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 *DC0* gene, which encodes the major catalytic subunit of PKA. *DC0*^{X4} hemizygotes died as third-instar larvae at 18°C, the restrictive temperature, but were viable when raised at 25°C. Shifting adult *DC0*^{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, *DC0*^{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 *DC0*^{X4} hemizygotes and controls but resulted in a steeper memory decay curve only in *DC0*^{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

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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 adenylyl cyclase, reduce calcium stimulation of adenylyl 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*, *rut¹*, 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 adenylyl cyclase isoform encoded by *rut* is important for learning, perhaps because it is able to respond to Ca^{2+} 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 (Bellen et al. 1989) have been used to isolate a P[*lacZ*] transposon insertion in the untrans-

lated leader of the *DC0* gene that reduces PKA activity severalfold. The combination of this insertional mutation, *DC0⁵⁸¹*, and a hypomorphic *DC0* missense allele, *DC0^{B10}*, 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/DC0^{B10}*) 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.

A third question is whether different aspects

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 *DC0* 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 *DC0* 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 *DC0* locus) were fed with 25 mM ethylmethanesulfonate (EMS) and mated to *bw; ry⁵⁰⁶* virgin females (as in Grigliatti 1986). F₁ males were singly mated to *DC0^{H2}S6cnbw/CyO; ry⁵⁰⁶* (*DC0^{H2}* is a strong *DC0* allele) virgin females at 18°C and at 29°C. The absence of white-eyed progeny of genotype [*S6cnbw*]^{*}/*DC0^{H2}S6cnbw* indicates a second chromosome carrying a mutation

that is lethal over *DC0^{H2}*. These chromosomes were converted to balanced stocks and retested at 18°C, 25°C, and 29°C for viability over *Df(2L)γ15* (which lacks the *DC0* gene) and several of the previously isolated *DC0* alleles (Lane and Kalderon 1993). To map the lethal mutation, [*S6cnbw*]^{*}/*CyO*; *ry⁵⁰⁶* flies were crossed to *ry⁵⁰⁶* flies. F₁ virgin females of genotype [*S6cnbw*]^{*}/+; *ry/ry* were mated to *Df(2L)γ15/CyO; ry⁵⁰⁶* and progeny flies were scored for *ry⁺* and *CyO* markers. In all cases, the lethal mutation mapped within 4 map units of the P[*ry⁺*] insertion, consistent with the map distance of 1.0 map unit obtained for previously isolated mutations shown to be *DC0* alleles by DNA sequence determination.

KINASE ASSAYS

Kinase assays were carried out as described previously using adult males at 2–5 days posteclosion (Lane and Kalderon 1993). Five time points in a 1-min reaction were taken to calculate the reaction rates. PKA activity in fly extracts from the mutants was always compared in the same experiment with that of *Df(2L)γ15/CyO* flies, which have 50% of wild-type PKA activity. Each extract was assayed twice, and several extracts were made for each genotype and temperature condition. Total protein concentration of extracts, assayed as in Bradford (1976), were very similar in all assays and were used to calculate specific PKA activities.

FLY CULTURE AND MAINTENANCE

Male flies of genotype *DC0^{X4}S6cnbw/Df(2L)γ15* were generated by crossing *DC0^{X4}S6cnbw/CyO; ry⁵⁰⁶* males to *Df(2L)γ15/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)γ15/CyO; ry⁵⁰⁶*. Flies were grown on the standard sugar–corneal–agar medium (Boyn-ton 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 *DC0^{X4}S6 cn bw/Df(2L)γ15* (mutant) or *S6 cn bw/*

Df(2L)γ15 (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).

OLFACTORY 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 $\alpha = 0.05$, 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 $\pm$ temperature shift). In these experiments, the experimenter was blind to genotype.

LEARNING AND MEMORY IN WILD-TYPE [*S6/Df(2L)γ15*] AND MUTANT [*DCO^{X4}/Df(2L)γ15*] FLIES AT PERMISSIVE (25°C) AND RESTRICTIVE (18°C) TEMPERATURES

PIs from two GENOTypes [*S6/Df(2L)γ15*] and [*DCO^{X4}/Df(2L)γ15*], 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 $\times$ TEMP [$F(1,120) = 0.023$, $P =$

