Effects of a Conditional Drosophila PKA Mutant on Olfactory Learning and Memory

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Abstract
The requirement for cAMP-dependent protein kinase (PKA) in associative learning of Drosophila was assessed in mutant flies hemizygous for a cold-sensitive allele, X4, of the DCO gene, which encodes the major catalytic subunit of PKA. DCO X4 hemizygotes died as third-instar larvae at 18°C, the restrictive temperature, but were viable when raised at 25°C. Shifting adult DCO X4 hemizygotes from 25°C to 18°C led to a decrease in PKA activity from 24% to 16% of wild-type without impairing viability. At 25°C, DCO X4 hemizygotes exhibited reduced initial learning relative to controls but normal memory decay in a Pavlovian olfactory learning assay. Shifting the temperature from 25°C to 18°C prior to training reduced initial learning to a similar extent in DCO X4 hemizygotes and controls but resulted in a steeper memory decay curve only in DCO X4 hemizygotes. These observations are suggestive of a role for PKA in medium-term memory formation in addition to its previously established role in initial learning.

Introduction
A very large number of physiological cellular responses to extracellular signals employ the second messenger, cAMP (Sutherland 1972). The rate of cAMP synthesis, catalyzed by adenylyl cyclase, can be modified via stimulatory (Gs) or inhibitory (Gi) G proteins that respond to ligand-induced activation of transmembrane receptors and also by changes in calcium concentration (Gilman 1984; Levitzki 1988; Cooper et al. 1995). Although intracellular cAMP has been shown to interact directly with ion channels in olfactory (Nakamura and Gold 1987; Dhallan et al. 1990) and muscle cells (Delgado et al. 1991), the vast majority of its effects in eukaryotes are mediated by binding to the regulatory subunit (R) of the inactive cAMP-dependent protein kinase (PKA) tetramer, R2C2 (Coffino et al. 1976; Toda et al. 1987; Francis and Corbin 1994). This dissociates monomeric catalytic subunit (C) that can now phosphorylate substrate proteins and thereby alter their activity. PKA is expressed at particularly high levels in the nervous system of mammals (for review, see Walaas and Greengard 1991) and Drosophila (Kalderon and Rubin 1988; Muller and Spatz 1989; Skoulakis et al. 1993), is responsive to a number of neurotransmitters (Uzzan and Dudai 1982; Deutsch and Sun 1992; Saudou et al. 1992; Tatsuno et al. 1992), and can alter the conductance properties of ion channels through phosphorylation (Greengard et al. 1991; Swope et al. 1992; Raymond et al. 1993; Wang et al. 1993). These properties, together with the potential to alter cell shape through phosphorylation of cytoskeleton-associated proteins (Geisler and Weber 1988; Reinhard et al. 1992; Scott et al. 1993) and to alter gene expression by phosphorylation of transcription factors (Gonzalez and Montminy 1989; Rehfuss et al. 1991), make PKA an excellent candidate for mediating short- and long-term changes in synaptic efficiency that underlie learning and memory.

The central importance of cAMP-mediated signal transduction in learning has already been demonstrated in Drosophila melanogaster and in the marine snail, Aplysia californica. In Aplysia, a molecular outline of the actions of cAMP at a defined
Drosophila promoter show marked reductions in learning relative to control flies following a heat shock (Drain et al. 1991), though in this case the effects of the inhibitors could not be resolved completely from the effects of heat shock and the temporal decay of the transiently expressed inhibitors to give a complete time course of memory retention.

A second question is whether PKA is required for all measurable facets of learning and memory in Drosophila. Loss-of-function DC0 mutations that reduce PKA activity to 20% of wild type show severe reductions in initial learning but no apparent reduction in memory for 3 hr after training (Skoulakis et al. 1993). Similarly, ectopic expression of PKA inhibitors reduced initial learning but did not appear to accelerate memory decay (Drain et al. 1991), though in this case the effects of the inhibitors could not be resolved completely from the effects of heat shock and the temporal decay of the transiently expressed inhibitors to give a complete time course of memory retention.

A third question is whether different aspects

PKA AND DROSOPHILA LEARNING

synapse has been developed to account for the sensitization of the gill withdrawal reflex by stimuli to the tail. Enhanced synaptic transmission between sensory and motor neurons is initiated by the modulatory transmitter serotonin (5-HT) that is released when the tail is stimulated. This causes an increase in cAMP concentration in the presynaptic neuron, followed by activation of PKA, closure of (Is) potassium channels, prolonged depolarization, and, hence, enhanced calcium entry and increased release of neurotransmitters (Kandel and Schwartz 1982). Long-term changes in synaptic efficiency are accompanied by changes in synaptic morphology (Bailey and Chen 1988) and have been shown to involve transcriptional induction of several genes mediated by PKA phosphorylation of the transcription factor CREB (Dash et al. 1991; Kaang et al. 1993; Alberini et al. 1994).

