CREB Transcription Factors and Long-Term Memory in *Drosophila*

by

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B.A., Amherst College 1987

Submitted to the Department of Brain and Cognitive Sciences in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

at the
Massachusetts Institute of Technology
June 1995

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Abstract

Studies presented here investigate the role of CREB-dependent cyclic 3',5'-adenosine monophosphate (cAMP)-responsive transcription in long-term memory (LTM) in *Drosophila*. *dCREB2*, a *Drosophila* gene encoding products similar to those mediating cAMP-responsive transcriptional activation in mammals was cloned in an expression screen of a head cDNA library. Amino acid sequence homology of the predicted protein suggested that *dCREB2* might be a *Drosophila* member of the subset of *CREB* family genes that produce cAMP-responsive transcriptional activators.

Proteins encoded by two different, alternatively-spliced *dCREB2* messages, *dCREB2-a* and *dCREB2-b*, were studied in F9 cell culture assays for the ability to mediate PKA-responsive transcriptional activation from a CRE-containing reporter construct. dCREB2-a was a PKA-responsive transcriptional activator. dCREB2-b was an antagonist of PKA-responsive transcriptional activation by dCREB2-a.

These results were used to test the possible role of CREB-mediated transcriptional events in LTM. Transgenic flies were made with a P-element containing the *dCREB2-b* coding region under *hsp70* promoter control. Cell culture results indicated that this would act as an inducible dominant-negative mutation blocking dCREB2-a activity. A regimen of ten cycles of spaced training in the Tully-Quinn olfactory classical conditioning paradigm was used to produce LTM. Acquisition, short-term memory, anesthesia-resistant memory, and behavioral controls were unaffected by expression of the transgene. In contrast, induction of the dCREB2-b transgene just prior to training specifically abolished LTM. The straightforward interpretation of these results is that overexpression of dCREB2-b blocks cAMP-responsive transcriptional activation that is required for LTM.

A gene encoding another previously unidentified CRE-binding protein, *dCREB1*, was found. The predicted protein sequence shows much stronger homology to the yeast GCN4 protein than to cAMP-responsive CREBs. Expressed dCREB1 is able to bind to CREs, but it does not appear to be a PKA-responsive transcriptional activator in cell culture.

Thesis advisor: Dr. William G. Quinn, associate professor

For my parents.

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Chapter 1

Introduction

1.1 The hippocampal memory system - progress in understanding the neural basis of learning and memory in mammals.

Investigations of the biology of learning and memory in mammals initially took the form of lesion studies. This work provided anatomical evidence for the involvement of the hippocampal system. Directed by these results, electrophysiological recordings from the hippocampus in behaving animals revealed learning-related changes in the activity of hippocampal neurons. In parallel with these correlative methods, it was found that applying specific patterns of electrical stimulation to hippocampal pathways could induce long-term potentiation (LTP) of synaptic transmission.

It is widely believed that learning and memory at the neural level involve changes in connectivity. Hippocampal LTP is characterized by persistent changes in synaptic efficacy in a part of an anatomically-defined memory system, suggesting that it could be a biological process involved in memory formation. Systems for studying LTP in vitro in hippocampal slices were developed, facilitating the study of the cellular and molecular basis of LTP. This model system is currently the richest source of molecular information about a candidate process for memory in mammals.

1.1.1. Hippocampal lesion studies.

The earliest systematic efforts to investigate the relation between memory and the substance of the brain were anatomical lesion studies. K. Lashley, studying the effects of cortical lesions, found that he was unable to localize specific sites for memory in rats after training for brightness discrimination or maze-running. In those experiments, the severity of memory loss was correlated with the amount, but not the region, of cortical tissue extirpated, which led him to conclude that the memory trace (the "engram") may be present as a widely distributed network of synaptically associated neurons in the cortex (Lashley, 1929; Lashley, 1950).

Localizing specific memories in the brain is a problem that remains to be solved, but the identification of neuroanatomical structures involved in memory processing has proven to be amenable to lesion studies. Early

studies with temporal lobe lesions in monkeys showed "psychic blindness", where, among other things, recognition of previously experienced objects was impaired (Kluver & Bucy, 1939). Insights from human brain lesions indicated that temporal lobe structures were crucial to memory processing.

1.1.1.1. Humans.

Studies on H.M., a patient with bilateral loss of temporal lobe structures, constitute the best-known work in this area (Corkin, 1984; Milner et al., 1968). A neurosurgical resection performed to treat H.M.'s intractable epilepsy included removal of his hippocampal formations, amygdalas and associated temporal cortex, leaving the patient with profound anterograde amnesia (Scoville and Milner, 1957) Patient H.M.'s tested intellectual functions were normal and his ability to recall events from before surgery was largely intact, although it was severely disrupted for a period spanning several weeks before his surgery and retrograde amnesia was detected for some events as much as eleven years before surgery. The specific nature of H.M.'s behavioral defect suggested that some or all of the disrupted temporal lobe structures were required to process new memories. It also suggests that these structures are not essential for retrieval of pre-existing memories over a few weeks old.

Squire's group studied patient R.B, who had less extensive temporal lobe injuries then H.M. An ischemic episode left R.B. with brain injuries that, in the temporal lobes, were limited to bilateral damage to the hippocampus proper, especially the pyramidal cells of CA1. The only cognitive deficit found in this subject was a moderately severe memory impairment, although it was less profound than the deficit seen in H.M. (Squire et al., 1990; Zola-Morgan et al., 1986). This pattern of relatively isolated CA1 damage is said to be a common post-mortem neuropathological finding in amnesic people. High-resolution NMRI studies done in living amnesic patients with medical histories and memory impairments similar to R.B.'s indicate apparent temporal lobe damage to their hippocampal formations (Press et al., 1989; Squire et al., 1990).

There appears to be more than one memory system in the human brain, at least one of which is spared in damage to the hippocampal system. This was

suggested by the preservation of H.M.'s ability to learn new motor and perceptual skills (reviewed in Ogden and Corkin, 1991). For example, H.M., though unable to form memories of events, persons, objects or scenes, was able to learn, over several days, to trace a pattern in an experimental situation where he could view the pattern (and his hand) only in a mirror (Milner, 1962). His performance improvement within and between sessions was indistinguishable from normal subjects, however at each new session he could not remember having performed the task before. The dissociation between conscious descriptive memory and unconscious performance memory has been seen in many other instances of human amnesia.

Historically, number of terminologies have arisen to describe this dichotomy of memory types (e.g., fact vs. skill, explicit vs. implicit). Cohen & Squire (1980) called these two types of memory declarative and procedural. Declarative memory is conscious, descriptive, flexible, relational and subject to generalization. Procedural memory, in contrast, is described as unconscious, involving skills and habits that are often fixed in the modalities in which they were learned (Cohen et al., 1985). Squire and Zola-Morgan (1991) currently favor uses the terms declarative and non-declarative memory. Declarative memory has the attributes of consciousness implied by the words "remembering" and "knowing". Non-declarative memory encompasses an enumerable but apparently heterogeneous set of mnemonic abilities that includes, for example, motor skill learning, habit learning, forms of associative conditioning, and priming phenomena. It is now widely believed that components of the hippocampal system are a necessary neuroanatomical substrate for declarative memory, but not non-declarative memory, at least in humans and monkeys (Mishkin and Appenzeller, 1987; Squire and Zola-Morgan, 1991; Zola-Morgan and Squire, 1993). but see Horel (1994).

1.1.1.2. Non-human primates.

The discovery that human medial temporal structures are involved in the processing of new declarative memories was followed by investigations to determine more precisely which temporal structures are required for normal memory function. Studies have continued in brain-damaged humans, but

this problem has been most aggressively pursued in lesion studies with monkeys.

Brain-lesioned monkeys can be behaviorally evaluated for memory impairment using a number of tests. The delayed non-matching to sample (DNMTS) test is generally accepted as a benchmark task for evaluating the monkey homolog of human declarative memory function, that is often referred to as recognition memory. In this test of visual recognition memory, a sample object is displayed to the subject and, after a delay interval, the sample object is displayed again, this time alongside a novel object. The animal has previously been trained to move aside the novel object (i.e., the one that does match the previously shown sample) to get a food reward. The duration of the delay interval over which the subject can remember (and thus correctly reject) the sample object is used as a measure of retention. Other tasks used in recognition memory evaluation include simple object discriminations and eight-pair concurrent object discrimination, where animals must learn which object of a pair is associated with a reward. In the latter task, eight pairs of objects must be learned simultaneously. Concurrent pattern discrimination and motor skill tasks are commonly used to test monkey correlates of types of memory that are preserved in human temporal lobe amnesias, for example, the Lifesaver™ task, where a monkey must learn to negotiate a small toroidal confection off of a rod with a 90^{o} bend.

A systematic nomenclature has been adopted to describe medial temporal lobe lesions used to explore memory function in monkeys. $H^{+}A^{+}$ represents a lesion including the hippocampus (H), the amygdala (A) plus brain tissue associated with each (+) that is damaged in a direct surgical approach through the ventral aspect of the temporal lobe to reach each of these deep structures. In lesions less extensive than $H^{+}A^{+}$, omitted symbols indicate sparing of tissue. Thus, H^{+} represents a lesion where the hippocampus plus associated cortex is lesioned, but the amygdala and its underlying cortices are spared. H^{++} describes a lesion that includes the tissue described for H^{+} and also the cortex superficial to the amygdala, but where the amygdala itself is left intact. Exceptions to this notation lesions of the perirhinal and parahippocampal cortices, that are denoted as PR and PH, respectively.

In early studies, Mishkin et al. (Mishkin, 1978; Mishkin, 1982) used what was essentially an H^+A^+ lesion in an effort to simulate the bilateral medial temporal resection of H.M. in monkeys in order that behavioral comparisons could be made. They found that this extensive lesion produced anterograde amnesia with characteristics very similar to that seen with medial temporal lesions in humans. Visual recognition memory function was severely disrupted (DNMTS performance reduce to the level of chance at ten-minute delay), but skill learning was left intact. They also investigated separate amygdalar and hippocampal lesions, each of which claimed associated brain tissue, and indicated that both hippocampus and amygdala contributed to recognition memory. The claim for amygdalar involvement in DNMTS performance was incorrect (see below).

Work by Zola-Morgan et al. (1989a), using more limited lesions, showed that on the DNMTS task, the H^{++} lesion was nearly as devastating as the H^+A^+ lesion, and the H^+ lesion also significantly impaired performance on this task (mean percent correct at ten-minute delay was 65% versus 80% for controls). The A lesion did not affect performance on DNMTS or other recognition memory tasks (retention of object discrimination or eight-pair concurrent discrimination), and the impairment from the H^+A lesion was much the same as for the H^+ lesion alone. These results indicate that the hippocampal formation and cortical structures surrounding both the hippocampal formation and amygdala, but not the amygdala itself, are critical for recognition memory (Squire and Zola-Morgan, 1991; Zola-Morgan, et al., 1989a; Zola-Morgan et al., 1989b).

In experiments designed to further delimit the cerebral substrate for memory required for DNMTS performance in monkeys, Squire's group used preoperative nuclear magnetic resonance imaging (NMRI) to guide stereotactic lesions of the hippocampal formation (hippocampus, dentate gyrus and subiculum) that spared adjacent cortex (Alvarez-Royo et al., 1991). The accuracy of these H lesions was confirmed post mortem by examination of histological sections. Such lesions impaired DNMTS performance to a level similar to that obtained by H^+ lesions, but only slightly degraded performance on acquisition of eight-pair concurrent discrimination and retention of simple object discrimination. Skill acquisition was undisturbed.

A monkey model of R.B.'s hippocampal lesion used intentionally-induced ischemia to produce damage limited mainly to areas CA1 and CA2 of the hippocampus and to some cells of the dentate gyrus (Zola-Morgan et al., 1992). This lesion resulted in moderate impairment on DNMTS tests, but only slight performance decrements on other tests of recognition memory, similar to the results seen with H lesions. These results suggest that the hippocampus proper is not solely responsible for recognition memory processing.

In support of this idea is the fact that the most debilitating specific lesions of the hippocampal system that have been identified to date are those made to the perirhinal and parahippocampal cortices, the PRPH lesion (Suzuki et al., 1993; Zola-Morgan, et al., 1989b) Surgical aspiration of these areas, which are the penultimate cortical connections for a majority of the neural traffic between the neocortex and the hippocampus, results in a profound impairment specific to recognition memory. The behavioral effect on memory is similar to that seen with H^+A^+ lesions, and are more severe than H lesions, suggesting that these cortical loci are more than mere waystations in the medial temporal memory system pathway.

The hippocampus and associated temporal cortices appear to be a substrate for encoding declarative memory, but not procedural memory, in humans and non-human primates. Results with lesions to the hippocampus proper, where effects on memory are only moderate, suggest two possible interpretations. Declarative memory may be composed of behaviorally dissociable components that are differentially detected by DNMTS versus other tests, or more likely, the hippocampus forms part, but not all, of the apparatus needed to encode declarative memories.

1.1.1.3. Rodents.

In rats, results from a wide variety of lesion studies indicate that damage to the hippocampus and associated cortices specifically impairs the ability to perform certain memory tasks (Jarrard, 1993; Squire, 1992). Further, hippocampal system lesions do not impair performance in tests of rat skill learning. For example, lesioned animals were able to learn the behaviors

required to perform conditional discrimination tests, but they were unable to make correct choices above the level of chance.

The rat hippocampal system has been strongly implicated in acquiring and retaining spatial information, a specialized form of recognition memory which is likely to be an important facility in many of the rat's everyday behaviors. A widely-accepted experimental paradigm for testing spatial learning and memory in rats is the Morris water maze (Morris, 1984). A circular pool, about one meter in radius is filled with opaque fluid. This is placed in a room that has visual cues at fixed positions, placed so that they can be seen by a rat swimming in the pool. In the "place navigation" version of the task, an escape platform is placed at a fixed position in the pool. The platform is designed so that its top is just beneath the surface of the opaque liquid in the pool, making it invisible to the swimming rat. In blocks of trials given over a few days, a subject animal is placed in the pool starting at various positions around the perimeter, each time swimming until he finds the hidden platform and climbs up onto it. The time required to find the platform, or escape latency, is measured. With normal rats, escape latency drops precipitously after the platform is located for the first time, and subsequently the rat can swim straight to the platform from any start position, apparently navigating by remembering and manipulating spatial relations among fixed distal cues in the room. Lesions of the hippocampal system impair performance in this task. Hippocampally-lesioned rats, released in successive trials from new positions around the circumference of the pool, perform as if the position of the platform is unknown to them. In the "cued navigation" version of the task, rats are trained with a visible platform. Both hippocampally-lesioned and control rats both quickly learn to swim to the platform and get out of the water, indicating that appetitive and motor behaviors required for Morris water maze tasks are intact. When the visible platform is replaced by a hidden one at the same location, however, unlesioned rats swim directly to the hidden platform, while hippocampally-lesioned rats show drastically increased latencies. In second measure of performance in the Morris water maze task, a "probe test" is administered in which a trained rat is released into the pool from which the escape platform has been removed. Its search pattern is videotaped and

analyzed for the proportion of time spent swimming in the quadrant where the platform was located during training. Normal rats spend a large part of their time searching in the quadrant where the platform was located during training, while hippocampally-lesioned rats spend approximately equal amounts of time in each quadrant, suggesting these animals do not know the location of the platform even at this approximate level.

Olton et al. (1978) devised a radial arm maze to test the effects of hippocampal damage on spatial learning and memory performance. The maze consists of a central arena from which radiate a number of identical arms, usually eight. At the end of each arm is a goal box that can be baited with a food reward. The maze is constructed so that distal visual cues outside of the maze can be seen by the rat. Animals are trained to visit each arm of the maze once per trial. Rats trained in this way and then hippocampally-lesioned (fornicotomized) re-enter arms they have already emptied, apparently unable to recognize these as previously-visited locations. This result has been confirmed elsewhere, using a modification of this task, where only a subset of the arms are baited (Jarrard, 1993; Olton and Papas, 1979). Olton and Feustle (1981) did similar experiments with a non-spatially cued version of the radial-arm maze. In this system, maze arms differed by intrinsic tactile and visual cues while relational spatial cues outside the maze were made unavailable by dim lighting and high outer walls. After training to criterion, fornix transection severely impaired performance on this cued task, suggesting that general recognition memory, and not just spatial memory, is impaired by hippocampal damage. Jarrard (1993), on the other hand, using a similar maze, found minimal effects of hippocampal damage in the cued situation, in contrast to a serious impairment in the spatial memory task, leading him to suggest that the rat hippocampus is specifically attuned to handle spatial map information and does not subserve general recognition memory.

At least part of the disagreement among behavioral results with rat hippocampal lesions turns on the specific lesion used (Jarrard, 1991). The term "hippocampal system" encompasses the hippocampal formation (hippocampus, dentate gyrus and subiculum), the adjacent cortices (parahippocampal, entorhinal and perirhinal) as well as the fornix/fimbria.

The vast majority of lesion studies indicating that the rat hippocampus mediates general recognition memory involve transection of the fimbria/fornix, or electrolytic or aspiration lesions that remove, at the very least, the entire hippocampal formation. Lesion results indicating that the hippocampus is limited to acquisition of spatial memory have usually employed multiple stereotactically-placed injections of ibotenic acid that precisely destroy the neurons of the hippocampus proper plus hilar and dentate granule cells, sparing surrounding cortical areas and fibers of passage (Jarrard, 1989). This limited lesion also spares the subiculum, which is ordinarily considered part of the hippocampal formation.

In support of the view that some of the reported differences in behavioral results are due to differences in the extent of lesions. Morris et al. (1990) found that although rats with ibotenate lesions of the hippocampus proper are impaired in performing the Morris water maze task, these animals eventually can learn it if given sufficient trials. This is also true of rats with damage limited to the subiculum. Rats with damage to both the hippocampus and subiculum, however, are unable to successfully learn this task, even with extended training. These results shows a similarity to monkey lesion studies, where damage to the hippocampus proper produces a milder impairment than damage to the hippocampus plus surrounding cortex.

Further work is required to resolve these specific issues but, at a minimum, results from rat hippocampal system lesions suggest the presence of more than one memory system. They support the idea that the hippocampus proper is important in rat spatial learning and memory, and that some elements of the hippocampal system, that includes adjacent cortical structures and fiber tracts, are required for rat recognition memory.

1.1.2. Correlates of learning and memory in hippocampal electrophysiology. Alterations in the firing patterns of hippocampal pyramidal neurons with classical condition have been noted in a number of species (Berger and Thompson, 1978a; Berger and Thompson, 1978b; Patterson et al., 1979; Segal and Olds, 1973). A situation where this was examined extensively was in the

delay form of classical conditioning of the nictitating membrane (NM) response in rabbits. In delay conditioning, the conditioned stimulus (CS; a 350-ms. tone) temporally overlaps the unconditioned stimulus (US; a 100-ms. puff of air to the eye). The unconditioned response (UR; a reflex eyeblink) was monitored electrically. Berger & Thompson (1978a,b) used chronically-implanted electrodes to make single- and multiple-unit extracellular recordings from rabbit hippocampal pyramidal cells during training. Large increases in firing were seen as early as the second paired training trial. Increases in unit activity were not seen either in response to unpaired exposure to the CS or US alone or as a concomitant of the NM reflex. Initially, firing was seen at the onset of the US, but as conditioning proceeded, the increased hippocampal response became time-shifted towards the onset of the CS in a way that tracked with the acquisition of the conditioned behavior, and this persisted through consecutive days of testing. This phenomenon was specific to conditioning and did not occur in explicitly unpaired trials. Thus, hippocampal activity appeared to be a correlate of learning and memory in this system, functioning as an indicator of stimulus association.

The hippocampal formation is not required for the acquisition or maintenance of delay conditioning of the eyeblink. Bilaterally hippocampectomized or, indeed, decerebrate animals can be successfully trained in this task (Mauk and Thompson, 1987; Norman et al., 1977; Schmaltz and Theios, 1972), Efforts by Thompson and his co-workers seem to have localized the rabbit engram for this behavior to specific deep nuclei of the cerebellum (Mauk et al., 1986; McCormick et al., 1982; McCormick and Thompson, 1984; Thompson, 1988) This raises the question of the significance of the observed correlation of hippocampal activity to learning in this system.

One answer to this is that the hippocampus appears to be required for more complex properties of associative learning. Rabbits with hippocampal system lesions show notably slow extinction of the conditioned response to CS (Schmaltz and Theios, 1972). They are also impaired in tests of discrimination reversal, latent inhibition and blocking (Berger and Orr, 1983; Orr and Berger, 1985; Solomon, 1977; Solomon and Moore, 1975). Solomon et al (1986) and Moyer et al (1990) have studied the trace form of classical

conditioning, where there is an interval between the end of the CS and onset of the US. They found that hippocampal pyramidal cell activity correlates with behavioral acquisition in this form of classical conditioning, and hippocampally-lesioned rabbits are unable to learn in the trace conditioning paradigm.

Unit recordings from complex-spike units, as slow-firing (<10Hz) hippocampal pyramidal cells are called, have shown that these neurons can be specifically tuned for particular stimuli or combinations of stimuli. A number of groups have reported that complex-spike neurons in CA1 have "place fields", such that they preferentially fire when the animal moves through a particular location in an explored environment (O'Keefe and Dostrovsky, 1971; O'Keefe, 1976; O'Keefe, 1979; Eichenbaum et al., 1989; Muller et al., 1987).

Recently, Wilson & McNaughton (1993) used multiple implanted electrode arrays to record activity from many tens of complex-spike cells simultaneously from a rat exploring a familiar environment. Analysis of the position-dependent firing of these ensembles of neurons allowed these workers subsequently to predict the rats position accurately from activity data alone.

The ability of hippocampal pyramidal cells to reflect position appears to be only one facet of the multidimensional responses of which these cells are capable. Location may be integrated with other variables to bias firing, but the position of the subject is not a necessarily a dominant component in many cases. In the classical conditioning experiments described earlier, for instance, there is no differential spatial component involved in the selective complex-spike cell response to specific stimuli, since the animals are maintained in a fixed position. This is also true in successive discrimination paradigms where, in experiments using either sound or odorants, pyramidal cells will acquire firing selectivity dependent on the temporal sequence of presentation of different stimuli. (Eichenbaum et al., 1987; Foster et al., 1987; Sakurai, 1990).

An extremely interesting property of complex-spike cells is that the same cell can show different tuning depending on the demands of a particular task. In

an experiment where unit activity of hippocampal cells was recorded across disparate tasks, a cell that fired preferentially at one location in a place task was active at another location in the same environment during a non-place task (O'Keefe and Speakman, 1987; Wiener et al., 1989). Thus, the properties of these hippocampal neurons appear to be quite unlike those of visual cortical cells, for example, that fire in response to a circumscribed domain of feature configurations in a single sensory mode. The triggers for these hippocampal cells can be multimodal , integrating sensory, positional, temporal and historical information, and they can simultaneously participate in several apparently independent representations, depending upon context (Cohen and Eichenbaum, 1993).

1.1.3. Hippocampal long-term potentiation (LTP).

The preceding sections present evidence from systems level anatomical studies and from electrophysiological recording of a role for the hippocampal system in learning and memory. At the synaptic level, learning and memory are though to be the result of use-dependent changes in connectivity among neurons. The well-regarded Hebbian model (Hebb, 1949) proposes that such changes occur specifically at those synapses where activity in the presynaptic terminal is repeatedly correlated with ongoing activity in the postsynaptic neuron.

LTP is probably the most intensively investigated form of use-dependent synaptic plasticity in the mammalian brain. Interestingly, it has features that conform to the Hebbian hypothesis. LTP refers to a long-lasting increase in postsynaptic response after patterned (usually tetanic) electrical stimulation. Lømo (1966) reported that tetanic stimulation of the perforant path (a major afferent pathway to the granule cells of the dentate gyrus) in rabbits induced persistent increases in field recordings of EPSPs and population spikes in the dentate gyrus. Increases could last for many days in chronically-implanted awake animals (Bliss and Gardner, 1973; Bliss and Lomo, 1973; Lømo, 1966). As a system that produces use-dependent increases in synaptic efficacy in the hippocampus, LTP suggested itself as a model process that might be involved in learning and memory.

Investigation of LTP revealed properties that conform to predictions of the Hebbian model. First, LTP is synapse-specific. HFS produces LTP only at synapses served by the stimulated axons, and not at other synapses on the same postsynaptic neurons (Larson and Lynch, 1986; Sastry et al., 1986). (Recent work indicates that LTP can spread from a tetanized synapse to nearby untetanized synapses, apparently via a diffusible chemical messenger, but this effect is limited to a small radius [Schuman and Madison, 1994]). Second, LTP induction by tetanic activation of a single input has a threshold stimulation requirement. Stimulation frequency and strength of activation of the input pathway act cooperatively in meeting this threshold (Bliss and Lomo, 1973; McNaughton et al., 1978). In terms of the Hebbian model, increasing the frequency or strength of the tetanic input increases the likelihood of summation of successive EPSPs, conforming to the Hebbian co-activity prediction and resulting in increasing depolarization of the postsynaptic neuron. Third, LTP induction using more than one input shows associative properties that again agree with Hebb's co-activity hypothesis. An input that is too weak to induce LTP at its synapses can be made effective by inducing temporally-contiguous electrical activity in the postsynaptic neuron. Sub-threshold stimulation of a small group of fibers can produce LTP when it occurs within about 100 milliseconds after a strong tetanizing stimulus on a different set of fibers that synapse on the same pyramidal neurons (Larson and Lynch, 1986; McNaughton, et al., 1978; Sastry, et al., 1986) Repeated depolarization of the postsynaptic neuron by current injection allows an otherwise ineffective synaptic input to produce LTP (Kelso and Brown, 1986; Wigstrom et al., 1986). Repeated depolarization of the postsynaptic cell alone did not induce LTP, indicating that presynaptic input plus depolarization are required. Besides its relationship to Hebb's theories, associativity is a reasonable candidate process for detecting temporal coincidence, which is in turn a plausible neural mechanism for associative learning.

Hippocampal LTP is a model system amenable to analysis in vitro, and in fact most work on the cellular and molecular basis of LTP is done at one remove from the whole animal. Transverse slices of the dissected hippocampal formation are cut a few hundred microns thick. These slices, which contain a cross-section through the laminar organization of the excitatory pathways of hippocampal formation, can be stably maintained for many hours in a culture

chamber and LTP can be induced in them (Alger and Teyler, 1976; Malinow et al., 1988; Schwartzkroin and Wester, 1975; Skrede and Westgaard, 1971; Spencer et al., 1976). This arrangement allows relatively precise manipulation of the tissue by electrophysiological, pharmacological and, most recently, molecular means (Collingridge et al., 1983a; Malinow, et al., 1988; Pettit et al., 1994)

Slice preparations have been used for work on LTP at hippocampal synapses in rat, rabbit, mouse and guinea pig, but the overwhelming majority of LTP studies are done on area CA1 of the rat hippocampus, where CA3 pyramidal cell axon collaterals of the Schaffer pathway and commissural fibers synapse on the dendritic trees of CA1 pyramidal cells. LTP can be induced by high frequency tetanic stimulation (HFS) from an electrode placed in the Schaffer collateral/commissural pathway. HFS typically consists of one or a more short bursts of 100Hz stimulation, spaced a few seconds apart, at an intensity usually about half that required to produce maximum EPSP slope in the CA1 neurons. (The slope of the EPSP is usually calculated for the region between 20% and 80% of the EPSP evoked by the test pulse.) Glass pipette electrodes, placed in the dendritic field or used in whole-cell clamp or intracellular recording arrangement, register postsynaptic activity. They can also be used to inject current or to deliver drugs by iontophoresis, dialysis or perfusion. As mentioned above, depolarizing the postsynaptic cell by current injection can reduce the requirement for HFS. HFS typically gives an increase in excitatory postsynaptic response that quickly stabilizes to a potentiated response level usually between 1.5- to 2.5-fold over baseline. This is measured as an increase in EPSP amplitude or, more commonly, EPSP slope. LTP ordinarily persists for 3-5 hours before decaying to baseline, but any stable potentiation lasting over one hour is considered LTP. Stimulation that is below the threshold for inducing LTP can induce short-term potentiation (STP), an enhancement of EPSPs that may be as large in amplitude as LTP but that, instead of stabilizing, falls back to baseline in less than an hour.

LTP can be induced in other synaptic fields of the hippocampus besides CA1, as well as at locations outside the hippocampus (Artola and Singer, 1987; Iriki et al., 1989; Komatsu et al., 1981; Racine et al., 1986; Teyler et al., 1989). Discussion of the cellular mechanisms here will deal mainly with LTP at

CA3-CA1, but each component of the hippocampal trisynaptic circuit (CA3-CA1, perforant path-dentate granule cells [PP-DG], mossy fiber-CA3 [MF-CA3]) can support LTP. Experimental evidence indicates that the mechanism for induction of LTP at CA3-CA1 and PP-DG synapses involves a type of glutamate receptor known as N-methyl-D-aspartate (NMDA)-receptor, while LTP induction at MF-CA3 LTP synapses appears to use a different mechanism, that at present is not as well understood.

1.1.3.1. N-methyl-D-aspartate (NMDA) receptor-dependent LTP induction.
The amino acid L-glutamate is a major excitatory neurotransmitter molecule in the mammalian CNS, and a number of different glutamate receptor types and subtypes have been identified by pharmacology and named for drugs to which they respond. One of these is the NMDA receptor, which is liberally represented in discrete areas of the telencephalon, and it is particularly abundant in area CA1 of the hippocampus, as assayed by autoradiographic receptor-binding experiments (Monaghan and Cotman, 1985; Monaghan et al., 1983). A second class of glutamate receptors, a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-receptors, are also abundant at CA1 synapses. AMPA receptors control ligand-gated channels that preferentially conduct Na^+ and K^+ ions and pass the bulk of the fast synaptic currents (Collingridge et al., 1983b). Post-synaptic NMDA receptors, on the other hand, control a channel that is a major route for Ca^{++} influx (Dingledine, 1983; MacDermott et al., 1986) and, although the NMDA receptor ionophore can also pass monovalent cations, it is not substantially involved in conducting fast synaptic currents of EPSPs (Herron et al., 1986).

The peculiarity of the NMDA receptor channel resides in its requirement for concurrent ligand-binding and membrane depolarization to achieve functional activation and pass a Ca^{++} current. A number of groups have shown that the apparent voltage-responsiveness of the NMDA receptor channel is not an intrinsic property of the receptor, but rather is secondary to a voltage-dependent block of the ionophore by magnesium ions (Ault et al., 1980; Mayer and Westbrook, 1987; Nowak et al., 1984). At resting potential, extracellular Mg^{++} ion is thought to be bound at a location deep in the transmembrane pore, which prevents the flow of Ca^{++} current through the open channel (Ascher and Nowak, 1988; Mori et al., 1992). The mechanics by

which the block is relieved by depolarization of the membrane is uncertain, but perhaps the changing electrical field makes it less favorable for Mg^{++} to remain in the pore, so that ion departs and allows Ca^{++} influx.

In current models of NMDA receptor-dependent LTP (see Bliss and Collingridge, 1993; Collingridge, 1987; Kennedy, 1989; Madison et al., 1991 for reviews), induction is thought to occur when glutamate release from the presynaptic terminal coincides with ongoing postsynaptic membrane depolarization (by temporal summation of EPSPs from HFS, activation of a second synaptic input to the postsynaptic neuron, or current injection via an electrode) that is sufficient to relieve the Mg^{++} block of the NMDA receptor channel. This leads to functional activation of the NMDA receptor channel and Ca^{++} influx, which is believed to be the intracellular trigger for downstream mechanisms leading to LTP.

The NMDA receptor is able to generate a potentially powerful multifunctional second-messenger signal (increased calcium) when presynaptic (processes leading to glutamate release) and postsynaptic (ongoing depolarizing activity) signals coincide. Hebbian properties of LTP induction can be explained in the context of the dual requirement for activation of the NMDA receptor calcium conductance. In this model, synapse specificity can be supported by the NMDA receptor, since regardless of the level of postsynaptic depolarization, only presynaptic terminals releasing glutamate can activate the ionophore. Cooperativity is a reflection of the necessity of producing EPSPs that are either close enough together or large enough to summate to sufficient level of depolarization to relieve the Mg^{++} blockade of NMDA receptor channels. In the case of associativity, spreading depolarization of dendrites by a second input to the postsynaptic neuron could ameliorate the Mg^{++} block of NMDA receptor channels at other synapses. A similar argument applies in the situation where even quite weak stimulation can induce LTP if the post-synaptic neuron is held depolarized by current injection. Malinow & Miller (1986) did an experiment where they held a postsynaptic neuron hyperpolarized and this prevented induction of LTP by strong HFS. In accordance with the model discussed here, hyperpolarization would prevent removal of the Mg^{++} block from the NMDA receptor channel, preventing LTP.

A huge assortment of pharmacological blocking experiments on hippocampal slices using a variety of NMDA receptor antagonists, with different potencies and modes of action, have convincingly shown that these compounds prevent the induction of LTP in CA1 and in the dentate (Bashir et al., 1990; Coan et al., 1987; Collingridge, et al., 1983b; Harris et al., 1984). These agents do not appear to affect baseline transmission, and in the case of the reversible inhibitor AP-5, the block is reversed by washout of the drug (Errington et al., 1987). These results are persuasive proof of the involvement of NMDA receptors in LTP induction. Once established, LTP is not disturbed by application of NMDA-receptor antagonists, indicating that NMDA receptors are involved in induction, but not maintenance, of LTP (Collingridge, et al., 1983a). NMDA receptor antagonists do not block LTP at mossy fiber synapses (Harris and Cotman, 1986), which is not a particularly surprising result in view of the scarcity of NMDA receptors in his region.

Applying glutamate or NMDA to slices without accompanying presynaptic activation until recently has not been found to produce LTP, although it does induce STP (Collingridge, et al., 1983b; Kauer et al., 1988). This led to the postulation of a requirement for presynaptic activity in LTP for purposes other glutamate release.

Although there may be presynaptic involvement, results from Cormier et al. (1993) call into question the necessity for concurrent presynaptic activity during induction of LTP by glutamate. They reported reasonably reliable induction of NMDA-receptor dependent LTP by a regimen of repeated pulsed iontophoresis of glutamate onto synapses while presynaptic activity is blocked. LTP induced by pulsed glutamate iontophoresis completely occluded induction of additional LTP by HFS. (Occlusion of further LTP induction is a commonly accepted criterion for sharing of LTP mechanisms.)

Cormier et al.'s pulsed iontophoretic method may produce a pattern of NMDA receptor activation and Ca^{++} influx more like that which occurs with tetanic stimulation than those used by others. Elsewhere, Aiba et al. (1994) propose a complex mechanism, based on results of LTP experiments in mutant mice made null for the metabotropic glutamate receptor 1 (mGluR1)

gene, involving activation of presynaptic mGluR1s. Cormier et al.'s result should be repeated and pursued, since it may provide a system for examining non-activity-dependent pre-synaptic event in LTP in isolation.

1.1.3.2. Calcium and downstream events in LTP.

Activity-dependent elevation of calcium levels in the postsynaptic neuron has been implicated in LTP induction. Both Lynch et al. (1983) and Malenka et al. (1988) have used injection of calcium-chelating agents into the postsynaptic neuron to prevent induction of LTP. Haas (1986) has shown that LTP induction is blocked LTP in the presence of cesium, a calcium channel blocker. Groups have employed various combinations techniques combining microfluorimetry, microscopy and single unit recording to observe transient increases in calcium concentration in dendrites and synaptic spines from tetanic stimulation (Malinow et al., 1994; Muller and Connor, 1991; Regehr and Tank, 1990) The increase in intracellular Ca^{++} probably occurs via NMDA receptor channels, since when non-NMDA receptor voltage-gated calcium conductances are blocked, HFS still produces elevated Ca^{++} levels and LTP, although there are indications that release of intracellular calcium stores may participate in the LTP process (Alford et al., 1993; Perkel et al., 1993). Bliss and Collingridge (1993) similarly indicated that calcium from intracellular stores may also be involved in LTP induction, since drugs that deplete or interfere with the release of these pools also appear to interfere with LTP.

Most attention to downstream effectors activated by increased calcium concentration has been focused on the involvement of kinases, especially the calcium-responsive serine/threonine protein kinases CaMKII and PKC. CaMKII is abundant in the hippocampus and postsynaptic densities are highly enriched in CaMKII (Erondu and Kennedy, 1985; Kennedy et al., 1990). After calcium/calmodulin (CaM)-dependent autophosphorylation, CaMKII complexes can become CaM-independent and persistently active (Miller and Kennedy, 1986). Theoretically, at least, CaMKII could maintain phosphorylation of neuronal substrates for an extended period of time after activation (Lisman and Goldring, 1988; Molloy and Kennedy, 1991). Interest in PKC was aroused by the observation of Routtenberg and his co-workers of a positive correlation between the duration of LTP the phosphorylation of

GAP-43, a synaptic substrate of PKC (Lovinger et al., 1986). They also found a correlation between translocation of hippocampal PKC from cytosolic to membrane fraction, involved in PKC activation, and the persistence of LTP (Akers et al., 1986).

The involvement of CaMKII and PKC in LTP has been extensively studied using protein kinase inhibitors. These agents can be applied extracellularly or by injection into the soma of postsynaptic neuron. The consensus of these results was that administering protein kinase inhibitors prior to HFS blocks induction of LTP, but not of STP (Lovinger et al., 1987; Malenka et al., 1989; Malinow, et al., 1988; Reymann et al., 1988b). Application after HFS did not disrupt established LTP. In an exception to this result, when Malinow et al. (1988,1989) applied the preferential PKC inhibitor H-7 to the bath at twenty minutes or 240 minutes after tetanic stimulation, it reversed existing potentiation. Injection of H-7 into the postsynaptic neuron did not produce this effect. This result was confirmed in later experiments by Colley et al. (1990), suggesting that protein kinase activity at a location outside the postsynaptic cell is involved in maintenance of LTP.

A problem with most protein kinase inhibitor studies is that the specificity of many of these agents for a particular kinase is often a far from absolute. In an attempt to address this problem and to determine whether PKC or CaMKII or both are required postsynaptically for LTP, Malinow et al. (1989) injected specific peptide inhibitors of either PKC or CaMKII into the postsynaptic neuron. Each peptide prevented the induction of LTP but not of STP, and mutated peptides were ineffective. This indicated that both kinases were involved in maintaining LTP, but in a recent paper, Hvalby et al. (1994) re-evaluated these peptide inhibitors. This group found that, in comparison to the PKC inhibitor peptide, the CaMKII inhibitor peptide had a relatively poor ratio of selectivity between CaMKII and PKC. It also showed much lower potency as an LTP blocker. The CaMKII inhibitor peptide's lower specificity and the higher dose of it required to block LTP suggest that its effect could be due to inhibition of PKC. This result does not rule out a role for CaMKII in LTP, and there is other good evidence for the involvement of that kinase. It is however, a useful cautionary tale.

More evidence linking protein kinases to LTP comes from targeted gene inactivation experiments in mice, often called knockouts (reviewed in Grant and Silva, 1994). LTP experiments in hippocampal slices from mouse genetic knockout mutants in a variety of protein kinases have generally supported the idea that these genes are required for normal LTP.

1.1.3.2.1. CaMKII.

In pharmacological experiments intended to interfere with another aspect of CaMKII activation, injection of calmodulin antagonist peptides into the postsynaptic cell blocks LTP induction (Malenka, et al., 1989; Reymann et al., 1988a). This treatment probably affects calmodulin-dependent processes besides CaMKII activation, but may avoid effects on PKC, since calmodulin antagonist peptides showed strong and specific inhibition of CaMKII compared to PKC in vitro.

A molecular genetic approach to examining the role of CaMKII in LTP was undertaken by Silva et al (1992b). He generated a mouse knockout in the α isoform of the CaMKII. This mutant is almost completely refractory to LTP induction in CA1 in hippocampal slice preparations. Neuroanatomy and NMDA receptor function in these mice appear to be undisturbed by the genetic lesion, and they are normal in general behavior, except for hyperreactivity.

The preceding experimental results indicate that disrupting CaMKII can interfere with LTP, but can specific activation of CaMKII induce LTP? Petit et al. (1994) have recently reported results that begin to answer this question. They used the novel technique of infecting the CA1 cell field in a hippocampal slice with a recombinant vaccinia virus vector programmed to produce a constitutively-active mutant form of CaMKII (CaMKII (1-290)VV) in postsynaptic structures. (They claimed that infection of CA3 axons was not consequential, citing literature indicating that axons and presynaptic terminals lack the protein synthesis machinery required for translation of the viral product. Experimentally, they show that no viral product was detected in the CA3 cell bodies when a recombinant vaccinia virus producing β-galactosidase (BGVV) was injected in CA1.)

CA1 neurons infected with BGVV were not different from uninjected neurons in tested electrophysiological properties. Infection with CaMKII(1-290)VV led to increased transmission in the CA1 neurons compared to BGVV-infected neurons. This was seen as a decrease in minimum threshold excitation and an increased response to that smaller stimulus and as a steeper slope of input-output curves in the CaMKII (1-290)VV-infected slices. This effect was also observed when the NMDA-receptor blocker (APV) was present throughout the experiment, indicating that the enhanced transmission was not LTP produced by NMDA-receptor activation. In a final experiment, they showed that tetanic stimulation was unable to produce further long-lasting enhancement of transmission in the CaMKII (1-290)VV-infected neurons.

A possible explanation of these results is that expression of the constitutively-active CaMKII- induced LTP, seen as enhanced transmission, and that the occlusion experiment shows that induction was maximal and shares mechanism(s) with HFS-induced LTP. Such an interpretation would mean that CaMKII is a necessary and sufficient for inducing LTP events downstream of NMDA receptor activation. It may be, however, that a process other than LTP is reflected in as enhanced transmission, and that overexpression of the constitutively-active kinase blocks, rather than occludes, subsequent induction of LTP by HFS. Given the background of LTP literature indicating the involvement of CaMKII in LTP, the answer probably lies somewhere in the middle. Whether or not CaMKII is sufficient for inducing maximal LTP, this is the first work directly indicating that CaMKII can induce LTP, and it introduces a valuable new technique that can be adapted for testing the role of other molecules in LTP.

1.1.3.2.2. PKC.

Experiments mentioned above, using inhibitor peptides, point to the involvement of PKC in the maintenance of LTP. Hvalby et al. (1994) found that a low dose of PKC inhibitor peptide blocks LTP but not STP. At higher doses, where this peptide could be affecting CaMKII, STP is blocked. This result is similar to those in experiments in the dentate gyrus, where iontophoresis of a low dose of polymyxin B (PMB, a kinase inhibitor that is relatively specific for PKC) before tetanic stimulation blocks LTP but not STP, and at a higher dose, at that CaMKII might also be affected, STP is also

abolished. Extracellular application at the lower dose of PMB up to thirty minutes after tetanic stimulation led to a decline and loss of LTP (Colley, et al., 1990). Wang & Feng (1992) found that infusing the postsynaptic neuron with a mixture of low doses of PKC pseudosubstrate inhibitor peptide and polymyxin B up to three hours after tetanic stimulation resulted in accelerated decline and loss of established LTP. These results suggest a requirement for long-lasting postsynaptic kinase activity in LTP.

Abeliovich et al. (1993a) performed thorough electrophysiological studies with a mouse knockout in the gene for the PKCγ isoform. Their outcomes were more complex and difficult to interpret than those in some other knockouts, but this careful work yielded interesting results. The PKCγ$^-$ mice were normal anatomically and in hippocampal synaptic transmission in slices. In addition to usual tests of synaptic function, PKCγ$^-$ hippocampal slices were investigated and found to be normal in long-term depression (LTD), a form of synaptic plasticity, independent from LTP, that can be induced by prolonged low-frequency stimulation (Dudek and Bear, 1992; Linden, 1994). PKCγ$^-$ mice were also normal in general behavior, except for some signs of cerebellar disturbances. LTP could not be induced in hippocampal slices from these mutant mice using an ordinary HFS protocol, but priming with an LTD-inducing stimulus pattern followed by tetanic stimulation produced robust LTP. These animals showed moderate impairments in hippocampus-dependent learning that could be overcome with extended training. The electrophysiological results indicate that these animals can support LTP, but only with priming. It is difficult to know whether to classify these mutants as severely or slightly abnormal in LTP.

While the mouse knockout of the gene for the γ isoform of PKC has effects on LTP, the mutation does not abolish it. In light of the apparent blocking effects of PKC inhibitors on LTP, this suggests that another PKC isoform may also be involved. Colley and Routtenberg (1993), based on evidence from their own protein kinase inhibitor experiments (Huang et al., 1992), present a scheme in which postsynaptically-located PKCγ is involved in events in the first few minutes after induction of LTP. At later times, up to one hour after induction, PKCβ in the presynaptic terminals is believed to be persistently active and is involved in LTP establishment and maintenance. This

hypothesis is consistent with the effects of extracellular application versus postsynaptic injection of H-7 and, if correct, may help explain the PKCγ knockout results.

Attempts to induce LTP by injection of activated PKC or activation of endogenous PKC by addition of phorbol esters have met with limited success (Gustafsson et al., 1988; Hu et al., 1987; Malenka et al., 1986; Muller et al., 1988). Although they have potentiating effects, these treatments appear not share the same mechanisms as tetanically-induced LTP. This could be due to technical and physiological difficulties arising from the mode of application and slow turnover of these agents.

1.1.3.2.3. Tyrosine kinases.

To investigate the involvement of tyrosine kinase(s) in LTP, O'Dell et al. (1991) used specific inhibitors of this family of kinases in slice preparations. Grant et al. (1992) then made mouse knockouts in each of four non-receptor tyrosine kinase genes. Hippocampal slice experiments with the mutant strains showed that among these, only the fyn $^-$ knockout affected LTP induction. The fyn$^-$ mice were histologically abnormal in the hippocampus, possessing larger-than-normal numbers of CA3 pyramidal neurons and dentate granule cells, but the mice were normal in general behavior (Grant and Silva, 1994). Baseline synaptic transmission in the hippocampus was indistinguishable from that of wild-type mice. LTP in hippocampal slices from fyn$^-$ mice is significantly abnormal. Low-intensity tetanus treatment that is sufficient to induce LTP in wild-type hippocampal slices was not effective in fyn$^-$ slices. A high-intensity train produced LTP in fyn$^-$ slices, but only half as large as that seen in wild-type slices (Grant, et al., 1992).

1.1.3.3. LTP induction by via metabotropic glutamate receptors.

A recent and very interesting development is the implication of metabotropic glutamate receptors (mGluRs) in LTP. There are currently seven known members of this gene family that codes for G protein-coupled glutamate receptors (Houamed et al., 1991; Masu et al., 1991; Nakanishi, 1994; Tanabe et al., 1992). Various members can activate phospholipases, affect cAMP metabolism through control of adenylyl cyclase activity or directly modulate ion channels. Preliminary work has indicated that mGluR-mediated effects

on kinase activation and release of calcium from intracellular stores might be involved in LTP (Bortolotto and Collingridge, 1993). A role for cAMP has also been indicated (Huang and Kandel, 1994). These and other effects could plausibly be mediated by second-messenger pathway coupled to the various mGluRs (Westbrook, 1994).