0.872], GENO $\times$ TIME [$F(5,120) = 1.64$, $P = 0.154$] and TEMP $\times$ TIME [$F(5,120) = 0.224$, $P = 0.055$] as two-way interaction terms and GENO $\times$ TEMP $\times$ 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 FLIES (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 $\pm$ S.E.M.s 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 $\text{PI Ratio}_i = (\text{mean } X4_i / \text{mean } S6_i) \times (1 + [\text{S.E.M.}^2 / (\text{mean } S6_i)^2])$, 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 *DCO^{X4}/Df(2L)γ15* mutants, mean *S6* = the mean PI of *S6/Df(2L)γ15* 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 $\text{S.E.M. PI RATIO} = [(\text{mean } X4)^2 / (\text{mean } S6)^2] \times [\text{S.E.M.}^2 / (\text{mean } X4)^2 + \text{S.E.M.}^2 / (\text{mean } S6)^2]^{1/2}$.

This calculation of the S.E.M. of a ratio of two S.E.M.s is derived from Tully and Hirsch (1982), where S.E.M.² 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 $P \leq 0.009$ 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)γ15* and *DC0^{X4}/Df(2L)γ15*], four ODOR/concentration groups (OCT 10⁻²; OCT 10⁰; MCH 10⁻²; or MCH 10⁰), and two TEMPeratures (25°C and 18°C) were subjected to a three-way ANOVA with GENOType [$F(1,112)=0.05$, $P=0.82$], 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)γ15* and *DC0^{X4}/Df(2L)γ15*], 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).

COMPARISONS OF RELATIVE LEARNING/MEMORY (PI RATIO) AT PERMISSIVE (25°C) AND RESTRICTIVE (18°C) TEMPERATURE

The PI ratios for *DC0^{X4}/Df(2L)γ15* mutants at 25°C and 18°C in Figure 4C, below, were plotted along with similar PI Ratios calculated as above using the data from Tully and Quinn (1985) for *amnesiac* (*amn*) mutants, deficient for MTM (Tully et al. 1990), and wild-type (Canton-S) flies. This set of PI Ratios, S.E.M.s, and associated degrees of freedom then were subjected to a one-way

ANOVA with GENO [$F(2,226)=4.23$, $P=0.016$] as the main effect. The two subsequent planned comparisons were deemed significant if $P \leq 0.025$ and are summarized in the legend to Figure 6, below.

Results

ISOLATION OF COLD-SENSITIVE ALLELES OF *DC0*

Ten EMS-induced alleles of *DC0* have been isolated previously as recessive lethals by test-crossing to the null *DC0* 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 *DC0* alleles, we screened for EMS-induced mutations that were lethal in combination with the strong *DC0* allele, *DC0^{H2}* at either 18°C or 29°C (see Materials and Methods). The lethality of all 32 newly isolated mutations, including *DC0^{T4}* and *DC0^{X4}*, and of the previously isolated and sequenced *DC0* alleles was found by recombination to map within 4 map units of a $P[ry^+]$ insertion at 30C. This indicates that all of the newly induced mutations are likely to be alleles of *DC0*.

All 32 new *DC0* alleles were tested for complementation of the *DC0* deficiency, *Df(2L)γ15* at 18°C, 25°C, and 29°C and for complementation of selected hypomorphic *DC0* alleles at 18°C and 25°C. Only 2, *DC0^{T4}* and *DC0^{X4}*, of the 32 mutations showed pronounced temperature-dependent complementation properties. Both *DC0^{T4}* and *DC0^{X4}* 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, *DC0^{B10}*, *DC0^{B12}*, and *DC0^{D30}*, at this temperature. Also the majority of *DC0^{X4}* and *DC0^{T4}* hemizygotes died as third-instar larvae at 18°C, characteristic of weak *DC0* alleles. Animals that are zygotically null for *DC0* die as first-instar larvae (Lane and Kalderon 1993).

BIOCHEMICAL PROPERTIES OF COLD-SENSITIVE ALLELES *T4* AND *X4*

The effects of *DC0^{T4}* and *DC0^{X4}* mutations on PKA activity were measured to verify that these

DC0 allele	DC0 T4		DC0 X4	
	% VIABILITY 18°C	25°C	% VIABILITY 18°C	25°C
Df(2L)γ15	0	91	0	57
B3	4	56	1	71
H2	8	66	1	44
G9	3	58	1	44
C4	6	80	1	65
A13	10	50	3	68
H3	16	61	21	66
B12	56	ND	53	69
D30	86	ND	80	87
B10	108	92	94	92