In Drosophila, genetic evidence indicates that normal control of cAMP concentration and PKA activity are important for associative learning. Mutations in the dunce (dnc) gene, a cAMP phosphodiesterase, lead to an increase in basal cAMP levels, whereas mutations in the rutabaga (rut) gene, a calcium/calmodulin-responsive adenyl cyclase, reduce calcium stimulation of adenyl cyclase activity without markedly altering basal cAMP concentration. Both mutations disrupt associative and nonassociative learning in a variety of behavioral assays (Byers et al. 1981; Livingstone et al. 1984; Davis and Dauwalder 1991; Tully 1991; Levin et al. 1992). Combining various dnc and rut mutations can restore basal levels of cAMP toward normal but does not restore learning ability when a null mutation of rut, rut+, is used (Livingstone et al. 1984; Tully and Quinn 1985; Feany 1990). This is consistent with the idea that modulation of cAMP concentration by the adenyl cyclase isoform encoded by rut is important for learning, perhaps because it is able to respond to Ca2+ signals as well as to G-protein-mediated activation and thereby respond synergistically to two temporally paired stimuli that converge on a single cell.

Genetic perturbations that alter the response of PKA to cAMP also disrupt associative learning in Drosophila. Transgenic flies that express inhibitors of PKA or additional PKA catalytic subunit (DC0) under the control of a heat-shock-inducible promoter show marked reductions in learning relative to control flies following a heat shock (Drain et al. 1991). Moreover, enhancer-trapping techniques (Bellon et al. 1989) have been used to isolate a P[lacZ] transposon insertion in the untranslated leader of the DC0 gene that reduces PKA activity severalfold. The combination of this insertional mutation, DC0581, and a hypomorphic DC0 missense allele, DC0P10, produced viable adult flies of grossly normal morphology that showed reduced Pavlovian olfactory learning (Skoulakis et al. 1993). Furthermore, the expression of lacZ, influenced by the DC0 enhancer in DC0581, and DC0 protein itself were found to be particularly abundant in the mushroom bodies of the brain, in support of the notion that this may be a key site of action of PKA in the learning process (Davis 1993; Skoulakis et al. 1993; deBelle and Heisenberg 1994).

Several questions regarding the role of Drosophila PKA in learning warrant further exploration to enhance our mechanistic understanding of learning and memory. One question is whether the effects of PKA perturbations on learning noted above are attributable to developmental defects or acute alterations in physiological responses. This issue is particularly relevant as hypomorphic mutations in the Drosophila PKA catalytic subunit gene DC0 are known to affect several developmental processes, whereas null mutations are lethal at larval stages (Lane and Kalderon 1993, 1994; Kalderon 1995). The impaired learning of adults with hypomorphic DC0 mutations (DC0581/DC0P10) was assumed to result from acute physiological defects because no obvious morphological changes in brain structure were seen (Skoulakis et al. 1993). More convincingly, learning defects associated with ectopic expression of DC0 and PKA inhibitors were manifest immediately following heat shock-induced expression (Drain et al. 1991). These measurements were, however, made against a background of substantially impaired learning owing to the heat shock treatment itself.

A second question is whether PKA is required for all measurable facets of learning and memory in Drosophila. Loss-of-function DC0 mutations that reduce PKA activity to 20% of wild type show severe reductions in initial learning but no apparent reduction in memory for 3 hr after training (Skoulakis et al. 1993). Similarly, ectopic expression of PKA inhibitors reduced initial learning but did not appear to accelerate memory decay (Drain et al. 1991), though in this case the effects of the inhibitors could not be resolved completely from the effects of heat shock and the temporal decay of the transiently expressed inhibitors to give a complete time course of memory retention.
of learning and memory require different thresholds of PKA activity. This might be expected if PKA participated in distinct learning processes by phosphorylation of different substrates. The only quantitative data correlating PKA activity and learning suggest a threshold somewhere between 60% and 20% of wild-type PKA activity for discerning any pronounced effects on learning (Skoulakis et al. 1993). The effects of reducing PKA activity below 20% remain to be explored.

We sought to identify conditional alleles of DCO to demonstrate acute requirements for PKA in learning and to investigate the effects of reducing activity to minimal levels on both initial learning and memory. Here, we report on the isolation of cold-sensitive alleles of DCO and the use of one of them in assays of associative learning. Initial studies using a courtship conditioning assay (Siegel and Hall 1979; Griffith et al. 1993) suggested that temperature-dependent reductions in PKA activity were associated with reduced memory over a 3-hr retention period without affecting initial learning (Li 1995). However, the statistical significance of these results was hard to assess because performance indices were not normally distributed. We therefore used the same cold-sensitive PKA mutant in a Pavlovian olfactory assay of learning and report the results here. Flies raised with reduced PKA activity (24% of wild type) were deficient in initial learning but exhibited memory decay rates similar to controls. Further reduction of PKA activity (to 16% of wild type) in adult flies produced little change in initial learning but was associated with an increased memory decay rate. These results suggest, for the first time, that there may be an acute physiological requirement for PKA in medium-term memory (MTM) in addition to its established role in initial learning.

