclase catalytic subunit (13, 19). Although the evidence provided by the analysis of alleles rut^2 and rut^3 does not rigorously distinguish between the two alternatives, it does support the second possibility as the mutations defined by rut^2 and rut^3 selectively alter adenylate cyclase activity without changing calcium/calmodulin stimulation.

Recent molecular analysis also indicates that rutabaga encodes one form of adenylate cyclase. Krupinski *et al.* (23) have isolated and characterized cDNAs encoding bovine adenylate cyclase. By using cross-hybridization to a bovine brain cDNA, a *Drosophila* genomic clone has been isolated. *In situ* hybridization to polytene chromosomes reveals hybridization to the same region (12E1–13A5) that contains the rutabaga locus (20, 23). Biochemical and perhaps behavioral rescue of the phenotypes mapping to the locus by the wild-type adenylate cyclase gene would conclusively establish the identity of the rutabaga gene product.

Biochemical and behavioral characterization of two rutabaga alleles, rut^2 and rut^3, provides further genetic evidence that the rutabaga locus does indeed encode an adenylate cyclase catalytic subunit. This analysis also emphasizes the similarity of cellular biochemistry thought to underlie associative learning in two simple, but distinct, organisms: *Aplysia* and *Drosophila*. In both systems calcium-dependent modulation of cyclic AMP levels seems a crucial, albeit not exclusive, mediator of neuronal plasticity.

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