Collingridge's group has demonstrated that bath application of the specific mGluR antagonist 1S,3R-aminocyclopentane dicarboxylate (1S,3R-ACPD) induces stable LTP in CA1 with slow (minutes) onset, and occlusion studies indicate that the LTP induced by 1S,3R-ACPD shares a common pathway with tetanically-induced LTP (Bortolotto and Collingridge, 1992; Bortolotto and Collingridge, 1993). Bashir et al. (1993) showed that the presence of a specific antagonist of mGluRs (a-4-methyl-carboxyphenylglycine, MCPG) blocked the induction of LTP by 1S,3R-ACPD and reversibly prevented tetanic induction of LTP in CA1 and also at MF-CA3 synapses, suggesting a requirement for mGluR activation in LTP. A report from Chinestra et al., describing similar experiments, specifically contradicts these results, but a recent paper by Bortolotto et al.(1994) affirmed their previous positive result. This newest work expands on their previous claims, inicating that MCPG is effective at blocking LTP only at synapses where LTP has not previously been induced. Once activation leading to even submaximal LTP has occurred, a long-acting switch required for LTP induction has been thrown, and thus eliminates the need for renewed mGluR activation for further LTP induction.

A mouse knockout in mGluR1 made by Tonegawa's group showed serious impairment, but not absence, of LTP in CA1 (Aiba, et al., 1994). These mice also were specifically deficient in one-day memory of contextual fear conditioning, a hippocampus-dependent form of learning that is sensitive to NMDA-receptor antagonists. Interestingly, MCPG has no effect on residual LTP in hippocampal slices from the mGluR1$^-$ mouse, suggesting that this isotype might be especially important among the metabotropic receptors in LTP.

1.1.3.4. The synaptic locus of expression of LTP.

An issue that was thought to be nearing resolution in the field of LTP, but which has one again become contentious (Malinow, 1994), is whether the

changes that result in enhanced transmission are presynaptic, postsynaptic or both. If the locus of change is postsynaptic, this would be reflected in increased sensitivity of the postsynaptic receptor population, either by addition of new receptors or modification of existing ones. If presynaptic changes that occur in LTP, one might expect to observe changes in neurotransmitter release.

At the gross level, levels of evoked labeled glutamate in the PP-DG synaptic field of cannulated anesthetized rats has been studied. After LTP induction, increased levels are detected upon test stimulation, and when LTP induction is blocked by APV treatment, this increase is not seen (Bliss et al., 1986; Dolphin et al., 1982; Errington, et al., 1987). Increases have been seen in CA1 and dentate in slice preparations, and were also observed in an intermediate preparation where hippocampal LTP was induced in the whole animal and the relevant region of the hippocampus (CA3) removed, sliced, loaded and examined for evoked glutamate levels (Feasey et al., 1986; Skrede and Malthe, 1981). Increased levels of evoked extrasynaptic glutamate might be due to increased release, or alternatively might be the result of decreased elimination of synaptic neurotransmitter by reuptake into neurons and/or glia (Hertz, 1979; Rothstein et al., 1994).

If LTP at potentiated synapses resulted in increased conduction through both the NMDA and non-NMDA classes of receptors that are present there, the simplest explanation would be enhanced transmitter release. If only non-NMDA current, which mediates most of the EPSP, were enhanced, then the presumption would be that the change was postsynaptic. In initial experiments, Kauer et al. (1988) and Davies et al. (1989) found a slowly-developing, LTP-dependent, specific increase in the synaptic response to agonists of non-NMDA glutamate receptors (that mediate most of the fast synaptic currents) in CA1. This argued for a post-synaptic component in LTP. More recent results, however, have shown have shown that NMDA-receptor currents are also potentiated in LTP (Bashir et al., 1991; Xie et al., 1992), suggesting enhanced transmitter release as a mechanism in LTP, and thus a presynaptic locus of change. The slow onset of both of these changes, however, could only explain later phases of LTP, and not the immediate

enhancement of transmission that is evident within seconds after application of an inducing stimulus.

Using a quantal model of neurotransmitter release, statistical analysis of whole-cell or patch-clamp electrophysiological observations of synaptic transmission, either spontaneously-occurring or induced by weak stimuli, can provide evidence about the locus of change in LTP. For example, weak synaptic stimulation results in occasional failures to induce a postsynaptic response. These events occur stochastically and a change in the frequency of such failures (in the absence of changes in response size on non-failure trials) has been taken as an indication of alterations in presynaptic biology governing release of transmitter. Postsynaptic alterations can also be teased out by quantal analysis, as increased EPSP response per quantum of neurotransmitter. Numerous quantal studies of hippocampal LTP have been undertaken, both in slices and in CA3-CA1 cell co-cultures.

In some studies, changes were judged to be the result of changes in parameters of quantal release (Bekkers and Stevens, 1990; Malgaroli and Tsien, 1992; Malinow and Tsien, 1990; Stevens and Wang, 1994). In others, indications of increased quantal amplitude were also seen, suggesting that both presynaptic mechanisms were at work (Kullmann and Nicoll, 1992; Larkman et al., 1992; Liao et al., 1992). Few groups favor a completely postsynaptic locus of expression for LTP, although recent experiments by Manabe & Nicoll (1994), using quantal analysis in combination with NMDA receptor antagonists, fail to detect changes in presynaptic release.

In a recent commentary, Malinow (1994) points out that reasonable changes in assumptions about the synapses involved in LTP can lead to opposite interpretations of the same quantal analysis data. In addition to being difficult to obtain, measurements are taken from only one side of the synapse, and they are made at a remote location. Such observations may not be adequate to give a complete picture of a process that involves both sides of the synapse and two interdependent processes (release and response). Wide variations are present in the measured properties of hippocampal synapses, even on the same hippocampal neuron, thus drawing conclusions from a response to which many synapses contribute may dilute synapse-specific

changes occurring in a subpopulation of the connections contributing to activity at the level of the whole neuron. Malinow concludes that the development of more synapse-specific methods, in combination with better molecular information about the relevant synapses, will be needed to finally solve the problem of assigning loci of synaptic change in LTP. There is as yet no true consensus on the locus of expression of LTP. The available evidence, like the scarecrow in the Wizard of Oz, points to both sides of the synapse. Furthermore, the locus of change may be different at different times in the LTP process.

1.1.3.5. Retrograde messengers in LTP.

The trigger for LTP induction at CA3-CA1 synapses appears to be postsynaptic, via activation of NMDA receptors and calcium influx. If some part of the LTP expression mechanism is presynaptic, as has been suggested, what is the retrograde messenger that the postsynaptic cell uses to communicate with the presynaptic cell? Several candidates have been suggested, and interference with these affects LTP, suggesting that they are, at least to some degree, involved. Most of this work has focused on nitric oxide (NO) and carbon monoxide (CO).

NO is a short-lived soluble gas that is known to act in vivo as a diffusible, membrane-permeant intercellular messenger over short distances (Bredt and Snyder, 1994; Moncada et al., 1989; Palmer et al., 1987) In the context of LTP, the evanescence of NO suggests a possible method for maintaining synapse specificity or for mediating very local spread of LTP (Schuman and Madison, 1994).

NO is synthesized from arginine by a family of CaM-dependent NO synthases (NOS's), which are represented in the hippocampus (Bredt et al., 1991; Bredt et al., 1990; Dinerman et al., 1994). NO can activate the cGMP second-messenger pathway via soluble guanylyl cyclase and application of NMDA to hippocampal slices elevates cGMP levels by a route that involves the generation of NO (East and Garthwaite, 1991). Chetkovich et al (Chetkovich et al., 1993) found that an increase in guanylyl cyclase activity that occurred after LTP induction could be blocked by NO synthase inhibitors. Antagonists of soluble guanylyl cyclase or antagonists of the cGMP-dependent kinase,

PKG, block LTP(Zhuo et al., 1994). No downstream targets of PKG have yet been identified in LTP.

LTP-blocking effects with antagonists of guanylyl cyclase and PKG were not observed by Schuman et al. (1994). This group did, however, detect a (putatively presynaptic) NO-responsive ADP-ribosyltransferase (ADPRT) activity in CA1, and bath application of inhibitors of this activity blocked LTP. There are indications that neuronal G-protein subunits are the targets of ADP-ribosylation by endogenous brain ADPRTs (Duman et al., 1991), and experiments where pertussis toxin (an ADPRT known to inactivate G-proteins) has been used to block LTP in CA1 have implicated (putatively presynaptic) G-proteins in LTP (Goh and Pennefather, 1989).

In experiments using guinea pig or rat hippocampal slices, a number of groups (Bohme et al., 1991; O'Dell, et al., 1991; Schuman and Madison, 1991) have shown that competitive inhibitors of NO synthase, added to the bath or injected into the postsynaptic neuron prior to tetanic stimulation, prevent LTP. These inhibitors do not interfere with established LTP. Extracellular application of hemoglobin, an avid NO scavenger that is not significantly membrane-permeant, also blocks induction of LTP. These data are consistent with the hypothesis that postsynaptically-produced NO acts early in LTP and that it must travel extracellularly to exert its effect, possibly by diffusing to neighboring neurons.

Some groups have reported that strong tetanic stimulation can overcome the presence of even high concentration of NOS inhibitors to induce LTP (Chetkovich, et al., 1993; Haley et al., 1993), suggesting that NO may not be necessary for LTP induction with HFS stimulation. (Most blocking experiments testing NO involvement in LTP use an induction protocol combining sub-threshold stimulation and depolarization of postsynaptic cells for induction.) In support of this idea, simply applying NO to the slice bathing solution fails to induce LTP. However, in combination with sub-threshold tetanic stimulation, treating the slice with NO produced long-lasting enhancement of synaptic potentials. This cooperatively-induced potentiation occluded subsequent LTP induction by a strong tetanus, indicating that it really is likely to be LTP (Zhuo et al., 1993). Thus, NO might

not be required for induction of LTP, but instead may increase the probability that a given stimulus will be effective. This could be an important role in vivo, where induction of a memory process analogous to LTP might involve weaker inputs than those used to produce LTP in slices.

Using chemical NO generators, Lonart et al. (1992) showed increased release of labeled neurotransmitters (not glutamate) from hippocampal slices. Analogous results were achieved by a brief application of NO to dissociated hippocampal cells in culture, where a rapidly-developing and long-lasting increase in the frequency of miniature excitatory postsynaptic currents (O'Dell, et al., 1991), again indicated enhanced probability of transmitter release. A recent paper by Meffert et al. (1994) indicates that NO can cause substantial calcium-independent release of synaptic vesicles from hippocampal synaptosomes. This raises the possibility that NO might increases the probability of LTP induction by activating or augmenting non-stimulus-coupled neurotransmitter release. Taken together, these results suggest that NO can increase synaptic connectivity via presynaptic effects.

A mouse knockout in the gene for a constitutively expressed, soluble isoform of NOS (so-called neuronal NOS) showed only slight reductions in LTP in slices, and an NO synthase inhibitor still blocked LTP from weak-, but not strong-intensity tetanus, as in wild-type mice (O'Dell et al., 1994). The authors suggest that the a different NOS isoform is likely to be involved in LTP, since there is still hippocampal NOS activity in the neuronal NOS knockout mouse (Huang et al., 1993). The endothelial form of NOS, which is membrane-associated and constitutively expressed, appears to be abundant in CA1 pyramidal cells (Dinerman, et al., 1994; O'Dell, et al., 1994).

CO is another candidate for an early retrograde messenger. Like NO, this a soluble gas molecule, but it is longer-lived than NO. Heme oxygenase-2, an enzyme that produces CO, is abundant in the brain, where its mRNA is co-localized with that for soluble guanylyl cyclase (Verma et al., 1993). They also performed experiments indicating that CO generated by heme oxygenase-2 can increase cGMP levels in primary olfactory neuron cultures. Inhibitors of heme oxygenase specifically blocked induction of LTP by a strong tetanus, but did not interfere with LTD. Furthermore, heme oxygenase inhibitors

reversed established LTP (Stevens and Wang, 1993). Hemoglobin serves as an extracellular sink for CO as well as NO, and as mentioned earlier its presence in the bath prevents LTP. CO acts like NO to produce long-lasting enhancement of synaptic transmission when applied along with weak electrical stimulation (Zhuo, et al., 1993). Thus, CO shares a subset of its LTP-related properties with NO, but appears to be involved in both early and later events in LTP.

1.1.3.6. Long-lasting LTP (L-LTP)

LTP induction protocols involving one tetanic pulse, or a few pulses massed given within a period of tens of seconds, give LTP that can last for from three to five hours. Much longer-lasting LTP can be obtained using a spaced protocol, where three tetanic pulses are given, with an interval of ten minutes between each stimulation. This produces a large LTP whose duration is apparently limited only by the longevity of the slice (Reymann et al., 1985). Using this system, the extended phase that appears with spaced stimulation has been called late-phase LTP (L-LTP), while the LTP ordinarily produced by a single stimulus is termed early LTP (E-LTP). Recent experiments suggest that L-LTP can be biologically dissected from E-LTP on at least two criteria. Recent experiments indicate that L-LTP, unlike E-LTP, is cAMP-dependent and its induction is prevented by the protein synthesis inhibitor, anisomycin.

Anisomycin sensitivity of L-LTP has been shown in the PP-DG synaptic field in awake rats (Krug et al., 1984) and in CA1 in slice preparations (Frey et al., 1988; Huang and Kandel, 1994). Application of anisomycin, an effective and reversible protein synthesis inhibitor in CA1, during or immediately after tetanic stimulation blocks induction of L-LTP but not E-LTP. Potentiation from spaced tetani that would ordinarily produce L-LTP began to decay within an hour. By three to five hours potentiation was significantly reduced compared to undrugged control slices, and eventually decayed completely.

The induction of E-LTP does not appear to require the protein synthesis blocked by anisomycin, but its magnitude does seem to depend on unidentified proteins with relatively short half-lives. Extended (30 minute) pre-incubation of slices with anisomycin before tetanic stimulation prevents

robust expression of E-LTP in CA1 but does not degrade baseline synaptic response (Deadwyler et al., 1987; Otani et al., 1992).

Induction of L-LTP appears to depend on early mobilization of the 3',5'-cyclic adenosine monophosphate (cAMP) second-messenger pathway resulting in activation of PKA. Spaced tetanization of CA1 results in a significant elevation of cAMP levels that does not occur with a single tetanus (Huang and Kandel, 1994). Inclusion of PKA inhibitors in the bathing solution from a time slightly before spaced tetanization until 45 minute afterward blocked the appearance of L-LTP but not E-LTP (Frey et al., 1993; Huang and Kandel, 1994; Matthies and Reymann, 1993). K-252a, a wide-spectrum kinase inhibitor that affects PKA, applied 90 minutes after the spaced tetanus protocol did not affect subsequent development of L-LTP (Matthies et al., 1991). KT-5720, a specific inhibitor of PKA and blocker of L-LTP at the concentration used, failed to block L-LTP when given 15 minutes after the third tetanus of the spaced protocol. Application of a functional cAMP analog (Sp-3',5'-cyclic adenosine monophosphothioate; Sp-cAMPS) in the bath for fifteen produced a brief synaptic depression followed by a long-lasting potentiation that peaked at 90 minutes and was maintained for at least eight hours. Furthermore, this effect was occluded by LTP induced by spaced tetanic stimulation, indicating that Sp-cAMPS-induced potentiation at least shares mechanisms with the tetanus-induced L-LTP.

1.1.3.7. LTP and learning in the behaving animal.

An extremely important issue in hippocampal LTP research is to show the relation between LTP and learning and memory in whole behaving animals. LTP is clearly a real neurophysiological phenomenon that can be observed in the intact brain and in slice preparations. Its phenomenology and molecular mechanisms are complex and interesting, and they have spurred the development of technical innovations for studying the mammalian brain at the molecular level. However, until there is some conclusive proof of a connection between LTP and memory function, it remains a model system with no strong attachment to the phenomenon that it attempts to explain.

1.1.3.7.1. Pharmacological experiments.

Intraventricular injection of the NMDA-receptor antagonist AP-5 blocked LTP induction in behaving rats. It also specifically impaired performance in hippocampus-dependent learning and memory tasks in rats, including the Morris water maze and contextual fear conditioning (Fanselow et al., 1994; Morris, 1989; Morris et al., 1986). Interference with LTP and behavioral performance in hippocampus-dependent water maze tasks in these animals was positively correlated with the dose of AP-5 (Butcher et al., 1990). Experiments employing administration of NMDA receptor blockers in vivo have been criticized mainly on the grounds that, in addition to their special role in LTP, NMDA receptors have roles in "ordinary" synaptic function in the hippocampus, cortex and thalamic nuclei. It is possible that drugs derange some other NMDA-receptor dependent function and not LTP, and these are responsible for the specific behavioral deficit.

Recent experiments indicate a role for mGluRs in LTP and memory in rats in vivo. (Richter-Levin et al., 1994) reported that infusion of the dentate gyrus of anesthetized rats with MCPG, an mGluR antagonist, repressed the induction of LTP in the dentate gyrus by tetanic stimulation. Injection of MCPG into the lateral ventricles of rats before training sessions in the Morris water maze had little effect on acquisition, but treated rats were severely impaired in remembering the platform location one day after training. Anesthetized trained animals from the MCPG group showed a significantly reduced level of potentiation in the dentate gyrus after tetanic stimulation.

1.1.3.7.2 LTP saturation.

If spatial learning in rats depend on the hippocampal formation, as suggested by lesion studies, and LTP is an underlying physiological mechanism, then maximally inducing LTP by electrical stimulation ("LTP saturation") of a set of hippocampal synapses prior to training might interfere with learning by rendering required synapses unavailable for further training-induced LTP. Addressing this idea, Castro et al. (1989) induced LTP at the dentate granule cell layer in live rats by repeated sessions of bilateral high-frequency stimulation in the perforant path until no further increment in LTP was seen. They found that learning in the Morris water maze was severely impaired by this treatment, and that this learning deficit recovered along with the decay of tetanically induced LTP.

McNaughton et al. (1986) and Barnes et al. (1994) found that repeated bilateral HFS of the perforant path that induces LTP in the dentate gyrus interferes with reversal learning in the Barnes open-platform paradigm. In the basic task, a rat is released in different orientations in the center of an uncomfortably brightly-lit arena with 18 identical exit holes spaced around its perimeter. Only one of the holes leads to a dark escape box, to which the rat has been familiarized, and distal visual cues are available to allow the rat to learn the spatial location of the "live" exit. Performance in this task is usually measured as the number of approaches to dead-end holes. In the reversal learning version, animals are trained for a fixed number of trials with the "live" hole at one position and then the location of the "live" hole changed and acquisition of the new position is measured.

Barnes et al. found that same HFS treatment that disrupted performance in the open-platform task did not appear to interfere with acquisition or probe trial performance in the Morris water maze, at odds with the result of Castro et al. (1989). They suggested that the two tasks might be differentially sensitive to the completeness of LTP saturation. LTP saturation was evaluated by two methods, looking at increases in EPSP caused by HFS from a second site near the first and at expression of the LTP-inducible zif268 gene (Worley et al., 1993) by in situ hybridization. By these tests, LTP induced by their HFS induction protocol did not appear to approach saturating levels. Experiments using maximal electroconvulsive stimulation (MECS), with or without HFS, produced larger-amplitude LTP and much more extensive expression of zif268 in the hippocampal formation than did bilateral HFS alone. After MECS treatment, performance in all aspects of the spatial version of the Morris water maze task (acquisition, reversal learning and probe test) were compromised compared to control mice. In the probe test, the amount of time spent searching for the absent platform outside of the target quadrant was positively correlated with the magnitude of MECS-induced LTP. McNaughton (personal communication) suggested that the disparity between Castro et al.'s results and those of Barnes et al. may have been due to differences in exact electrode placement or other difficult-to-control variables. Castro et al. may have achieved anatomically more

extensive or different LTP induction than did Barnes et al'. Castro et al. did not have an indicator for the anotomical extent of LTP.

In vivo experiments have indicated a role for NO in learning and memory performance. For example, systemic administration of an NOS inhibitor led to impairment in acquisition of the in the hippocampus-dependent Morris water maze task (Chapman et al., 1992). Intraperitoneal injection of NOS inhibitors has been reported to cause a dose-dependent disruption of spatial learning in a radial arm maze, and this parallels a dose-dependent suppression of hippocampal LTP in slices from treated animals (Bohm et al., 1993).

1.1.3.7.3. Genetically lesioned mice (knockouts).
Mutant mice that were examined for defects in LTP were also tested for impairments in learning and memory performance. The LTP deficit in CaMKII knockout mice was accompanied by a specific and severe impairment in hippocampal-dependent learning in the Morris water maze (Silva et al., 1992a). Hyperreactivity that has been noted in these animals did not appear to interfere with learning, since they were not different from the wild-type strain in tested non-hippocampal learning tasks. Mice made null for the fyn tyrosine kinase gene showed blunted LTP, and these mutants were specifically and profoundly impaired in hippocampus-dependent learning in the Morris water maze (Grant et al., 1992) . PKCγ mutant mice showed only a mild defect in learning in the Morris water maze that was detected in the probe trial with the platform removed from the pool (Abeliovich et al., 1993b). As mentioned earlier, these animals were refractory to LTP induction, unless they were previously primed with low-frequency stimulation. It may be that in the intact brain, normal ongoing electrical activity provides sufficient priming-like input to the hippocampus to allow the induction of near-normal LTP, leading to only a mild defect in learning. This needs to be further investigated in vivo. As mentioned earlier, a knockout in the mGluR1 gene has severely impaired LTP, and is also defective specifically in hippocampus-dependent contextual fear conditioning. Overall, the results from knockouts show a positive correlation between abnormalities in LTP in hippocampal slice preparations and impairments in hippocampal learning.

1.1.4. Conclusion.

The acknowledged goal of biological research on learning and memory is eventually to understand exactly how these processes operate in humans. At the systems level, results from work in humans and other mammals are the most likely to provide information directly applicable to this end, and indeed work in humans and other primates is largely limited to systems level structure-function studies and pharmacology.

In lower mammals, some headway has been made in understanding the molecular biology of LTP, a model process that may or may not be involved in hippocampally-dependent memory formation in vivo, but there are still strong disagreements in the field about a variety of basic issues of LTP biology. In evaluating results from mouse knockouts or constitutively-expressed dominant negative transgenes, it is hard to rule out developmental side-effects from genetic manipulation. These effects range from changes in brain structure to alterations in baseline properties of relevant neurons. Beyond the technical issues, a real connection between LTP and hippocampal memory remains to be made. Again, results from this mammalian system are naturally thought to be immediately relevant to humans, and probably rightly so. On the other hand, the complexity of mammalian brains and behaviors have few compensating technical advantages as systems for experimental biology, making them a tough place for obtaining information about the molecular mechanisms of learning and memory.

1.2. Studies of the neural basis of learning and memory in two vertebrate systems.

Invertebrate systems offer experimental advantages for studying the molecular neurobiology of learning and memory that can compensate for their evolutionary distance from humans. Even though invertebrate brain organization is radically different from mammals, some mechanisms for synaptic plasticity might be expected to be conserved through evolution. This is possible, and even likely, to be true of the molecular principles involved in basic non-associative and associative forms of memory that

invertebrates and vertebrates have in common. Thus, lessons learned from invertebrates are potentially instructive for mammalian research in this area (compare Yin et al. (1994) and Bourtchuladze et al. (1994)). Work on the molecular basis of behavioral plasticity that was carried out in the fruit fly, Drosophila, and the marine mollusk, Aplysia, are particularly relevant here, since they formed the basis for the work presented in later chapters.

1.2.1. Drosophila olfactory conditioning.

The tiny, densely structured brain of the adult fruit fly, containing about 10^5 neurons, is not an inviting place for single-cell studies of synaptic plasticity. Drosophila does, however, offer well-developed experimental systems for molecular genetic investigation of its relatively small and extensively catalogued genome that have been put to good use in neurobiological studies. The goal of this section to make two main points. First, there is persuasive evidence that the cAMP second-messenger pathway is important in learning and memory in Drosophila. Secondly, the Drosophila system has allowed us to genetically dissect memory phases, providing a convincing demonstration of their intrinsic biological authenticity.

Insect behavior, commonly thought of as consisting of the release of rigid fixed action patterns, is in fact quite plastic and subject to operant modification in natural situations (see Heisenberg, 1989; Menzel and Erber, 1978; Quinn and Greenspan, 1984). Importantly for studies of behavior in Drosophila, these dipterans can learn and remember in ways that bear remarkable behavioral similarity to higher species. A wide variety of experimental systems have been used in laboratory situations to measure Drosophila behavioral plasticity. These include demonstrations of various aspects of associative and non-associative learning in behaviors ranging from flight control to mating. Considered here are two odor discrimination learning paradigms.

1.2.1.1. Olfactory associative learning paradigms.

The development of a reasonably sensitive paradigm for group testing of associative learning in flies was a critical development for Drosophila neurogenetics, making it possible to test rapidly for differences in learning

and memory between populations without having to measure individual differences in single flies [see Tully (1986)].

Quinn, Harris and Benzer (1974) used an olfactory discrimination task in which two odors are presented separately, one of them with electric shock as a negative reinforcer (the QHB paradigm). From among a number of chemical odorants tested, 4-methylcyclohexanol (MCH) and 3-octanol (OCT) were adopted and are commonly used in Drosophila olfactory choice paradigms up to the present day. Both of these odorants are have some, but differing, intrinsic repellence to flies in a non-learning situation. In experimental situations, the amounts of the odorants presented are balanced so that groups of naive flies given a choice between them show no preference. Effects of training on olfactory choice can then be measured as deviations from this baseline.

The QHB paradigm follows operant conditioning models. In a dark environment room, a group of flies is drawn by positive phototaxis to migrate from an empty starting tube into each of a series of training and testing tubes, using the countercurrent device pictured in Fig. 1.1. For training, the first (training) tube, which contains an MCH-treated electrified grid, is set in register with the start tube. The apparatus is laid flat, with the bottom of the training tube near a light source. The flies are allowed 60 seconds during which they can migrate into the training tube, where they encounter odor paired with shock. The flies are then shaken back down into the start tube, where they are briefly rested. The process is then repeated with the second (training) tube, which contains an OCT-treated grid with no voltage on it. This training cycle (one presentation of each odor, the first one paired with shock) is repeated three times in succession before testing. The third and fourth tubes are testing tubes, identical to the first and second tubes, respectively, except that neither grid is electrified. The flies are given the opportunity sequentially to phototax from the start tube into each of two testing tubes without being shaken back to the start tube, and the number of flies in each tube is counted. Experiments are counterbalanced for order effects by repeating them on separate groups of flies with the roles of the odors swapped (i.e., OCT becomes the shocked odor). A learning index (λ) is calculated as the fraction of flies tested avoiding the shocked odor minus the

fraction avoiding the unshocked odor. The learning index ranges from zero (complete failure to learn) to one (perfect learning). The learning indices from the counterbalanced halves of the balanced experiment are averaged to give the learning index for the complete experiment. (A slightly different measure of learning, called the performance index (PI), subtracts the number of flies that do not choose either odor from the total. This is usually less than 5% of the flies thus the practical difference between the two measures is small, except that the performance index is finally expressed as a percentage between 0 and 100.) Wild-type Canton-S flies trained and tested in the QHB paradigm gave a mean learning index of about 0.3 when tested immediately after training, indicating that about two-thirds of the flies avoid the shocked odor and about one-third avoid the unshocked odor. To generate a memory curve, different groups are tested at different intervals after training. The memory from the training regimen described here remains at measurable levels for four to six hours after training.

The effect of training in the QHB paradigm is fairly weak. A learning index of 0.3 indicates that about a third of the wild type flies express learning. This behavioral change, however, shows properties of true associative learning. Quinn et al. (Quinn, et al., 1974) showed that the learned behavior extinguished with repeated testing in the absence of reinforcement. A group of flies trained to avoid one odor could, after forgetting, be re-trained to avoid the other odor, and flies could learn using a different reinforcer (unpleasant-tasting quinine in place of electric shock). Of paramount practical importance, the QHB paradigm was demonstrably good enough to allow learning and memory mutants to be identified in screens of ethyl methane sulfonate (EMS)-mutagenized lines (Aceves-Piña and Quinn, 1979; Quinn et al., 1979).

An odor discrimination assay developed later, the Tully-Quinn (TQ) paradigm, gives much more efficient associative learning. This system was used for training and testing for long-term memory in Drosophila in the experimental work presented in this thesis. The TQ paradigm is based on classical conditioning models (Tully and Quinn, 1985) and the apparatus differs from that used in the QHB assay (Fig. 1.2). A group of flies is placed into a training chamber, which contains a shock grid. Odors are carried on a continuous current of air drawn through the training chamber, and pulses of

electric current are applied to the shock grid during exposure to the conditioned odor. No shock is delivered during presentation of the control odor. An elevator chamber integral to the apparatus is used to move the flies from the training chamber to a T-maze in which the group is tested. Air currents carrying the two odorants, one from each arm of the T-maze, converge at the choice point. The trained flies are given two minutes to move into one or the other arm and the number of flies in each arm is then counted.

Wild-type Canton-S flies given a single cycle of TQ training ordinarily register an immediate performance index (tested within 3 minutes after training) of about 85, and retention lasts longer than that from three cycles of training in the QHB system. Learning and memory mutants detected by the QHB assay are also show abnormal performance in the TQ system.

1.2.1.2. Involvement of the cAMP pathway.

Analyses of dnc and rut mutants and the role of cAMP-dependent protein kinase provide strong indications that the cAMP second-messenger pathway is important in the biology of learning and memory in Drosophila. As assayed by measuring retention after a single cycle of training in the TQ paradigm, proper operation of this biochemical system is required at least for early events in learning and memory, probably starting within the first moments of memory formation during acquisition.

1.2.1.2.1 The dnc and rut mutants.

Drosophila learning and memory mutants were first isolated from EMS-mutagenized lines that were screened for defects in learning and memory after training in the QHB paradigm (Aceves-Piña and Quinn, 1979; Dudai et al., 1976; Quinn, et al., 1979). Eight X-linked mutations affecting learning and memory were isolated. From this crop of mutants, the most thoroughly studied from a molecular standpoint are dunce (dnc) and rutabaga (rut). Since the story of these two mutants provides the most complete example of the fruits of using classical genetics in Drosophila to study learning and memory and since they also point to the involvement of the cAMP pathway in behavioral plasticity, these results will be briefly reviewed here.

The X-linked dnc and rut mutants are healthy and normal in appearance, although homozygous dnc^2 females are sterile. With the exception of their learning and memory defects, both mutants seem to be behaviorally normal. They showed essentially no learning in the original QHB assay (dnc, λ=0.04; rut, λ=0.02), but were indistinguishable from wild-type in shock sensitivity, unconditioned odor avoidance and phototaxis, behaviors required for performance in this system (Aceves-Piña and Quinn, 1979; Dudai, et al., 1976). Testing in a QHB assay that had been modified to allow odors to be presented simultaneously during testing indicated that dnc actually achieved significant learning, but its memory decayed very quickly after training (Dudai, et al., 1976). In the TQ assay , these mutants were capable of a moderate, but substantially reduced, level of initial learning (Fig. 1.3). There was rapid memory loss occurred during the first thirty minutes after training, after which decay slowed substantially. There is a small, but measurable amount of retention at seven hours after training in dnc and at 24 hours after training in rut. In the TQ assay, the learning phenotype of the single rut allele tested is recessive, while the three dnc alleles that were tested appear to be semi-dominant (Tully and Quinn, 1985; Tully and Gold, 1993).

Tempel et al. (1983) tested the dnc and rut mutants in a positively-reinforced odor discrimination paradigm based on the QHB apparatus, where electric shock is replaced with "the opportunity to feed on sucrose". In wild-type flies, this reward learning arrangement leads to initial learning scores similar to those seen with negative reinforcement ($\lambda \approx$ 0.3), but the associative memory after training lasts much longer, at least a day as compared with a few hours. The dnc mutant showed learning comparable to wild-type flies, but forgot almost completely within two hours. rut showed below-normal, but moderate, learning in this system, and even more rapid forgetting than dnc.

In addition to testing in paradigms for associative conditioning of odor avoidance, the dnc and rut mutants (as well as others) have been evaluated across a variety of tasks. Non-associative forms of learning in individually trained flies were measured by Duerr & Quinn (1982), who demonstrated that, compared to wild-type flies, both dnc and rut flies showed low habituation and unusually fast recovery from sensitization of the proboscis extension reflex elicited by sucrose solutions. More recently, Corfas & Dudai (1989)

examined habituation and dishabituation of a cleaning reflex that occurs in response to stimulation of bristles on the back of the fly, and found normal habituation rates but faster-than-normal spontaneous recovery from habituation in dnc and rut. In an associative learning situation, Booker & Quinn (1981) found that they could operantly condition a flexed leg position using electric shock reinforcement in almost all (92%) individually-trained, headless wild-type flies , but only in 35% of dnc flies. The learning mutants are behaviorally impaired even in pre-imaginal stages. Wild-type Drosophila larvae can learn to associate odor with shock, but dnc larvae fail at this task (Aceves-Piña and Quinn, 1979). In a visual associative task, Drosophila can be conditioned to avoid a color associated with mechanical shaking (Folkers and Spatz, 1981). The dnc and rut mutants do learn in this system, but only about half as well as wild-type flies after 24 cycles of training. Retention, measured up to two hours after visual conditioning, does not appear to be worse in the mutants than in wild-type flies. Inhibition of courtship behavior in males who have approached already-fertilized females can last for hours, but in learning and memory mutants, the duration of this effect is significantly shorter (Hall & Siegel 1979). The priming effect of male courtship song on female receptivity lasts for five minutes in wild-type flies, but dnc females show essentially no memory for courtship-song priming (Kyriacou and Hall, 1984). This catalog of results shows that the effects of Drosophila learning and memory mutants are not specific for a single behavioral task and are likely to affect fundamental processes that are involved in many forms of learning and memory.

In these three associative olfactory discrimination tests, the consistent behavioral defect in both dnc and rut appears to be in early memory. The effect of these mutations on initial learning appears to vary with the behavioral task. The earliest time at which initial learning can practically be measured is at least a minute after the end of training in any of these paradigms. This makes it difficult to discern whether there is normal acquisition followed by immediate extremely fast decay as opposed to a defect in initial learning. The fact that dnc flies appear to have near wild-type levels of acquisition in the reward-learning paradigm and that memory decay still seems to be accelerated in this paradigm argues that the behavioral defect in that mutant may be in a short-term memory process rather than in initial

learning. Tully & Gold (1993) re-examined the behavioral defect in dunce mutants, using the TQ assay. Their statistical analysis of data from their own experiments and those of Tully & Quinn (1985) indicated that the memory decay rate in dnc during the first 30 minutes after training is not significantly different from wild-type, although the initial learning values are significantly different. This suggests that dnc is an acquisition mutant, but it does not strictly answer explanations that invoke memory loss before the initial learning measurement is made. Tully & Gold considered this and offered the idea that poor acquisition could be secondary to a failure of short-term memory retention during intertrial (intershock) intervals in training. This fits in with the fact that acquisition by dnc in the reward learning protocol is essentially normal, since in that situation the positive reinforcer is continuously present.

Two mutants recently identified from a P-element mutagenesis, latheo (lat) and linotte (lio), show attenuated learning in the TQ assay compared to wild-type flies, but little change in the shape of their memory curves (Boynton and Tully, 1992; Dura et al., 1993). This indicates that a genetic dissection between acquisition and memory can be made, making it unlikely that the defect in dnc and rut are on acquisition alone. At present, dnc and rut are best described as acquisition/short-term memory mutants.

The dunce mutations disrupt the structural gene for cAMP phosphodiesterase II (PDE II), an enzyme in Drosophila that specifically hydrolyzes cAMP to 5'-cAMP. The Drosophila X-chromosome is probably one of the most thoroughly studied objects in biology, so it was not particularly surprising to find that the chromosomal locus for a PDE activity had been mapped to the apparent dnc locus (Kiger and Golanty, 1978; Kiger and Golanty, 1977). A screen for mutations resulting in female sterility also isolated dnc alleles (Mohler, 1977; Salz et al., 1982). PDE II activity specifically is increased in a dose-dependent manner with the chromomere containing the dnc locus (Kiger and Golanty, 1977; Shotwell, 1983). dnc mutants show allele-specific biochemical defects in PDE II activity (Byers et al., 1981; Davis and Kiger, 1981; Kauvar, 1982; Kiger and Golanty, 1977; Shotwell, 1983). Furthermore, dnc flies appear to have elevated levels cAMP, as might be expected if the gene for enzyme responsible for its catabolism was lesioned (Byers, et al., 1981).

A number of cDNAs from the dunce gene have been cloned. Molecular analysis of the dnc genomic locus indicates a very large and complex gene, spread over more than 148 kb. At least ten different transcripts are generated from this gene, both by alternative splicing and by use of alternative transcription start sites (Chen et al., 1986; Chen et al., 1987; Davis and Davidson, 1986). Evidence from protein homology with other known phosphodiesterases and the activity of dnc cDNAs expressed in yeast (Chen, et al., 1986; Qiu et al., 1991), in combination with the biochemical evidence from the mutants, clinched the case that dnc encodes Drosophila PDE II. Nighorn et al. (1991) demonstrated by immunohistochemistry and in situ hybridization to RNA that products of the dnc gene are present at elevated levels in the neuropil of the mushroom bodies, a brain structure that is believed to be important in learning and memory in both bees and fruit flies (Davis, 1993; de Belle and Heisenberg, 1994; Erber et al., 1980). Qiu & Davis (1993) used a set of chromosomal rearrangements, mainly deletions with endpoints just 5' to dnc (in Notch) and 3' endpoints extending successively further into the gene, to genetically dissect the various aspects of dnc gene function. They found a positive relationship between elevated dnc expression in the mushroom body and RNAs originating from a particular transcription start site.

Transgenic flies with P-element constructs that express a dnc cDNA or a rat homolog under heat-shock promoter control in dunce background were made for rescue experiments. Expression of these transgenes resulted in improved learning scores, marking the first time a learning phenotype had been partially rescued by a transgene (Davis, 1993).

Defects in adenylyl cyclase activity in rutabaga were found by Dudai and Zvi (1984, 1985) and by Livingstone et al. (1984). Both groups detected reduced adenylyl cyclase levels in rutabaga extracts. Their results indicated that adenylyl cyclase activity in Drosophila is heterogeneous. Activity that is potentiated in response to the addition of low concentrations of calcium (~10^{-7} M) is disrupted in rutabaga, but not G-protein stimulated activity (Livingstone, 1985). Calcium stimulation of adenylyl cyclase is believed to be mediated through the calcium-binding protein, calmodulin (Salter et al.,

1981). Calmodulin levels in rut were normal and addition of calmodulin did not restore calcium-responsive adenylyl cyclase activity to calmodulin-stripped rutabaga extracts, however CaM-stimulated adenylyl cyclase activity increased with increasing rut^+ gene dose (Livingstone, et al., 1984). These results suggested that the rut lesion might be in a postulated CaM-responsive isoform of adenylyl cyclase, although such a molecule had not at that point been identified in Drosophila or any other organism.

This supposition was confirmed by Levin et al. (1992) who, in the course of cloning adenylyl cyclase genes from Drosophila by homology to already-cloned mammalian adenylyl cyclases, retrieved a cDNA that mapped to the rut locus. Their cDNA showed strong homology to mammalian type I adenylyl cyclase and it produced CaM-responsive adenylyl cyclase activity in cell culture. They also identified the single-base mutation in the null rut^- allele studied by Livingstone et al. (1984). They showed that introducing this mutation into their rut^+ cDNA abolished its expression of CaM-responsive adenylyl cyclase activity in cell culture.

Han et al (1992) showed by RNA in situ hybridization and antibody staining that the rut gene is preferentially expressed in the mushroom bodies. This group also examined behavior in a number of P-element stocks with insertions at the cytological locus of rut. Molecular and behavioral analysis showed that five stocks that showed reduced learning in the TQ assay had P-element insertions in the putative promoter region of the rut gene. and that at least three of these five did not behaviorally complement the rut^1 allele.

In a later section, non-associative learning in the gill-withdrawal reflex in Aplysia will be discussed in some detail. The biological basis of sensitization (increased response to a specific reflex-triggering stimulus, induced by prior exposure to a strong, noxious general stimulus) in the monosynaptic component of this reflex has been extensively investigated. Briefly, it appears to involve presynaptic facilitation of the sensorimotor connection by interneurons excited by the sensitizing stimulus. Neurotransmitter released by these facilitatory interneurons activates receptor-linked adenylyl cyclase activity that can lead to PKA activation. The kinase acts to reduce a K^+

conductance, resulting in action potential broadening and enhanced transmitter release.

Classical conditioning that specifically pairs a weak reflex-triggering stimulus with a sensitizing stimulus results. This results in greater enhancement of subsequent reflex responses than does unpaired sensitization treatment (Carew et al., 1981; Kandel and Schwartz, 1982). The cellular basis of this process has been studied in simplified Aplysia preparations in the gill withdrawal reflex and in the tail withdrawal reflex (Clark and Hawkins, 1988; Hawkins et al., 1983; Walters and Byrne, 1983). Cellular analogs of this classical conditioning treatment produced effects on the sensorimotor connection that were like those seen in sensitization but were of greater in magnitude. This suggested that part of the underlying process in classical conditioning is an amplified form of presynaptic facilitation that depends on temporal contiguity of the firing of the sensory neuron and the occurrence of the sensitizing stimulus (within 2 second of each other).

Experiments in which calcium concentration, depolarization and/or neurotransmitter application were manipulated suggested that, in addition to heterosynaptic facilitation by activation of receptor-coupled adenylyl cyclase activity, a homosynaptic activity-dependent component of presynaptic facilitation occurs. This is thought to involve recruitment of CaM-responsive adenylyl cyclase activity by Ca^{++} entry due to activity in the sensory neuron (see Abrams and Kandel, 1988; Byrne, 1987; Byrne et al., 1993 for reviews). Adenylyl cyclase activity in Aplysia central nervous tissue has been shown to be regulated by both G-protein and CaM, and it now appears that both forms of responsiveness result from allosterically distinct interactions with the same cyclase and that dual activation may be synergistic (Abrams et al. 1991).

In rats, CaM-responsive adenylyl cyclase accounts for about 50% of total adenylyl cyclase activity, and it has been detected in a number of tissues. Xia et al. (1991) have looked at the distribution of the rat CaM-responsive adenylyl cyclase specifically in the brain by RNA in situ hybridization. They found that its distribution was not general and that, among a small number of other brain locations, moderate or intense staining was observed in pyramidal cells of areas CA1, CA2 and CA3 of the hippocampus, in granule

cells of the dentate gyrus and in the entorhinal cortex. This pattern of localization in regions thought to be involved in learning and memory suggests that CaM-responsive adenylyl cyclase might be involved in this process in mammals as well as in Aplysia and Drosophila.

The products of the dunce and rutabaga genes are both involved in cAMP metabolism, one as a catabolic enzyme and the other as a synthetic one. Known dnc mutations result in increased cAMP levels, while rut tends to produce slightly lower than normal cAMP levels. Since dnc and rut mutations tend to drive cAMP in opposite directions, Livingstone et al. (1984) generated double-mutant flies carrying dnc^{M11}, rut^{1} X-chromosomes to test whether the opposite effects these two mutations could compensate for each other and result in a fly with normal learning. (The dnc^{M11} allele is a null.)

The homozygous double mutant had abdominal cAMP levels that were a bit above normal. (Adenylyl cyclase and PDE activity levels were similar to those seen in rut and dnc, respectively.) In the QHB assay, the double mutant learned no better than either dnc or rut, which is to say, not at all. (In a later test using the TQ assay, where dnc and rut show some learning, Tully & Quinn (1985) found that the double-mutant strain performed worse than either single mutant.) Females with one double-mutant X-chromosome and only rut^{1} on the other X were made to get cAMP levels even closer to wild-type, but these flies also failed to learn. It should be noted that although they did not appear to complement each other behaviorally, rut acted as a suppressor of dnc female sterility.

Feany (1990), did experiments to behaviorally rescue dnc^{M14}, using weak rut alleles that were recovered in a screen for suppressors of dnc^{M14} sterility by Bellen et al. (1987). These alleles, rut^{2} and rut^{3}, show baseline levels of adenylyl cyclase activity much nearer to wild-type than rut^{1}, with rut^{3} closest to wild-type. Importantly, both rut^{2} and rut^{3} retain normal calcium-responsive activation, unlike rut^{1}.

In the QHB assay, dnc^{M14}, rut^{2} flies learned about half as well as C-S, while dnc^{M14}, rut^{3} flies failed to learn. One interpretation of this data is that the Ca^{++}-responsive feature of rut function is important in learning and

memory, since rut^1 lacks this feature and even though dnc^{M11}, rut^1 flies have near-normal baseline cAMP levels, this double-mutant fails to learn.

Why did rut^2 restore learning while rut^3 failed to do so? It may be that both the baseline level of cAMP and calcium-responsiveness are important in learning. Although Feany did not assess cAMP levels in the double mutants, adenylyl cyclase was measured in the rut lines. Baseline activity is lower in rut^2 than in rut^3, suggesting that dnc^{M14}, rut^2 cAMP levels might be nearer to normal levels than those of dnc^{M14}, rut^3.

1.2.1.2.2. Genetic manipulation of the PKA catalytic subunit

The identification of dnc and rut as genes involved in cAMP metabolism, along with results from other systems implicating cAMP in learning and memory, suggested PKA as a promising target for investigation. The role of PKA, the cognate protein kinase of the cAMP second-messenger pathway, in learning and memory was first addressed experimentally by Drain et al. (1991). They used a reverse-genetic to create "dominant-negative" mutations targeted against PKA catalytic subunit function. This entailed making transformed lines using P-element constructs, each expressing a different product designed to disrupt PKA catalytic subunit activity. Each construct was placed under the transcriptional control of the Drosophila hsp70 promoter, so that it would be expressed at high levels only after the transgenic fly was subjected to heat-shock.

There are a number of advantages to this approach to examining gene function. The properties and effects of the transgenic product on its target often can be modeled in vitro. Expression of the transgene can be held in abeyance until it is required experimentally. A target with both pre-imaginal and adult roles can be studied in the adult by waiting to induce the dominant-negative transgene until after the imago has eclosed. Heat-shock transcription has fast onset and can produce a discrete pulse of transcription of the transgene. This, in some cases, could allow for testing for recovery of function after dissipation of the transgene product.

Among these inducible dominant-negative mutations that Drain et al. introduced were: a peptide inhibitor of PKA; a mutant N-terminal fragment

of mouse PKA type II regulatory subunit that constitutively binds PKA catalytic subunits; and the Drosophila PKA catalytic subunit. Heat-shock induction of these transgenes produced specific and significant reductions in two- or four-hour memory in the TQ paradigm, compared to heat-shocked controls. Memory in uninduced flies from any of these transgenic lines was not significantly different from uninduced, untransformed control flies. Induced control transgenes, such as an inactive mutant peptide inhibitor, did not significantly affect memory. These results suggest that molecules that interfere with the action of PKA, a key effector molecule of the cAMP second-messenger pathway, interfere with memory.