Materials and Methods

ISOLATION OF COLD-SENSITIVE MUTANTS

Male flies of genotype S6cnbw; ry506 (S6 represents a P[ry +] insertion at 30C, 1 map unit from the DCO locus) were fed with 25 mm ethyl-methanesulfonate (EMS) and mated to bw; ry506 virgin females (as in Grigliatti 1986). F1 males were singly mated to DCOH2S6cnbw/CyO; ry506 virgin females (as in Grigliatti 1986). F1 males were singly mated to DCOH2S6cnbw/CyO; ry506 males to Df(2L)g15/CyO; ry506 virgin females. Control flies of genotype S6cnbw/CyO; ry506 were generated by crossing S6cnbw; ry506 males to the same virgin females, Df(2L)g15/CyO; ry506. Flies were grown on the standard sugar-cormeal-agar medium (Boynton and Tully 1992) and kept at 25°C, except for tests at the restrictive temperature, 18°C.

FLY CULTURE AND MAINTENANCE

Male flies of genotype DCOX4S6cnbw/Df(2L)g15 were generated by crossing DCOX4S6cnbw/CyO; ry506 males to Df(2L)g15/CyO; ry506 virgin females. Control flies of genotype S6cnbw/CyO; ry506 were generated by crossing S6cnbw; ry506 males to the same virgin females, Df(2L)g15/CyO; ry506. Flies were grown on the standard sugar-cormeal-agar medium (Boynton and Tully 1992) and kept at 25°C, except for tests at the restrictive temperature, 18°C.

PAVLOVIAN OLFACTORY LEARNING

Flies were trained and tested with the odor-shock conditioning procedure of Tully and Quinn (1985) with modifications described in Tully et al. (1994). About 100 1- to 4-day-old flies of either DCOX4S6 cn bw/Df(2L)g15 (mutant) or S6 cn bw/
Df(2L)y15 (control) genotype were used in each trial. Flies of each genotype were trained simultaneously in two training machines set in parallel, with each genotype receiving an equal number of trainings in each machine to avoid bias. After each training, flies were either tested immediately (0-min time point; initial learning) or removed from the training chamber and stored in food vials in the dark for 10, 20, 30, 60, or 180 min before they were tested. Performance index (PI) was calculated as described in Boynton and Tully (1992).

OLFATORY ACUITY AND SHOCK REACTIVITY

Olfactory acuity was assessed as described in Tully et al. (1994) with two different odor concentrations, one normally used in training and the other at 100-fold dilution, at either 25°C or 18°C for each genotype. The ability of flies of different genotypes to avoid electrical shocks at 60 V (normally used in training) and 20 V was tested as described (Luo et al. 1992) at both 25°C and 18°C.

STATISTICAL ANALYSES OF BEHAVIORAL DATA

PIs are distributed normally (Tully and Gold 1993); so untransformed data were analyzed parametrically with the Macintosh software package JMP 3.1 (SAS Institute, Inc.). All pairwise comparisons were planned. To maintain an experiment-wise error rate of $a = 0.05$, the critical $P$ values were adjusted accordingly (Sokal and Rohlf 1981) and are listed below for each experiment. All behavioral experiments were performed in a balanced fashion, with $N = 2$ PIs collected per day per group (genotype ± temperature shift). In these experiments, the experimenter was blind to genotype.


PIs from two GENOtypes [S6/Df(2L)y15] and DCOX4/Df(2L)y15], two TEMPERATURES (25°C and 18°C), and six retention TIMES (0, 10, 20, 30, 60, and 180 min) were subjected to a three-way ANOVA with GENO $[F(1,120) = 216.29, P<0.001]$, TEMP $[F(1,120) = 151.16, P<0.001]$, and TIME $[F(1,120) = 58.58, P<0.001]$ as main effects and GENO×TEMP $[F(1,120) = 0.023, P = 0.872]$, GENO×TIME $[F(5,120) = 1.64, P = 0.154]$ and TEMP×TIME $[F(5,120) = 0.2.24, P = 0.055]$ as two-way interaction terms and GENO×TEMP×TIME $[F(5,120) = 0.84, P = 0.523]$ as the three-way interaction term (Fig. 4A,B, below). The 12 planned comparisons were deemed significant if $P<0.004$ and are summarized in the legend to Figure 4, below.

