The *Drosophila* mutants *amnesiac*, *dunce* (*dnc*), and *rutabaga* (*rut*), *amnesiac* (*amn*), and *linotte* (*lio*) (Dudai et al., 1976, 1984; Quinn et al., 1979; Dura et al., 1993) *dnc* and *rut* encode, respectively, a phosphodiesterase and an adenylyl cyclase (Chen et al., 1986; Levin et al., 1992), two enzymes involved with the cAMP pathway. *amn* encodes a putative neuropeptide that might regulate adenylyl cyclase activity (Feany and Quinn, 1995), and *lio* encodes a putative tyrosine kinase (Dura et al., 1995) involved in adult brain development (Simon et al., 1998). Despite recent progress (Zhong and Wu, 1991; Qiu and Davis, 1993; Dauwalder et al., 1995), the precise physiological roles of the *Drosophila* proteins *Amn*, *Dnc*, and *Rut* are not fully understood. In particular, although *Dnc* and *Rut* accumulate in the mushroom bodies (Nighorn et al., 1991; Han et al., 1992), an insect structure involved in learning and memory (Erber et al., 1980; Davis, 1993; Hammer, 1993; de Belle and Heisenberg, 1994), they are also expressed in other parts of adult brain (Nighorn et al., 1991; Han et al., 1992) in which their possible roles are unknown.

The conditioning protocols originally used to isolate and characterize most of *Drosophila* learning and memory mutants associate an odor with electric shocks (ES) (Quinn et al., 1974; Tully and Quinn, 1985). Naturally, before the abnormal performance of the mutants could be linked to a learning or memory defect, it was shown that untrained mutants could react normally to the stimuli used for the conditioning and, in particular, that their odor avoidance (OA) was normal (Dudai et al., 1976, 1984; Quinn et al., 1979; Dura et al., 1993). However, the possibility that ES presentation during conditioning could itself affect odor perception was never explored. This issue is crucial when one needs to compare a putative learning or memory mutant with a reference wild-type stock, because the ES might differentially affect the two groups. Thus, to characterize a mutant deficient in associative learning or memory, it is essential to separate a bona fide learning or memory defect (related to the association of stimuli) from behavioral deficits merely related to abnormal perception of the stimuli after the conditioning treatment. I show here that the mutants *amn*, *dnc*, and *rut* display a very strong decrease of their OA after ES. Thus, the lack of *Dnc* “general” expression is most likely responsible for the OA defect, which would be responsible for the apparent learning defect after conditioning. In contrast, the *Dnc* phosphodiesterase accumulated in the mushroom bodies would be involved specifically in memory formation.

**Key words:** *Drosophila melanogaster*; learning and memory mutants; cAMP; stress sensitivity; odor avoidance; conditioning controls

Several mutations affecting associative learning and memory have been characterized in *Drosophila*, including *dunce* (*dnc*), *rutabaga* (*rut*), *amnesiac* (*amn*), and *linotte* (*lio*) (Dudai et al., 1976, 1984; Quinn et al., 1979; Dura et al., 1993) *dnc* and *rut* encode, respectively, a phosphodiesterase and an adenylyl cyclase (Chen et al., 1986; Levin et al., 1992), two enzymes involved with the cAMP pathway. *amn* encodes a putative neuropeptide that might regulate adenylyl cyclase activity (Feany and Quinn, 1995), and *lio* encodes a putative tyrosine kinase (Dura et al., 1995) involved in adult brain development (Simon et al., 1998). Despite recent progress (Zhong and Wu, 1991; Qiu and Davis, 1993; Dauwalder et al., 1995), the precise physiological roles of the *Drosophila* proteins *Amn*, *Dnc*, and *Rut* are not fully understood. In particular, although *Dnc* and *Rut* accumulate in the mushroom bodies (Nighorn et al., 1991; Han et al., 1992), an insect structure involved in learning and memory (Erber et al., 1980; Davis, 1993; Hammer, 1993; de Belle and Heisenberg, 1994), they are also expressed in other parts of adult brain (Nighorn et al., 1991; Han et al., 1992) in which their possible roles are unknown.

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under both conditions. This experiment was performed blind as to genotype. Flies were transported to the choice point of a T maze, allowed to choose between the two odors in a vial with regular solid food. For memory testing, flies were transported which was not paired with ES. Flies were then kept for 30 or 90 min in a rest period, flies were exposed for 60 sec to the second odor (odor B), during which time they received ES (1.5 sec pulses of DC). After a 45 sec rest period, flies were exposed for 60 sec to the second odor (odor B), which was not paired with ES. Flies were then kept for 30 or 90 min in a vial with regular solid food. For memory testing, flies were transported to the choice point of a T maze, allowed to choose between the two odors for 120 sec, and counted. The performance index represents a normalized probability of correct choice. A score of 0 thus corresponds to a 50:50 distribution.

For OA tests, flies were treated in the upper chamber as for the associative conditioning, except that presentation of the second odor was omitted and replaced by exposure to air. For OA testing, treated animals were transported to the choice point of the T maze, allowed to choose between the new odor and air, and counted. A performance index was calculated as for associative conditioning. An index of 0 corresponds to a 50:50 distribution. An index of 100% corresponds to complete avoidance of the odor. Odors were used undiluted as by de Belle and Heisenberg (1994). Two groups of the same stock were run successively, and the side of the test tube with odor was alternated. To remove odor traces from the previous run before each new experiment and in the absence of flies, odor and fresh air, respectively, were aspirated through the relevant test tube for 1 min.