The involvement of PKA in Drosophila learning and memory has also been addressed in classical genetic experiments with mutations in DC0, the structural gene for the PKA catalytic subunit by Skoulakis et al. (1993). In studies of this gene by enhancer-trap β-galactosidase expression, RNA in situ and immunohistochemical methods they showed preferential expression in the mushroom bodies. Since homoallelic homozygotes are lethal, a heteroallelic double-mutant heterozygote that lacks about 80% of wild-type PKA activity was used for behavioral experiments. This mutant showed a large and significant reduction in initial learning scores compared to controls (λ=0.28 vs. 0.61). Indeed, this initial score is smaller than the learning index of the control or heterozygote flies measured even at three hours after training. The memory curve for the heteroallelic flies during three hours after training showed very little decline in learning index (0.20 at three hours), but at every time point tested its learning index is significantly below that of control flies. Thus, memory in the heteroallelic mutants is "different" than controls, but this may partially or wholly stem from differences in initial learning.

Skoulakis et al conclude that "it is possible that the memory is due to the 20% PKA activity remaining", but they find an explanation involving "learning and memory induced by a pathway other than cAMP signaling" more attractive. An alternative view is that the DC0 mutants have behavioral phenotypes consistent with those of rut and dnc, where acquisition and/or early memory are affected. Initial learning is reduced by about half, but retention of this moderate level of learning over three hours could hardly be

better. Testing of the mutants in the original TQ apparatus might allow direct behavioral comparisons to be made to dnc and rut.

1.2.1.3. Memory phases in Drosophila.

The formation of stable memory after training appears to be temporally graded in many species. This consolidation could be the result of a single underlying biological process, but experiments in both vertebrate and invertebrate systems indicate that this is not the case. Drosophila behavioral studies suggest that there may be as many as five genetically-dissectable processes are at work (Tully et al., 1990). Furthermore, the strength and duration of the memory trace depends not only on the number, but also the pattern of training trials administered. This suggests that different sets of biological memory processes may be recruited under different learning conditions.

1.2.1.3.1. Memory consolidation.

Consolidation from a labile to stable retention is an apparently universal property of memory phenomenon (DeZazzo and Tully, 1995). For a period of time ranging from a few minutes to a few hours after training (depending on the task and species tested), a variety of treatments including electroconvulsive shock, temperature shock, anoxia and anesthesia can disrupt memory consolidation by an unknown mechanism (Glickman, 1961; McGaugh, 1966; McGaugh and Herz, 1972; Pearlman et al., 1961; Quinn and Dudai, 1976; Folkers et al., 1993). Consolidated memory tested in these paradigms came to be known as "anesthesia-resistant" memory. The proportion of memory sensitive to these shocks declines with the interval between training and treatment.

In a representative study of anesthesia-resistant memory consolidation in rats, McGaugh & Alpern [reported in (McGaugh, 1966), p.1532] examined retrograde amnesia produced by electroconvulsive shock treatment (ECS) after giving rats a single training trial in passive avoidance footshock task. ECS within the first hour after training devastated retention measured 24 hours after training, and if given any time up to three hours after training, reduced 24-hour memory performance. They also found that with more intensive training regimens (i.e., two minutes of massed training trials or two

individual trials spaced two minutes apart), ECS given immediately after training reduced 24-hour memory performance to levels similar to those seen with ECS given at two minutes after single-trial training, but additional training attenuated the effect of ECS administered one hour after training on 24-hour retention.

Honeybees show good learning in both natural and laboratory situations, and these insects also show memory consolidation (Menzel et al., 1991). A commonly-used single-animal experimental paradigm for associative learning in this system is olfactory classical conditioning of the proboscis extension reflex (PER) in immobilized bees, which is triggered by sucrose applied to the foreleg or antenna. Most bees learn the association in single training trial, and the memory can last for a day or more. A course of three training trials usually results in lifelong retention of the conditioned behavior.

Honeybee memory after single-trial PER conditioning requires time to consolidate, since the memory trace is susceptible to chemical narcosis, cooling and electric shock disruption for up to seven minutes post-training . Remarkable insights on the progress of the time course of memory trace consolidated in successive anatomical structures were derived from experiments using reversible local cooling of specific regions within the honeybee brain. (Erber, et al., 1980; Menzel et al., 1974). Precisely-positioned cold needles were used to chill brain targets to 1^{o}C for 30 seconds at various intervals after training. Cooling the antennal lobes was effective in disrupting memory for about two minutes after training, while the mushroom bodies were susceptible for upwards of five minutes. Even within the mushroom bodies themselves, regional differences in susceptibility were revealed: efferent areas were susceptible for a longer time after training than were input areas. These experiments suggests that consolidation in bees reflects an anatomically sequential process, where the trace either becomes consolidated in or independent of each waystation along the circuit in turn. The antennal lobes directly receive antennal sensory afferents and are probably involved in a more primitive stage of processing than the mushroom bodies, which receives inputs from sensory areas, including the antennal lobes.

Memory consolidation can also be disrupted by drug treatments. Studies with drugs have the advantage of providing pointers to putative biological mechanisms. There is a large literature discussing the effects of inhibitors of gene expression on memory consolidation, suggesting that de novo transcription and translation are required for long-term memory (Agranoff et al., 1967; Barondes, 1965; Barondes and Cohen, 1967; Barraco et al., 1981; Flood et al., 1972; Flood et al., 1974; Kobiler and Allweis, 1974,; for reviews see Davis and Squire, 1984; Flood and Jarvik, 1976). Experiments have been carried out on a wide range of invertebrate and vertebrate species, using different training paradigms and regimens, and employing different kinds, doses and routes of administration of drugs inhibiting macromolecular synthesis. The results vary in their particulars, but generally indicate that there is a relatively brief window of time around training where strong repression of cerebral transcription or translation can impair long-lasting retention, without producing significant effects on acquisition or on short-term memory. It thus seems reasonable to differentiate short-term memory (STM) from long-term memory (LTM) on the basis of the requirement for de novo gene expression in the latter.

The mechanism and specificity of drug effects on LTM in vertebrates have been challenged. Since memory consolidation must be inferred from behavioral changes in whole animals, the usual caveats regarding unknown or indirect effects of drugs on neuronal physiology affecting the memory process are compounded by the possibility of drug action on other physiological systems that may influence behavior. For example, treatment with most inhibitors of transcription and translation eventually causes systemic illness, changes in locomotor activity, and altered levels of circulating adrenal hormones (Barraco and Stettner, 1976). Experiments have been done to dissociate many of the known side-effects of these drugs from their amnestic effects (reviewed in Davis and Squire, 1984), but controlling for all the myriad possible direct and indirect drug actions, especially in the absence of any kind of specific biological marker for memory consolidation, is essentially impossible.

More convincing results on the effects of transcription and translation blockers have been obtained from simplified or cell culture models of learning and memory where cellular correlates of learning and memory have been identified. In experiments in Aplysia, discussed more fully in a later section, application of inhibitors of transcription or translation during training blocked the appearance of long-term, but not short-term, facilitation and sensitization (Castellucci et al., 1989; Montarolo et al., 1986) and also block specific changes in physiology and cell biology that accompany them. Inhibition of protein synthesis in rat hippocampal slices by anisomycin treatment blocks the appearance of L-LTP, but not E-LTP, in this model system for vertebrate memory formation (Castellucci, et al., 1989; Frey, et al., 1988; Huang and Kandel, 1994; Montarolo, et al., 1986).

1.2.1.3.2. Mutants that dissect short-term memory from consolidated memory.

The amnesiac (amn) and radish (rsh) mutants were selected in a QHB screen for lines that learn well but forget quickly (Dudai, et al., 1976; Folkers, et al., 1993). These mutants learn nearly as well as wild type flies, but show fast forgetting during the first hour after training in both the QHB and TQ assay . This phenotype is unlike that of dnc and rut, which show greatly reduced initial learning scores. Elizabeth Folkers in the Quinn lab is preparing to identify genomic DNA containing radish by its ability to rescue the radish phenotype in P-element transformants and Mel Feany, working with Quinn, has discovered that *amn* codes for a neuropeptide transmitter similar to some known to have effects on the cAMP second-messenger system in mammals (Feany & Quinn, in press).

The formation of anesthesia-resistant memory in Drosophila was first described by Quinn and Dudai (1976). For these retrograde amnesia experiments, they used a training and testing system involving the QHB assay. Groups of flies were exposed to an odorant in the presence of electric shock for ten consecutive one-minute training sessions. At various time points up to forty minutes after training, different groups were subjected to cold anesthesia (one minute at 4^{o}C). Preferential odor avoidance was tested at 45 minutes after training.

Cold anesthesia given five or ten minutes prior to training did not significantly affect 45-minute retention. Treatment immediately after or five minutes after training essentially abolished memory measured at forty-five minutes. At 10, 15, 20 and 30 minutes after training, cold anesthesia still substantially impaired forty-five minute retention, but had progressively smaller effects. These results indicate that in Drosophila, as in vertebrates, an initially labile short-term memory is progressively consolidated into a more permanent form. This work, like the reports invertebrates, provides a phenomenological description of memory formation, but does not allow discrimination between a single or multiple underlying biological processes.

Boynton & Tully (1990) performed experiments similar to those of Quinn & Dudai (1976), except they used a single cycle of training in the TQ assay and tested retention three hours after training. Retrograde amnesia treatment consisted of 2-minute cold anesthesia at 1°C at various times after training. As in Quinn & Dudai, progressive consolidation of memory was seen. Compared to untreated flies, flies receiving cold-anesthesia at any of the time point within two hours after training tested showed impaired 3-hour memory, but the disruptive effect was progressively less severe when it was given at later times after training. In this system, the consolidation of memory into an anesthesia-resistant form probably begins during training and is substantially complete within the first two hours after training. At that time, it comprises just less than half of total memory. At about seven hours after training the total memory and ARM curves converge, indicating that by this time retention from single-cycle training consists mainly of consolidated memory.

Tully et al. (1990) used cold anesthesia to show that amn flies can form consolidated memory that is only slightly weaker than that seen in wild-type flies. The amn ARM curve is indistinguishable from wild-type, but the total memory and ARM curves from amn converge at two hours after training, indicating that total memory after this time is composed solely of consolidated memory. Wild-type total memory and ARM curves do not converge until about seven hours after training. This suggests that in amn, consolidated memory from single-cycle TQ training is near-normal, but a

middle-term (i.e., unconsolidated) memory component that is normally present between two and seven hours after training is lost.

Folkers et al (1993) performed a behavioral and genetic analysis of the rsh mutant. The learning and memory phenotype of rsh is indistinguishable from that of amn during the first two hours after single-cycle training in the TQ assay (Fig 1.4). Both mutants learn nearly normally and then show a rapid decline in memory over the next two hours. Beyond the first two hours after training, however, amn memory stabilizes, while rsh retention continues to decline at a rate paralleling the wild-type decay curve. Six hours after training, rsh has no measurable memory. This suggested a failure in memory consolidation. In retrograde amnesia experiments, rsh flies subjected to cold anesthesia two hours after single-cycle TQ training (when ARM is maximal in wild-type flies), showed almost no memory when they were tested an hour later. This indicates that the rsh mutation essentially abolishes ARM.

It might be that the rsh memory curve beyond two hours after training is revealing a short-term memory component(s) whose shape can be inferred from the difference between the ARM and total memory curve. Interestingly, if an adjustment is made for the small difference in initial learning between wild-flies and the amn and rsh mutants, adding up the portions of the two mutant memory curves beyond two hours after training produces something very much like the wild-type memory curve over this period. Behavioral experiments with double-mutants might tell us something about this intuition.

The relation between earlier memory phases and the formation of consolidated memory is not yet clear, but it appears that the acquisition/early memory process affected in rut can modulate the strength of ARM. Dudai et al. (1988) re-examined the performance of rut in the TQ assay and looked at memory consolidation using cold anesthesia. (Their measurements were made in a slightly different way than any of those described above. Instead of testing retention at a fixed time point after training, flies were tested one hour after the time of anesthetization.) They found that rut flies did indeed form consolidated memory, reduced in magnitude, but similar in time course to that of wild-type flies. This suggests a correspondence between initial

learning and the subsequent level of consolidated memory. (Surprise!) The amn mutant, which has near-normal learning combined with accelerated short-term memory loss, also has near-normal ARM. The fact that there is already some small amount of ARM present in wild-type, rut, and amn flies even at the earliest time points tested suggests that the formation of this kind of consolidated memory might begin during the training period.

1.2.1.3.3. Genetic dissection of consolidated memory.

It was unknown from experiments in vertebrates whether memory consolidation represented a unitary process or whether the traumas employed in the analysis of ARM were affecting different processes than those disrupted by inhibitors of de novo gene expression. It has been demonstrated on the basis of genetics that in Drosophila, ARM and LTM are biologically different phenomena.

Initial reports by Boynton & Tully (1990) indicated that feeding the protein synthesis inhibitor cycloheximide (CXM) or the transcription inhibitor actinomycin D to Drosophila did not interfere with long-term memory in the TQ assay (also see S. Boynton 1991, Ph.D. thesis, Brandeis University). A single cycle of TQ training produces memory that is detectable at 24 hours after training, long enough so that some component might involve new transcription or translation. As discussed earlier, retrograde anesthesia experiments in Drosophila indicated that by 7 hours after training, memory has been consolidated. To discover what proportion of this consolidated memory might be due to new gene expression, these workers fed flies with a solution of cycloheximide (CXM) or actinomycin-D in sucrose, or sucrose alone, for 14 hours before training. (Incorporation of tritiated leucine into brain protein was strongly depressed by this treatment.) Learning and memory of drug-treated flies was normal during the first seven hours after training and examination of consolidated memory using cold anesthesia showed no significant differences between drug-treated and control groups. These results argued against the operation of a transcription or translation-dependent LTM system after classical conditioning in flies. There was evidence for a such a system for retention after training in other tasks. Willmund et al. (1979) reported that CXM treatment blocked long-lasting

habituation of phototaxis, and Pruzan et al (1977) found that CXM blocked an acquired mating preference. In a more closely related behavioral task, Wittstock et al. (1993) investigated the link between protein synthesis and LTM in honeybees. They studied the effect of protein synthesis inhibition induced by CXM injection into the brain on one-day memory after PER classical conditioning. They found no effect of near-compiete brain protein synthesis inhibition on one-day memory, which in the context of the Drosophila result could be taken to suggest that protein synthesis-dependent LTM for classical conditioning is not a feature of insect memory after classical conditioning. On the other hand, PER conditioning is so strong that a ceiling effect in behavioral performance, combined with a strong, non-protein synthesis-dependent memory phase that lasts longer than expected, may occlude observation of the protein synthesis-dependent phase at one day after training. Weaker training conditions and/or examination of memory at later times after training might reveal a protein-synthesis dependent component of LTM in bees.

The opposite situation obtained in Drosophila. Among the reasons suggested by Boynton & Tully (1990) for the failure of their experiments to demonstrate a protein synthesis-dependent component of Drosophila memory was the possibility that a single cycle of TQ training might not be sufficient to give rise to this phase. Training regimens where groups of trials are punctuated by intervening rest periods have long been known to produce better retention than the same total number of training trials given en masse (Ebbinghaus, 1885; Hintzman, 1974). Examples of this difference between spaced and massed training are also seen in sensitization and habituation of the GWR in Aplysia (Carew et al., 1972; Frost et al., 1985). L-LTP in rat hippocampal slices also appears to conform to this rule, at least in concept, since induction of that protein synthesis-dependent process critically relies on spaced activation of the tetanizing input (Huang and Kandel, 1994; Reymann, et al., 1985).

Tully et al. (1994) found that ten cycles of spaced TQ training, using fifteen-minute rest intervals between cycles, produced memory in Drosophila that lasted for a week or more after training. Fig. 1.5 shows memory curves after single-cycle training, ten cycles of massed training, and 10 cycles of spaced training. All of the training protocols give similar initial performance

indices, but subsequent retention is different for each regimen. Retention after training in 10-cycle massed protocol is longer-lasting than that seen after single-cycle training, but it is qualitatively like memory after single-cycle training. It appears never to stabilize, falling steeply and continuously to a low level after two days and becomes negligible by four days post-training. Retention after 10-cycle spaced training is different. In addition to being about double the magnitude of 10-cycle massed training after one day, appears to stabilize beyond 48 hours after training. Quite good retention is seen even at seven days after spaced training (P.I. = 35-40), which is as late as it has been tested.

Is the long-lasting retention from massed or spaced training dependent on *de novo* gene expression? Tully et al. (1994) repeated their experiments with CXM on flies that had been given 10 cycles of training in the massed or spaced protocols. Groups of flies were allowed to feed on a 4% sucrose solution with or without 35 mM CXM for the 12 hours before training and for the one-day retention interval between training and testing. CXM-treated flies appear to be generally healthy and normal in immediate learning and peripheral behaviors at the times of training and testing. CXM produced about a 50% inhibition of protein synthesis in the brain. Behaviorally, CXM specifically reduced one-day retention after spaced training to less than half of that seen in identically-trained untreated flies, indicating that Drosophila probably has protein synthesis-dependent faculty for long-lasting memory. (Trials with other drugs that inhibit gene expression will be required for a stronger interpretation of this part of the experiment.) It is important to note that CXM had no significant effect on one-day retention after massed training, suggesting that long-lasting memory from this regimen involves a mechanism that is less sensitive, or perhaps insensitive, to CXM.

The fact that the block of one-day memory after spaced training by CXM was only partial might be explained by the incomplete block of protein synthesis. It was equally possible, however, that retention one day after spaced training might have biologically distinguishable CXM-sensitive and CXM-insensitive components. In this case, only some fraction of total retention would be susceptible to disruption. CXM treatment brought the level of one-day retention from spaced training down to the same level reached after massed

training, suggesting the second explanation was plausible. The difference between the CXM-insensitive component and total retention one day after spaced training could represent the entire contribution of the CXM-sensitive memory process.

The radish mutant was used to demonstrate that the CXM-insensitive component of one-day retention after either spaced or massed training is ARM. Folkers et al. (1993) showed that the rsh mutant is almost totally deficient in ARM. Later, Tully et al. (1994) observed that in wild-type flies, CXM treatment did not affect acquisition, total memory or memory consolidation into ARM during the first seven hours after single-cycle training. This strongly suggested that the CXM-insensitive component of one-day retention might in fact be ARM. Tully et al. found that rsh flies receiving ten cycles of massed training flies had zero retention at 24 hours, indicating that the CXM-insensitive component of retention and ARM were the same behavioral phenomenon.

Giving spaced training to radish flies results in a level of one-day retention of about half that seen in wild-type flies. Arithmetically, this lowered retention score was consistent with the hypothesis that one-day retention after spaced training consists of a CXM-insensitive (ARM) component, that radish flies cannot generate, and a CXM-sensitive component, which they can. To test this idea, CXM-treated radish flies were given spaced training. CXM treatment of rsh flies given ten cycles of spaced training abolished all memory one day later. The simplest interpretation of this result is that one-day retention after spaced training consists of both ARM and CXM-sensitive memory components, and that the effects of the rsh mutation and drug treatment, respectively, produce substantially complete blocks of these components.

We have defined LTM as long-lasting memory that is dependent on new gene expression. There is the possibility that the results observed with CXM were due to side-effects instead of its protein synthesis-blocking activity, but the flies appeared healthy and behaviorally normal in tested parameters. Tests with other drugs that interfere with gene expression are needed to help assure the correctness of these results. Even with assurances from such

experiments, however, block of macromolecular synthesis are still open to many alternative interpretations and criticisms, which range widely in plausibility. For instance, it has been suggested that CXM treatment indeed blocks de novo protein synthesis, but failure to express new memory-specific components is not the reason for the loss of LTM. Instead, unreplaced loss of labile housekeeping proteins during a critical period for LTM reversibly compromises the function of a set of brain cells involved in LTM, inm a way that interferes with LTM but not with learning or short-term memory, leaving the animal appearing healthy & behaviorally normal. Possible, but extremely difficult to address in a whole animal, even assuming that the proteins and cells involved were already known

The main known effect of CXM is to interfere with protein synthesis, which it was shown to do here in Drosophila brains. CXM does not interfere with ARM, but blocks the remainder of long-lasting memory. This is a sharp pharmacological dissection that directly parallels a genetic one. Finally, as the next section will describe, CXM blocks the form of retention with the longest duration. On these grounds, it is reasonable to suggest that CXM-sensitive memory is equivalent to LTM by our definition.

The one-to-seven-day memory curves after spaced training for CXM-treated and for rsh flies reinforce this conclusion. CXM-treated wild-type flies lack the LTM component of retention after spaced training, revealing the ARM component in isolation. This memory curve does, in fact, have all the characteristics of the wild-type ARM curve. Similarly, rsh flies should reveal the LTM curve in isolation. In this case, memory after the first day stabilizes at a level quite similar to that in wild-type flies. This is what might be expected, since ARM is only a minor component of total memory by the second day after spaced training.

1.2.1.4. Conclusion.

The development of a system for producing long-term memory in Drosophila, and the ensuing dissection of long-lasting memory into LTM and ARM provided the experimental test bed for examining the involvement of CREBs in long-term memory and an important context for the interpretation of our results.

1.2.2. Aplysia gill-withdrawal reflex.

The attractions of Aplysia are complementary to those of Drosophila. The complexity of vertebrate brains has led some researchers in the field of learning and memory to study the cellular basis of neural plasticity in invertebrate animals with simpler, more accessible nervous systems, and the sea slug Aplysia californica has become the paradigm for studies of this type. Systematic multilevel studies of neural systems in this marine mollusk have been going on for over twenty-five years. In his 1977 Grass Lecture, Kandel (1978) pointed out a number of the features of Aplysia, that "large, attractive, and obviously intelligent beast", which make it particular well-suited for neurophysiological and biochemical work on neural plasticity. It has a simple nervous system (about 20,000 neurons in four bilateral pairs of ganglia and a single unpaired abdominal ganglion). The structure of the nervous system, down to the locations of many tested neurons and connections, is stereotyped. Large neuronal cell bodies at the periphery of the ganglia, surrounding an interior neuropil, facilitate electrophysiology and biochemistry. Work with this organism has sought first to characterize the circuit mediating a specific modifiable behavior; second, to locate the sites of experience-dependent change within this network; and third, to investigate the nature and mechanisms of these changes at the cellular and molecular level. As this section describes, these goals are being met to a remarkable degree.

The first experiments clearly demonstrating a requirement for new gene expression in long-term synaptic plasticity were performed in this system, as were the first studies identifying and examining specific proteins whose levels change in the course of these events. Experiments implicating cAMP-responsive transcription and CREB proteins in long-term synaptic plasticity were also first performed in Aplysia

1.2.2.1. Neuronal circuit of the GWR.

An enormous amount of information about the cellular and molecular basis of learning and memory has come from studying plasticity in the defensive withdrawal reflex of the gill and siphon (GWR) in Aplysia. The fleshy parapodia on Aplysia's back pump water down through the mantle cavity

lying between them. Within the mantle cavity is the gill, ordinarily obscured beneath the mantle shelf which extends from one side to cover it. The posterior end of the mantle shelf forms a muscular tube, the siphon, through which water and waste is expelled. A moderate touch to the mantle shelf or siphon activates a reflex withdrawal of the siphon and gill. The withdrawal of the siphon and gill co-vary and both have been used to monitor the strength of the reflex (Kandel, 1978). The GWR shows two forms of non-associative learning: sensitization and habituation. Sensitization describes the potentiation of the GWR responses to subsequent gentle stimulation following a strong, presumably unpleasant mechanical stimulus to the head or tail. Habituation is a stimulus-specific decline in the reflex response (i.e., not due to sensory adaptation or motor fatigue) with repeated stimulation.

Behavioral, anatomical and electrophysiological studies were combined to elucidate in some detail much of the central nervous network underlying the GWR (Kandel, 1978). A large proportion of the central component of the reflex is mediated by a set of parallel monosynaptic connections made in the abdominal ganglion between sensory neurons innervating the skin of the mantle and siphon and motor neurons of the gill or siphon. A substantial collection of interneurons in the abdominal ganglion are also involved in the reflex network, including some that are implicated in neuromodulatory roles mediating behavioral plasticity. Partially-dissected preparations of Aplysia, containing only the abdominal ganglion connected by nerves to the bits of peripheral anatomy involved in the GWR, displayed normal behavioral habituation and sensitization of the reflex, indicating that the required central elements for these forms of plasticity are present in the abdominal ganglion (Castellucci et al., 1970; Pinsker et al., 1970).

Studies of the neurobiology underlying plasticity in the GWR focus on the monosynaptic component, particularly on the sensorimotor connection to the major motoneuron L7, which is estimated to mediate up to 50% of the withdrawal reflex. Monitoring this connection has provided a large amount of information about mechanisms of synaptic plasticity that may underlie learning.

1.2.2.2. Behavioral plasticity in the GWR.

Both short-term and long-term forms of habituation can be obtained in the GWR (Carew, et al., 1972). Forty stimuli spaced 30 seconds apart, given in one session (massed training), gave habituation of the reflex that lasted less than a day. The same total number of stimuli, given in a spaced training protocol of ten stimuli per day over four days, resulted in habituation that lasted over three weeks.

Kupfermann et al (1970) observed that the habituation was paralleled by reduction in the size of EPSPs seen in the L7 motor neurons, suggesting that depression of transmission at sensorimotor synapses was a neural substrate for habituation of the GWR. They ascertained that this effect was intrinsic to the sensory-motor synapse, and called it homosynaptic depression. Long-term habituation is also correlated with a depression of transmission at sensory-motor synapses (Carew and Kandel, 1973; Castellucci et al., 1978).

Castellucci & Kandel (1974) found that they repeated electrical stimulation of an impaled sensory neuron synapsing on L7 could produce homosynaptic depression in the connection. Using this system, they performed quantal analysis experiments that suggested homosynaptic depression results from a reduction in the amount of neurotransmitter release per action potential. The molecular mechanisms underlying homosynaptic depression have not been extensively investigated, nor have the changes that allow long-term habituation to endure for weeks.

Sensitization is a form of learning in which the animal learns to strengthen of defensive behaviors in response to a strong noxious stimulus. This makes ethological sense, since an attacker may remain on the scene and it pays to be prepared to respond vigorously to a renewed onslaught. A strong electric shock to the head or tail is commonly used as a sensitizing treatment in Aplysia. Sensitization of the GWR is usually measured as increased duration of siphon withdrawal. A single sensitizing stimulus increases siphon withdrawal duration by about 50% over baseline, and the effect lasts from a few minutes to an hour. Like habituation, sensitization occurs in short- and long-term forms. Effects of massed versus spaced training are seen, with spaced training producing sensitization of the GWR that can last for weeks

(Frost, et al., 1985; Pinsker et al., 1973). More progress has been made on the underlying neurobiology of sensitization than of habituation in Aplysia. An impressively detailed picture of sensitization, down to its molecular basis, has been assembled.

1.2.2.3. Presynaptic facilitation, a cellular basis for sensitization.

The neural mechanism underlying sensitization of the GWR involves pre-synaptic facilitation of transmitter release from sensory nerve terminals. A cellular model of sensitization was studied in the isolated abdominal ganglion. Strong simulation of afferent nerves that transmit sensitizing stimuli to the abdominal ganglion (left or right pleuroabdominal connectives for the tail, or the branchial nerve for the head) resulted in facilitation of the monosynaptic connections between sensory neurons and L7 (Castellucci and Kandel, 1976; Castellucci, et al., 1970). Stimulation of the sensitizing nerves, however, did not result in firing of the sensory neurons of the reflex circuit, indicating that a heterosynaptic, rather than homosynaptic, facilitation process was involved in the enhancement of the connection. Quantal analysis of the sensorimotor connection indicated that enhanced neurotransmitter release was responsible for the increased synaptic efficacy seen in sensitization. A similar case of neuromodulation in Aplysia was suggested to result from heterosynaptic facilitation (Kandel and Tauc, 1965a; Kandel and Tauc, 1965b). Castellucci and Kandel (1976) adopted this explanation for their observations, suggesting that the terminals of the facilitating neurons of the sensitizing pathway may "contact the presynaptic terminals of the sensory neurons and regulate their transmitter release."

Brunelli et al (1976) suggested that serotonin might be the facilitatory neurotransmitter in this system. Levels of several monoaminergic transmitters are elevated in the abdominal ganglion after sensitization treatment (Cedar et al., 1972), but only serotonin, among a panel of monoamines applied to the ganglion, enhanced synaptic transmission at monosynaptic sensory-motor connection of the GWR (Brunelli, et al., 1976).

It may be, however, that serotonin is not the actual neurotransmitter responsible for presynaptic facilitation in the natural situation in the GWR. The L29 facilitatory interneurons of the abdominal ganglion fulfill many of

the anatomical and functional requirements for an intrinsic system able to mediate heterosynaptic facilitation in the GWR, but these cells do not appear to release serotonin (Bailey et al., 1981; Hawkins et al., 1981a; Hawkins et al., 1981b; Ono and McCaman, 1984). Serotonin-containing terminals ending on siphon sensory cells of the GWR have been found and some of these appear to originate from serotonergic LCB1 and RCB1 cells in cerebral ganglion (Kistler et al., 1985; Mackey et al., 1989). In addition, the small cardiac peptides SCP_A and SCP_B, which are found in nerve terminals from unidentified neurons that contact the sensory neurons, have cellular effects on the GWR similar to those of serotonin (Abrams et al., 1984). While it is clearly important to identify all of the neurotransmitters involved naturally in presynaptic facilitation in the GWR, the discovery that serotonin is probably one of these provided a tool for examining the molecular mechanisms underlying sensitization.

1.2.2.4. A molecular model for facilitation.

Extensive work on the cellular and molecular mechanisms of presynaptic facilitation in the GWR supports a model in which the cAMP second-messenger pathway of the sensory neuron is activated by sensitizing stimuli. A facilitating interneuron releases serotonin or another neuromodulatory transmitter that activates receptor-coupled adenylyl cyclase in the presynaptic neuron, increasing intracellular cAMP levels and activating PKA. Free PKA catalytic subunit acts to decrease a specific outward K^+ conductance, slowing regeneration of the resting membrane voltage and prolonging action potentials. Broader spikes admit more Ca^{++} via voltage-gated channels at synaptic terminals. This enhances Ca^+-dependent excitation-coupled transmitter release, which is read out as a larger EPSP at the motor neuron. There is massive evidence consistent with this model, some of which is given below.

1.2.2.4.1. Involvement of cAMP second-messenger pathway.

Evidence for the involvement of the cAMP second-messenger pathway in facilitation is extensive. Application of serotonin to the abdominal ganglion resulted in a transient elevation of cAMP levels in the whole ganglion as well as in individual sensory neurons (Bernier et al., 1982; Cedar and Schwartz, 1972). The duration and amplitude of these cAMP increases were in the same

range as that produced by connective nerve stimulation that produces sensitization. Bath application of a permeant cAMP analog or injection of cAMP into the somata of sensory neurons augments the EPSP measured in the motor neuron (Brunelli, et al., 1976). In experiments to test the involvement of cAMP-dependent protein kinase in sensitization, Castellucci et al. (1980) found that injection of purified bovine PKA catalytic subunit into the sensory neuron increased transmitter release, decreased K^+ current and widened the action potential of the sensory neuron. In other experiments, injection of a peptide inhibitor of PKA into the sensory neuron could prevent spike broadening (an correlate of facilitation) caused by application of serotonin as well as facilitation from electrical stimulation of a sensitizing nerve (Castellucci et al., 1982).

1.2.2.4.2. Molecular mechanism of facilitation.

Klein & Kandel (1978) observed increased voltage-dependent Ca^{++} influx at the sensory cell body during presynaptic facilitation. Further studies, employing voltage clamp of the sensory neuron showed that the augmented Ca^{++} influx was secondary to a reduction in outward K^+ current that caused broadening of the action potential (Klein and Kandel, 1980). These studies also demonstrated that modest increases in spike width at a fixed extracellular Ca^{++} concentration could cause large increases in monosynaptic EPSPs in the follower motor neuron. Klein et al. (1982) identified a previously unknown K^+ conductance that they called the S-current. It was reduced after treatment with serotonin, and its current-voltage relationship showed it to be active at resting potential as well as at action potential voltages. The S-current could thus contribute to the electrical regeneration of the neuron and reduction in this conductance by serotonin would broaden the action potential.

Using single-channel recording techniques and cell-attached membrane patch clamping, Siegelbaum et al. (1982) demonstrated the existence of S-channels, K^+ ionophores that reflected macroscopic properties of the S-current. A dose-dependent proportion of these channels would close for minutes after bath application of serotonin. In patch clamping, the patch pipette forms an incredibly tight seal with the membrane, making it improbable that extracellularly applied serotonin was acting directly on the channels in the patch. This suggests that its effects were mediated by receptor-transduced

activation of an intracellular second messenger pathway, presumably cAMP. Consistent with this idea, experiments by Shuster et al. (1985) showed that intracellular injection of cAMP mimicked the effect of extracellular application of serotonin, as did exposure of the cytoplasmic side of cell-free inside-out patches to purified PKA catalytic subunits.

At the molecular level, the mechanism underlying behavioral sensitization thus involves cAMP-dependent phosphorylation of S-channels by PKA, or perhaps phosphorylation of a component of the cytoplasmic side of the cell membrane that is associated with the channels, resulting in their closure.

1.2.2.5. Long-term sensitization & facilitation.

One of the most important findings from research on GWR sensitization in Aplysia is that there are biological distinctions between short-term and long-term sensitization, although they share many properties. This provides an objective basis for differentiating between them beyond the arbitrary behavioral criterion of duration.

By a number of electrophysiological criteria, long-term facilitation appears to based on many of the same phenomena as short-term facilitation. Aplysia given spaced sensitization training (four sensitizing shocks to the tail daily for four days) were tested one day after training for differences versus untrained control animals in synaptic connectivity between individual sensory neurons and the L7 motor neuron. Long-term sensitized subjects showed significantly increased EPSP amplitude (over two-fold higher) and more detectable connections (but not statistically significant, 96% v. 75%). This result suggests that at least part of the memory for long-term sensitization is encoded as facilitation of the monosynaptic sensory-motor connection. Quantal analysis indicated that this long-term facilitation results from increased presynaptic release of transmitter, a mechanism shared with short-term facilitation (Dale et al., 1988). Scholz & Byrne (1987) showed that in tail sensory neurons, long-term sensitization present at 24 hours after training was correlated with a significant decrease in an outward ionic conductance with characteristics of the S-current. They also examined changes in membrane currents 24 hours after the injection of cAMP into tail sensory neurons and found that a K^+ conductance similar to the S current was significantly reduced compared to 5'-

AMP-treated controls (Scholz and Byrne, 1988). It appears, then, that changes in the electrophysiology of the sensory neuron similar to those seen in short-term sensitization are also involved in long-term sensitization.

1.2.2.5.1 Morphological changes at the synapse.

Bailey and Chen (1983, 1988) made electron micrographic reconstructions of serially-sectioned horseradish peroxidase-filled sensory neurons one day after long-term sensitization training and them compared to untrained controls. These studies revealed a doubling in the number of terminal varicosities in the neurons from sensitized animals and of these, a larger percentage had active zones. The active zones from sensitized animals were larger and these release sites had more synaptic vesicles associated with them, which could reflect the availability of more transmitter for immediate release. Total axon length of the sensory neuron was also increased in long-term sensitization. Nazif et al. (1991) saw increased vesicle number and axonal branching at 24 hours in sensory neurons of the pleural-pedal ganglia injected with cAMP, compared to cells injected with its immediate catabolite, 5'-AMP. These morphological changes suggest that at least part of the changes in long-term sensitization are presynaptic. Persistence of these alterations was investigated at one week by Bailey and Chen (1989), who found that the increase in the number of varicosities and the number of active zones per varicosity persisted, but the other modifications that they noted at 24 hours did not endure.

1.2.2.5.2. Requirement for *de novo* gene expression.

In a partially-dissected Aplysia preparation, Castelluci et al. (1989) used a gasketed ring to mechanically isolate the abdominal ganglion, while leaving its connections to the periphery intact They applied the protein synthesis inhibitor anisomycin to the ganglion for a period lasting from one hour before long-term sensitization treatment (4 groups of four tail shocks, with groups spaced 30 minutes apart) until one hour afterward. In this arrangement, the nervous system is largely intact and both monosynaptic and polysynaptic central components of the GWR can be exposed to the drug, while possible deleterious side effects of systemic anisomycin treatment are avoided. This treatment completely blocked the development of long-term sensitization, while leaving expression of short-term sensitization unaffected.

Rayport & Schacher (1986) showed that the monosynaptic component of the gill withdrawal reflex could be reconstituted in cell culture. In their co-culture system, using cells explanted from dissociated ganglia, sensory neurons form appropriate and functional synapses on L7 motor neurons. These connections display modulation comparable to that seen in the native synapse. Repeated firing of single spikes in the sensory neuron induced by an intracellular electrode results in homosynaptic depression of the connection. Facilitation can be obtained with serotonin, supplied either by direct addition or by stimulation of a co-cultured serotonergic modulatory cell. A single pulse of serotonin, results in short-term facilitation that lasts minutes, like behavioral sensitization from a single tail shock in the whole animal. Five 5-minute applications of serotonin, spaced 15 minutes apart (5x serotonin treatment) produced long-term facilitation, lasting over 24 hours (Montarolo, et al., 1986). This is similar to the duration of GWR sensitization obtained with five spaced tail shocks.

Montarolo et al. (1986) performed experiments indicating that long-term facilitation in this system required new gene expression, whereas short-term facilitation did not. The presence of transcriptional or protein synthesis inhibitors during and for a short time after 5x serotonin treatment prevented acquisition of long-term facilitation, but short term-facilitation was unaffected.

In the co-culture system, Schacher et al. (1988) showed that a 15-minute exposure to a permeant cAMP analog plus a phosphodiesterase inhibitor resulted in short-term facilitation of the monosynaptic EPSP, while prolonged exposure (1.75 hours) produced long-term facilitation. Inclusion of anisomycin in the bath for the period surrounding and during cAMP treatment specifically blocked long-term facilitation, again showing that a critical difference between long- and short-term facilitation is that long-term facilitation is protein synthesis-dependent.

Montarolo, et al. (1986) measured the time course of the effect of anisomycin on protein synthesis in the sensory neurons. It became effective in less than an hour, produced a 96% block, and protein synthesis took between two and four hours to recover after washout of the drug. Application of anisomycin

one hour before 5x serotonin treatment specifically and completely blocked long-term facilitation, while a 3-hour application of anisomycin given 12 hours before or four hours after 5x serotonin treatment had little effect. Anisomycin applied one-half hour after the end of 5x serotonin treatment for 4 or 22 hours each appeared to reduce long-term facilitation somewhat, but its effect was far from the complete block seen with application of anisomycin one hour before 5x serotonin treatment. These experiments indicate that expression of new proteins necessary for long-term facilitation occurs during a window of no more than a few hours from the start of 5x serotonin treatment. This finding, in a system which measures a neurobiological process underlying long-term memory for sensitization, is consistent with results from behavioral assays in vertebrates, where long-term memory is disrupted by the presence of protein synthesis inhibitors during and immediately after training [reviewed in (John, 1967) pp. 119-124 and Davis and Squire (1984)].

1.2.2.6. Gene-expression dependent changes in long-term facilitation.

1.2.2.6.1. Increased and persistent protein phosphorylation.
Sweatt and Kandel (1989) looked at protein phosphorylation in sensory neuron clusters from the pedal ganglia that had been exposed to a 2-minute pulse of serotonin using two-dimensional gel electrophoresis and computer-assisted image analysis. Compared to untreated controls, at least 17 proteins showed a transient (<24 hours) increase in phosphorylation. Two-hour exposure to serotonin led to increased phosphorylation of the same spots, and this was detectable 24 hours later. Application of anisomycin or actinomycin D (translation and transcription inhibitors, respectively) during serotonin treatment blocked the long-term changes in phosphorylation but not the short-term changes, suggesting that long-term alterations are dependent on new gene expression. Experiments analogous to those above, but using a permeant cAMP analog to induce facilitation, gave similar results.

Sweatt & Kandel reported that application of phorbol esters that increase PKC activity gave a different but slightly overlapping pattern of phosphorylation increases than that obtained with serotonin or cAMP treatment. This, in combination with evidence in these experiments and others for the

involvement of PKA downstream of serotonin in facilitation, suggested that the PKA was likely to be responsible for at least some of the increases in phosphorylation that were seen. Since cyclic AMP levels return to baseline fairly soon after serotonin treatment ends, mechanisms long-term maintenance of phosphorylation by PKA were examined. There were a number of reasonable candidates, including gene expression-dependent processes that causes persistent activation of PKA, sensitization of PKA to baseline cAMP levels and/or a decrease in an opposing phosphatase activity.

Looking at the levels of the subunits of PKA in sensory neurons of the gill withdrawal reflex 24 hours after long-term sensitization, Greenberg et al. (1987) observed a specific loss of regulatory subunits, while catalytic subunit levels were not different from untrained controls. (Regulatory subunit levels were quantitated by photoaffinity-binding of a cAMP analog. Catalytic subunit was assayed by its enzymatic activity.) They argue that at lower ratios of regulatory to catalytic subunit, the concentration of cAMP required to induce kinase activity should decrease. In the case of long-term facilitation, such a change might reduce the activation threshold sufficiently to maintaining phosphorylation of substrates at near baseline cAMP concentrations.

The loss of PKA regulatory subunits in sensory neurons is specific to long-term sensitization, and appears to be protein-synthesis dependent (Bergold et al., 1990; Greenberg, et al., 1987). Bergold et al. (1992) cloned N4, one of the neuronal isoforms of the Aplysia PKA regulatory subunit, and their experiments indicated that the persistent loss of these regulatory subunits in long-term facilitation was post-translational. Comparison of nuclease protection assays of RNA samples from control and long-term facilitated sensory neurons harvested 24 hours after treatment displayed no change in the ratio of steady-state levels of mRNAs for N4 regulatory subunit to catalytic subunit. Earlier results indicating the loss of regulatory subunits were confirmed using antiserum raised against the bacterially-expressed N4 protein.

Ubiquitin-mediated selective proteolysis was suggested as a cellular method for decreasing the level of PKA regulatory subunit. A 30-amino acid region

that is highly enriched in the amino acids proline, glutamate, serine and threonine (PEST) in the N-terminal portion of the predicted N4 protein, and such PEST domains are often found in proteins that turn over rapidly (Rogers et al., 1986). Hegde et al. (1993) performed experiments indicating that PKA regulatory subunits from Aplysia are subject to proteolysis by the ATP-dependent ubiquitination-proteasome pathway in rabbit reticulocyte extracts and in extracts from Aplysia tissue. Although the involvement of de novo protein synthesis in the elimination of regulatory subunits was not experimentally addressed, they speculate that ubiquitination-targeting proteins for the regulatory subunit may be induced in long-term facilitation.

1.2.2.6.2. Changes in protein expression.
Examination of two-dimensional (2-D) gels of ^{35}S-methionine-labeled protein extracts prepared from excised sensory and motor cells of the gill-withdrawal reflex one day after long-term sensitization of intact animals showed that the levels of four uncharacterized protein spots on two-dimensional gels were increased compared to controls (Castellucci et al., 1988).

Barzilai et al. (1989), did a more extensive study, using the sensory neuron cluster from the pleural ganglion involved in the tail-withdrawal reflex. (These cells have properties similar to the mechanosensory neurons of the gill-withdrawal reflex and have the advantage that they can be recovered without contamination by other cell types.) They examined the effect of serotonin treatment that produces short-term facilitation (5 minute exposure) or long-term facilitation (5x serotonin treatment or a continuous 1.5 hour exposure to serotonin) on general protein synthesis and on synthesis of individual proteins in these neurons.

A five-minute exposure of sensory neuron clusters to serotonin (5μM) did not increase TCA-precipitable counts measured 3 hours, whereas a 1.5-hour exposure increased protein synthesis by 94%. (For each treated cluster, the untreated contralateral cluster served as a matched control.) Two-dimensional gels loaded with equal amounts of protein showed only a small amount of labeling in untreated clusters, whereas 1.5-hour serotonin treatment appeared to cause a general increase in the labeling of proteins.

The time course of the protein synthesis response was tracked using thirty minute pulses of ^{35}S-methionine administered at successively later time points during and after 1.5-hour serotonin treatment. Three phases were apparent: a small decrease in synthesis at 30 minutes after the start of treatment, a small increase at one hour, and a large increase at three hours. Interestingly, the large late peak of protein synthesis appears to depend on earlier transcription events in response to serotonin, since the presence of actinomycin D for one hour before and throughout the 1.5-hour serotonin treatment results in the loss of this component. This might indicate the operation of some kind of cascade, with later waves of gene expression being dependent on transcription factors expressed in earlier waves [see Alberini et al. (1994)].

Computer-aided analysis of 2-D gels showed differences in the intensity of labeling of individual protein spots between treated and control extracts. Almost all of these changes were transcription-dependent, since they were blocked by the presence of actinomycin D one hour before and during serotonin treatment. The levels of fifteen proteins were found to change at 15 to 30 minutes. Ten were increased (E1-E10) and five were lower compared to controls (D1-5). These changes returned to baseline by 1 to 3 hours, and all were at least sensitive to, and most were blocked by, actinomycin D applied for 1 hour before and during serotonin treatment. Four other proteins (I1-I4) showed increased levels at three hours. (For the three of these for which it was determined, this increase was dependent on transcription, peaked at three hours, and had returned to baseline levels by about eight hours.) Two proteins, called L407 and L1603, levels of which were slightly increased at three hours, were even more elevated at 24 hours after the beginning of serotonin treatment. The increase in the level of these proteins was transcription-dependent.

Some proteins whose levels were observed to be changed by serotonin-treatment by Barzilai et al. have since been identified. For example, L1603, one of two "late" proteins whose level appeared to be increased at 24 hours, has been identified as the Aplysia homolog of BiP/GRP78 (Kuhl et al., 1992). BiP is, among other things, a major chaperone protein of the endoplasmic reticulum, participating in folding of nascent proteins. Long-term

sensitization training resulted in elevated levels of Aplysia BiP mRNA one day after the end of training, specifically in cells involved in the long-term sensitization of the reflex.

The most complete story for proteins whose levels change with sensitization are the Aplysia neural cell surface adhesion molecules (ApCAMs). Mayford et al. (1992) cloned Barzilai et al.'s D1-D4 and named them ApCAMs, since the predicted proteins show amino acid homology to cell adhesion molecules that are expressed on the surface of axons during development in other species. In the adult Aplysia, ApCAMs are expressed only on central neurons. These molecules appear on the neurites of sensory cells of the gill-withdrawal reflex, especially at synaptic varicosities, and is also present on the motor neuron. Application of antiserum raised against ApCAM disrupted the fasciculation of axonal fibers growing out from cultured Aplysia sensory neurons, suggesting that ApCAM is indeed a functional neuronal cell adhesion molecule.