LEARNING/MEMORY OF MUTANTS EXPRESSED AS A PROPORTION OF WILD-TYPE FIES (PI RATIO) AT PERMISSIVE (25°C) AND RESTRICTIVE (18°C) TEMPERATURES

Any difference in learning between mutant flies at permissive and restrictive temperatures can result from a specific effect of temperature on PKA activity encoded by the cold-sensitive X4 allele or from a nonspecific effect of temperature that is common to flies of all genotypes. To eliminate the latter, nonspecific effect of temperature, we divided the PI of mutant flies by the PI of control flies for each assay time at each temperature (Fig. 4C, below). Means ± S.E.Ms of PIs for each of the groups represented in Figure 4, A and B, below, were used to calculate "PI Ratios." At each temperature (25°C or 18°C), the PI Ratio $i = (\text{mean } X4i/\text{mean } S6i) \times (1 + [\text{S.E.M.}^2/(\text{mean } S6i)^2])$, where PI Ratio $i$ = the mean PI of mutant flies at retention time $i$ (0, 10, 20, 30, 60, or 180 min) expressed as a proportion of the corresponding mean PI of wild-type flies, mean $X4$ = the mean PI of DCOX4/Df(2L)y15 mutants, mean $S6$ = the mean PI of S6/Df(2L)y15 wild-type flies, and S.E.M. = the average S.E.M. of the groups in Figure 4, A and B, as determined from a one-way ANOVA on PIs (this value was determined as the square root of the "mean square error" divided by the average within-group sample size). Because this ratio was determined from two mean values, each of which have an associated S.E.M., then S.E.M. of a ratio of two S.E.M.s is derived from Tully and Hirsch (1982), where S.E.M. provides an estimate of the variance of group mean PIs. The retention curves of PI Ratios for 25°C and 18°C are plotted as the upper two lines of Figure 4C, below. PI Ratios, S.E.M.s, and associated degrees of freedom for each group then were subjected to a one-way ANOVA with GROUP $[F(11,84) = 4.84, P<0.001]$ as the main effect. The
six subsequent planned comparisons were deemed significant if P<0.009 and are summarized in the legend to Figure 4, below. These planned comparisons represent the differences between PI Ratios at 25°C vs. 18°C and are plotted, along with their corresponding SEMs, as the lower line in Figure 4C, below.

OLFACTORY ACUITY IN WIDE-TYPE AND MUTANT FLIES AT PERMISSIVE AND RESTRICTIVE TEMPERATURES

PIs from two GENotypes [S6/Df(2L)y15 and DCOx4/Df(2L)γ15], four ODOR/concentration groups (OCT 10^-2, OCT 10^-3, MCH 10^-2, or MCH 10^0), and two TEMPeratures (25°C and 18°C) were subjected to a three-way ANOVA with GENO type [F(1,112)=0.05, P=0.82], with ODOR [F(3,112)=95.11, P<0.001] and TEMP [F(1,112)=2.61, P=0.11] as main effects; with GENO×ODOR [F(3,112)=0.17, P=0.92], GENO×TEMP [F(1,112)=0.47, P=0.49], and ODOR×TEMP [F(3,112)=0.02, P=0.99] as two-way interaction terms; and with GENO×ODOR×TEMP [F(3,112)=0.30, P=0.83] as the three-way interaction term (Fig. 5A, below).

SHOCK REACTIVITY IN WILD-TYPE AND MUTANT FLIES AT PERMISSIVE AND RESTRICTIVE TEMPERATURES

PIs from two GENotypes [S6/Df(2L)T15 and DcoX4/Df(2L)T15], two VOLTages (20V or 60V), and two TEMPeratures (25°C and 18°C) were subjected to a three-way ANOVA with GENO [F(1,40)=0.08, P=0.78], VOLT [F(1,40)=153.83, P<0.001], and TEMP [F(1,40)=0.44, P=0.51] as main effects; with GENO×VOLT [F(1,40)=0.07, P=0.80], GENO×TEMP [F(1,40)=0.13, P=0.72] and VOLT×TEMP [F(1,40)=0.24, P=0.62] as two-way interaction terms; and with GENO×VOLT×TEMP [F(1,40)=0.11, P=0.74] as the three-way interaction term (Fig. 5B, below).

RESULTS

ISOLATION OF COLD-SENSITIVE ALLELES OF DCO

Ten EMS-induced alleles of DCO have been isolated previously as recessive lethals by test-crossing to the null DCO deficiency, Df(2L)γ15, at 25°C (Lane and Kalderon 1993). None of these original alleles complemented Df(2L)γ15 for viability at 18°C or 29°C, although the weakest alleles (B10, B12, D30) showed a mild cold-sensitive phenotype, partially complementing each other at 25°C but not at 18°C (Lane and Kalderon 1993). To isolate more strictly conditional DCO alleles, we screened for EMS-induced mutations that were lethal in combination with the strong DCO allele, DCOh2 at either 18°C or 29°C (see Materials and Methods). The lethality of all 32 newly isolated mutations, including DCOx4 and DCOy4, and of the previously isolated and sequenced DCO alleles was found by recombination to map within 4 map units of a P[ry + ] insertion at 30°C. This indicates that all of the newly induced mutations are likely to be alleles of DCO.