Figure 1: Complementation properties of cold-sensitive alleles, $DC0^{X4}$ and $DC0^{T4}$. 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 $DC0$ alleles used, other than T4 and X4, have been described previously and are written from top to bottom in descending order of allelic strength (Lane and Kalderon 1993).

mutations affect the $DC0$ 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 $DC0$ 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 het-

erozygous for a strong $DC0$ allele or the deficiency, $Df(2L)\gamma15$, have only 50% of wild-type PKA activity. Extracts of $DC0^{X4}/Df(2L)\gamma15$ adults showed large reductions in PKA activity relative to $Df(2L)\gamma15/CyO$ control flies that were raised in parallel under identical conditions (Fig. 2). At 25°C, $DC0^{X4}/Df(2L)\gamma15$ 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 $DC0^{X4}$ hemizygotes, is consistent with measurements on flies carrying other $DC0$ 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 $DC0^{X4}$ is a cold-sensitive allele of $DC0$ and that in vitro measurements of PKA activity reflect the change in activity of $DC0^{X4}$ -encoded enzyme in vivo. Similar measurements indicated that the PKA activity in $DC0^{T4}/Df(2L)\gamma15$ flies drops from 36% to 18% of wild-type upon shifting adult flies from 25°C to 18°C.

Quantitation of p40 DC0 protein concentration in $DC0^{X4}$ 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 $DC0^{X4}$ homozygotes (Fig. 3). Also, it was not possible to establish whether shifting from 25°C to 18°C reduced the concentration of $DC0^{X4}$ protein further.

Genotype	$\frac{S6cnbw}{S6cnbw}$		$\frac{DC0^{X4} S6cnbw}{DC0^{X4} S6cnbw}$		$\frac{DC0^{X4} S6cnbw}{Df(2L)\gamma15}$		$\frac{DC0^{T4} S6cnbw}{Df(2L)\gamma15}$		$\frac{Df(2L)\gamma15}{CyO}$	
	25	18	25	18	25	18	25	18	25	18
Temperature (°C)	25	18	25	18	25	18	25	18	25	18
% kinase activity	104	98	38	21	24	16	36	18	50	50
# of Assays	2	2	2	4	2	4	4	4	N.A.	N.A.
Standard Error of Mean	4	3	3	4	3	2	2	2	N.A.	N.A.

Figure 2: PKA activities encoded by $DC0^{X4}$ and $DC0^{T4}$. PKA activities of extracts were calculated as the rate of Kemptide phosphorylation in the presence of 10 μ M cAMP (see Materials and Methods). $Df(2L)\gamma15/CyO$ [rather than $S6cnbw/Df(2L)\gamma15$] flies were used as convenient controls in all assays because the wild-type $DC0$ gene on CyO and $S6cnbw$ chromosomes encodes equivalent activities; $Df(2L)\gamma15/CyO$ 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 $Df(2L)\gamma15/CyO$ measured in the same experiment. The activities for $DC0^{X4}$ 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 *DC0^{X4}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, *DC0^{X4}* 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 *DC0^{X4}*. 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-

Figure 4: Pavlovian olfactory learning and memory of PKA mutants. *DC0^{X4}/Df(2L)γ15* (□; lower curve) and control, *S6/Df(2L)γ15* flies (■; upper curve) were assayed for classical odor–shock conditioning at two different temperatures, 25°C (A) and 18°C (B). Performance indices (PIs) are plotted against time intervals between training and testing. Error bars represent s.e.m.; mean PIs ($n = 6$) for each genotype and time points (0, 10, 20, 30, 60, 180 min) are shown. (C) PI ratios of *DC0^{X4}* hemizygotes relative to controls at each memory retention point at 25°C (○) and 18°C (□) are plotted. (▲) The difference between PI ratios at the two temperatures. PI ratios and s.e.m.s are calculated as described in Materials and Methods.

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 P s > 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 $\times$ TEMP and/or GENO $\times$ TEMP $\times$ 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

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.