Immunofluorescence microscopy by Mayford et al. showed a protein synthesis-dependent reduction in the amount of ApCAM on sensory neurons, but not on motor neurons, which was evident as early as one hour after the beginning of serotonin treatment. Serial-section EM studies of anti-ApCAM immunogold-labeled sensory neurons by Bailey et al. (1992) found that ApCAM on the surface of serotonin-treated sensory neurons was reduced by 50% at 24 hours.

Serotonin treatment appeared to trigger a substantial and specific increase in internalization of ApCAM compared to controls. Endocytosis of ApCAM in coated pits was observed, occurring preferentially at areas of neurite-to-neurite contact (Hu et al., 1993). They also found an increase in the expression of clathrin light chain protein, which turned out to be protein E4 of Barzilai et al. They counted a ten-fold increase in the number of coated pits and vesicles within the first hour of serotonin treatment. The picture that emerged was one of a process very like a receptor-mediated endocytosis system for internalizing ApCAM. Labeled ApCAM molecules were seen to end up in structures strongly resembling sorting endosomes on the lysosomal pathway (Bailey, et al., 1992).

The down-regulation of cell-surface molecules that appears to be involved in maintaining neuronal contacts may be an early step in the remodeling of the terminals of the sensory neuron that is a hallmark of long-term facilitation. This is consistent with the fact that application of anisomycin during 5x serotonin treatment blocks the appearance of such morphological changes (Bailey et al., 1992). Evidence that serotonin treatment affects ApCAM on the sensory neuron without appearing to affect the motor neuron is in accord with previous observations that the morphological changes observed in long-term facilitation seem to be limited to the sensory neuron.

1.2.2.7. CREBs and long-term facilitation.

Investigation of sensitization of the Aplysia GWR provided convincing evidence of the necessity for new gene expression in long-term behavioral plasticity. Moreover, the combined involvement of the cAMP signaling pathway and changes in gene expression suggested a role for cAMP-responsive transcriptional control in this process.

The best-characterized metazoan system for control of cAMP-responsive gene expression is mediated by CREB-family transcription factors [for review, see deGroot and Sassone-Corsi, 1993)]. Briefly, many cAMP-responsive mammalian genes contain conserved enhancer sequences called cAMP-responsive elements (CREs, consensus sequence= TGACGTCA) in their upstream regulatory regions. CREs permit sequence-specific DNA binding by members of the CREB (CRE-Binding) transcription factor family, that can then influence transcriptional activation of the target gene. CREBs consist of a carboxyl-terminal domain involved in sequence-specific DNA binding to CREs and an amino-terminal domain that mediates transcriptional activation. Transcriptional activation by CREB is strongly potentiated by PKA phosphorylation of a specific phosphoacceptor serine (Ser133) in the transcriptional activation domain, providing the link between cAMP second-messenger pathway activation and activation of gene expression. This system will be treated in detail in a later section, but these facts are a sufficient context for comprehending the Aplysia experiments that follow.

Dash et al. (1990) performed the first experiments indicating that the involvement of CREBs in long-term facilitation in Aplysia.. Incubation of a radiolabeled CRE probe with a total protein extract from Aplysia ganglia resulted in the appearance of three gel-shifted bands in an electrophoretic mobility assay. The gel-shift effects could be competed out by addition of unlabeled CRE probes, but not by a non-specific linearized plasmid DNA. Competitor DNAs with mutated CREs did not interfere with the gel-shift, and addition of oligonucleotides containing AP-1 binding sites, which differ from CREs by the absence of a single nucleotide (TGAGTCA v. TGACGTCA) only weakly inhibited the gel shift. DNAse I footprint analysis with heparin-agarose purified Aplysia extract showed protection of a CRE-containing probe at and around the CRE site that was similar but not identical to that seen with a HeLa cell extract. These results indicated that proteins that specifically and competably bind to CREs, presumably CREBs, are present in the Aplysia nervous system, although none has yet been reported to be cloned.

The involvement of CREBs in Aplysia long-term facilitation was tested using the sensory neuron-L7 motor neuron co-culture system. Prior to 5x serotonin treatment for long-term facilitation, Dash et al. injected double-stranded oligonucleotides containing a CRE site into the nucleus of the sensory neuron. The idea here was that the exogenous CRE sites would compete for CREBs with chromosomal CRE sites in genes where CREB activation is required for long-term facilitation. This strategy is similar to that used by Gilman et al. (1988) who injected serum response element (SRE) oligonucleotides into fibroblasts to block serum-induction of c-fos expression.

Injection of about 10^6 CRE oligonucleotides before 5x serotonin treatment prevented long-term potentiation of EPSPs, measured 24 hours later. CRE oligonucleotides did not affect induction of short-term facilitation either immediately after injection or 24 hours after injection. Injection of $5x10^4$ CRE oligonucleotides resulted in a partial block of long-term potentiation. This graded effect suggests that the exogenous CRE sites do indeed titrate CREB activity. Injection of control oligonucleotides with a mutated CRE site, an NF-kappa B binding site or a consensus heat shock element all failed to interfere with long-term potentiation. The sequence-specificity of the block and its selective effect constitute persuasive evidence for the involvement of

one or more CRE-binding proteins in long-term facilitation. In the sensitization of the gill-withdrawal reflex, serotonin appears to activate the cAMP-second messenger system and increase phosphorylation of substrate proteins by PKA. It is possible that in long-term facilitation, CREB family members that are nuclear substrates for PKA are activated by phosphorylation and participate in activating the necessary gene expression.

Training to produce long-term facilitation requires multiple trials. Why might this be? Backsai et al. (1993) used fluorescent-labeled PKA catalytic subunits, so that their location in sensory neurons could be observed microscopically. After a single pulse of serotonin, free catalytic subunits appeared to be restricted to the cytoplasm of Aplysia sensory neurons, preferentially localized to the terminal regions. After 5x serotonin treatment, however, the catalytic subunit is found in the nucleus of the sensory neuron, an observation consistent with the idea that PKA phosphorylation of nuclear CREB is involved in long-term facilitation, and which also may explain why several training trials are required to obtain the long-lasting effect.

To show that stimuli that cause long-term facilitation activate cAMP-responsive genes, Kaang et al. (1993) microinjected a CRE-containing reporter construct into sensory neurons of the pleural ganglion of a semi-intact Aplysia preparation to demonstrate that treatments that result in long-term facilitation induce expression of CRE-containing reporters constructs. The reporter had a five tandem CREs placed just upstream of a fragment of the vasoactive intestinal peptide (VIP) promoter (-2000 to +146) that had been fused to the E. coli lac Z gene. A set of control reporters were also used. These included a reporter from which the CRE sites had been deleted, a reporter where the CRE sites were replaced by AP1 sites, a reporter with a fragment of the RSV promoter and a reporter with four TREs placed just upstream of the RSV promoter fragment. Treatments that cause long-term potentiation (prolonged application of a permeant cAMP analog, 5x serotonin treatment, 2-hour continuous exposure to serotonin) all produced large (from 3.4 to 6.0-fold) increases over baseline in reporter activity in ganglia injected with the CRE reporter, but no increases over baseline from any of the control reporters, suggesting that reporter activation was mediated through CRE sites. A single

serotonin pulse, which produces short-term but not long-term facilitation, did not affect expression of the CRE-containing reporter.

To show that a CRE-containing reporter could be activated by CREB in . e sensory neuron, Kaang et al. co-injected a plasmid expressing a fusion of the yeast GAL4 protein DNA-binding domain and the CREB transcriptional activation domain (GAL DBD-CREB TAD) fusion along with a reporter containing the GAL4 DNA-binding site. Exposing the injected neurons to 5x serotonin treatment led to massive activation of the reporter. The same experiment was done with the a GAL4 DBD-CREB fusion protein identical to the first, except the PKA phosphoacceptor serine in the CREB transcriptional activation domain was mutated to an alanine, a change that abolishes cAMP-responsiveness of CREB in mammalian systems. In this case, serotonin treatment did not activate the reporter substantially above baseline level. These results indicate that a cAMP-responsive mammalian CREB operates in its customary way in Aplysia sensory neurons.

1.2.2.8. An immediate-early gene downstream of CREB in Aplysia.
Alberini et al. (1994) cloned cDNAs from ApC/EBP, a gene for an Aplysia C/EBP. C/EBPs, or CCAAT-box enhancer binding proteins, are a family of transcription factors that are the products of multiple genes in mammals, where they have been implicated in terminal differentiation in a number of cell types, although not in neural cells (Johnson and McKnight, 1989; Landschulz et al., 1988a; McKnight, 1991). The ApC/EBP cDNAs predicted a protein with extensive similarity to other known C/EBPs, and in vitro-translated ApC/EBP bound specifically to DNA sites bound by mammalian C/EBPs. The ApC/EBP gene has a nearly perfect CRE a short way upstream from its putative TATA box.

Expression of ApC/EBP transcripts in the Aplysia nervous system could be induced by serotonin treatment, in the absence of protein synthesis. The time course of this induction, when the whole nervous system was exposed continuously to serotonin, looks like that of an IEG. Increased expression was seen after a few minutes of treatment, peaking at two hours and declining with continued exposure. This placed ApC/EBP induction within the time window for gene expression required for long-term facilitation. Induction of

ApC/EBP protein, localized to the nucleus, was detected in cultured Aplysia sensory neurons given 5x serotonin treatment.

To test for the involvement of in long-term facilitation, Alberini et al. injected oligonucleotides carrying an ApC/EBP binding site (ERE) into the nucleus of the sensory neuron in the co-culture system prior to 5x serotonin treatment. The ERE oligonucleotides blocked long-term, but not short-term facilitation. Mutated ERE oligonucleotides were ineffective in blocking long-term facilitation. This indicated that ApC/EBP was necessary for long-term facilitation, presumably by regulating expression of downstream genes containing EREs. Interestingly, injecting ERE oligonucleotides at times up to nine hours after 5x serotonin treatment still partially disrupts long-term facilitation, but progressively less so with larger intervals between 5x serotonin treatment and oligonucleotide injection. This suggests that the ApC/EBP protein must act over a surprisingly extended period of time to produce full long-term facilitation. (In other injection experiments in the co-culture system, ApC/EBP antisense RNA was introduced before 5x serotonin treatment to prevent translation of ApC/EBP mRNA into protein. This treatment specifically prevented long-term facilitation, as did injection of antibodies specific for the ApC/EBP protein.)

Injection of a 20-bp oligonucleotide representing the segment of the ApC/EBP promoter containing a putative CRE site (ApCRE) was also effective in blocking long-term facilitation in the co-culture system after 5x serotonin treatment. An identical oligonucleotide, but with random bases in place of the CRE, had no effect. Dash et al. (1990) had previously shown that injection of a canonical CRE oligonucleotide specifically blocked long-term facilitation. A plausible explanation for blocking of long-term facilitation by the naturally-occurring ApCRE is that the ApC/EBP gene is a downstream target of Aplysia CREB(s). CREB may thus sit at the head of a cascade of gene induction, where at each level there is expression both of products involved in control of gene expression at a lower tier and of the proteins involved directly in synaptic modifications for long-term potentiation.

1.2.2.9. Conclusion.

Experiments in the Aplysia system indicate that CREBs are involved in a model system representing a neural process underlying a form of non-associative learning in the GWR. Succeeding chapters of this thesis will present work characterizing CREBs in Drosophila and examining their involvement in long-term memory after associative training in a whole behaving animal.

1.3. CREB-dependent PKA-responsive transcription.

Both the investigation of CREB involvement in long-term facilitation and the examination of the role of cAMP-responsive transcription in Drosophila LTM were based on an understanding of the CREB system that was obtained from extensive work in mammals. CREB-mediated cAMP-responsive transcription, briefly sketched in an earlier section, will receive more thorough treatment here.

1.3.1. CREs.

A large group of mammalian genes whose expression can be affected by cAMP have been identified (Jameson et al., 1986; Meisner et al., 1982; Montminy et al., 1986a; Otani et al., 1982; Roesler et al., 1988; Wynshaw et al., 1984). Deletion analysis of the regulatory region of some of these genes has identified a common eight-nucleotide consensus sequence, TGACGTCA, which has been dubbed the cAMP-responsive element, abbreviated as CRE (Comb et al., 1986; Montminy et al., 1986b; Short et al., 1986; Tsukada et al., 1987). CREs are often located within the first few hundred bases upstream of the beginning of a transcription unit. Functional enhancers containing the CRE sequence also occur in viral promoters, notably those of some cAMP and E1a-inducible adenovirus early gene promoters (where they are called activating transcription factor [ATF] recognition sites) and in the HTLV-I long terminal repeat (Borrelli et al., 1984; Hardy and Shenk, 1988; Imperiale and Nevins, 1984; Lee et al., 1987; Yoshimura et al., 1990)

Cyclic AMP responsiveness can be reduced or abolished by deletion, mutation or methylation of CRE sequences (Comb et al., 1988; Deutsch et al., 1988;

Iguchi-Ariga and Schaffner, 1989). Moreover, addition of CRE sequences to promoters not normally regulated by cAMP can confer cAMP-responsiveness on them (Comb, et al., 1988; Deutsch, et al., 1988; Iguchi-Ariga and Schaffner, 1989; Leza and Hearing, 1989; Montminy, et al., 1986a; Wynshaw et al., 1986). There is evidence that sequences flanking the CRE can influence inducibility, but it seems clear that CREs are the principal cis-acting component in these regions (Benbrook and Jones, 1994; Bokar et al., 1988; Deutsch, et al., 1988; Knepel et al., 1990)

CREs are present in most cAMP-responsive genes. They are not, however, the only regulatory sequences that can confer transcriptional responsiveness to cAMP. The AP-2 binding site, which is quite different in sequence from the CRE, can mediate a transcriptional response to either cAMP or phorbol ester treatment (Imagawa et al., 1987). Finally, there are a few genes (e.g., chorionic gonadotropin β subunit, rat steroid hydroxylase gene CYP17) whose transcriptional response to cAMP appears to be dependent on non-CRE regulatory sequences (Albanese et al., 1991; Zanger et al., 1991). Nevertheless, cAMP-responsive transcriptional control via CREs is appears to account for the majority of cases and is currently by far the best understood.

1.3.2. PKA-responsive CREB family members.

The CREB family of proteins (sometimes called the CREB/ATF family) is numerous; in humans it includes the products of at least ten genes (deGroot and Sassone-Corsi, 1993; Habener, 1990; Hai et al., 1989; Hoeffler et al., 1990). CREB proteins are a diverse group, but, with very few exceptions, all have a carboxyl-terminal basic region-leucine zipper domain and show specific binding to CREs. Two CREB-family genes in particular have been the objects of close study: CREB, the first member of the family to be cloned, and the closely-related CREM gene. These and another less well-investigated close relative, ATF-1, are the mammalian CREB-family genes known to produce cAMP-responsive transcriptional activators, among all the genes that produce CRE-binding bZIP products. For this reason, discussion of CREB-family members here will, in the main, be limited to these three.

CREBα, a product of the CREB gene, is an archetype for describing features of the cAMP-responsive CREB transcriptional activators (Fig 1.6). The predicted CREBα protein is 341 amino acids long (Gonzalez et al., 1989). The carboxyl-terminal fifth of the protein consists of a basic region, involved sequence-specific DNA binding to CREs, and an adjacent leucine zipper, permitting selective dimerization with other leucine zipper molecules. (This basic region-leucine zipper (bZIP) motif marks CREB as a member of the bZIP gene superfamily that includes, among others, the jun, fos and C/EBP gene groups). The remainder of the protein is the transcriptional activation domain (TAD), made up of subdomains that appear to interact with various elements of the cellular transcription machinery. One of these subdomains, the P-box, contains consensus substrate sites for phosphorylation by a variety of protein kinases. The most well-studied of these phosphorylation sites is the PKA phosphoacceptor serine at amino acid 133 (Ser133). Phosphorylation of Ser133 is required to obtain PKA-responsive transcriptional activation. The TAD also contains domains called Q1 and Q2, one to either side of the P-box, which contribute to basal and PKA-stimulated transcriptional activation.

1.3.3. Implication of CREB in cAMP-responsive transcription.

Before CREB cDNAs were cloned, biochemical and cell culture experiments indicated that CREs and PKA were involved in mediating cAMP-responsive transcriptional activation. Montminy et al. (1986b) demonstrated that forskolin, a direct activator of adenylyl cyclase, specifically and powerfully increased expression from a CRE-containing reporter transfected into PC12 cells, but that this activation was not seen in a mutant PC12 subline lacking PKA activity. Grove et al. (1987) found that cAMP activation of a CRE-containing reporter in JEG-3 cells could be blocked by transfection of a plasmid expressing a peptide inhibitor of PKA. Riabowol et al. (1988) directly injected purified PKA catalytic subunit into C6 glioma cells, increasing expression of a CRE-containing reporter and an endogenous cAMP-responsive gene even more strongly than did forskolin or cAMP treatment. Mellon et al. (1989) showed that transfection into JEG-3 human placental cells of a construct overexpressing PKA catalytic subunit specifically activated expression from CRE-containing reporters. They also demonstrated that overexpression of a mutated PKA regulatory subunit that acts as an irreversible inhibitor of the

PKA catalytic subunit abolished forskolin activation of a CRE-containing reporter in this same system. In sum, these experiments showed that CRE-dependent transcriptional activation in response to cAMP depends on the action of PKA.

After CREB was isolated as a protein that binds to CREs by Montminy & Bilezikjian (1987), this molecule was integrated into the model of CRE-mediated cAMP-responsive gene regulation. The 43-kDa CREB protein was obtained by sequence-specific DNA affinity chromatography of PC12 cell extracts on a CRE column. In vitro and in vivo phosphorylation experiments showed that it was a substrate for PKA.

Experiments by Yamamoto et al. (1988) indicated that CREB binds to CREs primarily as a dimer, and that PKA phosphorylation did not appear to affect CREB homodimerization. Yamamoto et al. demonstrated that CRE-dependent transcriptional responses could be programmed by addition of CREB to in vitro transcription extracts that had been depleted by DNA affinity chromatography with CREs. Addition of purified PKA catalytic subunit to in vitro transcription extracts resulted in large increases in the level of transcription from CRE templates, but had little or no effect on transcription from templates from which the CRE had been removed.

Alberts et al. (1994) microinjected (recombinantly produced) CREB proteins, along with a CRE-lacZ reporter plasmid, into the nuclei of living cultured fibroblasts. Only co-injection of PKA-phosphorylated CREB resulted in significant activation of expression from the reporter. DephosphoCREB was ineffective. The PKA-phosphorylated CREB failed to activate a reporter containing the highly-related TRE site (TGACTCA), which is recognized by AP1-family transcription factors. Immunofluorescence studies of c-fos expression in microinjected REF252 cells indicated that expression of the cAMP- and CREB-inducible c-fos gene product was stimulated by phosphoCREB to approximately the same degree as with cAMP treatment. Injection of non-phosphorylated CREB or the mutant CREB had no effect on c-fos expression.

Taken as a whole, these results support a model in which CREB dimers bind to CREs and activate transcription from CRE-containing genes, and that the cAMP-responsive feature of this system depends on CREB phosphorylation by PKA. Further details of this model were developed with the cloning of the CREB gene.

1.3.4. Structure and function of cloned PKA-responsive CREBs.

Human and rat CREB cDNAs were cloned at about the same time by Hoeffler et al. (1988) and Gonzales et al. (1989), respectively. These cDNAs coded for essentially identical proteins, except that the rat cDNA (CREBΔ) predicted a 14-amino acid sequence called the a peptide, not present in the human clone (CREBα). (This difference was shown to be the result of alternative splicing. CREBα and CREBΔ forms occur in each species (Hoeffler, et al., 1990). The predicted product of the longer transcript, CREBα, was briefly described in an earlier section.

These cDNAs were the first representatives of a surprisingly extensive and disparate family of CRE-binding gene products. As mentioned previously, cDNAs that appear to represent at least ten other mammalian genes for CRE-binding factors have been identified (Foulkes et al., 1991; Gaire et al., 1990; Hai, et al., 1989; Kara et al., 1990; Maekawa et al., 1989; Meyer and Habener, 1993). All of these other CREB-family members predict bZIP proteins, although many differ substantially from CREB in details of structure (for instance, shorter and longer leucine zippers and in amino acid sequence. The region where they generally show most amino acid similarity to CREB is in the DNA-binding domain, which is not particularly surprising, since all of these molecules show binding specificity for the CRE site.

The CREB family includes a set of genes and cDNAs products have been named ATFs, for Activating Transcription Factor. These were cloned in expression screens of mammalian cDNA libraries to identify molecules involved in adenovirus early gene activation from consensus DNA-binding sites essentially identical to CREs (Hai, et al., 1989). One of the cloned molecules, ATF-2, participates in this function (Liu and Green, 1990).

Within the CREB gene family, CREB and two other genes, CREM and ATF-1, form a subgroup with a remarkably high level of amino acid identity in the bZIP region and good homology elsewhere (deGroot and Sassone-Corsi, 1993). Furthermore, these three are the only CREB-family genes that are known to express PKA-responsive transcriptional activators (Foulkes et al., 1992; Gonzalez and Montminy, 1989; Rehfuss et al., 1991). CREB and CREM are by far the most thoroughly-studied CREB-family members, and a significant amount of useful information has been accumulated about these genes.

The multi-exonic CREB and CREM genes show remarkable structural similarity (Fig. 1.7). (deGroot and Sassone-Corsi, 1993; Laoide et al., 1993). The most striking difference between the two structural genes is that CREM has an exon coding for a second, alternatively-spliced bZIP domain. As Fig. 1.7 indicates, CREB and CREM can generate numerous products. A comparison of long forms of CREB and CREM (CREBΔ vs. CREM-τ) shows the extensive amino acid identity between these two products of different genes (Fig. 1.8). (Although less is known about ATF-1 than either CREB or CREM, this product is extremely similar to CREBα in amino acid sequence, except that the predicted protein is shorter, essentially lacking the first 100 N-terminal amino acids of CREBα [Rehfuss, et al., 1991]).

Of the members of the CREB/CREM/ATF-1 subgroup, most is known about CREB. The strong amino acid similarity among these three suggested that they might be similar in functional performance, and this has largely been the case where it has been tested. Thus, the discussion below mainly addresses results with CREB (primarily CREBα), but where it's appropriate, differences or unusual results are known for CREM or ATF-1, these will be mentioned.

1.3.4.1. The bZIP region.

Landschutz et al. (1988b) suggested the name "leucine zipper" for stretches of amino acids that could form a continuous α helix with a leucine residue at every seventh position, such that the leucines would line up along the hydrophobic face of the coil. Such motifs could be found in predicted proteins of the C/EBP, fos, jun and GCN4 groups. Landschutz et al (1989) hypothesized that this leucine-studded hydrophobic surface could serve as a dimerization surface among molecules incorporating this motif. They adopted a model in

which the chunky heads of the leucine side chains from two antiparallel helices would interdigitate like the pins of a zipper, hence the term "leucine zipper".

Mutagenesis experiments in GCN4, Jun and C/EBP suggested that amino acid domains present in all these molecules, containing a high proportion of basic residues and positioned with consistent spacing just N-terminal to the leucine zipper, were involved in sequence-specific DNA binding (Agre et al., 1989; Hope and Struhl, 1986; Hope and Struhl, 1987; Neuberg et al., 1989). Vinson et al. (1989) formalized the notion that such "basic region" sequences predicted α helix with a positively charged face, attached to the leucine zipper, and coined the term "bZIP" to describe this arrangement.

Abundant experimental evidence from mutational and domain swapping experiments has confirmed that the basic region mediates sequence-specific DNA binding, and that the leucine zipper mediates selective dimerization among bZIP proteins that is required for optimal DNA-binding activity (Johnson and McKnight, 1989; Landschulz, et al., 1989) .

The most direct evidence for this bipartite functional organization of the bZIP domain comes from X-ray crystallographic studies of the yeast GCN4 protein. O'Shea et al. (O'Shea et al., 1991) found that the putative leucine zipper domain of the yeast GCN4 protein (the COO-terminal 33 amino acids) did indeed assume an a-helical conformation, but they formed dimers by parallel coiled-coil interactions, not by the "knobs-in-holes" model previously envisioned. Ellenberger et al. (1992) crystallized GCN4 bZIP domain homodimers with a DNA duplex 20-mer carrying a binding site for the GCN4 protein. They confirmed the leucine zipper observations of O'Shea et al, and also identified base-specific contacts between the GCN4 basic region and its DNA target.

Systematic mutational experiments showed the importance and specificity of leucine zipper interactions among bZIP transcription factors of the fos and jun families, which is a prerequisite for DNA-binding at phorbol 12-O-tetradecanoate 13-acetate (TPA)-responsive element (TRE site, also called the AP-1 site) and subsequent transcriptional activation (Busch and Sassone, 1990;

Gentz et al., 1989; Turner and Tjian, 1989). For instance, jun can dimerize with itself or with fos, but fos does not homodimerize. This suggests the potential combinatorial richness of leucine zipper complexes, and at the same time indicates that homo- and heterodimerization does not occur promiscuously (Ransone et al. 1989, Kouzarides & Ziff 1988,1989). The specific amino acid sequence of an individual type of leucine zipper molecule may embody a "dimerization code" that limits permissible combinations. The principles of this system are currently being worked out (Loriaux et al., 1993; Vinson et al., 1993).

CREB, CREM and ATF-1 have bZIP regions that are highly similar in amino acid sequence. Each predicts a four-repeat leucine zipper. Figure 1.9 shows a comparison of these sequences. Two different bZIP regions are represented for CREM because the gene expresses two different bZIP domains, CREM-I and CREM-II, by alternative splicing (Foulkes, et al., 1991).

The functions properties of the CREB bZIP region have been explored with respect to several of the properties described above. Yun et al. (1990) found that the sequences required for CREB dimerization and DNA-binding reside in the carboxy-terminal 66 amino acids of the protein that contain the basic region and the leucine zipper. Dwarki et al. (1990) demonstrated that in vitro translated CREBs bind to CRE sites as a homodimer, affirming the result obtained by Yamamoto et al. (1988) using affinity-purified CREB. Deletion of the CREB leucine zipper or mutation of any of the first three of the four zipper leucines to valines abolished or very substantially reduced homodimerization, as well as the binding of the mutated CREBs to CRE sites and the ability of the mutated CREBs to mediate PKA-dependent transcriptional activation in cell culture. Mutations in the basic region reduced or abolished the ability of CREB to bind to CRE sites, but did not affect dimer formation. Yun et al. also obtained results indicating that the bZIP domain of CREB is bipartite. CREB bZIP function is thus similar to that in other bZIP transcription factor families.

Waeber & Habener (1991) identified another important functional feature embodied in the bZIP domain of the CREB molecule - a nuclear localization signal. Their studies indicated that the full-length CREB protein was rapidly

translocated to the nucleus after synthesis, but a CREB isoform lacking the bZIP region accumulated in the cytoplasm. They performed transient transfection experiments in COS-1 cells using Western blot analysis of whole-cell and nuclear extracts, as well as immunostaining of cells to evaluate nuclear translocation. Fusion of a heterologous nuclear translocation signal rescued translocation-defective truncated CREBs. Deletion experiments indicated that a nine-amino acid sequence in the CREB basic region (residues 287-295, RRKKKEYVK) was required for normal nuclear localization. CREBs with an intact basic region but lacking the leucine zipper domain moved to the nucleus normally, indicating that nuclear translocation is independent of dimerization.

In experiments in vitro, CREBs display the selective heterodimerization that is characteristic of other bZIP proteins (Dwarki, et al., 1990; Hai and Curran, 1991; Hai, et al., 1989; Hoeffler et al., 1991; Hurst et al., 1991; Laoide, et al., 1993; Macgregor et al., 1990). For instance, CREB does not form heterodimers with jun- or fos-family proteins. The CREB-family member ATF-2 can combine with either jun or fos, while CRE-BP1 can form heterodimers with jun, but not with fos.

The two CREM bZIP regions have properties similar to the CREB bZIP. However, some functional differences between CREM-I and CREM-II have been described, though none of them are radical. In gel-shift experiments by Laoide et al. (1993) using bacterially-produced CREM proteins, a CREM-I-containing product appeared to recognize each of a ten different CRE-containing promoters more efficiently than did a CREM-II-containing molecule. A CREM-I-containing isoform homodimerized with CREB more readily than did the proteins with the CREM-II bZIP. These in vitro experiments show functional differences that could have biological consequences.

The DNA target sequence preferences of CREBs are strong but not absolute. Members of the fos- and jun families form dimers (AP-1 complexes) that bind to TREs, DNA sites that have the consensus sequence TGACTCA. Although TREs are only one nucleotide different than CREs in consensus sequence (TGACGTCA), CREB and AP-1 complexes can distinguish between them, each

showing strong preference for its own cognate site. Under some circumstances, however, CREB appears able to bind to a TRE and interfere with its function in vivo (Lamph et al. 1990, Masquilier and Sassone, 1992), Under the conditions ordinarily used in in vitro experiments, specific binding of CREB homodimers to TREs is not seen, but interfamily heterodimers between members of the CREB and AP-1 protein families can result in complexes with TRE-binding capacity (Benbrook and Jones, 1990; Benbrook and Jones, 1994; Hai and Curran, 1991; Ivashkiv et al., 1990; Macgregor, et al., 1990). It appears that at least some of these combinations are not cAMP-responsive transcriptional activators.

1.3.4.2. The transcriptional activation domain (TAD).

CREB is a transcriptional activator for CRE-containing genes, and this activity is strongly potentiated by PKA phosphorylation. Functional analysis reveals that these properties reside in the TAD, which has two major features (Fig. 1.6). First, there are two glutamine-rich regions, Q1 and Q2, at the amino- and carboxy-ends of the TAD, respectively. These are important in both basal and PKA-responsive transcriptional activation. Second, there is a central segment of about 60 amino acids between Q1 and Q2, called the P-box, that contains consensus substrate sites for a variety of protein kinases. Ser133, required for PKA-responsive activation of transcription by CREB, is part of a PKA target within this region. Also located in the P-box are residues necessary for interaction with a co-activator protein, CBP, that is involved in the mechanism of PKA-dependent enhancement of transcriptional activation via CREB. The experimental findings underlying this analysis are presented in following sections.

Many functional analyses of CREB transcriptional activation involve experiments where plasmids expressing wild-type or mutated CREBs are co-transfected into cell culture lines, along with reporters and various other constructs. Almost all cell culture lines have endogenous CREBs and intact cAMP-responsive transcriptional activation, which can confound results with transfected CREBs. Such experiments are therefore often performed using the F9 mouse teratocarcinoma cell line, since in the undifferentiated state, these cells do not have endogenous cAMP-responsive transcriptional activity (Masson et al., 1992; Strickland and Mahdavi, 1978). Gonzales & Montminy

(1989) found that if CREB, with and without PKA, was supplied by transfection of the appropriate expression constructs, CREB-dependent, PKA-responsive activation of a co-transfected CRE-containing reporter could be detected.

A second technique used to avoid confounds from endogenous CREBs in functional studies is to express CREB fusion proteins that incorporate a DNA binding domain (DBD) from a transcription factor not present in mammals, often the yeast GAL4 DBD (Sadowski and Ptashne, 1989). Transcriptional activation by the fusion protein is assayed using co-transfected reporter constructs with GAL4 DNA recognition sites instead of CREs (see (Berkowitz and Gilman, 1990) for an example of this system).

1.3.4.2.1. Q1 and Q2 domains.

A number of proven or putative transcription factors have glutamine-rich domains (Mitchell and Tjian, 1989). Glutamine-rich domains in the mammalian transcription factor Sp1 are known to contribute to transcriptional activation, and a glutamine-rich region from the Drosophila Antennapedia gene product can activate transcription when fused to the isolated DNA-binding domain of Sp1 (Courey and Tjian, 1988). There is no apparent amino acid sequence conservation between the glutamine-rich region from Antennapedia and those of Sp1, suggesting that glutamine content per se is a major determinant for the activity of these domains.

The N-terminal glutamine-rich domain of CREB, Q1, is contained in the first 87 amino acids. Gonzales et al. (1991) examined the effect of deleting amino acids 1-67 from CREB on basal and PKA-stimulated transcriptional activation from a CRE-containing reporter in F9 cells. This caused an 85% reduction in basal activation, but stimulation of activation by PKA was not affected. A deletion to amino acid 87 reduced basal activity to 5% of wild-type and also compromised stimulation of activity by PKA by approximately 50%, from 36-fold to a still-impressive 17-fold. These results indicated that Q1 was important for basal activation and possibly for PKA-responsive transcriptional activation, and also that basal activation by Q1 could be disrupted separately from PKA-responsive stimulation.

CREB has a single alternatively-spliced exon called the α peptide, coding for a 14 amino acid helical stretch at the COOH-terminal of the Q1 domain (amino acids 88-101 in CREB-α). Each CREB isoforms can mediate some level PKA-responsive transcriptional activation from CREs, however Yamamoto et al. (1990) found that CREBΔ was much less efficient than CREBα in stimulating PKA-dependent transcription from a CRE reporter in F9 cells. Others have failed to reproduce this difference either in F9 cells or in other cell culture lines (Berkowitz and Gilman, 1990; Quinn, 1993; Ruppert et al., 1992). The reasons for this are unclear, but F9 cells can subtly differentiate, which might explain the disparate results of Yamamoto et al. and Ruppert et al.

Another member of the group of PKA-responsive CREBs, the predicted ATF-1 protein, in form and transcriptional activating potential closely resembles CREB-Δ minus its Q1 domain. Rehfuss et al. (1991) and Liu et al. (1993) both tested transcriptional activation by ATF-1 from a CRE-containing reporter in the absence and presence of PKA in F9 cell transfection experiments. Both groups found that ATF-1 gave lower basal activation than did CREB. Liu et al. found approximately equal fold increases over basal transcriptional activation by ATF-1 and CREB in response to PKA. The lower basal activation observed for ATF-1 and the minimal effect on relative PKA-stimulation of activation agrees with results from Gonzalez et al. (1991), who tested a CREB N-terminal deletion similar to ATF-1.

A second glutamine-rich domain in CREB, Q2, was examined by Brindle et al. (1993). Q2 spans the carboxyl-terminal section of the activation domain, lying between the P-box domain and the bZIP (i.e., amino acids 160 to 284 of the predicted CREBα protein). Nested deletions in Q2, functionally tested in F9 cells, showed marked reduction or complete loss of basal and PKA-stimulated transcriptional activity, suggesting that this glutamine-rich domain may be required for both types of activity. The degree of impairment was positively correlated with the size of deletions, which makes some sense if the number of glutamine residues (spread fairly evenly throughout this domain) is an important determinant for transcriptional activation. Quinn (1993) found effects generally similar to those seen by Brindle et al., using GAL4-CREB fusions with deletions in the Q2 domain. Brindle et al. also found that a GAL4 DBD-Q2 fusion was a constitutive, but not PKA-responsive,

transcriptional activator from a GAL4-CAT reporter in F9 cells, indicating that this domain probably cooperates with other regions for PKA-dependent potentiation of CREB activity.

In CREM, two alternatively spliced glutamine-rich exons, q1 and q2, code for large parts of the Q1 and Q2 protein domains of CREM, respectively. Although not co-extensive with the Q1 and Q2 protein domains as defined experimentally in CREB, functional studies with naturally-occurring CREM isoforms with and without these exons are substantially in agreement with results on Q1 and Q2 from CREB. A variety of naturally occurring CREB isoforms (α, β, and γ, for example) lacking both q1 and q2 are not activators (Foulkes, et al., 1991). Cell culture experiments by Foulkes et al (1992) and Laoide et al (1993) indicated that GAL4 DBD fusions with isoforms lacking one or the other of q1 or q2 (naturally-occurring CREM isoforms τ1 and τ2) were functional, but were substantially poorer basal and PKA-responsive activators than an isoform containing both q1 and q2. Furthermore, q1 and q2 were not equally potent, and the effects of q1 and q2 on both basal and PKA-responsive transcription appeared to be additive. However, Brindle (1993) tested a CREM isoform lacking the q2 domain and found it to be inactive in basal and PKA-activated transcriptional function in F9 cell culture, indicating that a conclusive functional description of these domains is not yet in hand. CREMs lacking both q1 and q2 are not PKA-responsive transcriptional activators in cell culture (Foulkes, et al., 1991).

Recent results from Ferreri et al. (1994) provide a molecular insight into the functional role of the Q2 domain. Addition of a mutant CREB protein with only the Q2 and bZIP domains was able to stimulate transcription from a CRE-containing reporter in a CREB-depleted HeLa cell in vitro transcription extract. Other in vitro transcription experiments involving manipulation of basal transcription factors indicated a possible requirement for transcriptional co-activator proteins (TAFs) in transcriptional activation by CREB. TAFs are found in the TFIID fraction of nuclear extracts, associated with TATA-binding protein (Goodrich and Tjian, 1994; Hernandez, 1993). Ferreri et al. noted similarities between Q2 and the glutamine-rich activation domain B in the mammalian Sp1 transcription factor. Transfected mammalian Sp1 is active in Drosophila Schneider cells (which lack Sp1), and domain B appears to

interact with the cloned Drosophila co-activator $dTAF_{II}110$ (Ferreri, et al., 1994; Hoey et al., 1993). In affinity chromatography and yeast two-hybrid system experiments, Ferreri et al. found that the CREB Q2 domain interacts specifically with $dTAF_{II}110$.

1.3.4.2.2. The P-box.

In the inferred amino acid sequence of CREB, Gonzalez et al. (1989) found a consensus PKA phosphorylation site, overlapped with a consensus PKC phosphorylation site (RRPSYRK, amino acids 130-136). Nearby is another consensus phosphorylation site for PKC, plus six for casein kinase II, and one for glycogen synthase kinase III (Lee et al., 1990). This indicates that the CREBα P-box may extend N-terminally as far as amino acid 92.

Gonzalez & Montminy (1989) mutagenized the putative phosphoacceptor serine of the CREB PKA substrate site (Ser133), and tested its effect on PKA-dependent transcriptional activation in F9 cell transfections. When Ser133 was mutated to alanine (a change that eliminates the PKA phosphorylation site but maintains charge balance) the mutant CREB molecule was no longer able to mediate PKA-responsive transcriptional activation from a CRE-containing reporter. A second mutant, Ser133 --> Asp133, designed to provide the negative charge ordinarily provided by phosphorylation, was unable to activate transcription from the CRE reporter, either constitutively or in the presence of PKA. These results strongly indicate that phosphorylation of Ser133 is necessary for transcriptional activation by PKA, and that the effect of phosphorylation is probably not due merely to the local addition of negative charge. They also made mutations that disrupted the consensus PKC site in a way that left the overlapping PKA phosphorylation site intact. This did not affect PKA inducibility, suggesting that they were not observing the effects of transcriptional activation by PKC. Lee et al. (1990) obtained similar results in experiments in JEG-3 cells with GAL4-CREBΔ mutant fusions where Ser119 (the CREBΔ homolog of Ser133) was replaced with an alanine or glutamate residue.

PKA-phosphorylation dependence of transcriptional activation has also been evaluated in CREM, which has a P-box similar in organization to the one in

CREB (deGroot et al., 1993). Ser117 in the consensus PKA phosphorylation site of CREMτ is the homolog of the Ser133 in the PKA site of CREBα. Changing CREMτ Ser117 to glutamate or alanine severely reduces the ability of the mutagenized molecule to mediate PKA-dependent transcriptional activation compared to the wild-type protein.

Regions adjacent to the PKA phosphorylation site of PKA also participate in cAMP-responsive activation, indicating that Ser133 and its homologs are necessary, but not sufficient, for full activation (Gonzalez, et al., 1991). In CREBΔ, short segments N-terminal (pDE-1; aa 92-108) and C-terminal (pDE-2; aa 121-131) to the PKA phosphoacceptor Ser119, severely impaired cAMP-responsive transcriptional activation. The N-terminal sequence contains multiple consensus phosphorylation substrate sites for non-PKA kinases and was phosphorylated in vivo and in vitro. One possible interpretation of this observation is that non-PKA phosphorylation events may be permissive for functional activation of CREB by PKA. Mutation, deletion or spacing changes in a specific five-amino acid sequence (DLSSD) within PDE-2 abolished PKA-dependent activation without affecting expression, nuclear localization or PKA-phosphorylation of CREB. This small sequence within the P-box appears to cooperate with phosphorylation to effect cAMP-responsive transcriptional activation by CREB, possibly by conformational change in the activation domain.

Dash et al. (1991) showed that CREB can be phosphorylated at Ser133 in vitro by purified calcium/calmodulin-dependent (CaM kinase), and that this phosphorylation can potentiate CREB activation of transcription. PC12 cell culture experiments by Sheng et al. (1990, 1991) indicated that Ca^{++}-responsive induction of the immediate early gene (IEG) c-fos involved a CRE containing-region in the c-fos promoter, and occurred by a CREB-dependent mechanism involving phosphorylation of Ser133. In transient transfection experiments with GAL4-CREB fusion constructs, this group showed that Ca^{++} influx due to membrane depolarization or application of the Ca^{++} ionophore A23187 led to activation of a GAL4-fos reporter protein by a wild-type CREB fusion, but not by a Ser133 mutant. Induction by Ca^{++} did not appear to involve a general increase in intracellular cAMP level, which suggested that PKA might not be involved. Noting that the CREB P-box has

good consensus phosphorylation sites for CaMK I and II, both with Ser133 as the phosphoacceptor, they demonstrated in vitro that each of these kinases can phosphorylate CREB on Ser133 and elsewhere. These results are all consistent with a model in which direct phosphorylation of Ser133 by CaM-kinases could be involved in Ca^{++}-dependent induction of c-fos.

Experiments by Ginty et al. (1991) in PKA-deficient PC12 mutant lines are at odds with these results, indicating that direct CaM kinase phosphorylation of Ser133 is not an important mechanism in Ca^{++}-dependent IEG expression. In the three such cell lines that they tested, Ca^{++}-dependent induction of c-fos and a second IEG, egr1, was almost completely suppressed. These lines showed a greatly diminished transcriptional response to forskolin, but they had normal activation in response to phorbol ester or nerve growth factor. Baseline CaM kinase activity and activation of CaM kinase in response to membrane depolarization were not compromised in these lines, indicating that calmodulin and CaM kinase systems were intact. These experiments suggest a requirement for PKA in Ca^{++}-dependent transcriptional activation of IEGs, but they do not rule out a role for direct CaM kinase phosphorylation of CREB.

These studies are not conclusive. Sheng et al. (1990, 1991) do not strictly establish a connection in vivo between CaM kinase and CREB phosphorylation in Ca^{++}-responsive transcription. Ginty et al. (1991) failed to measure important properties of the mutant cell lines (most notably, CREB expression). Thus, while it seem clear that CREB phosphorylation is involved in Ca^{++}-responsive gene induction, the important question of mechanism is incompletely determined. Because calcium is an important transsynaptic messenger for gene expression in nerve cells, this problem demands continuing attention.

A recent report by Ginty et al. (1994) suggests that transcriptional activation of c-fos by nerve growth factor (NGF) involves CREB phosphorylation on Ser133 by an apparently novel Ras-dependent protein kinase. They found that CREs in the c-fos promoter region were required for full induction of this gene in response to NGF treatment in PC12 cells. In this same system, NGF treatment resulted in a rapid increase in CREB Ser113 phosphorylation, that apparently

was not mediated by PKA or CaM kinase, since NGF could induce Ser133 phosphorylation in PKA-deficient PC12 cells and NGF treatment did not seem to induce CaM kinase activity.

NGF is believed to activate a Trk receptor tyrosine kinase, which then stimulates Ras activity (Qiu and Green, 1991). Phosphorylation of CREB Ser133 in response to NGF was greatly reduced in a PC12 line that expressed an inducible dominant-negative Ras mutant, supporting the suspicion that a Ras-dependent protein kinase may be involved. (Induction of c-fos by forskolin treatment was undisturbed by expression of the Ras dominant-negative mutant.) By fractionating extracts from NGF-treated PC12 cells, Ginty et al. (1994)identified an activity that phosphorylated CREB on Ser133 and that was absent in extracts made from cells not treated with NGF. Furthermore, the appearance of this "CREB kinase" activity in response to NGF treatment was suppressed in extracts from PC12 cells that expressed dominant-negative mutant Ras. Ginty et al. characterized an apparently novel 105kD polypeptide as the molecule responsible for this activity.

Brindle et al. (1993) reported that the loss of PKA-responsive activity in a CREBα mutant, missing the Q2 domain but retaining its P-box, could be rescued by inserting an acidic activation domain from the yeast GAL4 protein either upstream or downstream of the P-box. This result suggested that the P-box could endow a heterologous activation domain with PKA-responsiveness, and that the position of the activation domain relative to the P-box was flexible. They tested the ability of the ability of the P-box to confer PKA-responsiveness in trans by co-expressing a GAL4 DBD-CREB P-box fusion along with a GAL4 DBD-GAL4 acidic domain fusion, a GAL4 DBD-CREB Q2 fusion or a GAL4 DBD-GAL4 acidic domain-CREB Q2 fusion. In co-transfection experiments in F9 cells, using a reporter containing a string of five GAL4 DNA recognition sites, the presence of the isolated P-box resulted in substantial PKA-responsive activation with each of the activation domains in trans. The P-box fusion caused no PKA-responsive activation when co-transfected with a construct expressing the GAL4 DBD alone. This suggests a mechanism by which the CREB isoforms missing Q1 and/or Q2, but possessing a P-box, might confer cAMP-inducibility on heterologous transcription factors that might be bound to nearby recognition sites.

1.3.5. CREB-binding-protein (CBP), a probable CREB co-activator .
Molecules that interact specifically with PKA-phosphorylated CREB might cooperate in stimulation of transcriptional activation. To detect such proteins, Chrivia et al. (1993) screened a λgt11 human thyroid cDNA expression library, using as a probe CREB protein that had been labeled by PKA-phosphorylation with radioactive phosphate in vitro. A single positive clone was and this contained a cDNA predicting a previously unidentified 2441-amino acid protein, CBP.

The predicted CBP features a carboxyl-terminal glutamine rich domain and a consensus PKA phosphorylation site, as well as several consensus CaMKII substrate sites. It also has two predicted zinc-finger domains. The ability of CBP to interact with CREB was further evaluated in sandwich binding assays. CBP protein, immobilized on nitrocellulose filters, was probed with PKA-phosphorylated or unphosphorylated CREB that had been previously incubated with a radiolabeled CRE oligonucleotide. CBP was detected only by the PKA-phosphorylated probe complex. In co-immunoprecipitation experiments using anti-CBP antisera, CBP was again found to interact only with PKA-phosphorylated CREB, and not with unphosphorylated or CKII-phosphorylated CREB. Chrivia et al. determined that CREB's CBP-binding activity required sequences in the P-box domain, between amino acids 101 and 160, including some outside the PKA phosphorylation site. The CREB-binding domain of CBP was mapped to a region in the N-terminal quarter of the predicted protein (amino acids 462-661).