All 32 new DCO alleles were tested for complementation of the DCO deficiency, Df(2L)γ15 at 18°C, 25°C, and 29°C and for complementation of selected hypomorphic DCO alleles at 18°C and 25°C. Only 2, DCOx4 and DCOy4, of the 32 mutations showed pronounced temperature-dependent complementation properties. Both DCOx4 and DCOy4 mutations showed no complementation of Df(2L)γ15 at 18°C but almost full complementation for viability at 25°C (Fig. 1). These alleles clearly encode some PKA activity at 18°C as they were able to complement the three weak alleles, DCOw10, DCOw12, and DCOw30, at this temperature. Also the majority of DCOx4 and DCOy4 hemizygotes died as third-instar larvae at 18°C, characteristic of weak DCO alleles. Animals that are zygotically null for DCO die as first-instar larvae (Lane and Kalderon 1993).

BIOCHEMICAL PROPERTIES OF COLD-SENSITIVE ALLELES T4 AND X4

The effects of DCOx4 and DCOy4 mutations on PKA activity were measured to verify that these
mutations affect the DCO gene and to investigate the severity of PKA dysfunction at 25°C and 18°C. It has been shown previously that there is a close correlation between DCO gene activity and the PKA activity measured in adult fly extracts in the presence of saturating amounts of cAMP (Lane and Kalderon 1993). Thus, adult flies that are heterozygous for a strong DCO allele or the deficiency, Df(2L)γ15, have only 50% of wild-type PKA activity. Extracts of DCO\(X^4\)/Df(2L)γ15 adults showed large reductions in PKA activity relative to Df(2L)γ15/Cyo control flies that were raised in parallel under identical conditions (Fig. 2). At 25°C, DCO\(X^4\)/Df(2L)γ15 flies had 24% of wild-type PKA activity. When these flies were shifted to 18°C, the kinase activity was further reduced to only 16% of the wild-type level. The same reduced level of PKA activity (16%) was measured at both 3 and 16 hr after shifting adult flies from 25°C to 18°C (Fig. 2). The change in PKA activity, from 24% to 16% in DCO\(X^4\) hemizygotes, is consistent with measurements on flies carrying other DCO alleles that establish a level of ~15%–20% as a threshold required for viability in Drosophila (W. Li and D. Kalderon, unpubl.). These results therefore support the idea that DCO\(X^4\) is a cold-sensitive allele of DCO and that in vitro measurements of PKA activity reflect the change in activity of DCO\(X^4\)-encoded enzyme in vivo. Similar measurements indicated that the PKA activity in DCO\(X^4\)/Df(2L)γ15 flies drops from 36% to 18% of wild-type upon shifting adult flies from 25°C to 18°C.

Quantitation of p40 DCO protein concentration in DCO\(X^4\) homozygous flies by Western blot indicated an approximately twofold reduction in protein levels relative to wild-type at 25°C but was not sufficiently accurate to determine if this fully accounts for the reduced kinase activity of DCO\(X^4\) homozygotes (Fig. 3). Also, it was not possible to establish whether shifting from 25°C to 18°C reduced the concentration of DCO\(X^4\) protein further.