The correct perception of ES requires the presence of humid air (Tully and Quinn, 1985), and various voltage–humidity set-ups have been adopted as regular working conditions. In the present study, unless specified, 120 V–70% humidity was used (medium-humidity condition). A 60 V–90% humidity set-up was also used (high-humidity condition).

Statistical significance of the differences between two means, corresponding to mutant and control, were assessed with Student’s t test.

**RESULTS**

The olfactory associative conditioning protocol that produces the strongest learning scores consists of the following sequence (Fig. 1A) (Tully and Quinn, 1985). A first odor is presented to a group of flies paired with pulses of ES. After a rest period, a second odor is presented in the absence of shock. To measure the association between the first odor and ES, flies are brought to a choice point from which they have free access to two compartments, each filled with one of the odors previously used during training. Animals, having learned and remembered the odor–ES association, will tend to avoid the corresponding odor.

A major drawback of this procedure is that flies make their olfactory choice after having received strong ES, which might itself induce behavioral changes unrelated to learning and memory, only indirectly affecting learning and memory performances. In particular, because unimpaired olfaction is a prerequisite for the correct interpretation of results in the olfactory associative conditioning protocol, I tested the OA of wild-type and mutant flies after presentation of ES (Fig. 1B). In this test, a first repellent odor is presented to the flies in association with ES. The flies are then brought to the choice point, where their reactivity to a second repellent odor is measured. This protocol is thus similar to the associative conditioning procedure, but the second odor is not presented during the first phase, whereas the first odor, which has been associated with the shocks, is not presented during the
test. Such control of the OA of the mutants is more relevant to the associative conditioning protocol than the direct test of naive animals. The fact that the second odor, used to test OA, is novel to the flies at the time of testing precludes interference from phenomena related to multiple presentations, such as habituation.

After presentation of ES combined with the first odor, the mutants amn, dnc, rut, and rutP230 displayed strongly reduced avoidance of a second odor compared with normal flies (Fig. 1B). On the contrary, lio1 and lio2 behaved in the same way as wild-type flies. Thus, although naive amn, dnc, and rut mutants have been shown in previous studies to react normally to odors (Dudai et al., 1976, 1984; Quinn et al., 1979), their OA is dramatically reduced after presentation of ES, the stimulus normally used for aversive conditioning.

The amn mutant was chosen, together with the reference wild-type stock Canton-S, to analyze in more detail the effect of ES on OA. amn displays an abnormal OA to odor B after presentation of ES, whether ESs are delivered in combination with fresh air or with odor A (Fig. 2). This result indicates that ES presentation is the main cause of the abnormal OA and not preexposure to a first repellent odor. In the case of Canton-S, avoidance of odor B is less efficient when ES is associated with air rather than with odor A (Fig. 2). A plausible explanation for this is that Canton-S flies, having learned the association between the air flow and ES, tend to avoid air during the test. Consequently, to measure any learning-independent effects of ES on OA, it is imperative to present ES associated with odor A, a condition which will not be one of the options in the test.

If amn displays a strong OA decrease after a 120 V ES presentation, Canton-S is also affected at this voltage (Fig. 3). This is apparently attributable to the degree of sensitivity, because...
of the ES on some aspect of perception in the animals. It is possible that ES alters faculties in the mutants required not only for the test but also for the training phase of associative conditioning, and it is likely that these faculties are progressively deteriorated as the shock pulses are delivered. In summary, an altered physiology in the mutants after ES presentation could lead to an apparent defect in associative conditioning because of weaker acquisition during training and/or because of poorer performance during the test.

This work highlights the need for two-step controls in associative learning and memory experiments, especially in which a strong stimulus is used to condition the animals. In particular, the effect of the aversive unconditioned stimulus on the ability of the animals to perceive the conditioned stimulus should be investigated. Thus, although the mutants studied here have been shown to perform poorly under many different associative conditioning protocols (Aceves-Pina and Quinn, 1979; Folkers, 1982; Mariath, 1985), the unconditioned stimulus was generally stressful.

Two strategies can now be adopted to differentiate learning and memory defects from unspecific deficits. First, genetic dissection might reveal that a specific protein isoform and/or expression in a particular subdomain is involved in only one type of behavior. Such an approach was performed successfully with dnc (Table 1) by testing two previously characterized deficiencies (Qiu and Davis, 1993). Thus, a dramatic and specific decrease in Dnc mushroom body product correlates with normal OA after ES. These animals showed a specific memory defect. On the contrary, elimination of the entire brain expression of Dnc leads to a strongly reduced OA after ES. The fact that only the latter animals showed an apparent learning deficit suggests that this might be a secondary consequence of the nonassociative deficit induced by ES. In this hypothesis, the Dnc phosphodiesterase accumulated in the mushroom bodies would be involved specifically in memory formation.

Second, the mutants could be studied under conditions designed to reduce stress to prevent the occurrence of nonspecific defects which might interfere with the conditioning procedure. Thus, it has been seen that dnc, which apparently displays a learning defect when conditioned with a negative stimulus (ES), learns normally when a positive stimulus (sugar) is used in association with odors (Tempel et al., 1983). This observation supports the idea that Dnc is specifically required for associative memory but not for associative learning.

### REFERENCES

Aceves-Pina EO, Quinn WG (1979) Learning in normal and mutant Drosophila larvae. Science 159:93-95


Dauwalder B, Davis RL (1995) Conditional rescue of the dunce