CBP appears to be a PKA-responsive transcription factor in its own right. Expression of GAL4 DBD fusion gene with the amino terminus of the CBP in F9 cells resulted in a 10-fold increase in transcription from a GAL4-CAT reporter, which increased to 45-fold in the presence of a co-transfected PKA expression plasmid (Chrivia, et al., 1993). Kwok et al. (1994) found that transfection of a CBP expression construct into F9 cells, with or without an accompanying PKA expression plasmid, failed to activate transcription from a CRE-CAT reporter. CREB is unavailable in F9 cells, suggesting that this molecule is required for CBP to activate transcription. To test whether CREB

and CBP cooperate in PKA-stimulated transcriptional activation by CREB, and the further possibility that CBP might be limiting in F9 cell transfection experiments, Kwok et al. added a CBP expression plasmid to a co-transfection experiment with CREB and PKA. Addition of CBP resulted in a dose-dependent increase in activation, up to five- fold over CREB plus PKA alone. A similar experiment using a non-PKA-responsive CREB Ser133-->Ala133 mutant showed minimal activation and no effect of CBP addition, indicating that the effect of CBP addition involves phosphorylated CREB and that CBP can be limiting in F9 cell transfection experiments with CREBs.

Arias et al. (1994) injected an antiserum that interferes with CREB-CBP interaction into the nuclei of CBP-producing NIH-3T3 cells. This treatment specifically blocked activation of a CRE-lacZ reporter that normally occurs in response to cAMP treatment, indicating that direct interaction between CREB and CBP proteins are involved in PKA-responsive transcription via CREs. A possible model from the results with CBP might be that phosphorylated CREB acts as a DNA-bound scaffold for CBP docking at the promoter, putting the CREB-anchored CBP in a position to interact with component(s) of the general transcription machinery. This is similar to the current model for the multifunctional adenoviral E1a protein, which does not itself bind to DNA and in the context of CRE/ATF sites is thought to use ATF-2 as its DNA-bound scaffold (Liu and Green, 1990). The complete E1a picture, however, is known to be more complicated. In a separate, but related, development, the recently-cloned p300 protein, a member of E1a complexes that interacts directly with the E1a protein, appears to be a member of the protein family that includes CBP (Eckner et al., 1994).

With regard to interactions between CBP and the general transcription machinery, deletion experiments using GAL4 DBD-CBP fusions indicated that the carboxyl-terminal 758 amino acids of CBP (containing the second zinc-finger domain, the consensus PKA phosphorylation site and the C-terminal glutamine rich domain) constituted an even better transcriptional activator than a similar fusion containing the entire CBP protein, suggesting that this portion of the molecule might logically be a place to look for domains interacting with general transcription factors (Kwok, et al., 1994). There appears to be an interaction between the carboxy-terminal segment of CBP

and immobilized fusion proteins containing the general transcription factor, TFIIB.

1.3.6. Expression patterns.

Although the CREM and CREB genes are similar in genomic structure, they are remarkably different in their patterns of expression. CREB expression is, in the overwhelming majority of tissues, ubiquitous, and involves only two isoforms that are functionally indistinguished PKA-responsive activators. In contrast, CREM expression is complex at every possible level. At least ten different products of the CREM gene have been identified. Expression of CREM isoforms are tissue- and cell-type specific, and isoform-specific expression is known to be inducible. Furthermore, an important functional division exists among CREM products: some are PKA-responsive activators of transcription, others are antagonists of PKA-responsive transcription.

1.3.6.1. CREB.

In the vast majority of cell types, the "full-length" CREBα and CREBΔ isoforms described earlier are expressed ubiquitously and at low levels. Expression of these products does not appear to be modulated by cAMP (Berkowitz and Gilman, 1990; Hai, et al., 1989; Hoeffler, et al., 1990). The 5' flanking genomic sequence of the human gene has a TATA-less GC-rich promoter region characteristic of many constitutively expressed genes (Bird, 1986; Meyer et al., 1993).

The testis appears to be a special case for CREB gene expression. Waeber et al. (1991) and Ruppert et al. (1992) found that high levels of full length CREB transcripts were differentially expressed in Sertoli cells and spermatocytes, in rhythm with hormonal signals involved in germ cell maturation. Furthermore, in contrast to other tissues, where only the full length isoforms are expressed, studies in rat testis identified at least five alternatively-spliced CREB mRNAs that predicted a class of carboxyl-terminal truncated proteins lacking bZIP domains. Waeber et al. studied CREB-W, a representative of this class. The CREB-W protein appears to be abundant in the testis, but could not be detected in any of a wide selection of other tissues. CREB-W mRNA contains an alternatively spliced exon (W) that is omitted from the full-

length CREB transcripts. Exon W is located just upstream of the region coding for the bZIP domain, and it contains a number of in-frame stop codons. The CREB-W message thus predicts a truncated CREB molecule missing the region that mediates DNA-binding and dimerization in other CREB isoforms. (Other truncated CREB isoforms use different splice sites to the same effect). As would be expected of an isoform lacking the nuclear localization signal present in the bZIP, CREB-W protein appears to be concentrated in the cytoplasm.

Interestingly, rat spermatogenesis is governed by hormones that activate the cAMP cascade, suggesting that expression of CREB in the testis might be governed by some autoregulatory mechanism. The CREB gene does, in fact, have upstream CRE sites that can produce modest increases in gene expression in cell culture (Meyer, et al., 1993).

Although CREBs are ubiquitously expressed, a specific function of CREB function examined in a physiological context using transgenic rats that express a CREB protein that cannot be phosphorylated by PKA, due to an engineered mutation at Ser133 (Struthers et al., 1991). In cell culture, such a mutant acted as a dominant negative blocker of PKA-responsive activation. It may work by binding to CREs as homo- or heterodimers that are unresponsive to PKA. Spatially-specific expression of the transgene was achieved by placing it under the control of the growth hormone (GH) promoter. GH is expressed by somatotrophic cells in the pars distalis of the adenohypophysis, and cAMP is a mitogenic signal for these cells. The transgenic mice were dwarves, indicating a loss of growth hormone activity. A massive reduction of somatotroph cells was also seen. Presumably, their normal cAMP-dependent proliferation was mediated by CREB activity that was disrupted by the dominant-negative transgene.

1.3.6.2. CREM.

Earlier sections discussing the functional anatomy of CREM isoforms gave some inkling of the blizzard of isoforms produced by CREM, but the most important known functional difference among the many CREM products is that some are PKA-responsive activators (i.e., CREMτ, CREMτ1, CREMτ2) and others are dominant-negative antagonists of PKA-responsive transcriptional

activation (e.g., CREMα, CREMβ, CREMγ, s-CREM and ICER) (Foulkes, et al., 1991; Foulkes, et al., 1992; Laoide, et al., 1993; Molina et al., 1993). Unlike CREB, CREM shows isoform, tissue- and cell-specific expression, as well as inducible expression of its various isoforms. Inducible expression of CREM isoforms has been examined in some interesting physiological contexts (Foulkes, et al., 1991; Laoide, et al., 1993; Mellstrom et al., 1993).

The large number of CREM products derives in part from the many isoforms generated by combinations of alternative splicing of the q1 and q2 exons, as well as a 12 amino acid exon called γ, just carboxyl-terminal to the q2 exon. In addition, splicing differences in the TAD can occur in combination with each of the two CREM bZIP regions. (The functional difference between such pairs in vivo is yet a mystery.) Other strategies besides alternative splicing are also used for control of expression of CREM isoforms, including alternative transcription initiation, alternative translation initiation, and use of multiple polyadenylation sites that affect transcript stability to cause differential expression of protein isoforms.

mRNA in situ hybridization studies by Mellstrom et al. (Mellstrom, et al., 1993), using probes that can discern between CREMα, -β and -τ forms, indicated that there is constitutive expression of CREM transcripts in the adult rat brain. mRNAs for blocking isoforms of CREM are expressed in widespread but regionally-specific patterns (including preferential expression in cornu Ammoni, especially in CA-3). Transcripts for CREM activator isoforms appear to be generally and diffusely expressed in the brain at low levels, reminiscent of CREB. They also showed that CREM-α and -β mRNAs were induced in suprachiasmatic nuclei of the pituitary by osmotic stimulation from salt-water feeding.

Working in mice, Delmas et al. (1992) discovered a short CREM protein, s-CREM, that appears to be expressed only in the brain and only after birth. The s-CREM product predominates over CREM-α and -β in abundance in the brain by adulthood, the reverse of the situation that obtains during prenatal and early postnatal period. Functionally, s-CREM is a repressor of PKA-responsive transcriptional activation from CREs. It is produced by the CREM gene. but no specific s-CREM transcript exists. Instead, alternative translation

initiation from an internal AUG in the CREMτ transcript results in a CREM molecule that contains only the Q2 domain and bZIP region. The mechanism of s-CREM induction (i.e., the signal and process mediating the change in translational start site preference) is unknown, but could be of general biological interest.

Molina et al. (1993) identified another short CREM product, ICER, that is a powerful antagonist of PKA-responsive transcriptional activation. ICER is the only known cAMP-inducible CREM isoform, and it has properties of an immediate early gene. Unlike s-CREM, which is produced by alternative translation initiation in a complete CREM transcript, ICER is translated from short transcripts that are produced by alternative transcription initiation from an internal promoter located in an exon just 5' to the γ exon of CREM. ICER mRNAs predict a protein that is essentially a disembodied CREM bZIP domain.

ICER mRNA expression can be induced in vivo in the rat pituitary gland and in neuroendocrine cell culture lines by treatments that elevate cAMP. In cell culture experiments, this induction does not require de novo protein synthesis, and has a time course characteristic of an IEG, appearing within minutes and decaying, in a protein synthesis-dependent manner, within a few hours.

Even more interesting is evidence suggesting that the protein synthesis-dependent reduction in ICER expression may occur by ICER acting as a repressor at its own promoter. The intronic ICER alternative promoter region has a number of interesting features. It has a TATA box (which is lacking in the GC-rich promoter region 5' to the full-length CREB and CREM transcription units)and, most strikingly, four well-conserved CRE sites within 100 amino acids of the ICER TATA box. Bacterially-generated ICER protein can bind to the CRE-containing fragment of this promoter and in cell culture, ICER can antagonize PKA-responsive activation of a reporter construct containing this promoter.

Stehle et al. (1993) studied circadian cycling of ICER transcript in the rat pineal gland, the physiological context where it was discovered. Levels of ICER

increase dramatically in this structure in the subjective nighttime, and essentially vanish during the daylight hours. Release of the hormone melatonin by the pineal gland is known to follow a similar cycle, increasing at night and waning in the daytime, triggered by norepinephrine stimulation. Although Stehle et al. make no molecular connection between these two, both rhythms appear to be governed by a light-entrained internal clock. A pulse of light in the nighttime, known to result in reduction of melatonin production, also results in a reduction of ICER expression. At the level of physiological mechanism, adrenergically-triggered cAMP pathway activation leads to ICER expression in the pineal gland. Interestingly, melatonin production in recently-isolated pineal glands can be induced by β-adrenergic agonists at any time, while these drugs can only induce ICER expression during subjective night, indicating that some entrained mechanism exists for restricting competence to express ICER in the pineal gland.

The testis is a site for cyclic differential expression of CREM, as well as for CREB. The non-activator CREMα and -β isoforms are expressed in immature germ cells and in non-germ cell types of the seminiferous tubules, the Leydig and Sertoli cells. Postmeiotic spermatocytes, but not the testicular somatic cells or the immature stem cells, begin predominantly to express the PKA-responsive activator form CREMτ in response to pituitary release of follicle-stimulating hormone (FSH), a hormone that activates the cAMP pathway (Foulkes et al., 1993). Although the CREMα and -β repressor isoform differ from the activator by the presence of the q1 and q2 exons in the activator form, alternative splicing does not appear to be the mechanism for the change in the steady-state level of CREMτ. Instead, FSH stimulation triggers an unknown, but presumably cAMP-dependent, mechanism that results in selection of an alternative downstream polyadenylation site in the CREMτ transcript, which results in enhanced stability. Otherwise the transcript is constitutively expressed in a quickly-degraded form. In addition to being fascinating as a part of the CREM story, this system might provide insight into an aspect of general biology, that is, cAMP-responsive control of alternative polyadenylation.

1.3.7. Conclusion.

The previous section gives some indication of the complexity of cAMP-responsive gene expression via CREBs in mammals. As the recent discovery of CBP shows, only an elementary understanding of the CREB system has been achieved. The reason for the existence of so many CREB genes and products is not understood, and the relevant function of many of the non-cAMP-responsive CREBs is completely unknown. Combinatorial interactions between CREBs and other transcription factors has scarcely been examined. However, the lineaments of a pathway mediating cAMP-responsive transcription are in hand, and information available for the mammalian CREB system was a sufficient guide for characterizing a seemingly analo gous (but perhaps less complex) system from Drosophila, and to allow us to test the involvement of this system in LTM.

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Figure 1.1 legend. QHB training apparatus. The start tube (6) can be aligned with each of the other tubes by sliding the block along a dovetail groove attaching the two blocks. Tube 1 is the rest tube, which is perforated for ventilation. Tubes 2 and 3 are for training. Tubes 4 and 5 are for testing. A and B represent two different odorants. Horizontal stripes in tubes indicate electric grids and lightning bolt over tube 2 indicates voltage on that grid. (Adapted from Quinn et al., 1974).

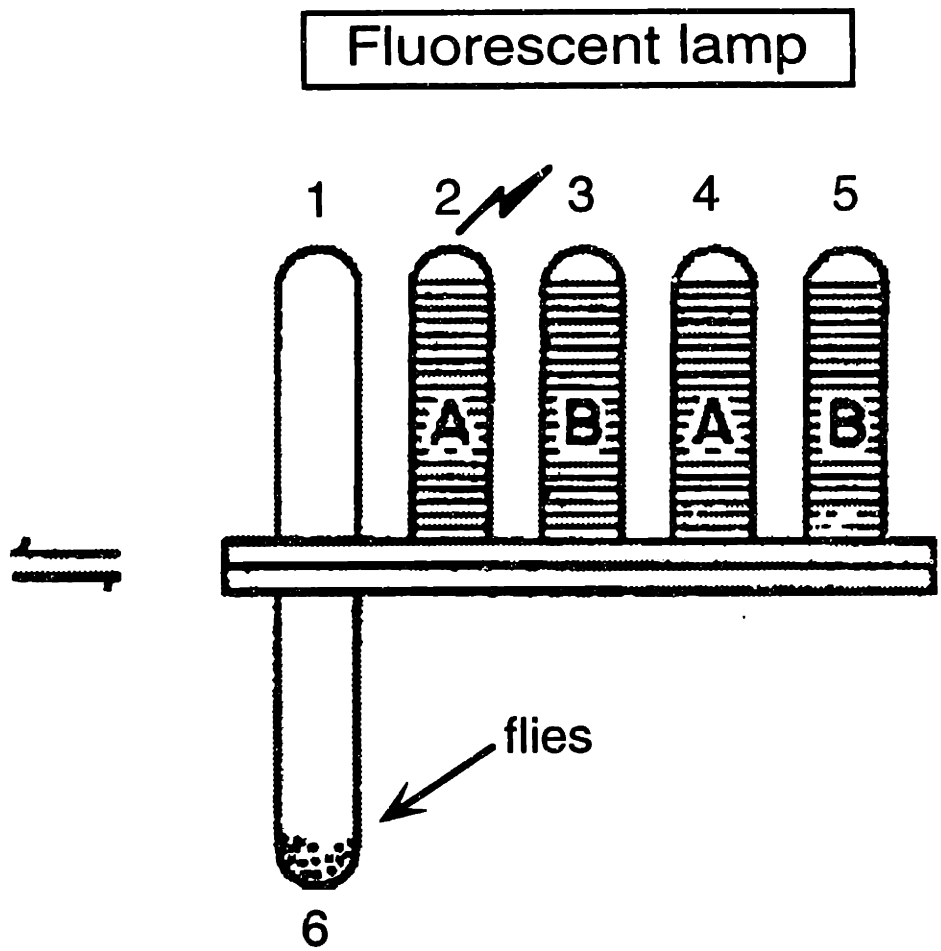

Figure 1.1

Figure 1.2 legend. TQ training apparatus. The training tube (a) contains an electrifiable grid. Fresh air can be drawn throught the training tube by connecting it to a regulated vaccum line via the upper port. Odor blocks (d) containing cups of different odorants are fitted over the end of the training tube to present the conditioned (CS^+) and unconditioned (CS^-) olfactory stimuli on the stream of air . After training, the flies are trapped in a compartment (c) in the sliding body of the machine. For testing, collection tubes (b) are snapped into place on either side of a choice point to serve as arms of a T-maze. Each of the odor blocks used for training is fitted over the end of one of the two collection tubes and the vacuum line is connected to the lower port. The chamber containing the flies is then slid in register with the choice point of the T-maze. Streams of air are drawn simultaneously via the two odor blocks, to converge at the choice point. The flies are allowed to assort themselves in the collection tubes. (Adapted from Tully and Quinn, 1985).

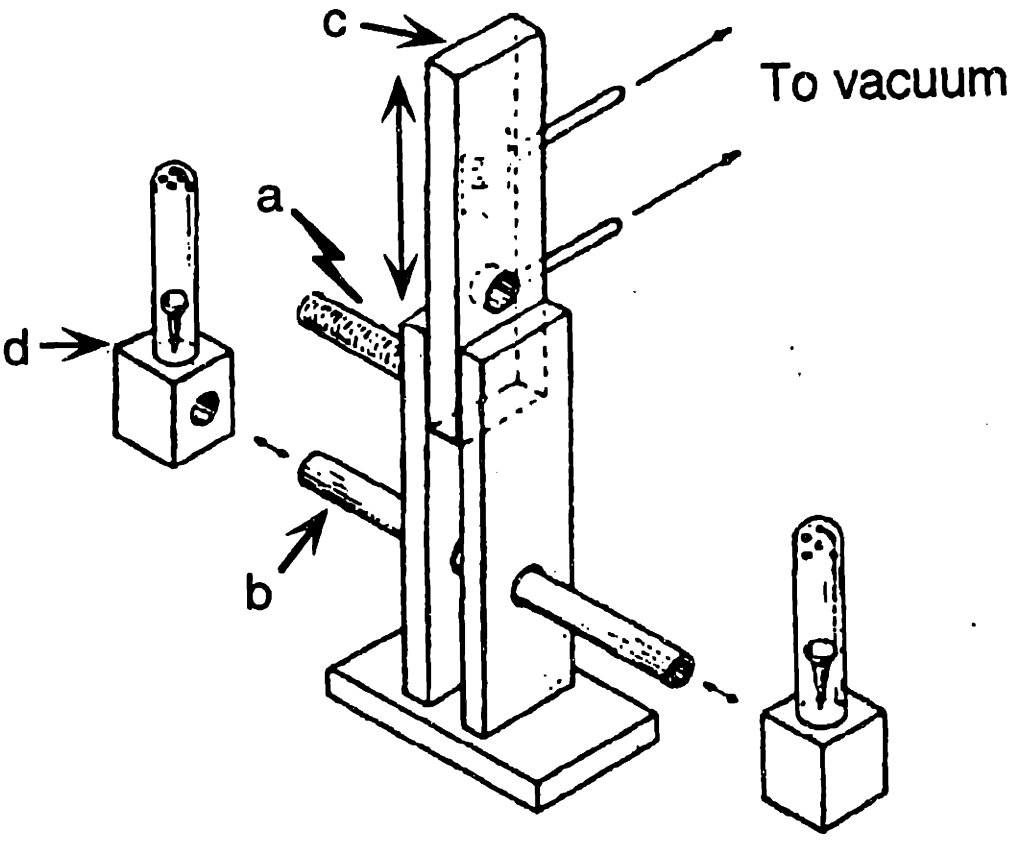

Figure 1.2

Figure 1.3 legend. Memory retention in wild-type (C-S), rutabaga and dunce flies in the TQ paradigm. Groups of flies were given a single cycle of training (12 x [1.25 sec @ 60 VDC, 3.75 sec voltage off]). Odorants were 3-octanol and 4-methylcyclohexanol. Different groups were tested for each of the time points indicated. Flies were returned to food vials between training and testing. Flies were given two minutes at the choice point to enter a collection tube. Each point represents four balanced experiments. (Adapted from Tully and Quinn, 1985).

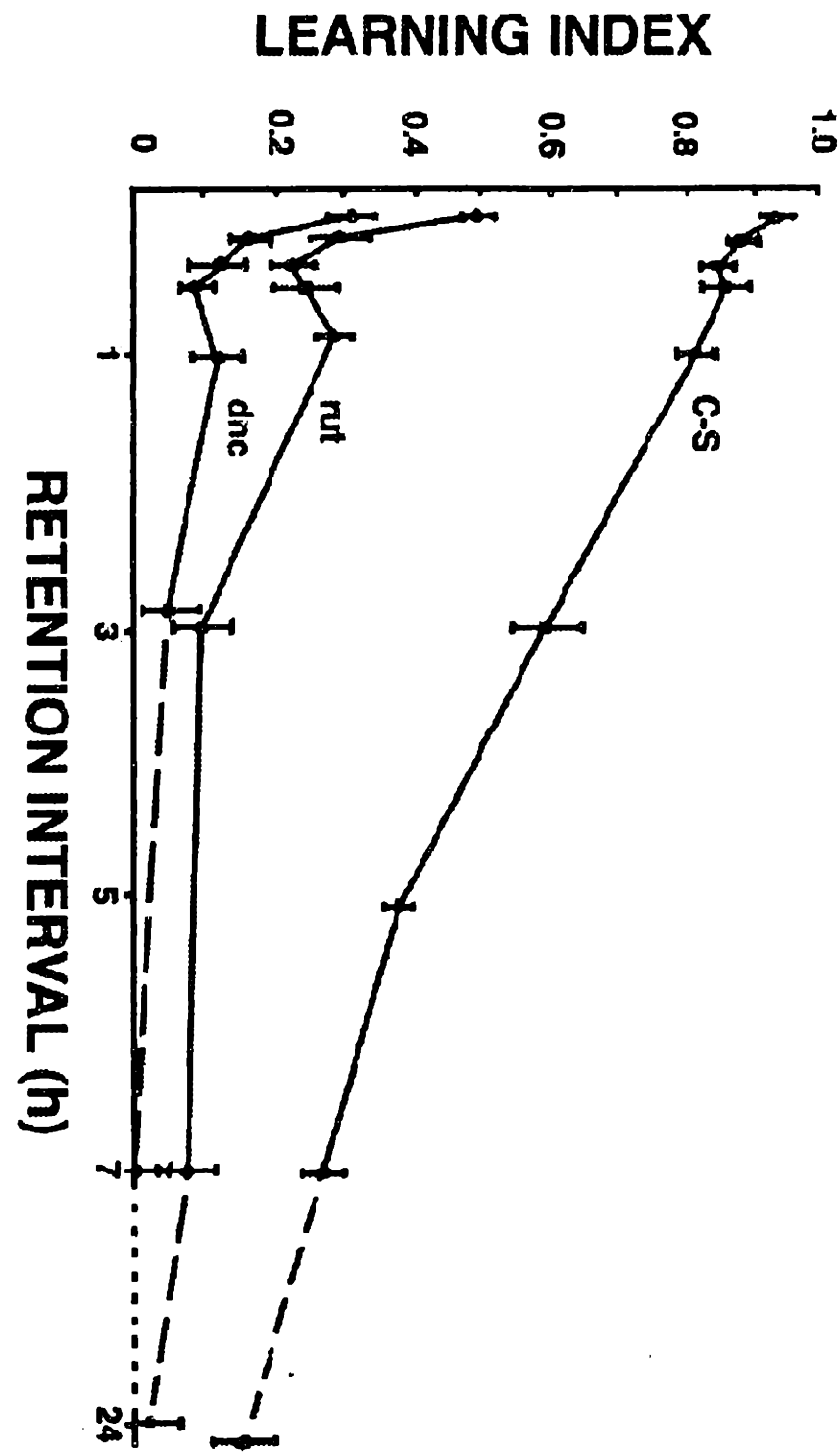
LEARNING INDEX
0
0.2
0.4
0.6
0.8
1.0
1
3
5
7
24
RETENTION INTERVAL (h)
dnc
rut
C-S

Figure 1.3

Figure 1.4 legend. Memory retention in wild-type (C-S), radish and amnesiac flies in the TQ paradigm. Groups of flies were trained as decribed in Fig. 1.3. The three genotypes wsere assayed in parallel. Learning indices for wild-type and radish flies represent averages from the same number of determinations (n=8-13) at a given time. For amnesiac n=4-8. SEM's for immediate learning scores are smaller than the symbols. The learning indices of radish flies are significantly lower than those of C-S flies (P <.01) at all intervals shown. Learning indices of radish flies are significantly lower than those of amnesiac flies at 6 and 8 hours (P <.01). (Adapted from Folkers et al., 1993).

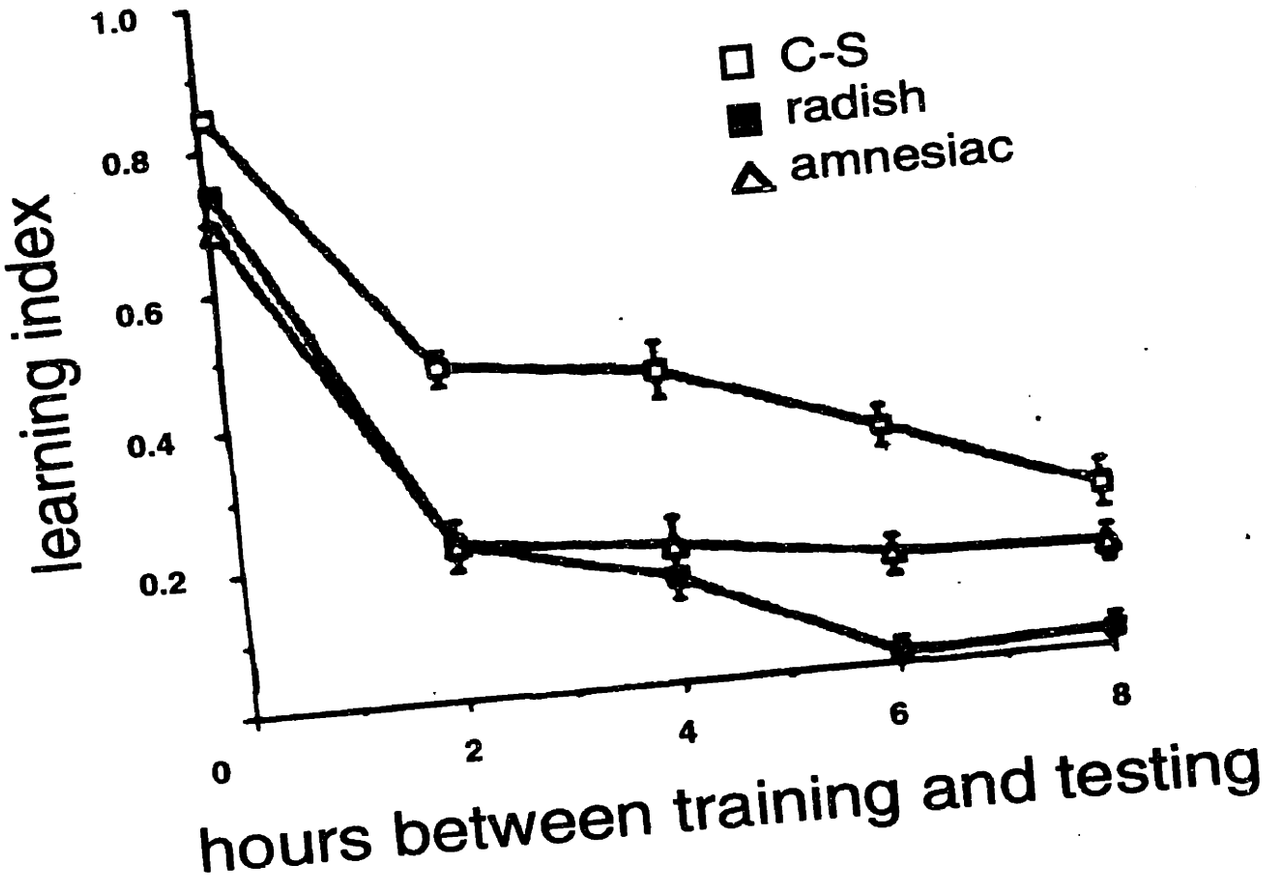
1.0
0.8
0.6
0.4
0.2
learning index
C-S
radish
amnesiac
0
2
4
6
8
hours between training and testing

Figure 1.4

Figure 1.5 legend. Long -lasting memory after training the TQ paradigm. Different groups of wild-type (C-S) flies were tested for each treatment and time point. Groups received either a single cycle of TQ training; ten cycles of massed training, in which successive cycles immediately follow one another; or ten cycles of spaced training, where the flies are allowed to rest for fifteen minutes between cycles. Flies were stored in their usual food vials during the retention interval, and then returned to the TQ apparatus for testing.

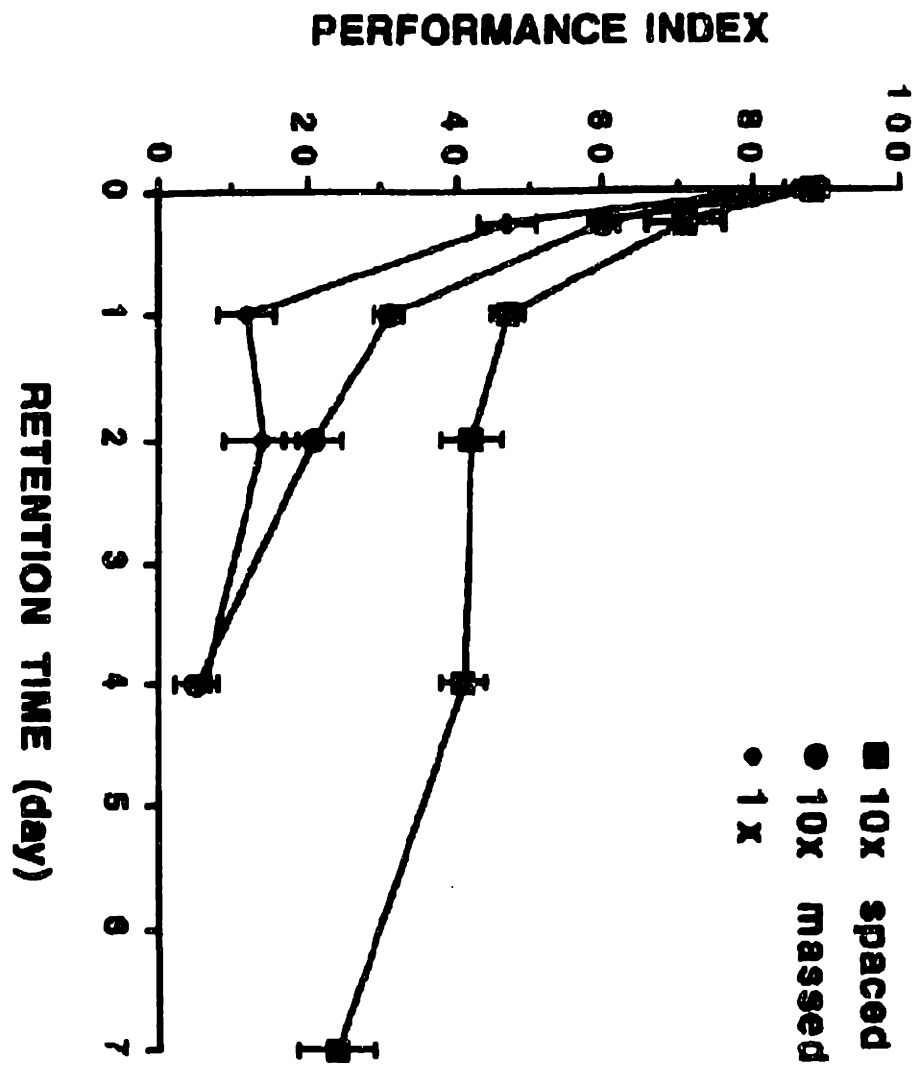
PERFORMANCE INDEX
0
20
40
60
80
100
RETENTION TIME (day)
0
1
2
3
4
5
6
7
■ 10x spaced
● 10x massed
• 1x

Figure 1.5

Figure 1.6 legend. Domain organization of CREB.

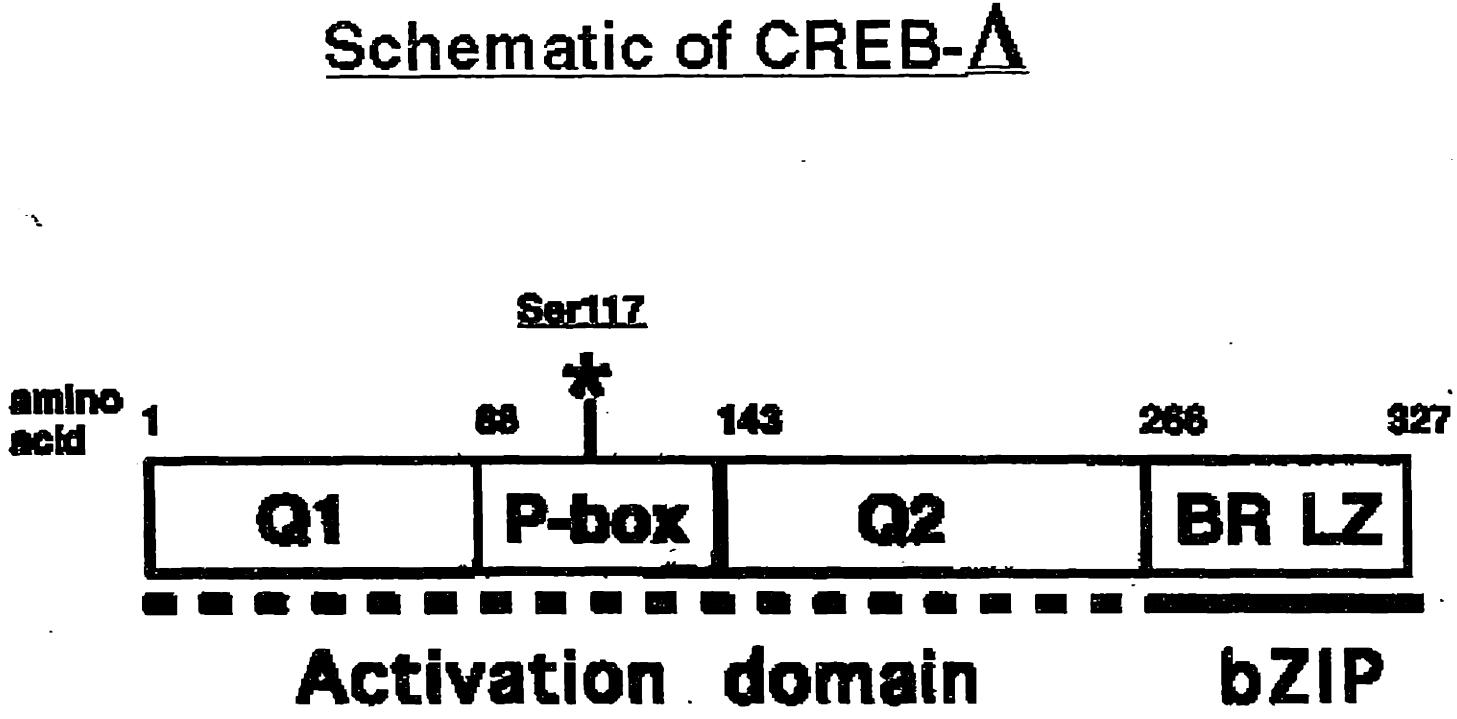
Schematic of CREB-Δ
Ser117
amino acid
1
68
143
266
327
Q1
P-box
Q2
BR LZ
Activation domain
bZIP

Figure 1.6

Figure 1.7 legend. Schematic representation of genomic regions and product isoforms of the CREM and CREB genes. Genomic regions of CREM and CREB are at center, labeled CREM and CREB at left end. The different exons are not drawn to scale. Open boxes indicate non-coding regions of exons. ATG = start codon of CREM and CREB in exon 2; Q = glutamine-rich domains; bZIP = basic region/leucine zipper; TAA = stop codon. (Adapted from de Groot and Sassone-Corsi, 1993).

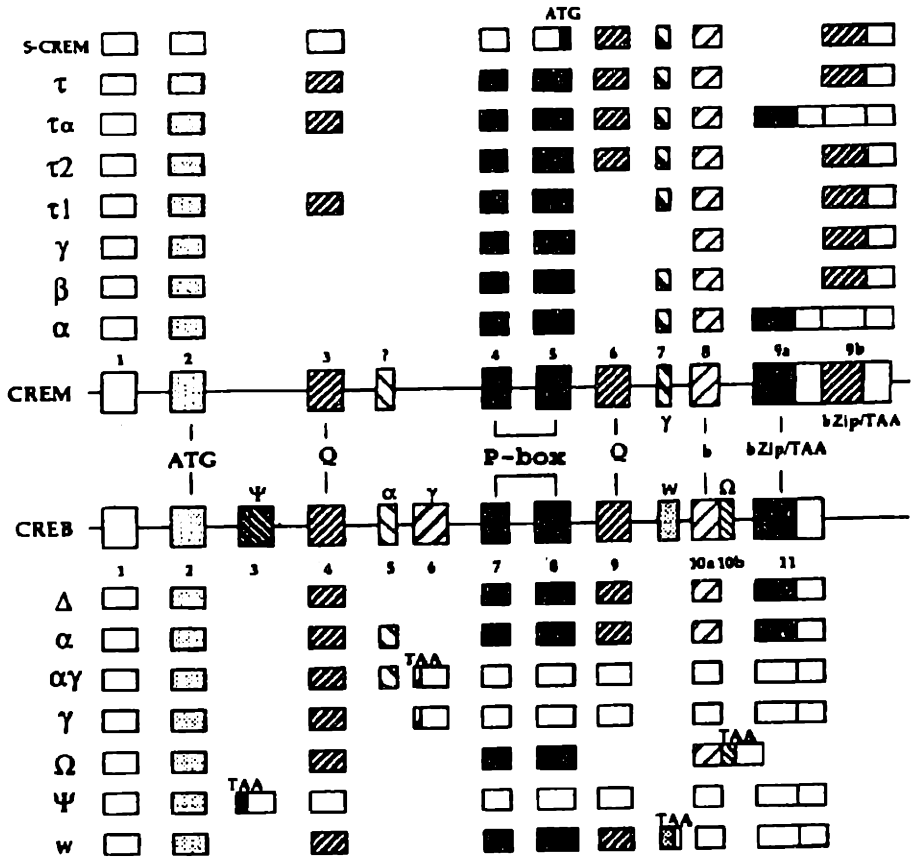
ATG
S-CREM
τ
τα
τ2
τ1
γ
β
α
CREM
ATG
Q
P-box
Q
γ
b
bZip/TAA
bZip/TAA
CREB
Ψ
α
γ
w
Ω
TAA
TAA
TAA
TAA
Δ
α
αγ
γ
Ω
Ψ
w

Figure 1.7

Figure 1.8 legend. Amino acid sequence comparison of CREBα and CREM-τ. Predicted protein sequences are aligned starting at amino acid 1. Amino acid number of last residue in each row is indicated to right. Identities between sequences are shaded. Gapping introduced to maintain alignment is indicated by dashes.

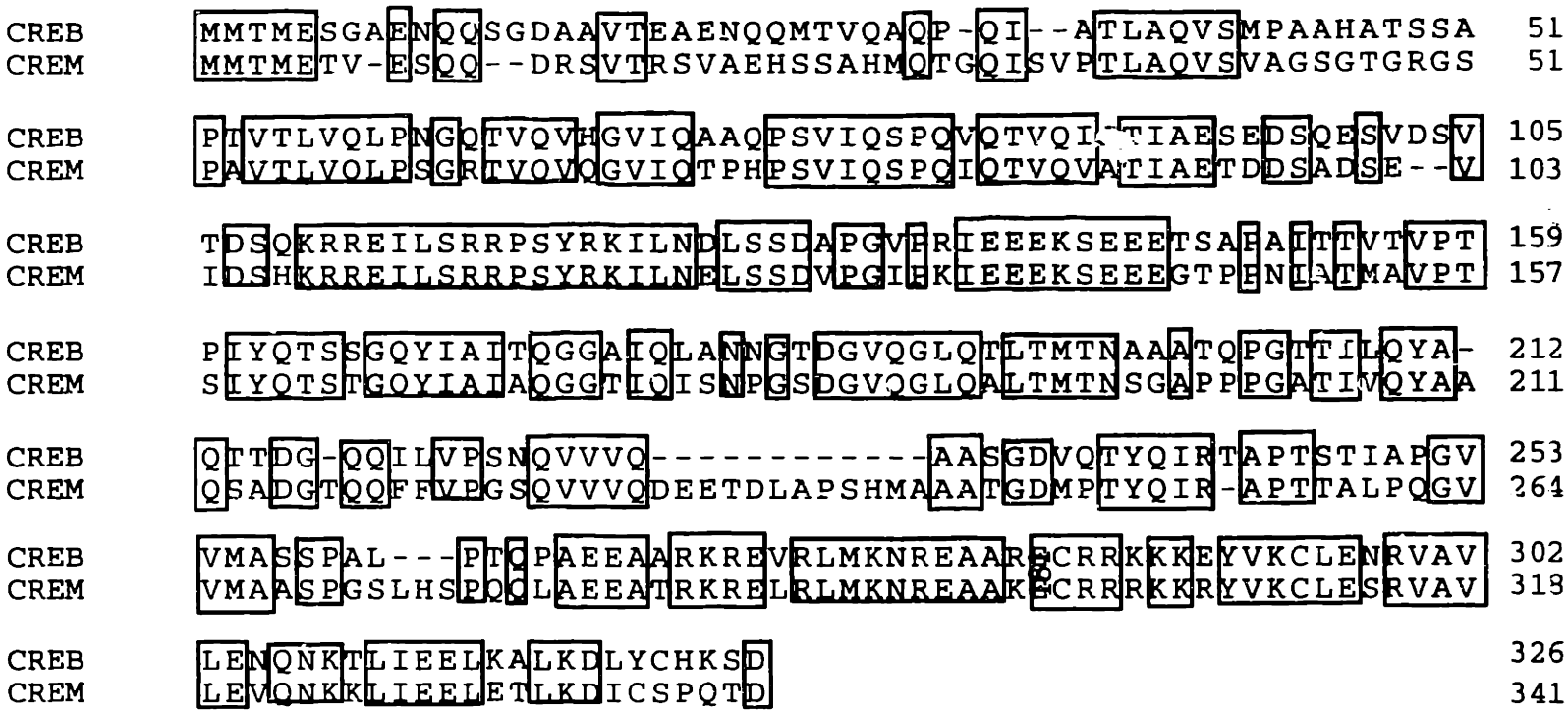
CREB MMTMESGAENQQSGDAAVTEAENQQMTVQAQP-QI--ATLAQVSMPAAHATSSA 51
CREM MMTMETV-ESQQ--DRSVTRSVAEHSSAHMQTGQISVPTLAQVSVAGSGTGRGS 51
CREB PIVTLVQLPNGQTVQVHGVIQAAQPSVIQSPQVQTVQI--TIAESEDSQESVDSV 105
CREM PAVTLVQLPSGRTVQVQGVIQTPHPSVIQSPQIQTVQVATIAETDDSADSE--V 103
CREB TDSQKRREILSRRPSYRKILNDLSSDAPGVERIEEEKSEEETSAEAITTVTVPT 159
CREM IDSHKRREILSRRPSYRKILNELSSDVPGIERKIEEEKSEEEGTPENIATMAVPT 157
CREB PIYQTSSGQYIAITQGGAIQLANNGTDGVQGLQTLTMTNAAATQPGTTILQYA- 212
CREM SIYQTSTGQYIAIAQGGTIQISNPGSDGVQGLQALTMTNSGAPPPGATIVQYAA 211
CREB QTTDG-QQILVPSNQVVVQ----------AASGDVQTYQIRTAPTSTIAPGV 253
CREM QSADGTQQFFVPGSQVVVQDEETDLAPSHMAAATGDMPTYQIR-APTTALPQGV 264
CREB VMASSPAL---PTQPAEEAARKREVRLMKNREAARECRRKKKEYVKCLENRVAV 302
CREM VMAASPGSLHSPQQLAEEATRKRELRLMKNREAAKECRRKKKRYVKCLESRVAV 318
CREB LENQNKTLIEELKALKDLYCHKSD 326
CREM LEVQNKKLIEELETLKDICSPQTD 341
181

Figure 1.8

Figure 1.9 legend. Amino acid comparison between the bZIPs of CREB, CREM and ATF-1. Residues at which one zipper differs from the other three are circled. Locations where more differences occur are marked by an arrow.

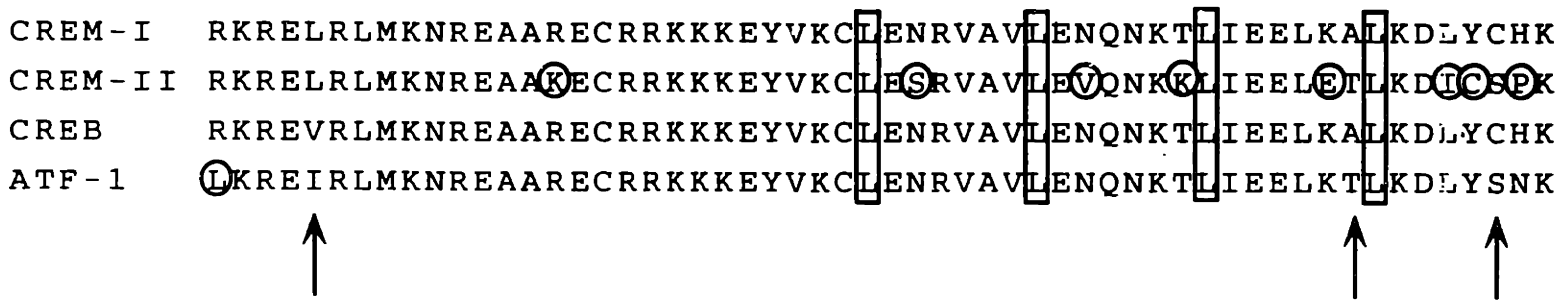
CREM-I RKRELRLMKNREAARECRRKKKEYVKCLENRVAVLENQNKTLIEELKALKDLYCHK
CREM-II RKRELRLMKNREAAKECRRKKKEYVKCLESRVAVLEVQNKKLIEELETLKDICSPK
CREB RKREVRLMKNREAARECRRKKKEYVKCLENRVAVLENQNKTLIEELKALKDLYCHK
ATF-1 LKREIRLMKNREAARECRRKKKEYVKCLENRVAVLENQNKTLIEELKTLKDLYSNK

Figure 1.9

Chapter 2

Drosophila CREB/CREM homolog encodes multiple isoforms including a PKA-responsive transcriptional activator and antagonist

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(Submitted to Molecular and Cellular Biology)

Abstract

We have characterized a *Drosophila* gene that is a highly conserved homologue of the mammalian cyclic AMP-responsive transcription factors CREB and CREM. Uniquely among *Drosophila* genes characterized to date, it codes for a cAMP-responsive transcriptional *activator*. An alternatively-spliced product of the same gene is a specific *antagonist* of cAMP-inducible transcription. Analysis of the splicing pattern of the gene suggests that the gene may be the predecessor of the mammalian CREB and CREM genes.