**Figure 1:** Complementation properties of cold-sensitive alleles, DCO\(X^4\) and DCO\(T^4\). The viability of various allelic combinations was determined by counting adult progeny from a cross between five pairs of heterozygotes (over CyO) in a vial. The tabulated percentage viability was calculated as the number of non-CyO progeny divided by the number of CyO progeny and multiplied by 50. (ND) Not determined. The figures given are averages of many such experiments. The DCO alleles used, other than T4 and X4, have been described previously and are shown previously (Lane and Kalderon 1993). All other activities are expressed relative to that of DCO\(X^4\)/CyO adults.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>S6cnbw</th>
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<th>DCO(X^4) S6cnbw</th>
<th>DCO(X^4) S6cnbw</th>
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<td>18</td>
<td>25 18</td>
<td>25 18</td>
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<tr>
<td>% kinase activity</td>
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<td>98</td>
<td>38 21</td>
<td>24 16</td>
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<tr>
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**Figure 2:** PKA activities encoded by DCO\(X^4\) and DCO\(T^4\). PKA activities of extracts were calculated as the rate of Kemptide phosphorylation in the presence of 10 μM cAMP (see Materials and Methods). Df(2L)γ15/Cyo [rather than S6cnbw/ Df(2L)γ15] flies were used as convenient controls in all assays because the wild-type DCO gene on CyO and S6cnbw chromosomes encodes equivalent activities; Df(2L)γ15/Cyo flies are assumed to encode 50% of wild-type activity, as shown previously (Lane and Kalderon 1993). All other activities are expressed relative to that of Df(2L)γ15/Cyo measured in the same experiment. The activities for DCO\(X^4\) flies at 18°C were measured after shifting flies to 18°C for 2–3 hr (15.8%) or ~12–16 hr (16.4%). As the results were very similar, the average of the two is tabulated. (N.A.) Not applicable.
Figure 3: Western blot of Drosophila Extracts. The protein concentrations in homogenates of adult flies were measured (Bradford 1976) so that the same amount of total protein was loaded in each lane for SDS-PAGE. After transfer to nitrocellulose, DC0 p40 antigen was detected using a primary DC0 antibody purified as described previously (Lane and Kalderon 1993) and alkaline-phosphatase-conjugated second antibody. Extracts of control, S6cnbw homozygotes (lane 1) have considerably more DC0 protein than Df(2L)γ15/CyO flies (lane 2), which are expected to have 50% of wild-type levels, and DC0X4 S6cnbw homozygotes at 25°C (lane 3) and 18°C (lane 4). This experiment was repeated several times using different amounts of extract. In each case, DC0X4 homozygotes showed less protein than wild type and similar levels to Df(2L)γ15/CyO flies, as in the example shown.

EFFECTS OF PKA MUTATIONS ON PAVLOVIAN OLFACTORY LEARNING

We measured initial learning and memory retention after one training session of flies with various reductions in PKA activity using a Pavlovian odor avoidance assay (Tully and Quinn 1985; Tully et al. 1994). The control flies used in these experiments were heterozygous for the DC0 deficiency, Df(2L)γ15, and S6cnbw, the parent chromosome of DC0X4. These flies have 50% of normal PKA activity and were therefore not expected to show marked defects in learning (cf. Skoulakis et al. 1993). At 25°C the control flies showed robust initial learning that decayed roughly exponentially over a 3-hr period to an apparent asymptote in excess of 50% of the initial learning score (Fig. 4A), as described previously for wild-type flies (Tully and Quinn 1985; Tully et al. 1990; Boynton and Tully 1992; Dura et al. 1993; Tully and Gold 1993). At 18°C, control flies showed a similar time course of memory retention but all learning scores were reduced by ~25% relative to the values at 25°C (Fig. 4B).

A three-way ANOVA of the combined data summarized in Figure 4, A and B, revealed a significant effect of GENOtype (P<0.001), a signifi-
cant effect of TEMPerature ($P<0.001$), and a significant effect of retention TIME ($P<0.001$) but no significant interactions among these experimental variables (all $Ps>0.05$). Thus, the performance of mutant $DCO^{X4}$ hemizygotes was subnormal at all retention times, and temperature lowered performance similarly in mutant and control flies.

In the above analysis of performance differences, a specific effect of temperature shift on mutant $DCO^{X4}$ hemizygotes would be detected in the GENO×TEMP and/or GENO×TEMP×TIME interactions terms. However, this type of statistical analysis has notoriously low power for detecting even moderately large effects (Wahlsten 1990). Consequently, we have also analyzed the temperature-shift effects that are specific to mutant $DCO^{X4}$ hemizygotes by expressing mutant mean PIs as a proportion of the corresponding control mean PIs to produce a PI Ratio for each time and temperature (Fig. 4C; see Materials and Methods). This treatment of the data revealed that reducing PKA activity from 24% to 16% of wild type in adult flies had no effect on initial learning but led to a more rapid decay of memory in the first hour following training. The difference between the memory curves of flies with 24% and 16% PKA activity peaked at 30 min ($P=0.008$) and then decreased again to zero by 180 min after training (Fig. 4C).

The nonspecific and allele-specific effects of temperature shift on performance in the Pavlovian learning assay were not attributable to changes in task-relevant sensorimotor responses, because experimental and control flies behaved similarly in tests of olfactory acuity and shock reactivity at both 18°C and 25°C (Fig. 5A,B; see Materials and Methods).

These data suggest that the reduction in PKA activity owing to temperature shift of adult mutant $DCO^{X4}$ hemizygotes disrupted the normal function of MTM (see Discussion). Previous behavior-genetic studies also suggested the involvement of $amn$ mutants in MTM (Tully et al. 1994). Thus, we expressed the $amn$ memory retention data from Tully and Quinn (1985) as PI Ratios (using wild-type Canton-S flies as controls) and plotted the resulting memory curve for $amn$ along with those for mutant $DCO^{X4}$ hemizygotes assayed at 18°C or 25°C (Fig. 6). Visual inspection and statistical analysis reveals a striking similarity between the memory curves of $amn$ mutants assayed at 25°C and $DCO^{X4}$ mutants assayed at 18°C ($P=0.451$), in contrast to the improved performance of $DCO^{X4}$ mutants assayed at 25°C ($P=0.024$).