Figure 6: The temperature-shift-specific effect of $DC0^{X4}$ disrupts MTM. Comparison of PI ratios of $DC0^{X4}$ hemizygotes at 18°C or 25°C with *amn* flies at 25°C. The PI ratios of $DC0^{X4}$ hemizygotes at 18°C (□) or 25°C (○) are plotted with those of *amn* mutants (●; recalculated from the data of Tully and Quinn 1985). Note the PI Ratios for $DC0^{X4}$ hemizygotes at 18°C decay at a similar rate to those for *amn* mutants, and both decay rates are faster than that for $DC0^{X4}$ hemizygotes at 25°C. PI ratios of $DC0^{X4}$ hemizygotes are the same as shown in Fig. 4C.

allele of the major catalytic subunit gene *DC0*. This allowed us to investigate the effects of different levels of PKA activity on learning and memory using a Pavlovian olfactory learning assay. Animals with at least 50% of normal PKA activity throughout their development showed roughly normal learning and retention over 3 hr, whereas animals with <25% of normal PKA activity throughout their development were defective in initial learning (Fig. 4A,B). After development was complete, further reduction of PKA activity (from 24% to 16%) did not produce any additional reduction in initial learning relative to controls assayed at the same temperature (Fig. 4C). However, a more rapid decay of memory was seen in animals with 16% of normal PKA activity relative to animals with 24% or 50% of normal PKA activity (Fig. 4C), when the temperature-shift-specific effects of the conditional allele were resolved. From these data we suggest an acute, physiological role for PKA in MTM.

CHARACTERIZATION OF THE COLD-SENSITIVE MUTATIONS $DC0^{X4}$ AND $DC0^{T4}$

We would ideally like to assay *Drosophila* learning in the complete absence of PKA activity.

A single gene, *DC0*, 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 *DC0* probably do not eliminate all PKA activity in *Drosophila*. Furthermore, *Drosophila* requires *DC0* 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 *DC0* alleles, $DC0^{T4}$ and $DC0^{X4}$, 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 $DC0^{T4}$ or $DC0^{X4}$ 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 $DC0^{X4}$ 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 $DC0^{X4}$ 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 $DC0^{X4}$ larvae pupariate and eclose at 25°C but not at 18°C. A variety of other *DC0* 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^{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^{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^{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^{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^{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^{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 *amn* gene (Quinn et al. 1979; Tully et al. 1990). If we compare the memory curves of *amn* mutants at 25°C with those of *DCO^{X4}* hemizygotes at 18°C using PI Ratios, we see almost identical curves (Fig. 6). If, as recently suggested (Feany and Quinn 1995), *amn* 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^{X4}* hemizygotes and controls or by assessing MTM in *amn*, *DCO^{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 *amn* gene may encode a precursor of several peptides including a homolog of mammalian pituitary adenylyl cyclase-activating peptide (PACAP) (Feany and Quinn

1995), so it may also act by modifying the state of PKA activation. *amn* mutations have little effect on initial learning but reduce retention of conditioned behavior measured at any time from 10 to 180 min following conditioning (Quinn et al. 1979; Tully et al. 1990; Tully and Gold 1993; Feany and Quinn 1995).

Longer-term memory can only be induced in normal flies by repeated training and can be separated into two components. The most durable memory, long-term memory (LTM), requires spaced training, is disrupted by inhibitors of protein synthesis, and may be eliminated or enhanced by manipulation of the activity of *dCREB-2* gene products (Tully et al. 1994; Yin et al. 1994, 1995). Mammalian homologs of *dCREB-2* encode transcription factors that change their activity in response to phosphorylation by PKA or calcium-dependent protein kinases (Gonzalez and Montminy 1989; Foulkes and Sassone-Corsi 1992; Sun et al. 1994). A second long-lasting form of memory is resistant to anesthesia and various other forms of disruption and, therefore, is termed ARM (anesthesia-resistant memory). ARM is disrupted by mutation of the *radish* gene (Folkers et al. 1993; Tully et al. 1994), but LTM is not. It is not known whether the *radish* gene product is involved in cAMP signal transduction.

For those aspects of learning and memory that appear to require normal cAMP signal transduction, we would like to define targets of signal transduction and investigate how alterations in their activity correlate with different memory phases. In particular, Is prolonged PKA activation necessary for any form of memory? and Do different memory phases depend on phosphorylation of different substrates by PKA? Presumably, during associative learning, neurotransmitter initially elevates cAMP levels and hence PKA activity via adenylyl cyclase, either through activation of a G protein or elevation of calcium concentration. It is possible that PKA reverts to its initial activation state shortly thereafter as neurotransmitter is rapidly removed or degraded. However, activated PKA may be maintained for several minutes or hours, perhaps through continued presence of an activator of adenylyl cyclase, such as PACAP, or through proteolytic modification or degradation of regulatory subunit (Greenberg et al. 1987; Muller and Spatz 1989; Aszodi et al. 1991). In each case, the PKA signal could be extended further according to the decay rates of PKA substrates and other downstream effectors.

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 LTM 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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