Introduction

Activation of the cyclic 3',5'-adenosine monophosphate (cAMP) signal transduction pathway can have long-lasting global consequences through its influence on the expression of specific genes. In mammalian systems, many of the known cAMP-responsive genes serve important neural and endocrine roles (38, 54, 63, 70).

Some members of the CREB family of transcription factors in mammals are known to participate in the control of gene expression by cAMP (13, 25, 32, 36). Proteins of this family consist of two major domains. The carboxyl-terminal portion contains a basic region-leucine zipper domain (bZIP), involved in sequence-specific DNA binding and protein dimerization (9, 33, 40, 74, 79). The remainder of the protein comprises the activation domain. This contains regions that can presumably interact with other components of the transcription machinery and with signal transduction pathways to influence gene expression (10, 25, 27, 43). CREB proteins can bind as dimers to a conserved palindromic DNA sequence, the cAMP-responsive element (CRE, consensus sequence: 5' TGACGTCA 3'). CRE sequences have been characterized from upstream control regions of cAMP-responsive genes (15, 39, 55, 63, 80) and have been identified as the *cis*-acting component of the CREB-mediated transcriptional response to cAMP.

cAMP-responsive transcriptional activation from CREs appears to require the presence of cAMP-dependent protein kinase (PKA) in the nucleus (50). Persistent elevation of cAMP levels can lead to the migration of free catalytic subunits of PKA to the nucleus (5, 22, 32, 34, 56). Experimentally, elevation of PKA levels or injection of PKA into the nucleus can substitute for all earlier cytoplasmic steps in this signal transduction pathway (7, 32, 51, 60, 61). In the nucleus, PKA is believed to phosphorylate and activate nuclear substrates that include a subset of CREB family proteins (3, 50). Phosphorylation of a particular serine residue by PKA is obligatory for cAMP-dependent transcriptional activation by the best characterized of the cAMP-responsive activator proteins, CREB (28, 43).

Only two other genes in the *CREB* family, besides *CREB* itself, are known to code for PKA-responsive activators: *CREM* (23, 25) and *ATF-1* (46, 59). Other CREB family members that have been tested do not appear to respond to PKA (47, 67). The *CREM* gene, besides producing PKA-responsive transcriptional activators, gives rise to isoforms that explicitly antagonize cAMP-dependent transcription (23, 24).

We undertook the cloning of *Drosophila* CREB/ATF family members with the specific aim of obtaining tools for examining the involvement of cAMP-dependent gene expression in *Drosophila* long-term memory. We have isolated and characterized a *Drosophila* CREB-family gene that produces a number of alternatively-spliced isoforms. This gene, which we call *dCREB2*, is the same gene previously reported as *dCREB-B* and partially characterized by Usui et al. (73), based on one cloned cDNA isoform. That isoform (corresponding to the dCREB2-c isoform illustrated in figure 2.5) did not encode a PKA-responsive transcriptional activator. Among the isoforms we have characterized is one that is a PKA-responsive transcriptional activator (dCREB2-a) and another that is an antagonist of PKA-responsive transcriptional activation (dCREB2-b). In our communications, we have used the nomenclature dCREB2-x to differentiate the various alternatively spliced forms, where x is a letter code signifying the particular isoform (see figure 2.5). Furthermore, our analysis of the genomic organization of *dCREB2* identified additional coding exons, requiring a different numbering scheme than in (73). For these reasons, we will employ our own nomenclature for the gene here.

dCREB2 appears to be closely related to, and perhaps an ancestral form of, both of the mammalian genes *CREB* and *CREM*. The presence of a PKA-responsive transcriptional activator from the CREB family in *Drosophila* suggests that this organism may share mechanisms for the control cAMP-dependent gene expression with the CREB-mediated system of mammals. The characterization of the various gene products from this gene will provide useful biological tools for examining the involvement of cAMP-dependent gene expression in long-term memory (76, 77) and in other areas of *Drosophila* biology where cAMP is an important second messenger.

Methods

Expression cloning of *dCREB2*. Standard protocols for expression cloning by DNA-binding activity (4, 66) were followed, except as noted. A double-stranded 3xCRE oligonucleotide based on an adenovirus E4 CRE (45), was synthesized and cloned in between the XbaI and KpnI sites of pGEM7Zf+ (Promega). The sequence of one strand of the oligonucleotide was- 5' CGTCTAGATCTATGACGTAATA TGACGTAATATGACGTAATGGTACCAGATCTGGCC 3' (CREs underlined). The oligonucleotide was excised as a BglII/HindIII fragment and labeled by filling-in the overhanging ends with Klenow fragment in the presence of [α^{32}P]dGTP, [α^{32}P]dCTP and unlabeled dATP and dTTP (4). Just prior to use, the labeled fragment was pre-absorbed to blank nitrocellulose filters to reduce background binding. All other steps were as described (4). After secondary and tertiary lifts, positive clones were subcloned into Bluescript pKS+ (Stratagene) and sequenced. Standard hybridization and screening methods (4) were used in the oligonucleotide-based screen of a Drosophila head cDNA library.

Gel shift analysis. Gel-mobility shift assays were performed as in (4), with the following modifications. The 4% polyacrylamide gel (crosslinking ratio 80:1) was cast and run using 5x Tris-glycine buffer supplemented with 3mM $MgCl_2$. The oligonucleotides used as the DNA probes were boiled and slowly cooled to room temperature at a concentration of 50 µg/ml in 0.1M NaCl. 50 ng of double-stranded probe was end-labeled using polynucleotide kinase in the presence of

100 μCi of [γ^{32}P]ATP. The double-stranded oligonucleotides were purified on a native polyacrylamide gel and used in a mobility shift assay at about 0.5 ng per reaction.

A *dCREB2-b* cDNA was subcloned and subjected to site-directed mutagenesis to introduce restriction sites immediately 5' and 3' of the open reading frame. This open reading frame was subcloned into the pET11A expression vector (Novagen) and used to induce expression of the protein in bacteria. The cells containing this vector were grown at 30° C to an approximate density of $2x10^8$/ml and heat-induced at 37° C for 2 hours. The cells were collected by centrifugation and lysed according to (8). The crude extract was clarified by centrifugation and loaded onto a DEAE column that had been previously equilibrated with 50mM Tris-HCl, pH 8.0, 10% sucrose, 100mM KCl. dCREB2-b protein was eluted by steps with increasing concentrations of KCl in the same buffer. Fractions were evaluated by gel mobility-shift assay. The peak fraction was dialyzed against the loading buffer and used in the binding experiment. The specific competitor that was used was the wild-type CRE oligonucleotide. The sequence of one strand of the double-stranded oligonucleotides used in the gel shift analysis are listed. For the first two oligonucleotides, wild-type and mutant CREs are underlined.

Wild-type 3xCRE:

5′ AAATGACGTAACGGAAATGACGTAACGGAAA TGACGTAACG 3′

Mutant 3xmCRE:

5′ AAATGAATTAACGGAAATGAATTAACGGAAATGAATTAACGG 3′

Nonspecific competitor #1:

5' TCGACGGGTTTTCGACGTTCACTGGTAGTGTCTGATGAGG -
CCGAAAGGCCGAAACGATGCCCATAACCACCACGCTCAG 3′

Nonspecific competitor #2:

5' TCGACCCACAGTTTCGGGTTTTCGAGCAAGTCTGCTAGTGTCTGA-

TGAGGCCGAAAGGCCGAAACGCGAAGCCGTATTGCACCACGCTCAT-
CGAGAAGGC 3'

3′ Nonspecific competitor #3:

5' CTAGAGCTTGCAAGCATGCTTGCAAGCAAGCATGCTTGCAAGCA-
TGCTTGCAAGC 3′

Nonspecific competitor #4:

5′ CTCTAGAGCGTACGCAAGCGTACGCAAGCGTACG 3′

Northern blots. Total head and body RNA was isolated from adult flies according to the protocol of (16). Total RNA from other developmental stages was a gift from Eric Schaeffer. All RNA samples were selected twice on oligo-dT columns (5 Prime-3 Prime) to isolate poly A^+ RNA. Aliquots containing 2μg of poly A^+ RNA were fractionated on 1.2% formaldehyde-formamide agarose gels, transferred to nitrocellulose and the filters probed using a uniformly labeled, strand-specific antisense RNA probe. The template for the synthesis of the probe was pJY199, which contains a partial *dCREB2* cDNA coding for the carboxyl-terminal 86 amino acids of the dCREB2-b protein plus about 585 bp of 3' untranslated mRNA, subcloned into Bluescript pKS^+ (Stratagene). All Northern blots were washed at high stringency (0.1% SDS, 0.1xSSC, 65° C).

***In situ* hybridization to tissue sections.** Frozen frontal sections of *Drosophila* heads were cut and processed under RNAse-free conditions, essentially as described in (57) , with modifications for riboprobes as noted here. Digoxigenin-labeled riboprobes were transcribed from the pJY199 template, using the Genius kit (Boehringer-Mannheim). One microgram of Xba-linearized template in a T3 RNA polymerase reaction was used to make the antisense probe. One microgram of HindIII-linearized template was transcribed by T7 RNA polymerase to generate the control sense probe. Alkaline hydrolysis in 1M calcium carbonate solution, pH 10 for 30 minutes at 60° C was used to reduce the average probe fragment size to about 200 bases. The hydrolyzed probe was diluted 1:250 in hybridization solution (57), boiled, quickly cooled on ice, added to the slides and hybridized at 42° C overnight. The slides then were then treated with RNase A (20 mg/ml RNAse A in 0.5M NaCl/10mM Tris pH 8.0 for 1 hour at 37°C) prior to two 50° C washes. Digoxigenin detection was as described in (57).

RT-PCR analysis of dCREB2 and identification of alternatively spliced exons. The template for RT-PCR was total RNA or poly A^+ RNA isolated from *Drosophila* heads as in (16). Total RNA used was exhaustively digested with RNase-free DNase I (50 μg of RNA digested with 50 units of DNase I for 60-90' at 37° C followed by phenol, phenol/chloroform extraction, and ethanol precipitation) prior to use. Results from separate experiments (J.C.P.Y., unpublished) indicate that this DNase-treatment effectively eliminates the

possibility of PCR products derived from any contaminating genomic DNA. Two rounds of selection using commercial oligo-dT columns (5 Prime-3 Prime Inc.) were used to isolate poly A^+ RNA from total RNA. The template for an individual reaction was either 100-200 ng of total RNA, or 10-20 ng of poly A^+ RNA.

The RT-PCR reactions were performed following the specifications of the supplier (Perkin-Elmer) with a "Hot Start" modification (Perkin-Elmer RT-PCR kit instructions). All components of the RT reaction, except the rTth enzyme, were assembled at 75°C, and the reaction was initiated by adding the enzyme and lowering the temperature to 70°C. At the end of 15 minutes, the PCR components (including trace amounts of [α^{32}P]dCTP), preheated to 75°C, were added quickly , the reaction tubes were put into a pre-heated thermocycler, and PCR amplification was begun. Cycling parameters for reactions (100l total volume) in a Perkin-Elmer 480 thermocycler were 94°C for 60 seconds, followed by 70°C for 90 seconds. For 50µl reactions in an MJ Minicycler (MJ Research), the cycle parameters were 94°C for 45 seconds and 70°C for 90 seconds.
All primers used in these procedures were designed to have 26 nucleotides complementary to their target sequences. Some primers had additional nucleotides for restriction sites at their 5' ends to facilitate subsequent cloning of the products. Primers were designed to have about 50% GC content, with a G or C nucleotide at their 3' most end and with no G/C runs longer than 3. For RT-PCR reactions with a given pair of primers, the Mg^{+2} concentration was optimized by running a series of pilot reactions, at Mg^{+2} concentrations ranging from 0.6 mM to 3.0 mM. Reaction products were analyzed on urea-polyacrylamide denaturing gels by autoradiography. Any product that appeared larger than the band predicted from the cDNA sequence was purified from a preparative native gel, re-amplified using the same primers, gel-purified, subcloned and sequenced.

To verify that a given RT-PCR product was actually derived from RNA, we ran control reactions to show that the appearance of the product was eliminated by RNase A treatment of the template RNA. Also, products generated from reactions using total RNA as the template were re-isolated from reactions using twice-selected polyA^+ RNA as template.

Plasmids. Construction of the plasmid *RSV-dCREB2-a* entailed a number of cloning steps and details are available upon request. In brief, the *dCREB-a* open reading frame was first reconstructed in the plasmid Bluescript pKS$^+$ by sequentially adding each of the three exons (exons 2, 4 and 6) into *dCREB2-b* cDNA which had been subcloned from phage into pKS$^+$. Site-directed mutagenesis was used to introduce unique restriction enzyme sites both 5' and 3' of the *dCREB2-b* open reading frame to facilitate the subcloning process and allow removal of 5' and 3' untranslated sequences. Once the activator was assembled, the resulting open reading frame was checked by sequencing and moved into a modified RSV-SG vector, RSV-0, that contains a polylinker located between the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter and the SV40 polyadenylation sequences. *RSV-dCREB2-b* was made by moving the *dCREB2-b* cDNA, which had been subcloned into pKS$^+$, into RSV-0.

Other constructs used in transfection experiments were: pCaEV (51) which contains the cDNA for mouse PKA catalytic subunit cloned under the mouse metallothionein 1 promoter, RSV-bgal (21) which expresses the *lacZ* gene under control of the RSV LTR promoter (30), RSV-CREB (29), which is a CREB cDNA fragment containing the complete 341-amino acid open reading frame under the RSV LTR-promoter in RSV-SG, and the D(-71) CAT reporter (55), which is a fusion of a CRE-containing fragment of the rat somatostatin promoter and the bacterial chloramphenicol acetyltransferase (CAT) coding region.

F9 cell culture and transfection. Undifferentiated F9 cells were maintained and transfected using the calcium phosphate method as described in (11), except that chloroqine was added to cultures to a final concentration of 100μM immediately before transfection. Precipitates were washed off ten hours after transfection, at which time the dishes received fresh, chloroquine-free medium. Amounts of DNA in transfections were made equivalent by adding RSV-0 where required. Cells were harvested 30 hours after transfection. Extracts were made by three cycles of freeze/thawing, with brief vortexing between cycles. Particulate matter was cleared from extracts by ten minutes of centrifugation in the cold. β-galactosidase activity assays were performed as described in (52). CAT activity assays were performed as described in (65) using aliquots of extract which were heat-treated at 65^o C for ten minutes and then centrifuged for ten minutes to remove debris. Results reported are from three experiments run on different

days with at least three dishes per condition within each experiment. Error bars represent standard error of the mean, with error propagation taken into account (31).

Results

Expression cloning of *dCREB2*. Numerous cDNAs from *dCREB2* were obtained in a DNA-binding screen of an expression library of *Drosophila* head cDNAs, using a radiolabeled duplex oligonucleotide probe, 3xCRE, containing three copies of a functional, non-canonical CRE from the Ad5 E4 promoter (37). Sequencing of the *dCREB2* clones revealed two alternatively-spliced forms, *dCREB2-b* and *dCREB2-c*. As illustrated in figure 2.4, these differ in the presence or absence of exon 4. They also had differences in their 5′ and 3′ untranslated sequences.

Chromosomal *in situ* hybridization using a *dCREB2* probe resulted in diffuse labeling centered at 17A2 on the X chromosome, in agreement with the results of Usui et al. (73). This region contains several lethal complementation groups (18).

DNA binding properties of *dCREB2-b*. The DNA binding activity of dCREB2-b was assayed using a gel mobility-shift assay (figure 2.1a). Bacterial extracts expressing the dCREB2-b protein retarded the migration of a triplicated CRE probe (3xCRE). The protein had lower, but detectable, affinity for a mutated 3xCRE oligonucleotide (compare lanes 1 and 2). Competition experiments using unlabeled competitor oligonucleotides showed that the binding of dCREB2-b to 3xCRE was specific (lanes 3-12) with higher affinity for CRE sites than to nonspecific DNA. Together with the conserved amino acid sequence, this functional similarity suggested that *dCREB2* was a CREB family member.

Expression pattern of *dCREB2*. Northern blot analysis of poly A^+ RNA from larval stages and heads and bodies of adult flies (figure 2.2a) showed a complex pattern of bands, with at least 12 different-sized transcripts apparent. Two bands of approximately 0.8 and 3.5 kb were common to all of the stages. The adult head contained transcripts of at least six sizes (0.8, 1.2, 1.6, 1.9, 2.3, and 3.5 kb). *In situ* hybridization to RNA in *Drosophila* head tissue sections, using a riboprobe

with antisense sequences for the dCREB2 bZIP region, resulted in signal in most or all cells. In the brain, cell bodies were stained, but not neuropil (figure 2.2b).

***dCREB2* has alternatively-spliced forms.** Our initial transfection experiments showed that the dCREB2-c isoform was not a PKA-responsive transcriptional activator (data not shown), a result confirming preliminary observations in (73). The complex developmental expression pattern of transcripts from *dCREB2*, plus information from the mammalian CREM gene that alternative splicing was required to generate PKA-responsive activators (23, 25, 42), suggested that additional domains might be required for activating isoforms from *dCREB2*.

Reverse transcription coupled with the polymerase chain reaction (RT-PCR) was used to identify new coding exons. Comparison of dCREB2 genomic DNA sequence with that of cDNAs indicated the general exon/intron organization and assisted in the search for additional exons. Sense and antisense primers spaced across the *dCREB2-b* cDNA were synthesized and used pairwise in RT-PCR reactions primed with *Drosophila* head mRNA to look for these new coding regions. Positions of the PCR primers used in this analysis are shown in figure 2.3. Reactions with primer pair A and B, hybridizing in exons 5 and 7, generated two products, one of the size predicted by our cDNA clones and one that was larger. Cloning and sequencing of the larger product suggested the presence of exon 6. A primer within exon 6 (primer C) was synthesized, end-labeled and used to screen a *Drosophila* head cDNA library. Two clones were isolated, sequenced and found to be identical. This splicing isoform, *dCREB2-d*, confirmed the splice junctions and exon sequence inferred from the RT-PCR products.

Initial attempts to isolate exon 2 proved difficult. We conceptually translated the genomic sequence that separated exons 1 and 3 in all frames and identified one relatively long open reading frame (ORF). Three antisense primers (D, E & F) were synthesized based on the genomic sequence, only one of which fell within the ORF (primer D). Three sets of RT-PCR reactions were run in parallel, each using a different one of the three antisense primers paired with primer G, a sense primer in exon 1 . Only the reaction that used primer D produced a PCR product. The sequence of this product matched a continuous stretch of nucleotides from the genomic sequence, extending 3' from exon 1 past the splice

junction in the *dCREB2-b* cDNA to the location of primer D. This fragment suggested that exon 1 might be extended in some mRNAs by use of an alternative 5' splice site located 3' to the site used to make *dCREB2-b*. Based on the newly-identified exon sequences, we made primer H which, when used in a PCR reaction with primer I in exon 3, generated a new product whose sequence established the location of the alternative 5' splice site. The sequence added to exon 1 by alternative 5' splice site selection is denoted as exon 2. The exon 2 sequence also showed that the same 3' splice site was used both in the cDNAs we originally isolated and for the RT-PCR product. To independently verify this alternative splicing pattern, we carried out RT-PCR using primer J, which spans the 3' splice junction of exon 2, in combination with primer G in exon 1. The sequence of the product corroborated the splice junctions of exon 2 shown in figure 2.3.

To determine if exons 2 and 6 could be coordinately spliced into the same molecule, we carried out an RT-PCR reaction with primers H and K, located in exons 2 and 6, respectively. The reaction produced a product of the size predicted for a fragment containing both exons and the identity of this product was confirmed by extensive restriction analysis.

***dCREB2* is a *Drosophila* CREB gene.** Figure 2.4a shows the DNA sequence and inferred amino acid sequence of *dCREB2-a*, the ORF which results from combining all of the identified *dCREB2* exons. The translation start site indicated for *dCREB2-a* is likely to be authentic because: i) stop codons occur upstream from this ATG in all reading frames in our *dCREB2* cDNAs (sequences not shown), ii) this ATG was selected by computerized analysis (68) as the best ribosome binding site in the DNA sequence that contains the ORF, and iii) use of the next ATG in the open reading frame (480 nucleotides downstream) would not predict a protein that would be a PKA-dependent activator (see below). This does not exclude the possibility that internal translation initiation sites may be used in this transcript, as happens with the *CREM* gene's S-CREM isoform (14).

The *dCREB2-a* open reading frame predicts a protein of 360 amino acids. A computerized amino acid sequence homology search (71) with the predicted

dCREB2-a protein sequence identifies CREB, CREM and ATF-1 gene products as the closest matches to dCREB2-a. As noted in (73), amino acid homology is particularly striking between dCREB2 and these three mammalian CREBs in the carboxyl-terminal bZIP domain (figure 2.4b). Amino acid homology is much less strong, but still present, in the activation domain. The predicted dCREB2-a product has a region of amino acids containing consensus phosphorylation sites (58) for PKA, calcium/calmodulin-dependent kinase II (CaM kinase II) and protein kinase C (PKC), analogous to the more extensive P-box or kinase-inducible (KID) domains defined in CREB, CREM and ATF-1. Like the P-boxes in CREB and CREM-t, the dCREB2-a P-box is located carboxyl-terminal to a glutamine-rich region. As in CREM-τ, the dCREB2-a P-box lies between a pair of alternatively-spliced domains implicated in transcriptional activation (42).

Figure 2.5 shows the exons present in each of the *dCREB2* alternative splice forms that we have detected, both as cDNAs and by RT-PCR. The splice products of *dCREB2* fall into two broad categories. One class of transcripts (*dCREB2-a, -b,-c, -d*) employs alternative splicing of exons 2, 4 and 6 to produce isoforms whose predicted protein products all have the bZIP domains attached to different versions of the activation domain. Members of the second class of transcripts (*dCREB2-q, -r, -s*) all use alternative splice site selection. Splice forms q and s are generated from alternative 5' and 3' splice sites respectively, while form r is a direct splice from exon 1 to exon 7. These splicing variations change the reading frame and result in translation termination at various positions 5' of the bZIP domain. Thus, they predict a set of truncated activation domains lacking the DNA binding or dimerization activity that the basic region and leucine zipper provide.

Two different *dCREB2* isoforms, *dCREB2-a* and *dCREB2-b*, have opposite roles in PKA-responsive transcription. The ability of dCREB2 isoforms to mediate PKA-responsive transcription was tested in F9 cells. These cells have been used extensively to study CREB-dependent activation because their endogenous cAMP-responsive transcription system is inactive (28, 49). In this system, expression constructs for candidate cAMP-responsive transcription factors are transiently transfected with and without a construct expressing the PKA catalytic subunit. CREB-dependent changes in gene expression are measured using a

cotransfected reporter construct that has a CRE-containing promoter fused to coding sequences of the bacterial CAT gene.

In this assay, dCREB2-a is a PKA-dependent activator (figure 2.6). Transfection of expression constructs for PKA or dCREB2-a alone gave only modest activation of the CRE reporter above baseline values. Cotransfection of these constructs together, however, gave levels of activation 5.4-fold greater than the activation seen with PKA alone. This level of PKA-dependent activation was only slightly less than that obtained with mammalian CREB-341 in parallel experiments (data not shown).

dCREB2-b did not function as a PKA-dependent activator in this assay. It failed to stimulate CRE reporter activity in the presence or absence of PKA (data not shown). Instead, dCREB2-b worked as direct antagonist of PKA-dependent activation by dCREB2-a (figure 2.7). Cotransfection of equimolar amounts of the *dCREB2-a* and *dCREB2-b* expression constructs, along with a construct expressing PKA, resulted in a nearly complete block of the PKA-dependent activation produced by dCREB2-a.

Near-identity between the predicted leucine zippers of dCREB2 and mammalian CREB suggested that the effects of mutations in CREB in this region could serve as a guide for making mutations in dCREB2. A DNA coding for a mutant dCREB2 molecule, *mLZ-dCREB2-b*, was made by introducing two single-base changes that convert the middle two leucines of the predicted leucine zipper to valines. An identical mutation in CREB abolishes homodimerization *in vitro* (17). In cotransfection experiments, expression of a construct for mLZ-dCREB2-b failed to block PKA-dependent activation by dCREB2-a (figure 2.7).

Discussion

We have investigated the *dCREB2* gene and found that it encodes the first known PKA-responsive CREB transcriptional activator in *Drosophila*. Previously, the mammalian *CREB, CREM* and *ATF-1* genes were the only CREB-family members known to express PKA-responsive transcriptional activators. These three mammalian genes form a CREB subfamily defined by this shared function and by amino acid homology, which is especially strong in the bZIP region. A

protein database homology search indicated that dCREB2-a is most similar to CREB, CREM and ATF-1 proteins, especially in the bZIP region, where up to 90% amino acid identity was found. For these reasons, we propose that *dCREB2* is a member of this subgroup of CREB-family genes that produce PKA-responsive transcriptional activators, and thus might play roles in *Drosophila* analogous to those served by the mammalian genes in this group.

The *dCREB2* transcript undergoes alternative splicing. *dCREB2-a,-b,-c* and *-d* are splice forms that predict variants of the activation domain attached to a common basic region-leucine zipper. These alternative splice forms result in seemingly minor changes in the size and spacing of parts of the activation domain. Nevertheless, alternative splicing of the activation domain has profound effects on the functional properties of *dCREB2* products. dCREB2-a is a PKA-responsive transcriptional activator in cell culture, whereas dCREB2-b, lacking exons 2 and 6, produces a specific antagonist. This *dCREB2* splicing pattern (and its functional consequences) is virtually identical to that seen in the *CREM* gene. In *CREM*, alternative splicing of exons flanking the P-box determine whether a particular isoform is an activator or an antagonist (23, 25, 42).

In contrast to the *dCREB2* splicing variants that encode isoforms with a basic region-leucine zipper domain, the *dCREB2-q, -r* and-*s* splice forms incorporate in-frame stop codons, resulting in predicted proteins which are truncated amino-terminal to the bZIP region. Isoforms of this type have been identified among the products of the *CREB* gene (13, 64) but not among those of the *CREM* gene. The function of these truncated molecules is not known, but at least one such *CREB* mRNA is cyclically regulated in rat spermatogenesis (75).

Other CRE-binding proteins from *Drosophila* have been identified, but dCREB2-a is, so far, the only cAMP-responsive *Drosophila* CREB transcription factor. These other *Drosophila* proteins, BBF-2/dCREB-A (1, 67) and dCREB1 (unpublished results, J.Y. and J.S.W, see chapter 4 of this thesis) have substantially less amino acid homology to mammalian CREB, CREM and ATF-1 than does dCREB2. It may be that in *Drosophila, dCREB2* is the only true representative of this family of genes.

Protein homology and structural gene similarity between the mammalian *CREB* and *CREM* genes has led some to suggest that they may be the result of a gene duplication (48, 64). The *Drosophila dCREB2* gene has mRNA splicing isoforms similar to exclusive products of *CREB* and *CREM*. In combination with amino acid sequence homology and the functional similarity between the predicted proteins from *dCREB2* and those from *CREB*, *CREM* and *ATF-1*, the variety of *dCREB2* splice products suggests that it may be an ancestral form of the mammalian family of PKA-responsive CREB genes.

We believe that the net level of gene activation by CREB family members in response to activation of the cAMP pathway depends on the ratio of the amounts and activities of activator isoforms to blocking isoforms, a proportion we call the A/B ratio. In mammals, three known genes *(CREB, CREM* and *ATF-1)* can contribute to this ratio, making it difficult to evaluate the function of any single gene in a particular process. For instance, *CREB* knockout mice appear normal, but at the molecular level they show increased levels of *CREM* expression (37), indicating that compensation can occur among genes of this group. In other experiments, expression of a CREB blocker transgene under control of the somatostatin promoter in mice results in dwarfism (70), while the knockout mice are normal in size, further suggesting that such compensation is functional.

If *dCREB2* is the only PKA-responsive CREB in *Drosophila*, experiments aimed at disrupting CREB-mediated cAMP-responsive transcription may be easier to perform and interpret here than in the multigene mammalian systems. Athough *Drosophila* apparently uses only a single gene, the *dCREB2* gene retains many of the subtleties of its mammalian counterpart, including the production of both activators and blockers. Combining the well-developed molecular genetic techniques available in *Drosophila* with the ability to make inducible transgenic flies and isolate mutations, *dCREB2* could provide insights not currently available elsewhere into the role of cAMP-responsive transcription in a variety of biological processes.

One such process is the possible involvement of CREB in long-term memory formation. Results from *Aplysia* pointed to a requirement for a CREB factor in a cellular model for long-term memory (2, 12). Recently we have performed experiments in *Drosophila* to test the effect of blocker or activator *dCREB2*

isoforms on long-term memory in the intact, behaving animal. Using a conditionally-expressed transgene we have shown that induced *dCREB2-b* expression specifically abolishes long-term memory (77). Most recently, we have obtained a complementary result in *dCREB2-a* transgenic flies, where induction of the activator actually *potentiates* formation of long-term memory (76). We believe that long-term memory depends on PKA-responsive gene activation via CREBs, and that this transcriptional switch is de[endenty on the balance of blocker to activator in the relevant brain cells during and after behavioral training.

Discovery of a cAMP-responsive transcriptional control system in *Drosophila* can also be rapidly integrated into the framework of ongoing molecular genetic investigations. Two examples where this is occurring are in the area of biological rhythms and in development. Rhythmic phosphorylation of CREB and cyclic expression of a blocking CREM isoform, ICER, is correlated with a phase of a biological clock in mammals (26, 69, 72). Mutations directly affecting cAMP metabolism are reported to alter behavioral rhythms in *Drosophila* (44), where a clock gene has been cloned (20, 78). Experiments evaluating the effects of inducing *dCREB2* transgenes on behavior or expression of the clock protein may prove informative.

Lane & Kalderon (41) showed the general involvement of the cAMP second-messenger system in *Drosophila* development using mutants in the catalytic subunit of PKA. Recently, the *hedgehog (hh)* developmental pathway has become the subject of intense interest in both *Drosophila* and mammalian systems (6, 19, 35, 53, 62), and results in *Drosophila* suggest a role for cAMP in this system (S. Cohen, G. Struhl, D. Kalderon, G. Rubin personal communications). The possible involvement of CREB-mediated transcription in these processes now can be tested using reverse genetic strategies.

Acknowledgements

J.Y. and J.S.W. contributed equally to this work. We thank Bob Rosenberg for generously providing materials and facilities for the synthesis of oligonucleotides used in this work. We thank Russell Grove, Paul Brindle, Andrew Darrow and Marc Montminy for advice on cell culture transfections, and Eric Schaeffer, Kevin

Lee, Marc Montminy, Don Rio, Stan McKnight, Paul Salvaterra, Peter Cherbas and Jacqueline Norris for reagents, plasmids and cell lines. We also thank Jim Dezazzo and Chris Jones for comments on the manuscript.

J.Y. was a postdoctoral fellow of the American Cancer Society and a CSHLA fellow. J.S.W. was supported by an NIH traineeship and funds from the Lucille P. Markey Charitable Trust. This work was also supported by a National Cancer Institute postdoctoral fellowship (to E.W.), by predoctoral funds from the Lucille P. Markey Trust (to J.K.), by a grant from the March of Dimes (N.P.), by a John Merck Scholarship and CSHL start-up funds (to T.T.), and by NSF grants BNS 9021698 and BNS9410934 (to W.G.Q.). N.P. is an investigator of the Howard Hughes Medical Institute.

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Figure 2.1 legend: Gel mobility-shift assays of dCREB2. Bacterially-expressed dCREB2-b protein was used in mobility-shift assays. All reactions contained labeled, 3x wild-type CRE (3xCRE) DNA probes except the reaction in lane 2, which contained labeled, 3x mutant CRE (3xmCRE) probe. Competitors were added at two different molar ratios. Reactions in lanes 3 and 4 contained specific (3xCRE) competitor oligonucleotides, while the reactions in lanes 5-12 contained nonspecific competitors.

dCREB2 GEL SHIFT ANALYSIS

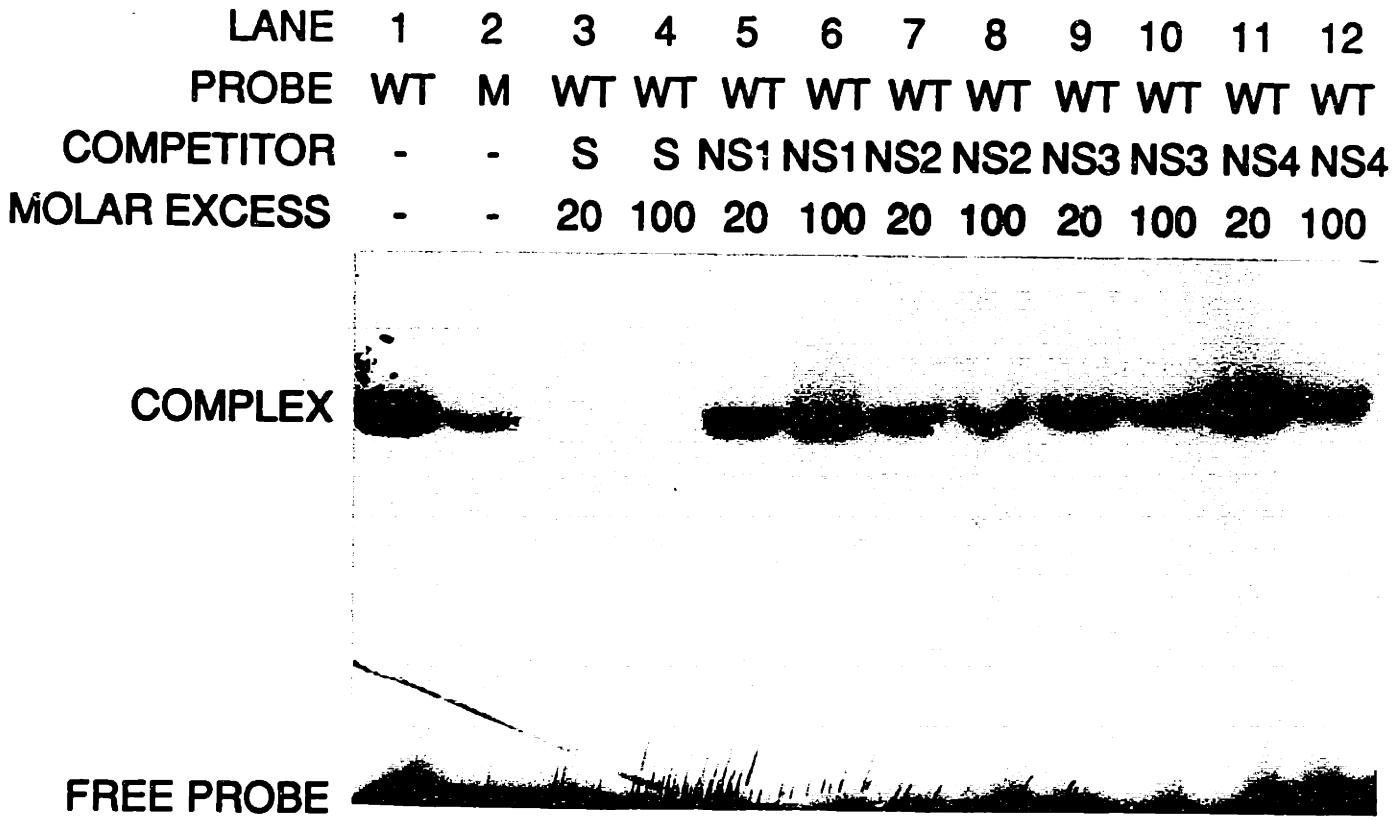

Figure 2.1

Photo side is page 213.
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Figure 2.2 legend: A. Northern blot analysis of *dCREB2* transcripts. Poly A+ RNA samples from different developmental stages were separated and probed with an antisense *dCREB2* probe. B. *dCREB2* RNA in a representative (medial) tissue section from *Drosophila* head. An antisense riboprobe to *dCREB2* was hybridized to serial frontal sections of wild-type (Can-S) flies. Sections hybridized with the corresponding sense riboprobe showed no signal.

dCREB2 DEVELOPMENTAL NORTHERN

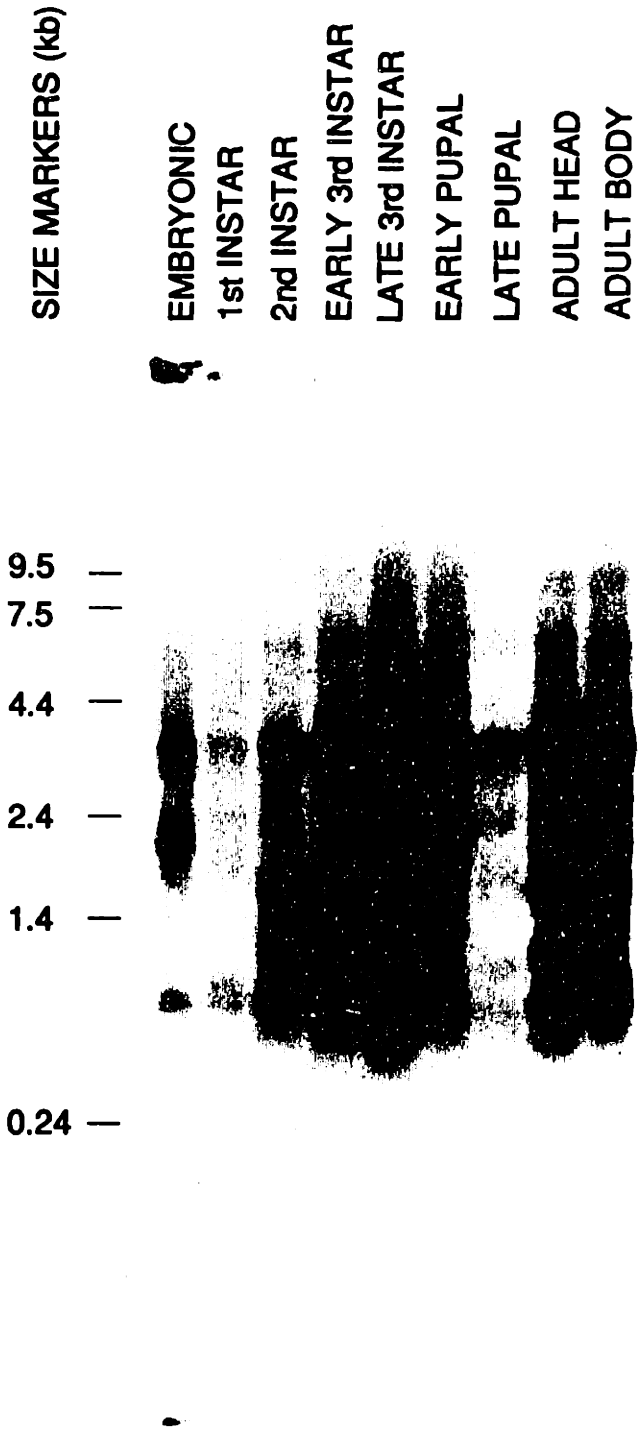

Figure 2.2a

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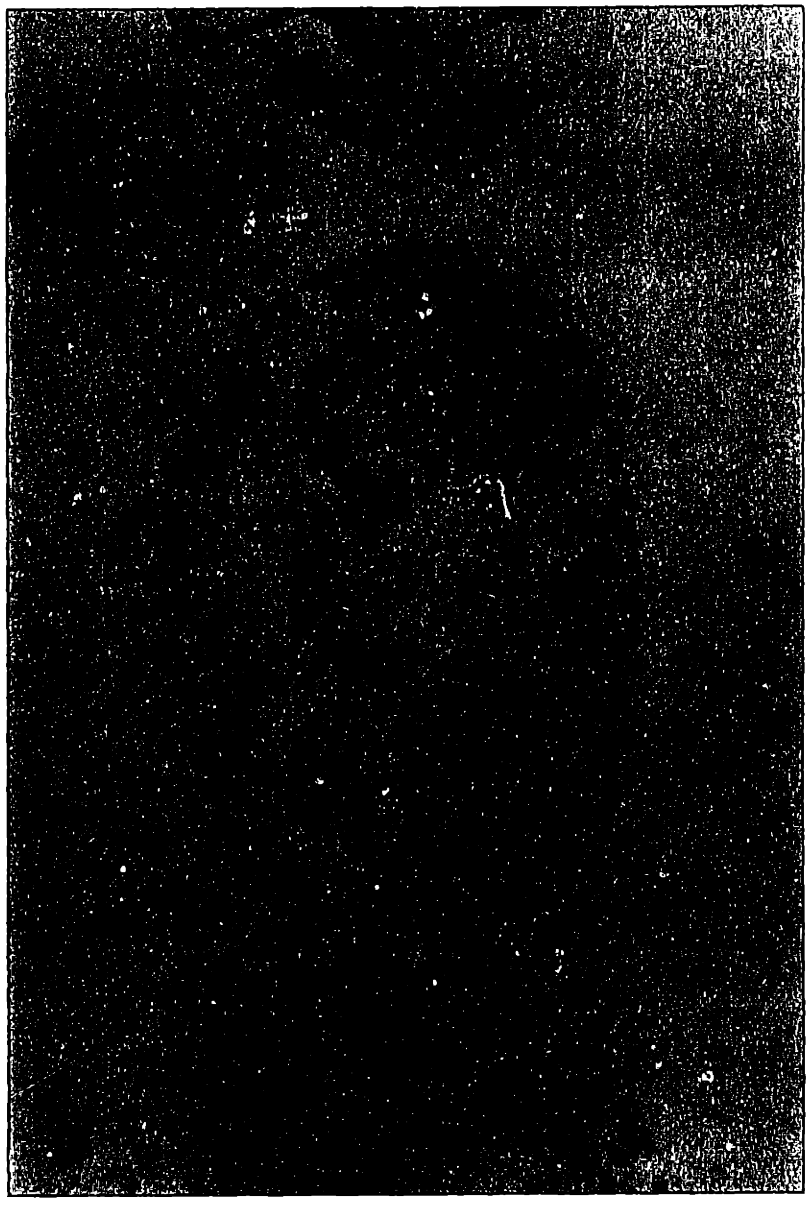

Figure 2.2 B

Figure 2.3 legend: Schematized dCREB2 genomic region showing PCR primer locations. Boxes represent exons containing known coding sequences. Enclosed numbers indicate length of coding regions in base pairs. Lines between boxes indicate introns and approximate lengths in base pairs. Horizontal arrows represent PCR primers discussed in text.

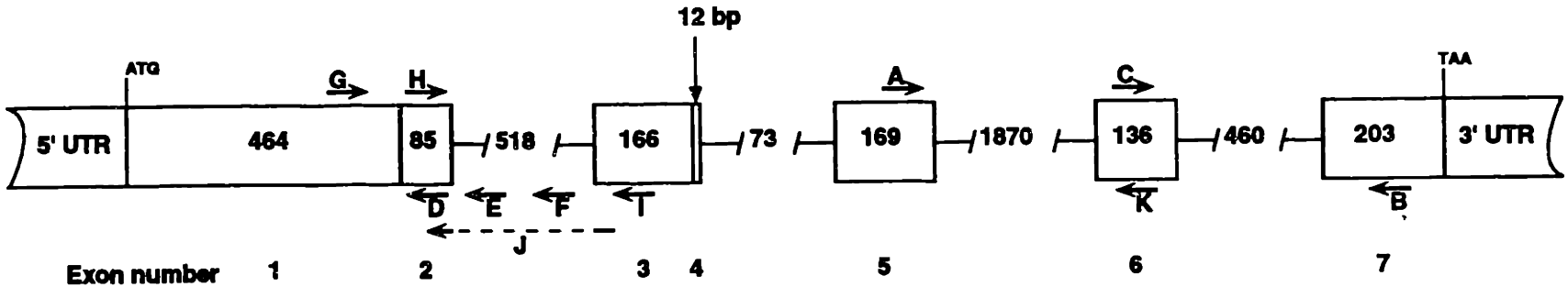
12 bp
ATG
TAA
G
H
A
C
5' UTR
464
85
518
166
73
169
1870
136
460
203
3' UTR
D
E
F
I
J
K
B
Exon number
1
2
3
4
5
6
7

Figure 2.3

Figure 2.4 legend: A. DNA sequence and predicted amino acid sequence of the *dCREB2-a* coding region. The basic region and leucine zipper domains are indicated by solid and broken bold underlining, respectively. Positively-charged residues in the basic region are circled. Periodic leucines in the zipper motif are boxed. Glutamines in the activation domain are underlined. The short amino acid motif with target sites for kinases, starting at residue 227, is indicated by a bold outline. Sequences specified by alternatively-spliced exons 2, 4 and 6 are shaded. B. Amino acid sequence comparison of bZIP domains of dCREB2, mammalian CREB, CREM and ATF-1. Differences between dCREB2 and CREB are boxed.