**Discussion**

We have investigated the role of PKA in associative learning in *Drosophila* using a conditional

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**Figure 5:** Task-relevant sensorimotor behaviors are normal in mutant flies. (A) Olfactory acuity and (B) shock reactivity of $DCO^{X4}$ hemizygous flies (shaded bars) vs. control (open bars) flies were tested at 25°C and 18°C, respectively. Mean PIs ($n=8$) are shown with s.e.m. No significant differences were detected between the two genotypes at either temperature.
characterization of the cold-sensitive mutations DCoX4 and DCoT4

We would ideally like to assay Drosophila learning in the complete absence of PKA activity. A single gene, DCo, can account for all measurable PKA activity in homogenates of whole adult Drosophila (Lane and Kalderon 1993; this paper). However, this does not exclude the possibility that other catalytic subunits are expressed at low levels or in small groups of cells. We have recently shown that a second Drosophila gene, DC2 (Kalderon and Rubin 1988), can behave as a PKA catalytic subunit in vitro (Melendez et al. 1995). Thus, null mutations in DCo probably do not eliminate all PKA activity in Drosophila. Furthermore, Drosophila requires DCo gene activity for oogenesis, embryogenesis, and survival past larval stages. We can therefore only test the effect of eliminating PKA on adult behavior by reducing PKA activity once development is complete or by limiting PKA dysfunction to specific cell types.

We have isolated two cold-sensitive DCo alleles, DCoT4 and DCoX4, that provide enough PKA activity for apparently normal development at 25°C but that do not allow development beyond larval stages at 18°C. Transferring adult DCoT4 or DCoX4 hemizygotes raised at 25°C to 18°C did not affect their continued viability and allowed a behavioral analysis of learning in animals that have very low PKA activity.

Although the temperature-dependent change in PKA activity of DCoX4 hemizygotes is modest (from 24% to 16% of wild type), it appears to cross a threshold for several PKA-dependent processes. Mild defects in oogenesis of hemizygous DCoX4 adult females that are apparent at 25°C are markedly exaggerated at 18°C (M.E. Lane and D. Kalderon, unpubl.) and are accompanied by a reduction in egg laying (W. Li and D. Kalderon, unpubl.). Hemizygous DCoX4 larvae pupariate and eclose at 25°C but not at 18°C. A variety of other DCo allelic combinations also respect a threshold of 15%–20% of wild-type PKA activity that is apparently required for larval viability and normal oogenesis.

The quantitative requirement for PKA in a given cellular process may depend on a number of factors including the change, if any, in cAMP concentration to which it responds, the affinity of PKA for the relevant substrates, the concentration of substrate, and the activity of antagonistic phosphatases. Other studies have already provided empirical evidence for a threshold somewhere between 20% and 50% of wild-type PKA activity for normal initial learning (Skoulakis et al. 1993) and also for expression of circadian rhythms (Levine et al. 1994). We suspected that a second, lower...
threshold, which we know to be critical for larval viability and oogenesis, might reveal a second distinct role of PKA in adult memory formation.

BEHAVIORAL DEFECTS ASSOCIATED WITH REDUCTION OF PKA ACTIVITY

The initial 25% learning deficit of DCO\textsuperscript{X4} hemizygotes relative to controls at 25°C in Pavlovian olfactory learning is most likely attributable to a reduction of PKA activity from 50% to 24%. It is formally possible that other EMS-induced differences between the DCO\textsuperscript{X4} chromosome and its parent chromosome could have produced a dominant effect on learning behavior, but this is unlikely. A somewhat larger decrement in initial learning (~50%) was previously observed to result from an ostensibly similar change in PKA activity (Skoulakis et al. 1993). The quantitative difference in the learning defect seen in these two studies may reflect differences in the learning assays or, alternatively, in the nature of the PKA mutations used. The PKA biochemical assays measure the activity of PKA in whole adult or adult head extracts (with similar results) when maximally stimulated by cAMP rather than the activation state of PKA in vivo in the very cells that are responsible for associative learning. Hence, measurements that show similar reductions in PKA activity might mask important differences in the distribution, abundance, and specific activity of the kinase.

The substantial levels of initial learning of DCO\textsuperscript{X4} hemizygotes allowed us to monitor the time course of memory decay and also to examine the effects of a further reduction of PKA activity once development was complete. At 25°C, memory decay in DCO\textsuperscript{X4} hemizygotes was very similar to that observed in wild-type flies. Previous learning assays had indicated that the performance of flies with altered PKA activity was very stable over a period of a few hours (Drain et al. 1991; Skoulakis et al. 1993). However, initial learning scores in those assays were low, possibly precluding a sensitive measurement of memory decay.