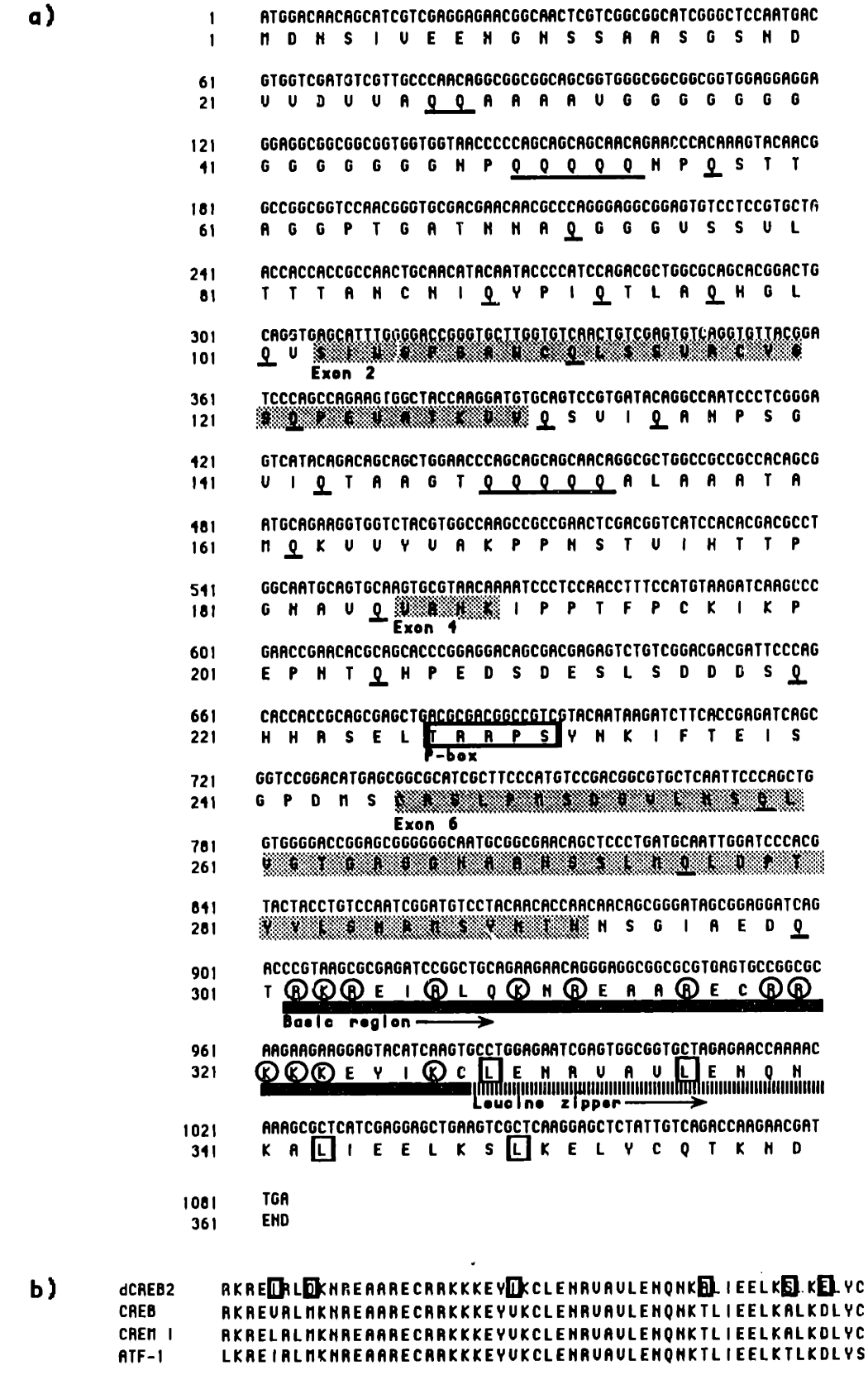
a)
Exon 2
Exon 4
P-box
Exon 6
Basic region
Leucine zipper
TGA
END
b)
dCREB2 RKREIRLDKNREAARECRRKKKEYIKCLENRVAVLENQNKTLIEELKSLKELYC
CREB RKREVRLMKNREAARECRRKKKEYVKCLENRVAVLENQNKTLIEELKALKDLYC
CREM I RKRELRLMKNREAARECRRKKKEYVKCLENRVAVLENQNKTLIEELKALKDLYC
ATF-1 LKREIRLMKNREAARECRRKKKEYVKCLENRVAVLENQNKTLIEELKTLKDLYS

Figure 2.5 legend: Diagram of *dCREB2* isoforms. Exon boundaries are defined with respect to *dCREB2-a*. Diagram is not drawn to scale.

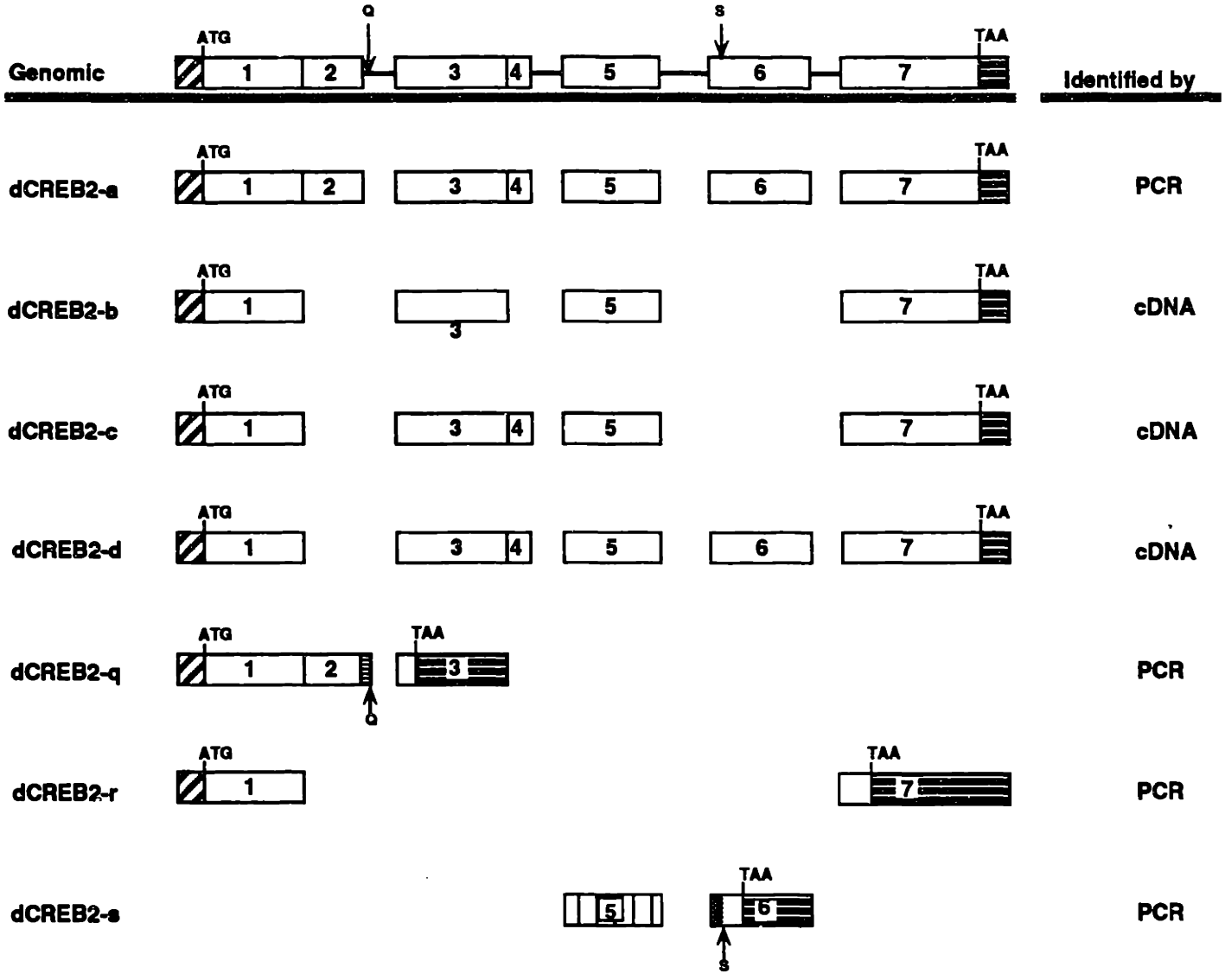
Genomic
ATG
Q
S
TAA
1
2
3
4
5
6
7
Identified by
dCREB2-a
PCR
dCREB2-b
cDNA
dCREB2-c
cDNA
dCREB2-d
cDNA
dCREB2-q
PCR
dCREB2-r
PCR
dCREB2-s
PCR

Figure 2.5

Figure 2.6 legend: PKA-responsive transcriptional activation by dCREB2-a. F9 cells were transiently transfected with 10 μg of Δ(-71) CAT plasmid as a CRE-directed reporter. 5 μg of RSV-βgal reporter was included in each dish as a normalization control for transfection efficiency. Different groups received 8 μg of dCREB2-a expression vector and 4 μg of PKA expression vector, separately or in combination, as indicated. All results are expressed as CAT/β-gal enzyme activity ratios, standardized to values obtained with PKA-transfected dishes.

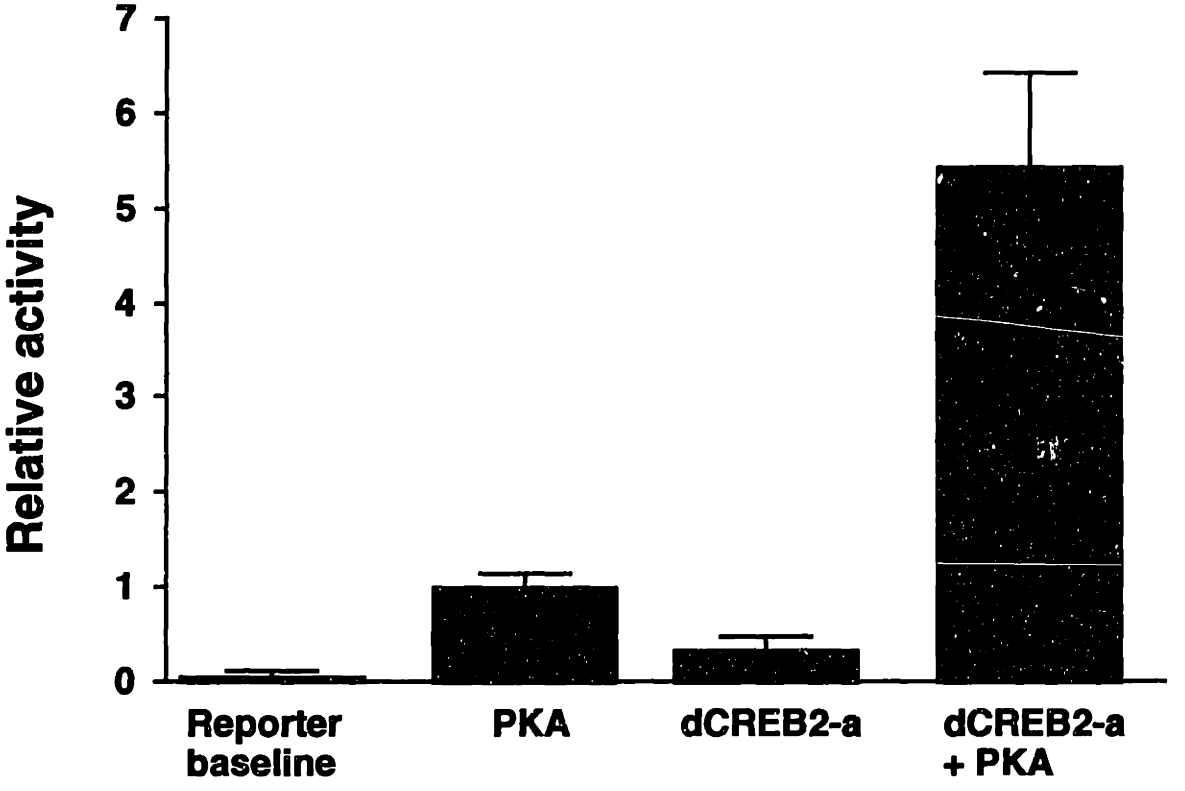
Relative activity
7
6
5
4
3
2
1
0
Reporter baseline
PKA
dCREB2-a
dCREB2-a + PKA

Figure 2.6

Figure 2.7 legend: Transcriptional effect of dCREB2-b and a mutant variant on PKA-responsive activation by dCREB2-a. F9 cells were transiently cotransfected with 10 μg of Δ(-71) CAT and 5 μg of RSV-βgal, along with the indicated combinations of the following expression constructs: RSV-dCREB2-a (5 μg), pMtC (2 μg), RSV-dCREB2-b (5 μg), and RSV-mLZ-dCREB2-b (5 μg). The DNA mass for each dish was made 27 μg with RSV-0. Other experimental conditions are as described in Figure 2.6.

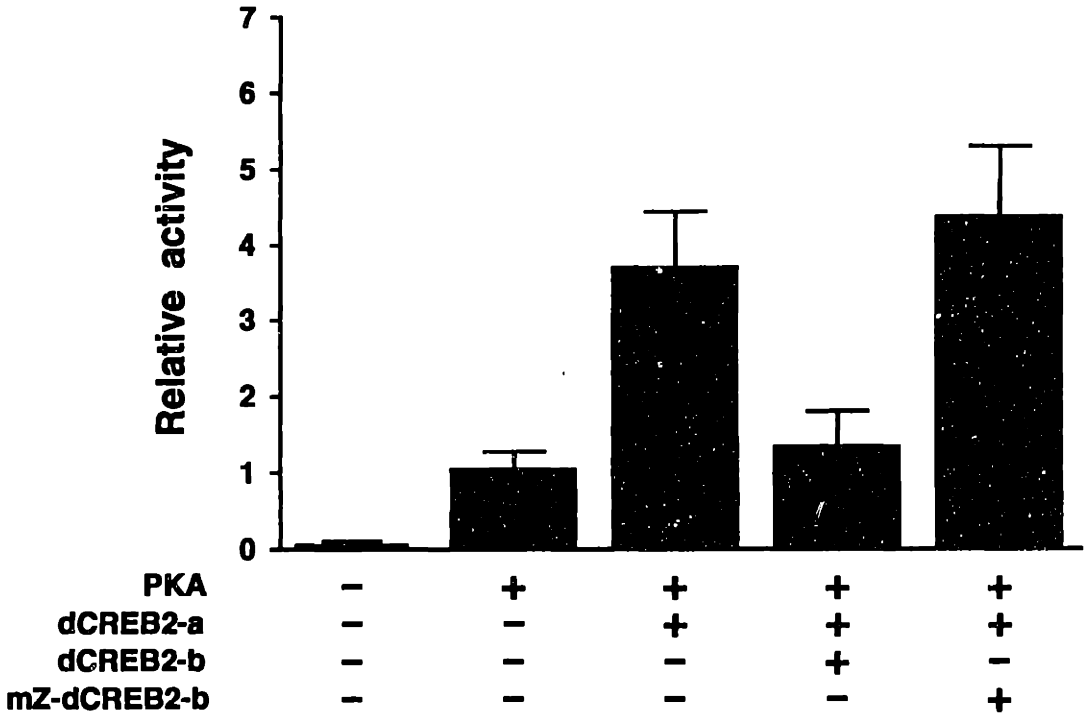
Relative activity
7
6
5
4
3
2
1
0
PKA
dCREB2-a
dCREB2-b
mZ-dCREB2-b
– + + + +
– – + + +
– – – + –
– – – – +

Figure 2.7

Chapter 3

Induction of a dominant-negative CREB transgene specifically blocks long-term memory in Drosophila

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(Published: Cell (1994) 79:49-58)

Summary

Consolidated memory after olfactory learning in Drosophila consists of two components, a cycloheximide-sensitive, long-term memory (LTM) and a cycloheximide-insensitive, anesthesia-resistant memory (ARM). Using an inducible transgene which expresses a dominant-negative member of the fly CREB family, LTM was specifically and completely blocked only after induction, while ARM and learning were unaffected. These results suggest that LTM formation requires *de novo* gene expression probably mediated by CREB family genes.

Introduction

A recurrent finding from work on the biology of learning and memory is the central involvement of the cAMP signal transduction pathway. In Aplysia, the cAMP second-messenger system is critically involved in neural events underlying both associative and non-associative modulation of a behavioral reflex (Kandel and Schwartz, 1982; Kandel et al., 1987; Byrne et al., 1993). In Drosophila, two mutants, *dunce* and *rutabaga*, were isolated in a behavioral screen for defects in associative learning and are lesioned in genes directly involved in cAMP metabolism (Quinn, et al., 1974; Dudai et al., 1976; Byers et al., 1981; Livingstone et al., 1984; Chen et al., 1986; Levin et al., 1992). These latter observations were extended with a reverse-genetic approach using inducible transgenes expressing peptide inhibitors of cAMP-dependent protein kinase (PKA) and with analyses of mutants in the PKA catalytic subunit (Drain et al., 1991; Skoulakis et al., 1993). Recent work on mammalian long-term potentiation (LTP) also has indicated a role for cAMP in synaptic plasticity (Frey et al., 1993; Huang and Kandel, 1994; Bourtchuladze et al., 1994).

The formation of long-lasting memory in animals and of long-term facilitation in Aplysia can be disrupted by drugs that interfere with transcription or translation (Agranoff et al., 1966; Barondes and Cohen, 1968; Davis and Squire, 1984; Rosenzweig and Bennett, 1984; Montarolo et al., 1986). This suggests that memory consolidation requires *de novo* gene expression.

Considered along with the involvement of the cAMP second-messenger pathway, this requirement for newly synthesized gene products suggests a role for cAMP-dependent gene expression in long-term memory (LTM) formation.

In mammals, a subset of genes from the CREB/ATF family are known to mediate cAMP-responsive transcription (Habener, 1990; deGroot and Sassone-Corsi, 1993). CREBs are members of the basic region-leucine zipper transcription factor superfamily (Landschulz et al., 1988). The leucine zipper domain mediates selective homo- and hetero-dimer formation among family members (Hai et al., 1989; Hai and Curran, 1991). CREB dimers bind to a conserved enhancer element (CRE) found in the upstream control region of many cAMP-responsive mammalian genes (Yamamoto et al., 1988). Some CREBs become transcriptional activators when specifically phosphorylated by PKA (Gonzalez and Montminy, 1989; Foulkes et al., 1992), while others, isoforms from the CREM gene, are functional antagonists of these PKA-responsive activators (Foulkes et al., 1991; Foulkes and Sassone-Corsi, 1992).

Work in Aplysia has shown that cAMP-responsive transcription is involved in long-term synaptic plasticity (Schacher et al., 1988; Dash et al., 1990). A primary neuronal co-culture system has been used to study facilitation of synaptic transmission between sensory and motor neurons comprising the monosynaptic component of the Aplysia gill-withdrawal reflex. Injection of oligonucleotides containing CRE sites into the nucleus of the sensory neuron specifically blocked long-term facilitation (Dash et al., 1990). This result suggests that titration of CREB activity might disrupt long-term synaptic plasticity.

We cloned a Drosophila CREB gene, *dCREB2*, to facilitate genetic manipulation of cAMP-responsive transcription in flies (Yin et al., submitted). This gene produces several isoforms that share overall structural homology and nearly complete amino acid identity in the basic region-leucine zipper with mammalian CREBs. The dCREB2-a isoform is a PKA-responsive transcriptional activator whereas the dCREB2-b product *blocks* PKA-responsive transcription by dCREB2-a in cell culture (Yin et al., submitted). These molecules with opposing activities are similar in function

to isoforms of the mammalian *CREM* gene (Foulkes et al., 1991; Foulkes and Sassone-Corsi, 1992; Foulkes et al., 1992). The numerous similarities in sequence and function between *dCREB2* and mammalian CREBs suggest that cAMP-responsive transcription is evolutionarily conserved.

To investigate the role of CREBs in LTM formation in Drosophila, dominant-negative transgenic lines which express *dCREB2-b* under the control of a heat-shock promoter (*hs-dCREB2-b*) were generated. Groups of flies, which had been heat-shock induced or left uninduced, were tested for memory retention after Pavlovian olfactory learning. This acute induction regimen minimized potential complications from inappropriate expression of dCREB2-b during development and allowed a clear assessment of the effect of *hs-dCREB2-b* induction on memory formation.

In Drosophila, consolidated memory after olfactory learning is composed of two genetically distinct components: anesthesia-resistant memory (ARM) and long-term memory (LTM) (Tully et al., 1994). ARM decays to zero within four days after training, and formation of ARM is insensitive to the protein synthesis inhibitor cycloheximide (CXM) but is disrupted by the *radish* mutation (Folkers et al., 1993). In contrast, LTM shows essentially no decay over at least seven days, its formation is cycloheximide-sensitive and it is not disrupted by *radish*. Tully et al. (1994) employed two different training protocols involving massed and spaced sessions (Ebbinghaus, 1885; Baddeley, 1976) to dissect memory formation. The massed training procedure consists of ten consecutive training cycles with no rest interval between them, while the spaced training protocol consists of the same number of sessions but with a 15-minute rest period between each. Their genetic dissection revealed that the massed protocol produced only ARM, while the spaced protocol produced memory retention composed of both ARM and LTM.

We used the inducible transgene approach combined with these training protocols to investigate the effect of inducing *hs-dCREB2-b* on LTM formation. We show here that induction of this transgene specifically and completely blocks LTM, while leaving learning and ARM intact.

Results

Increased transgene expression after heat-shock induction

In order to interpret the effects of transgene induction on behavior, we measured dCREB2-b expression in transgenic flies (*17-2*) after heat-shock induction. Northern blot analysis revealed elevated levels of *hs-dCREB2-b* message in the *17-2* flies immediately and three hours after heat-shock (Fig. 3.1A). This induction was also detectable in brain cells using *in situ* hybridization (data not shown). Western blot analysis showed increased dCREB2-b protein immediately after induction (Fig. 3.1B). Elevated levels of the dCREB2-b protein were seen nine hours later and were still detectable twenty four hours after induction. These data indicate that increased amounts of dCREB2-b existed in brain cells throughout spaced training, which ended about six hours after heat induction (see Experimental Procedures).

Our behavioral experiments also used transgenic flies (*A2-2*) which expressed a mutated dCREB2-b protein (dCREB2-mLZ, Yin et al., submitted). These mutations changed the two internal leucine residues of the leucine zipper to valine residues, and these changes have been shown to result in a protein which is unable to form dimers (Dwarki et al., 1990). In transient co-transfection assays, the mutant protein was unable to block PKA-dependent transcription mediated by dCREB2-a, while the wild-type protein had blocking function (Yin et al., submitted). Western blot analysis showed that the wild-type and mutant blocker are expressed at similar levels beginning immediately after heat-shock induction and lasting for at least 6 hours (Fig. 3.1C). Therefore it is unlikely that these two proteins have large differences in expression levels or stability in the transgenic flies.

Northern blot analysis of two different housekeeping genes, *myosin light chain* (Parker et al., 1985) and *elongation factor a* (Hovemann et al., 1988), showed that steady-state levels of their RNAs were unaffected after transgene induction for at least 3 hours (data not shown). Gel shift analysis using two different consensus DNA binding sites showed that there was no large effect on the gel shift species which formed after transgene induction for at least 9 hours (data not shown). Cotransfection of the blocker did not interfere with

the activity of a transcription factor from a different family in cell culture (data not shown). Considered together, *hs-dCREB2-b* probably had fairly specific molecular modes of action after induction.

Cycloheximide feeding immediately before or after spaced training disrupts one-day memory

To confirm and extend the results of Tully et al. (1994), we fed flies 35 mM cycloheximide (CXM) for 12-14 hours before, or for the 24-hr retention interval immediately after, massed or spaced training. Each of these CXM feeding regimens significantly reduced one-day memory after spaced training but had no effect on one-day memory after massed training (Fig. 3.2). These results suggest that protein synthesis is required soon after training for the formation of long-lasting memory.

Induction of *hs-dCREB2-b* disrupts one-day memory after spaced training

One-day retention after spaced training was unaffected in uninduced (**-hs**) transgenic flies (*17-2*) but was significantly reduced in induced (**+hs**) transgenic flies. In contrast, one-day retention after massed training was normal in both uninduced and induced transgenic flies (Fig. 3.3A). Comparisons of one-day retention after spaced or massed training between wild-type flies with (**+hs**) or without (**-hs**) heat-shock indicated that the heat-shock regimen itself did not have a non-specific effect on memory after either training protocol. Thus induction of the *dCREB2-b* transgene only affected one-day memory after spaced training.

One-day retention after spaced or massed training in *M11-1*, a second line carrying an independent *hs-dCREB2-b* insertion, also was tested. Results with *M11-1* were similar to those obtained with *17-2* (Fig. 3.3B). These results show that the effect of induced *hs-dCREB2-b* does not depend on any particular genomic insertion site of the transgene.

Induction of *hs-dCREB2-b* does not affect learning

If induction of the transgene specifically affected LTM via disruption of gene expression, then learning should not be affected, since it does not require new protein synthesis (see Tully et al., 1994). Different groups of flies were trained using one-cycle training either without heat-shock, or three or twenty four

hours after heat-shock. These time points after induction were selected to correspond to the times when flies were trained and tested in the previous experiments (see Figs. 3.3A and 3.3B). Induction of the transgene in the *17-2* line had no effect on learning in either case (Fig. 3.3C).

Induction of the mutant blocker does not affect one-day memory after spaced training

Induction of the transgene which contained the mutant blocker (*A2-2*) did not affect one-day retention after spaced training, while the wild-type blocker (*17-2*) had a dramatic effect (Fig. 3.4). The *w* (*iso CJ1*) flies, whose one-day retention also was unaffected by heat induction, is the isogenic control for the mutant blocker transgenic flies (see Experimental Procedures). Since Western blot analysis showed that wild-type and mutant blockers probably have similar expression levels, this result suggests that the blocker requires an intact leucine zipper to function effectively.

***hs-dCREB2-b* induction does not affect olfactory acuity or shock reactivity**

Olfactory acuity and shock reactivity are component behaviors essential for flies to properly learn odor-shock associations. Table 3.1 shows the scores for these peripheral behaviors for *Can-S* versus *17-2* flies. With or without heat-shock, olfactory acuity and shock reactivity were normal in *17-2* transgenic flies.

Induction of *hs-dCREB2-b* disrupts long-term memory (LTM)

If induction of *hs-dCREB2-b* blocks LTM, then long-lasting memory also should be blocked. In wild-type flies, seven-day retention after spaced training consists solely of the CXM-sensitive LTM because the CXM-insensitive ARM component has decayed away (Tully et al., 1994). In uninduced transgenic flies (*17-2*), seven-day retention after spaced training was similar to retention in uninduced wild-type flies (P=0.83; Fig. 3.5). Seven-day retention was severely disrupted, however, in transgenic flies which were trained three hours after heat-shock (P=0.001) and did not differ from zero (P=0.91). In contrast, the heat-shock protocol had no detectable effect on seven-day memory in wild-type flies (P=0.39).

If induction of the *hs-dCREB2-b* transgene specifically blocks LTM, then it should only affect the CXM-sensitive component of consolidated memory after spaced training. For both transgenic lines, *17-2* and *M11-1*, the effect of transgene induction looked similar to the effect that CXM had on wild-type flies (compare Fig. 3.2 with Figs. 3.3A and B). This similarity suggested that the induced dCREB2-b protein completely blocked CXM-sensitive memory, leaving ARM intact.

The *radish* mutation disrupts ARM (Folkers et al., 1993), leaving only LTM one day after spaced training (Tully et al., 1994). Thus a *radish hs-dCREB2-b* "double mutant" (*rsh;17-2*) was constructed to allow examination of LTM in the absence of ARM. In the absence of heat-shock, *rsh;17-2* double-mutants and *radish* single-gene mutants yielded equivalent one-day retention after spaced training (Fig. 3.6). In contrast, when these flies were heat-shocked three hours before spaced training, one-day retention was undetectable in *rsh;17-2* flies but remained at mutant levels in *radish* flies. The double mutant also showed normal (*radish*-like) learning (P=0.59; data not shown) and normal (wild-type) olfactory acuity and shock reactivity in the absence of heat-shock versus three hours after heat shock (see Table 3.1).

Discussion

Our behavioral results show that formation of LTM is completely blocked by induced expression of *hs-dCREB2-b*. This effect is remarkable in its behavioral specificity. ARM, a form of consolidated memory genetically distinguishable from LTM, but co-existing with it one-day after spaced training, was not affected. Learning and peripheral behaviors likewise were normal. Thus the effect of the induced *hs-dCREB2-b* transgene is specific to LTM.

Induction of the mutant blocker did not affect LTM. This result, together with the molecular data which showed that induction of the wild-type blocker did not have widespread effects on transcription, suggests that the blocker is reasonably specific at the molecular level when it specifically blocks LTM. The wild-type blocker may disrupt cAMP-dependent transcription *in vivo*, since it can block cAMP-responsive transcription in cell culture (Yin et

al., submitted). We infer that dimerization is necessary for blocker function and that the wild-type blocker could interfere with cAMP-responsive transcription either by forming heterodimers with dCREB2-a, the activator, or by forming homodimers and competing for DNA binding with homodimers of dCREB2-a. This interpretation is not yet proven, however, and experiments are underway to address this issue. In any case, the molecular target(s) of dCREB2-b are likely to be interesting because of the behavioral specificity of the block of LTM.

In Drosophila, consolidation of memory into long-lasting forms is subject to disruption by various agents. In an accompanying paper, Tully et al. (1994) use a single-gene mutation *radish* and the pharmacological agent CXM to show that long-lasting memory in flies is dissectable into two components, a CXM-insensitive ARM, which is disrupted by *radish*, and a CXM-sensitive LTM, which is normal in *radish* mutants. The work in this paper shows that CREB-family members are likely to be involved in the CXM-sensitive, LTM branch of memory consolidation. Together these two papers show that only one functional component of consolidated memory after olfactory learning lasts longer than four days, requires *de novo* protein synthesis and involves CREB-family members.

Based on work in Aplysia, a model has been proposed to describe the molecular mechanism(s) underlying the transition from short-term, protein synthesis-independent to long-term, protein synthesis-dependent synaptic plasticity (Alberini et al., 1994). Our work in Drosophila on long-term memory extends this model to the whole organism. Important molecular aspects of this transition seem to involve migration of the catalytic subunit of PKA into the nucleus (Backsai et al., 1993) and subsequent phosphorylation and activation of CREB-family members (Dash et al., 1990; Kaang et al., 1992). In flies, it is likely that the endogenous dCREB2-a isoform is one of these nuclear targets (Yin et al., submitted). Activated dCREB2-a molecules then might transcribe other target genes, including the immediate early genes--as is apparently the case in Aplysia. (Alberini et al., 1994).

The possible universality of this model is strengthened by results reported in an accompanying paper (Bourtchuladze et al., 1994). Here, using mouse gene-

knockout techniques, data are presented which show the requirement for CREB in various behavioral tasks and in hippocampal LTP. It is remarkable that the cAMP signal transduction pathway, including its nuclear components, seem to be required for memory-related functions in each of these species and behavioral tasks. Taken together with cellular analyses of a long-lasting form of LTP in hippocampal slices (Frey et al., 1993; Huang and Kandel, 1994), the emerging picture is that cAMP-responsive transcription is a conserved molecular switch universally involved in the consolidation of short-term memory to long-term memory.

Experimental Procedures

Isolating transgenic flies

EcoRI restriction sites were added (using PCR) just 5' to the putative translation initiation site and just 3' to the translation termination site in the *dCREB2-b* cDNA. This fragment was sequenced and subcloned into CaSpeR hs43, a mini-white transformation vector which contains the *hsp70* promoter, in the orientation so that the dCREB2-b open reading frame is regulated by the *hsp70* promoter. Germ-line transformation was accomplished using standard techniques (Spradling and Rubin, 1982; Rubin and Spradling, 1982). Two transgenic lines, *17-2* and *M11-1*, each with one independent P-element insertion were generated and characterized. They appeared normal in general appearance, fertility and viability. These transgenic lines were outcrossed for at least five generations to *w*(CS-10) (Dura et al., 1993), which itself had been outcrossed for ten generations to a wild-type (Can-S) stock. This extensive series of outcrossing is necessary to equilibrate the genetic background to that of Canton-S. Flies homozygous for the *17-2* transgene were bred and used for all experiments.

The mutant blocker has been described previously (Yin et al., submitted). The mutations were substituted into an otherwise wild-type blocker construct and germ-line transformants were made by injecting into *w(isoCJ1)* embryoes. Flies homozygous for the *A2-2* transgene insertion were bred and used for all experiments. *w(isoCJ1)* is a subline of *w(CS10)* (see above) carrying isogenic X, 2nd and 3rd chromosomes and was constructed by Dr. C. Jones in our

laboratory. Originally 40 such sublines were bred from *w(CS10)* using standard chromosome balancer stocks. Olfactory acuity, shock reactivity, learning and three-hr memory after one-cycle training then were assayed in each isogenic subline. As expected, a range of scores among the sublines was obtained. *w(isoCJ1)* yielded scores that were most like those of *w(CS10)* on each of these assays. By injecting DNA into the relatively homogeneous genetic background of *w(isoCJ1)*, outcrossing of the resulting germ-line transformants to equilibrate (heterogeneous) genetic backgrounds was not necessary.

Cycloheximide feeding and heat-shock regimen

Flies were fed cycloheximide as reported (Tully et al., 1994) except that the feeding period was limited to 12-14 hours prior to training, or to the 24-hr retention interval after training. Flies which were fed prior to training were transferred directly to the training apparatus after feeding, subjected to massed or spaced training, then transferred to test tubes containing filter paper strips soaked with 5% glucose during the 24-hr retention interval. Flies which were fed after training were trained, then transferred immediately to test tubes containing filter paper strips soaked with 5% glucose solution which was laced with 35 mM CXM. Flies remained in the test tubes for the duration of the 24-hr retention interval.

For heat-shock induction, flies were collected within two days of eclosion, placed in glass bottles in groups of about 600, and incubated overnight at 25° C and 70% relative humidity. The next day, three hours before training, groups of approximately 100 flies were transferred to foam-stoppered glass shell vials containing a strip of filter paper to absorb excess moisture. The vials then were submerged in a 37° C water bath until the bottom of the foam stopper (inside the vial) was below the surface of the water, thereby insuring that the flies could not escape heat-shock. The vial remained submerged for 30 min, after which the flies were transferred to a standard food vial for a 3-hr recovery period at 25° C and 70% relative humidity. Training began immediately after the recovery period.

Pavlovian learning and memory

Training and testing: Flies were trained with an automated version of the learning procedure of Tully and Quinn (1985; Tully et al., 1994). Groups of about 100 flies were exposed sequentially to two odors [either octanol (OCT) or methylcyclohexanol (MCH)] for 60 s with 45-s rest intervals after each odor presentation. During exposure to the first odor, flies also were subjected to twelve 1.5-s pulses of 60 V DC with a 5-s interpulse interval. After training, flies were transferred to food vials for a particular retention interval. Conditioned odor-avoidance responses then were tested by transferring flies to the choice point of a T-maze, where they were exposed simultaneously to OCT and MCH carried in the distal ends of the T-maze arms and out the choice point on converging currents of air. Flies were allowed to distribute themselves in the T-maze arms for two minutes, after which they were trapped in their respective arms, anesthetized and counted. The "percent correct" then was calculated as the number of flies avoiding the shock-paired odor (they were in the opposite T-maze arm) divided by the total number of flies in both arms. (The number of flies left at the choice point, which usually was less than 5%, were not included in this calculation). Finally, a performance index (PI) was calculated by averaging the percent corrects of two reciprocal groups of flies -- one where OCT and shock were paired, the other where MCH and shock were paired--and then by normalizing the average so that a PI=0 represented a 50:50 distribution in the T-maze and a PI=100 represented 100% avoidance of the shock-paired odor. For these studies, three different training protocols were used: 1. **One-cycle training** consisted of the training session just described. 2. **Massed training** consisted of 10 of these training cycles delivered one right after the other. 3. **Spaced training** consisted of 10 training cycles with a 15-min rest interval between each. One-cycle training was used to assay learning, while massed and spaced was used to assay consolidated memories (Tully et al., 1994).

Olfactory acuity and shock reactivity

Odor avoidance responses to OCT or to MCH at two different concentrations -- one (10^0) usually used in conditioning experiments and a 100-fold (10^{-2}) dilution thereof -- were quantified in various groups of flies in the absence of heat shock and 3 hr or 24 hr after heat shock with the method of Boynton and Tully (1992). Briefly, flies are placed in a T-maze and given a choice between an odor and air. The odors are naturally aversive, and flies ususally choose

air and avoid the T-maze arm containing the odor. For shock reactivity, flies are given a choice between an electrified grid in one T-maze arm, and an unconnected grid in the other. After the flies have distributed themselves, they are anesthetized, counted and a PI is calculated.

Statistical analyses of behavioral data

Since each PI is an average of two percentages, the Central Limit Theorem predicts that they should be distributed normally (see Sokal and Rohlf, 1981). We have determined empirically, with data from Tully and Quinn (1985) and Tully and Gold (1993), that this expectation is true. Thus, we analyzed untransformed (raw) data parametrically with JMP2.1 statistical software (SAS Institute Inc., Cary NC). Since preliminary experiments preceded all of the experiments summarized herein, all pairwise comparisons were planned. To maintain an experimentwise error rate of alpha = 0.05, the critical P values for these individual comparisons were adjusted accordingly (Sokal and Rohlf, 1981) and are listed below for each experiment.

All experiments were designed in a balanced fashion with N = 2 PIs per group collected per day; then replicated days were added to generate final Ns. In each experiment, the experimenter (M.D.) was blind to genotype.

One-day memory in wild-type flies fed CXM before or immediately after massed or spaced training (Fig. 3.2): PIs from these four drug treatments (-CXM before, -CXM after, +CXM before and +CXM after) and two training procedures (massed and spaced) were subjected to a TWO-WAY ANOVA with DRUG ($F_{(3,56)} = 8.93$; $P < 0.001$) and TRAINing ($F_{(1,56)} = 18.10$, $P < 0.001$) as main effects and DRUG x TRAIN ($F_{(3,56)} = 4.68$, $P = 0.006$) as the interaction term. P values from subsequent planned comparisons are summarized in Fig. 3.2. The six planned comparisons were judged significant if $P \leq 0.01$.

One-day memory after massed or spaced training in *dCREB2-b* transgenic flies (Figs. 3.3A and 3.3B): In experiments with the *17-2* transgenic line, PIs from two strains (Can-S and *17-2*) and four training-regimens (spaced-hs, spaced+hs, massed-hs and massed+hs) were subjected to a TWO-WAY ANOVA with STRAIN ($F_{(1,40)} = 1.57$; $P = 0.22$) and TRAINing-regimen ($F_{(3,40)} = 25.81$, $P < 0.001$) as main effects and STRAIN x TRAIN ($F_{(3,40)} = 6.62$, $P = 0.001$) as the interaction term. A similar analysis was done with data from the *M11-1* transgenic line, yielding STRAIN ($F_{(1,40)} = 2.81$; $P = 0.10$), TRAINing-regimen ($F_{(3,40)} = 11.97$, $P < 0.001$) and STRAIN x TRAIN ($F_{(3,40)} =$

3.37, P = 0.03) effects. P values from subsequent planned comparisons are summarized in Figs. 3.3A and B. In each experiment, the seven planned comparisons were judged significant if $P \leq 0.01$.

Learning after one-cycle training in *17-2* transgenic flies (Fig. 3.3C): PIs from two strains (Can-S and *17-2*) and three heat-shock regimens [-hs, +hs (3 hr) and+hs (24 hr)] were subjected to a TWO-WAY ANOVA with STRAIN ($F_{(1,30)} = 0.69$; P = 0.41) and HEAT-shock regimen ($F_{(2,30)} = 10.29$, $P < 0.001$) as main effects and STRAIN x HEAT ($F_{(2,30)} = 0.71$, P = 0.50) as the interaction term. P values from subsequent planned comparisons are summarized in Fig. 3.3C. The three planned comparisons were judged significant if $P \leq 0.02$.

One-day memory after spaced training in *A2-2* transgenic flies (Fig. 3.4): PIs from these three strains [*w(isoCJ1)*, *17-2* and *A2-2*] and two heat-shock regimens [-hs and +hs (3 hr)] were subjected to a TWO-WAY ANOVA with STRAIN ($F_{(2,30)} = 9.43$, $P < 0.001$) and HEAT-shock regimen ($F_{(1,30)} = 9.84$, P = 0.004) as main effects and STRAIN x HEAT ($F_{(2,30)} = 5.71$, P = 0.008) as the interaction term. P values from subsequent planned comparisons are summarized in Fig. 3.4. The six planned comparisons were judged significant if $P \leq 0.01$.

Olfactory acuity in *17-2* flies (Table 3.1): PIs from these two strains (Can-S and *17-2*), four different odor-levels (OCT-10^0, OCT-10^{-2}, MCH-10^0 and MCH-10^{-2}) and three heat-shock regimens [-hs, +hs (3 hr) and +hs (24 hr)] were subjected to a THREE-WAY ANOVA with STRAIN ($F_{(1,184)} = 0.12$, P = 0.73), ODOR-level ($F_{(3,184)} = 126.77$, $P < 0.001$) and HEAT-shock regimen ($F_{(2,184)} = 3.55$, P = 0.03) as main effects, STRAIN x ODOR ($F_{(3,184)} = 1.23$, P = 0.30), STRAIN x HEAT ($F_{(2,184)} = 0.33$, P = 0.72) and ODOR x HEAT ($F_{(6,184)} = 3.14$, P = 0.006) as two-way interaction terms and STRAIN x ODOR x HEAT ($F_{(6,184)} = 0.48$, P = 0.83) as the three-way interaction term. P values from subsequent planned comparisons are summarized in Table 3.1. The twelve planned comparisons were judged significant if $P \leq 0.005$.

Shock reactivity in *17-2* flies (Table 3.1): PIs from these two strains (Can-S and *17-2*), two shock groups (60V and 20V) and three heat-shock regimens [-hs, +hs (3 hr) and +hs (24 hr)] were subjected to a THREE-WAY ANOVA with STRAIN ($F_{(1,84)} = 0.50$, P = 0.48), SHOCK ($F_{(1,84)} = 97.78$, $P < 0.001$) and HEAT-shock regimen ($F_{(2,84)} = 3.36$, P = 0.04) as main effects, STRAIN x SHOCK ($F_{(1,84)} = 1.12$, P = 0.29), STRAIN x HEAT ($F_{(2,84)} = 1.06$, P = 0.35) and SHOCK x HEAT ($F_{(2,84)} = 6.66$, P = 0.002) as two-way interaction terms and

STRAIN x SHOCK x HEAT ($F_{(2,84)}$ = 1.75, P = 0.18) as the three-way interaction term. P values from subsequent planned comparisons are summarized in Table 3.1. The six planned comparisons were judged significant if $P \leq 0.01$.

Seven-day memory after spaced training in *17-2* flies (Fig. 3.5): PIs from two strains (Can-S and *17-2*) and two heat-shock regimens [-hs and +hs(3 hr)] were subjected to a TWO-WAY ANOVA with STRAIN ($F_{(1,20)}$ = 6.09; P = 0.02) and HEAT-shock regimen ($F_{(1,20)}$ = 16.30, P = 0.001) as main effects and STRAIN x TRAIN ($F_{(1,20)}$ = 7.73, P = 0.01) as the interaction term. P values from subsequent planned comparisons are summarized in Fig. 3.5. The three planned comparisons were judged significant if $P \leq 0.02$.

One-day memory after spaced training in *rsh;17-2* double mutants (Fig. 3.6): PIs from hree strains (*17-2*, *rsh* and *rsh;17-2*) and two heat-shock regimens [-hs and +hs (3 hr)] were subjected to a TWO-WAY ANOVA with STRAIN ($F_{(2,30)}$ = 32.05; P <).001) and HEAT-shock regimen ($F_{(1,30)}$ = 59.68, P < 0.001) as main effects and STRAIN x TRAIN ($F_{(2,30)}$ = 11.59, P < 0.001) as the interaction term. P values from subsequent planned comparisons are summarized in Fig. 3.6. The five planned comparisons were judged significant if $P \leq 0.01$.

Learning after one-cycle training in *rsh;17-2* mutants (see text): PIs from these two strains (Can-S and *rsh;17-2*) and two heat-shock regimens [-hs and +hs (3 hr)] were subjected to a TWO-WAY ANOVA with STRAIN ($F_{(1,20)}$ = 86.85, P < 0.001) and HEAT-shock regimen ($F_{(1,20)}$ = 0.02, P < 0.89) as main effects and STRAIN x HEAT ($F_{(1,20)}$ = 0.86, P = 0.37) as the interaction term. P values from subsequent planned comparisons are summarized in Table 3.1. The two planned comparisons were judged significant if $P \leq 0.03$.

Olfactory acuity in *rsh;17-2* flies (Table 3.1): PIs from these two strains (Can-S and *rsh;17-2*), four different odor-levels (OCT-10^0, OCT-10^{-2}, MCH-10^0 and MCH-10^{-2}) and two heat-shock regimens [-hs, and +hs (3 hr)] were subjected to a THREE-WAY ANOVA with STRAIN ($F_{(1,112)}$ = 0.02, P = 0.88), ODOR-level ($F_{(3,112)}$ = 50.03, P < 0.001) and HEAT-shock regimen ($F_{(1,112)}$ = 29.86, P < 0.001) as main effects, STRAIN x ODOR ($F_{(3,112)}$ = 2.15, P = 0.10), STRAIN x HEAT ($F_{(1,112)}$ = 0.34, P = 0.56) and ODOR x HEAT ($F_{(3,112)}$ = 6.41, P = 0.001) as two-way interaction terms and STRAIN x ODOR x HEAT ($F_{(3,112)}$ = 1.12, P = 0.35) as the three-way interaction term. P values from

subsequent planned comparisons are summarized in Table 3.1. The eight planned comparisons were judged significant if $P \leq 0.01$.

Shock reactivity in *rsh;17-2* flies (Table 3.1): PIs from these two strains (Can-S and *rsh;17-2*), two shock groups (60V and 20V) and two heat-shock regimens [-hs and +hs (3 hr)] were subjected to a THREE-WAY ANOVA with STRAIN ($F_{(1,56)} = 0.51$, $P = 0.48$), SHOCK ($F_{(1,56)} = 88.14$, $P < 0.001$) and HEAT-shock regimen ($F_{(1,56)} = 0.08$, $P = 0.77$) as main effects, STRAIN x SHOCK ($F_{(1,56)} = 0.12$, $P = 0.73$), STRAIN x HEAT ($F_{(1,56)} = 0.03$, $P = 0.86$) and SHOCK x HEAT ($F_{(1,56)} = 0.39$, $P = 0.53$) as two-way interaction terms and STRAIN x SHOCK x HEAT ($F_{(1,84)} = 1.58$, $P = 0.21$) as the three-way interaction term. P values from subsequent planned comparisons are summarized in Table 3.1. The four planned comparisons were judged significant if $P \leq 0.01$.

Northern Analysis

For RNA collection, the heat-shock regimen was the same as for behavioral experiments. For any indicated time interval between heat-shock and collection, flies rested in food-containing vials at 25°C. Flies were collected and quickly frozen in liquid nitrogen. All Northern analyses used head RNA. The tube of frozen flies was repeatedly rapped sharply on a hard surface, causing the heads to fall off. The detached frozen heads were recovered by sieving on dry ice. Approximately 1000 heads were pooled for RNA preparation. Wild-type and transgenic flies for each individual time point always were processed in parallel . Flies that were not induced were handled in a similar manner to induced flies, except that the vials were not placed at 37°C. Total head RNA was isolated from each group of flies, and poly A+ RNA was isolated using oligo dT columns according to the instructions of the manufacturer (5'--->3' Inc.). The concentration of poly A+ mRNA was measured spectrophotometrically, and 0.5 µg of mRNA per lane was loaded and run on 1.2% formaldehyde-agarose gels. Northern blots were prepared, probed and washed (0.1x SSC at 65°C) as described (Ausubel et al.,1994). For detection of the transgene, an 843bp *dCREB2-b* cDNA fragment was subcloned into pKS+ and used to generate a uniformly-labeled antisense riboprobe. This fragment codes for the carboxyl-terminal 86 amino acids of the *dCREB2-b* protein plus 3' untranslated mRNA.