Shifting flies to 18°C prior to training reduced learning indices for all flies examined but showed some evidence of more rapid memory decay in DCO\textsuperscript{X4} hemizygotes compared with controls. A three-way ANOVA did not reveal a temperature-shift-dependent effect of reducing PKA activity from 24% to 16% on memory decay among its interaction terms. Interaction terms in ANOVAs, however, possess low statistical power to detect even moderately large effects (Wahlsten 1990). We therefore devised a way to separate the temperature-shift-specific effects of the DCO\textsuperscript{X4} mutation from the more general effects of genetic disruptions of DCO or of temperature-dependent behavioral performance. We did this by calculating PI Ratios, which express mutant scores at each retention time as a proportion of their corresponding control scores. Then, we compared the memory curves for PI Ratios from different temperatures. This treatment of our data indicated that reducing PKA activity from 24% to 16% had no effect on initial learning but resulted in a transient memory deficit that was maximal 30 min after training and gone within 180 min after training (Fig. 4C).

The overall time course of the memory deficit appears similar to that ascribed previously to MTM, a form of memory that can be defined empirically by the use of "reversal" training procedures (Tully and Quinn 1985; Tully et al. 1990) and that appears to be absent from flies carrying mutations in the ann gene (Quinn et al. 1979; Tully et al. 1990). If we compare the memory curves of ann mutants at 25°C with those of DCO\textsuperscript{X4} hemizygotes at 18°C using PI Ratios, we see almost identical curves (Fig. 6). If, as recently suggested (Feany and Quinn 1995), ann encodes a product that acts by raising intracellular cAMP concentration, we might expect to find a requirement for PKA in MTM formation, just as studies of rut and dnc had implied a likely role for PKA in initial learning. In the future, our hypothesis that PKA is required for MTM may be tested more definitively by using a reversal training protocol on mutant DCO\textsuperscript{X4} hemizygotes and controls or by assessing MTM in ann, DCO\textsuperscript{X4} double mutants.

ROLE OF PKA IN DROSOPHILA LEARNING

Many genetic studies testify directly or indirectly to the importance of PKA in Drosophila learning and suggest that PKA is involved in a variety of memory phases. Mutations in dnc, a cAMP phosphodiesterase, rut, a calcium-dependent adenylyl cyclase, and PKA can each reduce learning at the earliest time measured but in no case reduce learning to zero (Tully and Quinn 1985; Dudai 1988; Han et al. 1992; Tully and Gold 1993; Dauwalder and Davis 1995). The ann gene may encode a precursor of several peptides including a homolog of mammalian pituitary adenylyl cyclase-activating peptide (PACAP) (Feany and Quinn...
whether the formation of different memory phases is restricted to specific cell types or subcellular compartments.

The use of more than one PKA substrate would allow for the generation of a variety of temporal memory phases from a single time-course of PKA activation. We have found that different PKA activities generate different memory curves, suggesting that there are indeed different threshold requirements for PKA in initial learning and memory. Changing PKA activity from 50% to 24% of wild type (throughout development) affected initial learning but not the subsequent time course of memory decay, whereas reducing PKA from 24% to 16% (in adults) was associated with more rapid memory decay without affecting initial learning. These observations are most easily accommodated by postulating that different PKA substrates are pertinent to the initial expression of learning and to memory. Clearly, the identification of the relevant PKA substrates and other effectors that selectively affect short- or medium-term memory would provide more definitive evidence in favor of this notion. Although the DCO mutations we have used might be expected to affect PKA activity similarly in all cells, the precise numerical value of our PKA activity measurements in whole-fly extracts can only be taken as an approximation of the effects of mutations on PKA activity in the relevant subcellular microenvironment of those neurons pertinent to olfactory learning or memory storage.

Previous studies using single-gene mutants and different training protocols have suggested that longer-lasting memories (ARM and LTM) are, in part, dependent on the normal function of earlier memory phases (Tully et al. 1994). The use of a common biochemical component in several memory phases would provide a parsimonious explanation of this effect. It was assumed previously from the requirement for normal modulation of cAMP levels during acquisition and short-term memory (STM) and from the key role in LTM formation of the PKA substrate, CREB, that PKA activation was likely to be one such common component. Our use of a conditional PKA mutation to demonstrate separable roles for PKA in initial learning and in MTM provides further support for this hypothesis. In the future, it will be interesting to define how many distinct biochemical consequences relevant to learning and memory are initiated by PKA, whether these changes depend on additional signal transduction pathways, and whether the formation of different memory phases is restricted to specific cell types or subcellular compartments.
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