Western Blot Analysis and Antiserum

Western blot analysis was performed using a rabbit antiserum raised against a peptide corresponding to 16 amino acids in the basic region of the *dCREB2-b* cDNA with an additional COOH terminal Cys. The sequence of the peptide was:NH_2-RKREIRLQKNREAAREC-COOH. The peptide was synthesized and coupled to Sulfo-SMCC (Pierce) activated keyhole lympet hemocyanin. The antigen was injected into rabbits (100 μg) and boosted at two week intervals. Sera was bled and tested for immune reactivity towards bacterially-expressed dCREB2-b protein. The antiserum was passed through a CM Affi-gel Blue column (Biorad), and the flow-through was concentrated by ammonium sulfate precipitation, resuspended and dialyzed against PBS (0.14 M NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_47H_2O$, 1.4 mM KH_2PO_4, pH7.3). The dialyzed serum was affinity-purified using a peptide column made using an Ag/Ab Immobilization kit (Immunopure from Pierce). After the antiserum was eluted using a 4M $MgCl_2$, 0.1 M HEPES pH 6.0 buffer, it was dialyzed into PBS and frozen.

Each data point represents approximately 5 fly heads. Groups of about 25-50 flies were collected and quickly frozen on liquid nitrogen until all of the time points had been collected. Heads were isolated resuspended in approximately 200ul of 1x Laemmli sample buffer, allowed to thaw and homogenized with a Dounce type B pestle. Samples were boiled for 5 minutes, and centrifuged for 10 minutes at room temperature in an Eppendorf microcentrifuge. The supernatants were collected and boiled again just prior to loading onto protein gels. Standard procedures were used to separate equal amounts of proteins from each sample on 12% polyacrylamide-SDS gels and to transfer them to PVDF membranes by electroblotting (Ausubel et al., 1994).

The membranes were blocked for 60 minutes with a 5% BSA solution made up in TBST (10 mM Tris pH 7.9, 150 mM NaCl, 0.05% Tween 20). The primary antibody was diluted 1:1000 in TBST and incubated with the filter for 30 minutes. The membranes were washed three times with TBST for 5 minutes each time and then incubated for 30 minutes with an alkaline phosphatase-conjugated anti-rabbit IgG second antibody (Promega) diluted 1:7500 in TBST. The membranes were washed three more times as before and

developed using a chromogenic alkaline phosphatase reaction according to manufacturers suggestions (Promega).

Acknowledgements

We thank M. Gilman, M. Montminy, P. Brindle, and K. Lee for reagents and unpublished information during the course of this work, and the above and G. Enikolopov, E.R. Kandel, R. Greenspan, J.C. Hall, B. Stillman, Y. Zhong, N. Hernandez, C. Jones and B. Mihalek for helpful discussions and comments on the manuscript. This work was supported by a Cold Spring Harbor Laboratory Association Fellowship (to J.C.P. Yin), a National Cancer Institute postdoctoral fellowship (to E.L. Wilder) and NIH grants NS25621 and HD32245, a McKnight Scholars Award, a John Merck Scholarship for the Study of Developmental Disabilities in Children and start-up funds from Cold Spring Harbor Laboratory (to T.T.).

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Table 3.1. Olfactory acuity and shock reactivity in Can-S (wild-type), *17-2* (*hs-dCREB2-b* transgenic) and *rsh;17-2* (*radish, hs-dCREB2-b* "double mutant") flies[a].

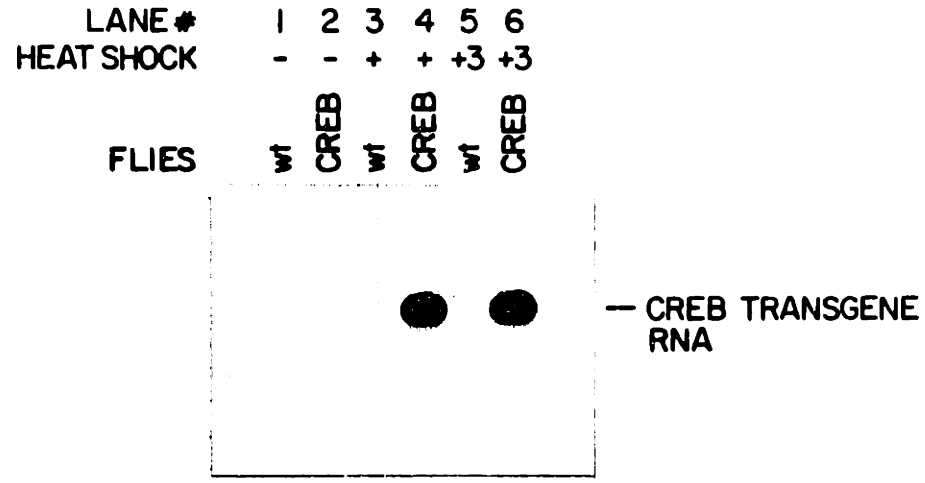
HEAT SHOCK	GROUP	OLFACTORY ACUITY				SHOCK REACTIVITY	
		OCT		MCH			
		10^{0*}	10^{-2}	10^{0}	10^{-2}	60V	20V
-hs	Can-S	58 ± 3	32 ± 3	80 ± 2	33 ± 7	79 ± 5	52 ± 5
	17-2	60 ± 3	34 ± 8	77 ± 3	37 ± 5	87 ± 3	43 ± 2
+hs (3 hr)	Can-S	69± 4	41± 4	77± 2	25± 9	74± 5	58± 6
	17-2	71± 4	37± 3	76± 5	26± 3	78± 3	67± 5
+hs (24 hr)	Can-S	66± 2	56± 8	79± 4	33± 2	84± 3	63± 3
	17-2	65± 3	42± 6	76± 3	41± 5	85± 2	60± 6
-hs	Can-S	51± 4	39± 5	72± 5	33± 7	87± 3	52± 5
	rsh; 17-2	57+ 3	39± 5	74± 5	29± 4	82± 4	53± 6
+hs (3 hr)	Can-S	72± 4	48± 3	66± 2	60± 3	80± 4	58± 6
	rsh; 17-2	68± 4	46± 6	78± 2	49± 4	83± 1	50± 5

[a]Olfactory acuity and shock reacitivity were assayed in untrained flies with the methods of Boynton and Tully (1992) and Dura et al., (1993), respectively (see Experimental Procedures for more details). N = 8 PIs per group. Planned comparisons between Can-S vs. mutant flies failed to detect any significant differences with any heat-shock regimen.
*10^0 is manual concentration and corresponds to 10^{-3} for bubbler (see Tully et al., 1994).

Figure 3.1 legend: Analysis of Transgene Induction after Heat-Shock A. Northern blot analysis of mRNA from the heads of wild-type flies (**wt**) or *17-2* transgenic flies (**CREB**) in the absence (lanes 1 and 2) of heat-shock, or immediately (lanes 3 and 4) or three hours (lanes 5 and 6) after heat shock. **B.** Western blot analysis of total head protein from wild-type (**wt**) or *17-2* (**CREB**) flies in the absence of heat shock (lanes 1 and 2), immediately (lanes 3 and 4), one (lanes 5 and 6), three (lanes 7 and 8), nine (lanes 9 and 10), or 24 (lanes 11 and 12) hours after heat-shock. Total proteins from each group were run on a polyacrylamide gel, electroblotted to PVDF membranes, probed with affinity-purified antibodies raised against a peptide from the basic region of the *dCREB2* gene, and detected with an AP-conjugated second antibody. C. Western blot analysis of total head protein from *17-2* (**wt** blocker) and *A2-2* (**mutant** blocker) transgenic flies in the absence of heat-shock (lanes 1 and 2), immediately (lanes 3 and 4), three hours (lanes 5 and 6) and six hours after heat-shock. Samples were treated as in **B**.

HEAT SHOCK INDUCTION OF THE CREB TRANSGENE

A.



B.

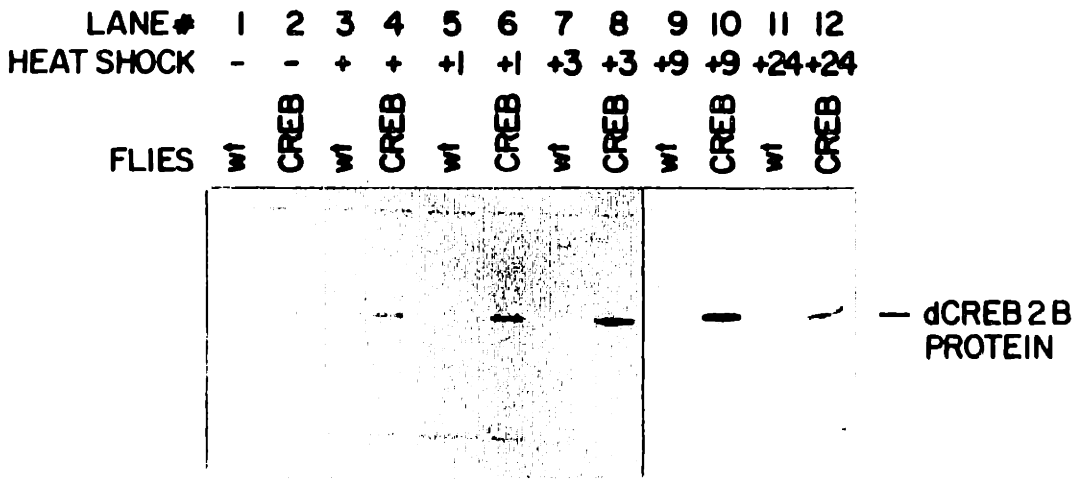


Figure 3.1 (A & B)

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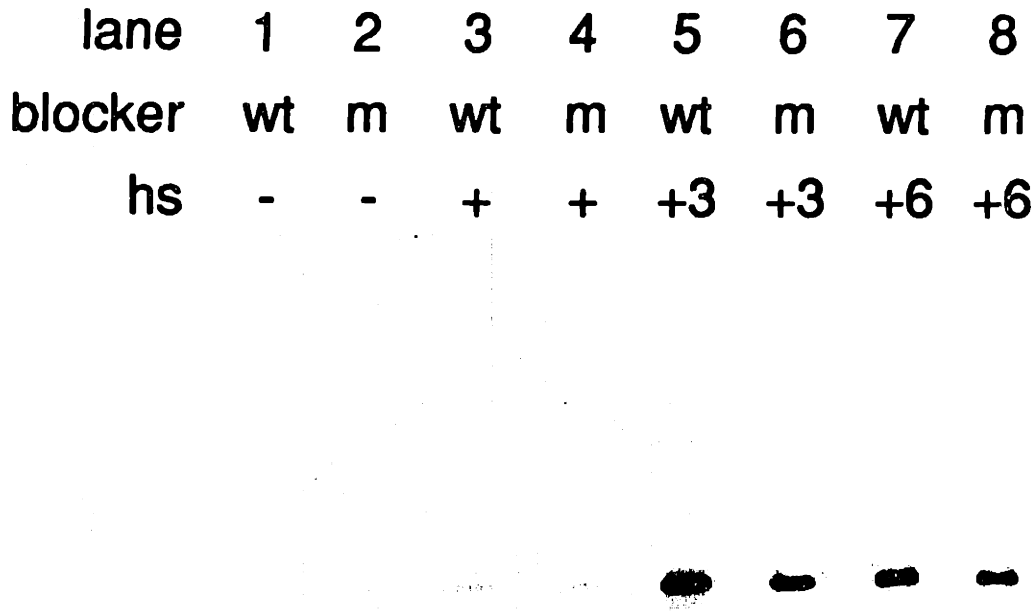


Figure 3.1 C

This is page 272.
Photo side is page 271.

Figure 3.2 legend: Cycloheximide feeding affects one-day retention after spaced training but not massed training. Different groups of wild-type (Can-S) flies were fed 5% glucose solution alone (hatched bars) or laced with 35 mM CXM (striped bars) either for 12-14 hr overnight before **massed** or **spaced** training or for the 24-hr retention interval immediately after training. One-day memory retention was significantly lower than normal in flies fed CXM before ($P < 0.001$) or after ($P < 0.001$) spaced training. In both cases, one-day retention in CXM-fed flies was reduced to a level similar to one-day memory after massed training in glucose-fed flies ($P = 0.88$ for CXM before training and $P = 0.71$ for CXM after training). In contrast, no difference was detected between CXM-fed and control flies for one-day memory after massed training ($P = 0.49$ and $P = 0.46$, respectively).

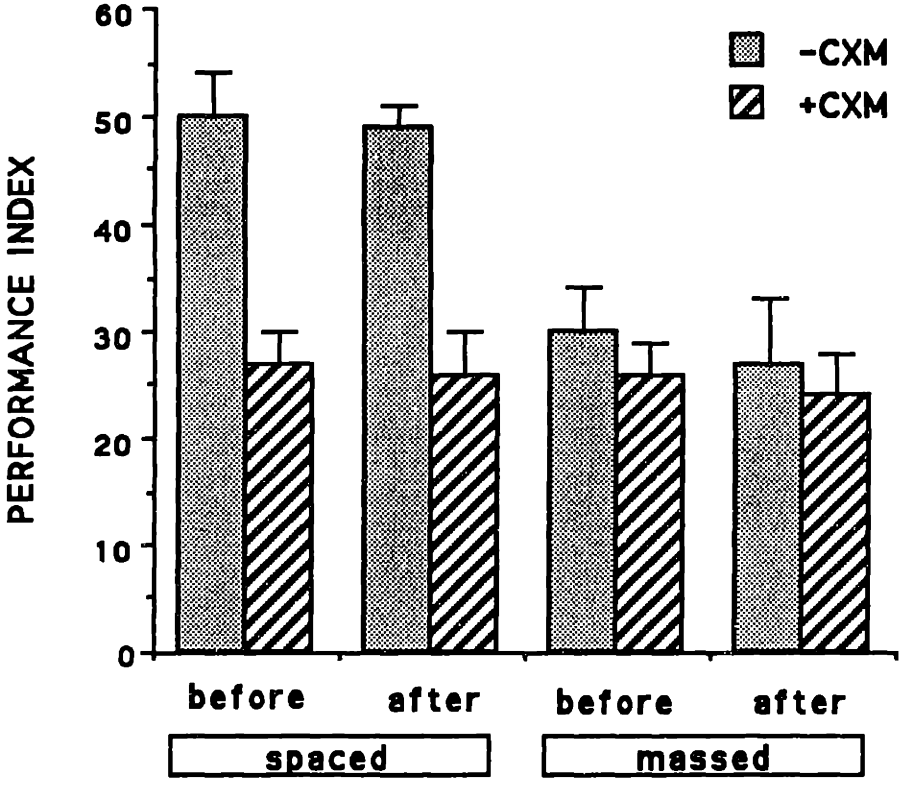
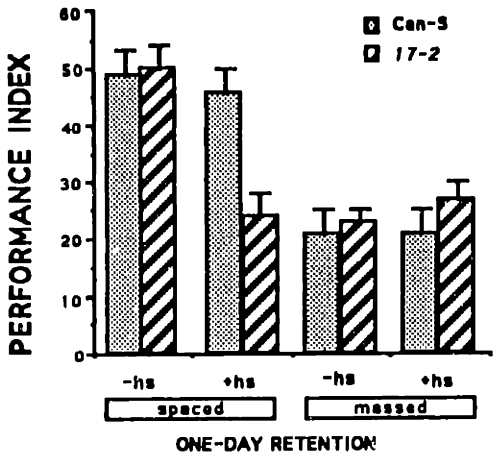

60
50
40
30
20
10
0
PERFORMANCE INDEX
-CXM
+CXM
before
after
before
after
spaced
massed
ONE-DAY RETENTION

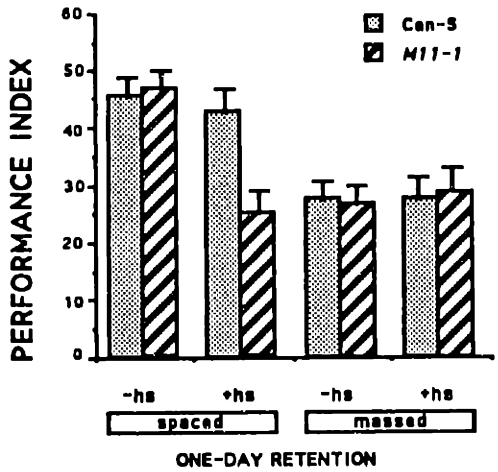
Figure 3.2

Figure 3.3 legend: Induction of the *dCREB2-b* transgene disrupts one-day memory after spaced training, while one-day memory after massed training and learning are normal. A. Different groups of wild-type (Can-S) flies (hatched bars) or *hs-dCREB2-b* transgenic (*17-2*) flies (striped bars) were given **spaced** training or **massed** training in the absence of heat shock (**-hs**) or three hours after heat shock (**+hs**). After training, flies were transferred to standard food vials and stored at 18° C until one-day memory was assayed. No differences in one-day memory after spaced or massed training were detected between Can-S vs. *17-2* flies in the absence of heat shock (**-hs**; P = 0.83 and 0.63, respectively). When flies were trained three hours after heat shock (**+hs**), however, one-day memory was significantly different between Can-S vs. *17-2* flies after spaced training (P < 0.001) but not after massed training (P = 0.23). In fact, the one-day memory after spaced training was no different than that after massed training in induced *17-2* flies (P = 0.59). In addition, the heat-shock regimen did not produce a non-specific effect on one-day retention after spaced (P = 0.59) or massed (P = 1.00) training in Can-S flies. N = 6 performance indices (PIs) per group. **B.** The same experiment as in A was repeated with a second, independently derived *dCREB2-b* transgenic line, *M11-1* (striped bars). Here again, a) no differences in one-day memory after spaced or massed training were detected between Can-S vs. *M11-1* flies in the absence of heat-shock (**-hs**; P = 0.83 and 0.86, respectively), b) a significant difference between Can-S vs. *M11-1* for one-day memory after spaced training (P < 0.001) but not after massed training (P = 0.85) when trained three hours after heat-shock (**+hs**), c) one-day memory after spaced training was no different than that after massed training in induced *M11-1* flies (P = 0.43) and d) the heat-shock regimen did not produce a non-specific effect on one-day retention after spaced (P = 0.59) or massed (P = 0.94) training in Can-S flies. N = 6 PIs per group. **C.** Different groups of Can-S flies (hatched bars) or *17-2* transgenic flies (striped bars) received one-cycle training in the absence of heat shock (**-hs**) or three (**+hs 3hr**) or 24 (**+hs 24hr**) hours after heat-shock and then were tested immediately afterwards. In each case, no differences between Can-S vs. *17-2* flies were detected (Ps = 0.28, 0.64 and 0.42, respectively), indicating that learning was normal in induced or uninduced transgenic flies. N = 6 PIs per group.

A.


Can-S
17-2
PERFORMANCE INDEX
60
50
40
30
20
10
0
-hs
+hs
-hs
+hs
spaced
massed
ONE-DAY RETENTION

B.


Can-S
M11-1
PERFORMANCE INDEX
60
50
40
30
20
10
0
-hs
+hs
-hs
+hs
spaced
massed
ONE-DAY RETENTION

C.

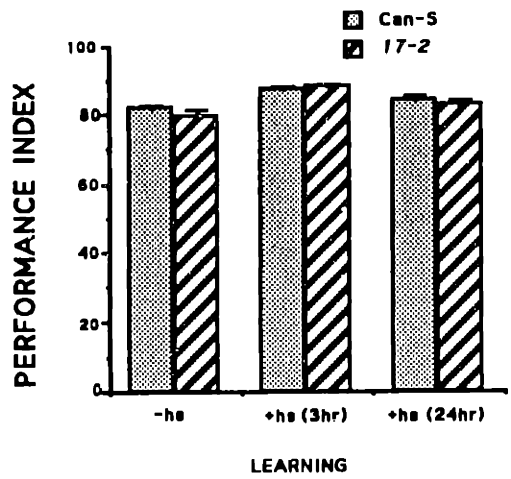

Can-S
17-2
PERFORMANCE INDEX
100
80
60
40
20
0
-hs
+hs (3hr)
+hs (24hr)
LEARNING

Fig. 3.3

Figure 3.4 legend: Induction of the *hs-dCREB2-mLZ* mutant blocker does not affect one-day retention after spaced training. Different groups of wild-type [*w (iso CJ1)*], *hs-dCREB2-b* transgenic (*17-2*) or mutant *hs-dCREB2-mLZ* transgenic flies (*A2-2*) received spaced training in the absence of heat-shock (**-hs**) or three hours after heat-shock (**+hs**). The flies were then handled and tested as in Fig. 3A. No differences in one-day memory after spaced training were detected between *w(isoCJ1)* vs. *17-2* flies or between *w(isoCJ1)* vs. *A2-2* flies in the absence of heat shock (**-hs**; P = 0.38 and 0.59, respectively). When flies were trained three hours after heat shock (**+hs**), however, one-day memory after spaced training was significantly different between *w(isoCJ1)* vs. *17-2* flies (P < 0.001) -- as in Fig. 3A -- but was not different between *w(isoCJ1)* vs. *A2-2* flies (P = 0.78). In addition, the heat-shock regimen did not produce a non-specific effect on one-day retention after spaced training in *w(isoCJ1)* or *A2-2* flies (P = 0.40 and P = 0.97, respectively). N = 6 performance indices (PIs) per group.

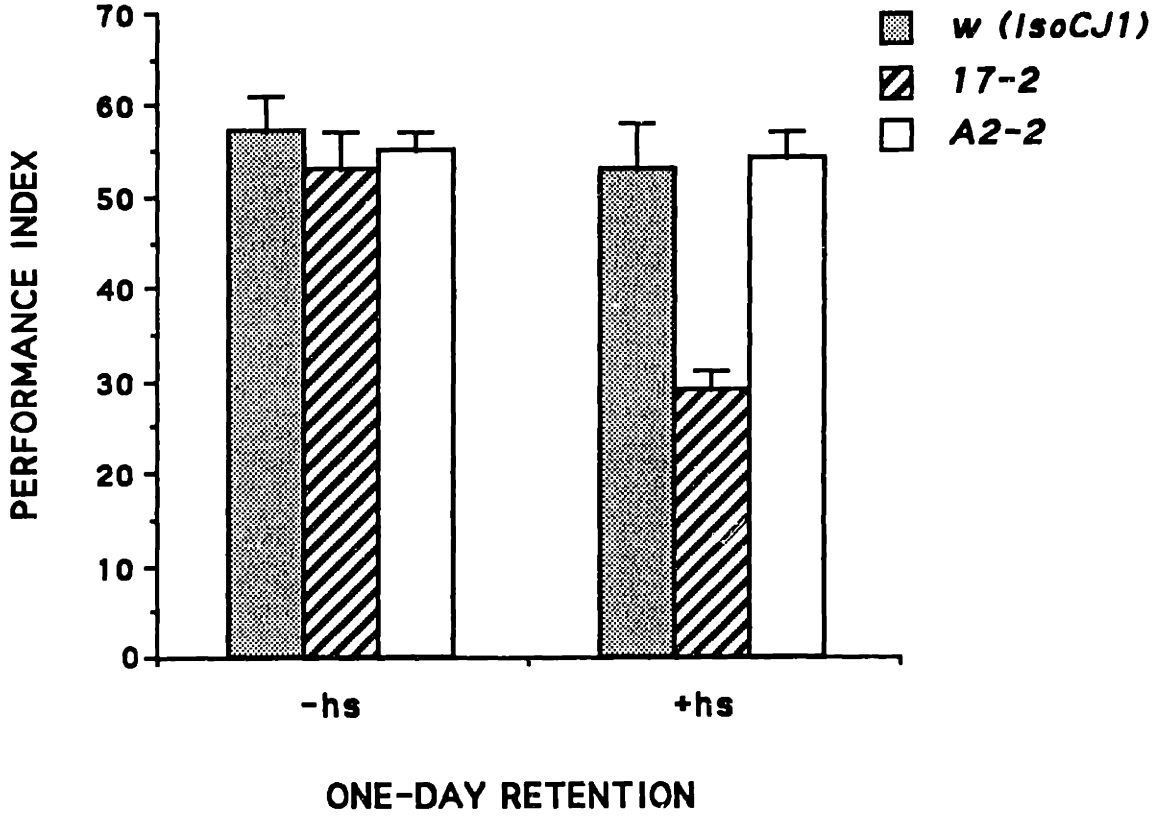

70
60
50
40
30
20
10
0
PERFORMANCE INDEX
w (IsoCJ1)
17-2
A2-2
-hs
+hs
ONE-DAY RETENTION

Figure 3.4

Figure 3.5 legend: Induction of *hs-dCREB2-b* completely abolishes 7-day memory retention. Previous analyses of *radish* mutants indicated that memory retention four or more days after spaced training reflects the sole presence of LTM. Thus, the effect of induced *hs-dCREB2-b* on LTM was verified by comparing 7-day retention after spaced training in Can-S (hatched bars) vs. *17-2* transgenic (striped bars) flies that were trained in the absence of heat shock (**-hs**) or three hours after heat shock (**+hs**). Flies were stored in standard food vials at 18^{o} C during the retention interval. N = 6 PIs per group. Seven-day retention after spaced training did not differ between Can-S and *17-2* flies in the absence of heat shock (P = 0.83) and but was significantly lower than normal in *17-2* flies after heat shock (P = 0.002). In fact, 7-day retention after spaced training in induced *17-2* transgenic flies was not different from zero (P = 0.92). In addition, the heat-shock regimen did not affect 7-day retention after spaced training non-specifically in Can-S flies (P = 0.39).

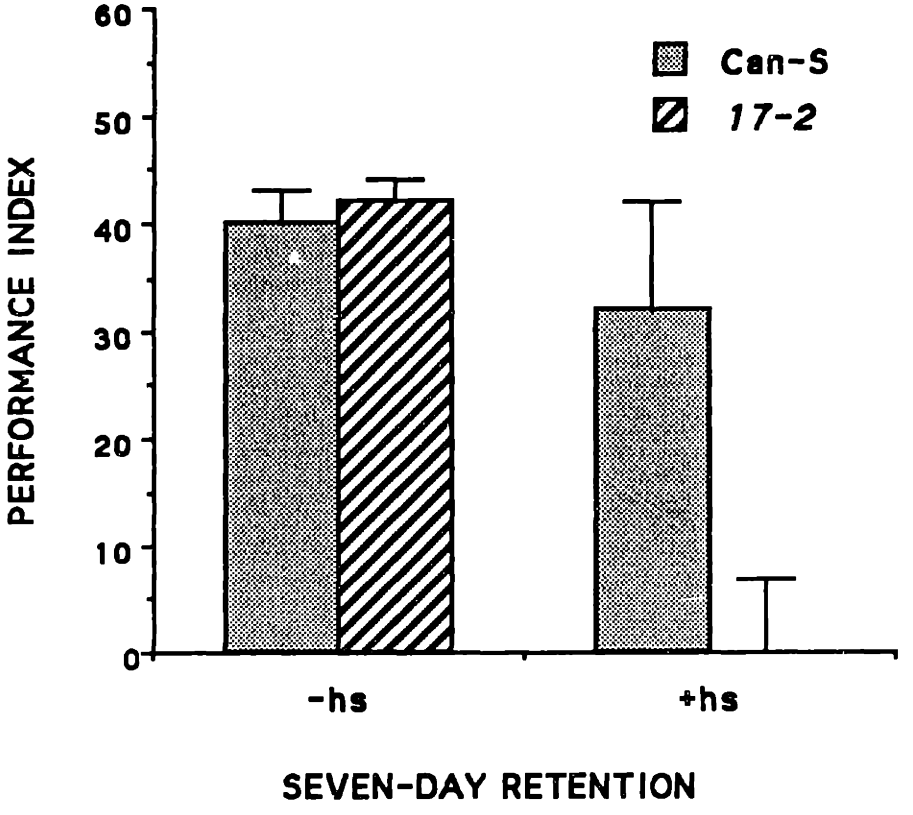

60
50
40
30
20
10
0
PERFORMANCE INDEX
Can-S
17-2
-hs
+hs
SEVEN-DAY RETENTION

Figure 3.5

Figure 3.6 legend: Induction of *hs-dCREB2-b* completely abolishes one-day memory after spaced training in *radish; 17-2* "double mutants." Since *radish* is known to disrupt ARM, a clear view of the effect of *hs-dCREB2-b* on LTM was obtained in *radish; 17-2* flies. One-day retention after spaced training was assayed in *rsh;17-2* double mutants and in *17-2* and *rsh* single-gene mutants as controls. Flies were trained in the absence of heat-shock (hatched bars) or three hours after heat-shock (striped bars) and stored at 18° C during the retention interval. As usual, induction of *hs-dCREB2-b* produced significantly lower one-day memory after spaced training in *17-2* flies ($P < 0.001$; cf. Fig. 1A). The heat shock regimen, however, had no effect on such memory in *radish* mutants ($P = 0.52$), which reflects only the presence of LTM (see Tully et al., 1994). In contrast, heat shock produced significantly lower scores in *rash;17-2* double mutants ($P < 0.001$), which were not different from zero ($P = 0.20$). N=6 PIs per group.

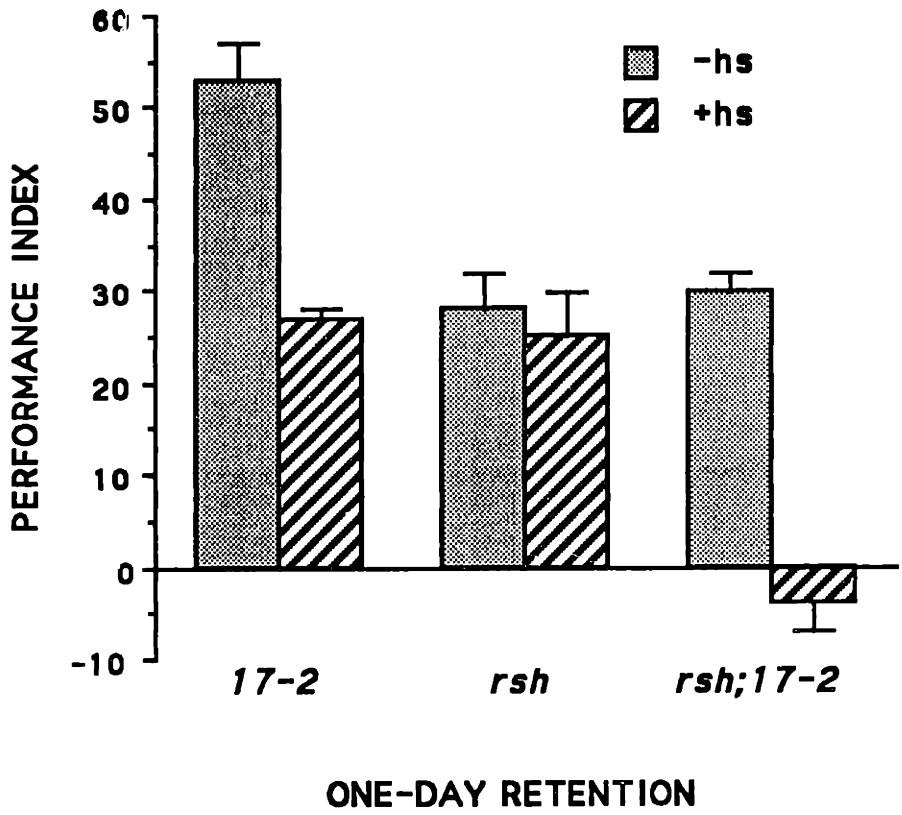

60
50
40
30
20
10
0
-10
PERFORMANCE INDEX
-hs
+hs
17-2
rsh
rsh;17-2
ONE-DAY RETENTION

Figure 3.6

Chapter 4

dCREB1, another gene for a CRE-binding protein from Drosophila

Introduction

The screen for cDNAs producing CRE-binding proteins that led to the identification of *dCREB2* also led to the cloning of a second gene, *dCREB1*. The *dCREB1* cDNA was subcloned and sequenced before any substantial work had been done on *dCREB2*. As *dCREB2* emerged as the better candidate for a gene coding for a PKA-responsive CREB activator, our work shifted away from *dCREB1*. Nonetheless, we have accumulated a certain amount of information about *dCREB1*. Since our experiments with *dCREB2* indicate that CREBs are critical in LTM, any *Drosophila* protein that can potentially interact with CREs or CREBs will merit further examination.

Methods

Methods used were as described in chapter 2, except as noted below.

Plasmid construction

Expression constructs for *Drosophila* transient transfection experiments were made in the expression vector pAct5CPPA (Han et al., 1989) or in pAcQ, which differ only in their polylinker sites. The polylinkers of these vectors are situated between a 2.5kb *Drosophila* actin 5C promoter fragment and a 1.1kb fragment of actin 5C 3' untranslated sequence containing a polyadenylation signal. pAc-*dCREB1* was made by subcloning a KpnI-SacI fragment containing the complete *dCREB1* open reading frame from Bluescript pKS into pAct5CPPA. pAc-PKA was constructed by subcloning an EcoRV fragment encoding the 352-amino acid Drosophila PKA catalytic subunit (Foster et al., 1988) from a modified pHSREM1 construct (Drain et al.,

1991) into pAct5CPPA, and identifying the sense-oriented fragment by restriction digest. To make the p3x(wtCRE)-lacZ reporter construct for *Drosophila* cell culture, a pair of complementary synthetic deoxyribonucleotides were synthesized, identical to the wild-type 3xCRE oligonucleotides used in gel-shifts (see chapter 2), but with 5'-XbaI and 3'-KpnI overhangs. The double-stranded oligonucleotide was ligated into the large KpnI-XbaI fragment of HZ50PL (Hiromi and Gehring, 1987), a reporter construct made for enhancer testing which has cloning sites upstream of a minimal *hsp70* promoter-*lacZ* fusion gene.

***Drosophila* cell culture and transient transfection**

Schneider L2 cells were maintained in Schneider's medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS). For transient transfections, $5x10^6$ L2 cells were plated in 5 ml of medium in a 60 mm tissue culture dish sixteen to twenty-four hours prior to their intended use. Calcium phosphate-DNA coprecipitates for transient transfections were prepared as described in Krasnow et al. (1989) except that a total of 10µg of plasmid DNA per dish was used. Transfections involving less than these amounts of experimental plasmids were made up to the required mass with pGEM (Promega). Thirty-six to forty-eight hours after transfection, cells were harvested by scraping with a rubber policeman, transferred to tubes and pelleted (10 minutes at 400x*g* @ 4°C). The pelleted cells were resuspended in cold phosphate-buffered saline solution (7.4g NaCl/ 0.940g Na_2HPO_4 / 0.414g NaH_2PO_4 per liter. pH 6.7) and centrifuged again. Pelleted cells were resuspended in 250µL of 0.25M Tris pH7.8 and transferred to 1.5ml microfuge tubes. Cytoplasmic extracts made as described for F9 cells in chapter 2. Results reported for transfections are averages of two or three experiments run on different days, with at least duplicate dishes for each condition within experiments. (L2 cells were a gift from D. Rio, U. Cal., Berkeley.)

Results

In the same expression screen that yielded the *dCREB2* cDNAs (detailed in chapter 2), J. Yin isolated a single clone representing a second, previously uncharacterized gene, *dCREB1*. Chromosomal *in situ* hybridization produced

a single signal localized the *dCREB1* gene to band 54A on the right arm of chromosome 2.

A hybridization screen of the lgt11 cDNA library, using the *dCREB1* cDNA as a probe, failed to identify any additional *dCREB1* cDNAs. Northern analysis was performed Dr. Yin with a blot containing 2μg of poly-A-selected adult mRNA, probed with a nick-translated probe made from the subcloned *dCREB1* cDNA. No bands were detected after a week of exposure to film. No signal above background was seen in sections of adult heads in my *in situ* hybridization studies.

In gel shift analyses (done by J. Yin) with bacterially expressed dCREB1, probe retardation was seen with labeled wild-type 3XCRE probe, but not with the mutant 3xmCRE probe. Retardation of the labeled 3xCRE probe was successfully and completely outcompeted by an excess of unlabeled wild-type 3xCRE probe, but was only marginally reduced by unlabeled mutant 3xmCRE competitor (Fig. 4-1).

I tested transcriptional activation by the *dCREB1* product in transient cotransfection experiments in *Drosophila* cell culture. In the Schneider L2 cell line, dCREB1 activates transcription from a CRE-containing reporter (Fig. 4-2). Cotransfection of a construct that expresses PKA catalytic subunit did not enhance transcriptional activation by dCREB1.

We sequenced the *dCREB1* cDNA. It contained a complete open reading frame, specifying a 266 amino acid protein (Fig. 4-3). The predicted dCREB1 protein has a basic region and a four-repeat leucine zipper at its carboxyl terminus. The majority of the amino-terminal portion of the inferred protein consists of a putative acid-rich transcriptional activation domain (Mitchell and Tjian, 1989), containing abundant glutamate and aspartate residues, with prolines spaced throughout. Computerized examination of the predicted dCREB1 amino acid sequence (GeneWorks, Intellegenetics,Inc.) indicated the presence of a number of consensus phosphorylation target sites for CaM kinase II and casein kinase II (CKII) dispersed throughout the protein.

There is some amino acid sequence homology between dCREB1 and dCREB2 in the bZIP domain, but this is quite limited. Computerized amino acid sequence comparisons were made between protein databases and the inferred dCREB1, using the BLAST program at The National Center for Biological Information. dCREB1 was by far most similar to the yeast GCN4 transcriptional activating protein (Thireos et al., 1984), based on extensive homology in the eighty-five carboxyl-terminal residues (75% similarity overall in this domain, and nearly complete identity in the basic region).

Discussion

The predicted dCREB1 protein possesses a carboxyl-terminal bZIP, and it is able to bind to CREs and activate transcription. These are features of members of the CREB family, but closer examination indicates that dCREB1 is more closely related to the yeast GCN4 protein than to CREBs.

The predicted dCREB1 bZIP shows very limited amino acid sequence homology to any other CREB product, including dCREB2, and its presumed transcriptional activation domain is of the acid-rich variety , quite different from the glutamine-rich class of activation domain found in the PKA-responsive CREB transcription factors (Mitchell and Tjian, 1989). In contrast, dCREB1 and GCN4 show strong amino acid sequence similarity over the carboxyl-terminal third of the proteins, including nearly perfect conservation in the basic region. The more amino-terminal portions of both proteins contain acidic activation domains (Struhl 1992).

Yeast GCN4 is a transcriptional activator that coordinately regulates the expression of genes involved in amino acid synthesis (Hinnebusch, 1984). Its activity does not appear to depend on direct regulatory interactions with other proteins, such as kinases. In structure and sequence, GCN4 is most similar to Jun among the mammalian bZIP transcription factors. A fusion protein containing the Jun DNA-binding domain fused to the GCN4 activation domain is able to activate transcription of GCN4-responsive promoters in yeast, thus GCN4 is, at some level, a *Jun* family member (Struhl, 1987).

The GCN4 bZIP is probably the best-studied example of this structure from any transcription factor. Particularly relevant here is the observation that the flexibility of the GCN4 DNA-binding domain allows it to use both TREs (TGACTCA) and CREs (TGACGTCA) as binding sites (Oliphant et al., 1989; Sellers et al., 1990; Struhl 1992). This dual DNA-binding specificity differentiates it from Jun, whose homodimers do not recognize CRE sites. Near-identity to GCN4 in the basic region probably explains the ability of dCREB1 to bind to a CRE-containing probe.

In the *Drosophila* L2 cell culture line, dCREB1 mediates substantial transcriptional activation from a CRE-containing reporter. This activation is not dependent on or substantially increased by cotransfection of the catalytic subunit of PKA. This is not particularly surprising, since the predicted dCREB1 protein has consensus sites for phosphorylation by casein kinase II and CaM kinase II, but none for PKA.

In early experiments, the ability of mammalian CREB to activate expression of CRE-containing reporters in L2 cells was tested. Robust activation by CREB was seen but, as was the case later with dCREB1, cotransfection of a PKA catalytic subunit expression construct did not substantially enhance activity. Experiments from Michael Gilman's lab with mammalian CREB in L2 cells suggested that a ceiling effect from endogenous PKA activity is not the reason that cotransfection of PKA failed to stimulate dCREB1 transcriptional activation. Transfections of a Ser133 -> Ala133 mutant mammalian CREB, which is unresponsive to PKA in F9 cell culture, activated transcription nearly as well as the wild-type protein, indicating that only basal activation was occurring with the wild-type CREB. Furthermore, cotransfection of a peptide inhibitor of PKA had no effect on transcriptional activation by CREB in these cells (S. Natesan and M. Gilman, personal communication). These results suggest that mammalian CREB is not acting in a PKA-responsive manner in L2 cells. Similarly, the dCREB2-a molecule failed to show PKA-responsive activity in *Drosophila* cells, but was later found to be a PKA-responsive activator in F9 cell culture experiments (see chapter 2). Thus, L2 cells do not appear to be an appropriate line for testing PKA-responsive transcription. For completeness, dCREB1 will be re-tested for PKA-responsive activity in F9 cells. The constructs necessary for this experiment

are complete. In addition to testing for PKA-responsive transcriptional activation, dCREB1 will also be tested for its effect on transcriptional activation by dCREB2-a.

The issue of whether dCREB1 is a functional homolog of *Drosophila* homolog of GCN4 remains to be resolved. As mentioned earlier, the known role of GCN4 in yeast is coordinate activation of genes for enzymes in amino acid biosynthesis. We have not attempted to identify any gene targets of dCREB1, and we know very little about its physiological role. With regard to its expression, the rarity of the dCREB1 cDNA in a *Drosophila* head cDNA library and the failure to detect dCREB1 mRNA by Northern analysis or tissue *in situ* hybridization indicate that steady-state levels of *dCREB1* expression is ordinarily at extremely low levels in the cells of the head. However, we have no information on the amount of protein present, nor do we know under what conditions *dCREB1* might be induced. The overriding question on which our further investigation of dCREB1 would be predicated is: might dCREB1 play a role in LTM? In our system, the litmus test for this is whether induction of a dCREB1 transgene influences memory formation. An inducible transgenic dCREB1 fly will be made and tested in the LTM paradigm.

An observation from our cell culture experiments indicates that dCREB1 could be an interesting molecule apart from any involvement in learning and memory. dCREB1 is apparently a constitutive transcriptional activator in L2 cells, but we have found that it fails to activate expression from a CRE reporter in the *Drosophila* Kc167 cell line. This failure could be occurring at any level, from insufficient protein expression in Kc167 cells to the absence of necessary transcriptional coactivators. Another possibility is that dCREB1 is not a constitutive activator, but is responsive to a potentiating or repressing cellular signal present in one of the cell lines but not the other. In view of the presence of consensus CaMKII phosphorylation sites on dCREB1, for example, it might be informative to mutagenize these sites or to cotransfect expression constructs for this kinase or its peptide inhibitor and observe the effects on transcriptional activation.

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Figure 4.1 legend. Gel shift analysis of dCREB1. Induced bacterial lysogens were used to make crude extracts for gel mobility-shift analysis of dCREB1. All reactions contained protein from an induced lysogen containing the dCREB1 open reading frame, except reaction 2, which contained protein from an induced lysogen that did not originally bind to CRE in the expression screen. All reactions contained wild- type 3xCRE oligonucleotide as the labeled probe, except reaction 3, which contained the mutant 3xmCRE (see chapter 2 for probe sequences). Increasing amounts of unlabeled competitor wild-type CRE (reactions 4-8) or mutant CRE (reactions 9-13) were used.

dCREB1 GEL SHIFT ANALYSIS

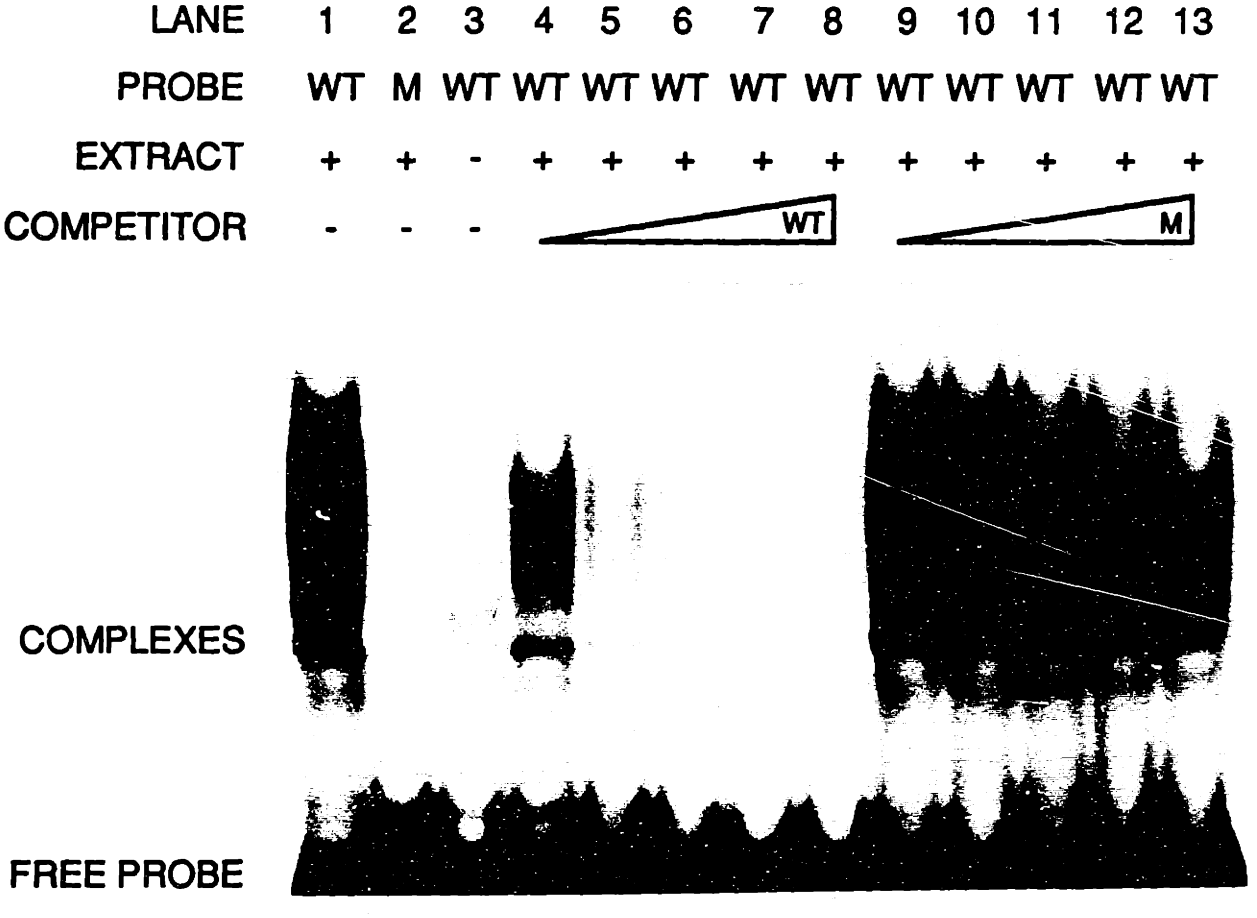

Figure 4.1

This is page 304.
Photo side is page 303.

Figure 4.2 legend. CRE reporter activation by dCREB1. *Drosophila* Scneider l2 cells were transiently transfected with Ac-dCREB1 expression construct (1μg), with or without pAC-PKA (1μg). 3xCRE-βgal reporter and Ac-CAT reporter were included in each dish. Expression vectors not present in any particular dish were replaced by the same weight of pAct5PPA. pGEM plasmid was added to make each transfection mass 10 μg.

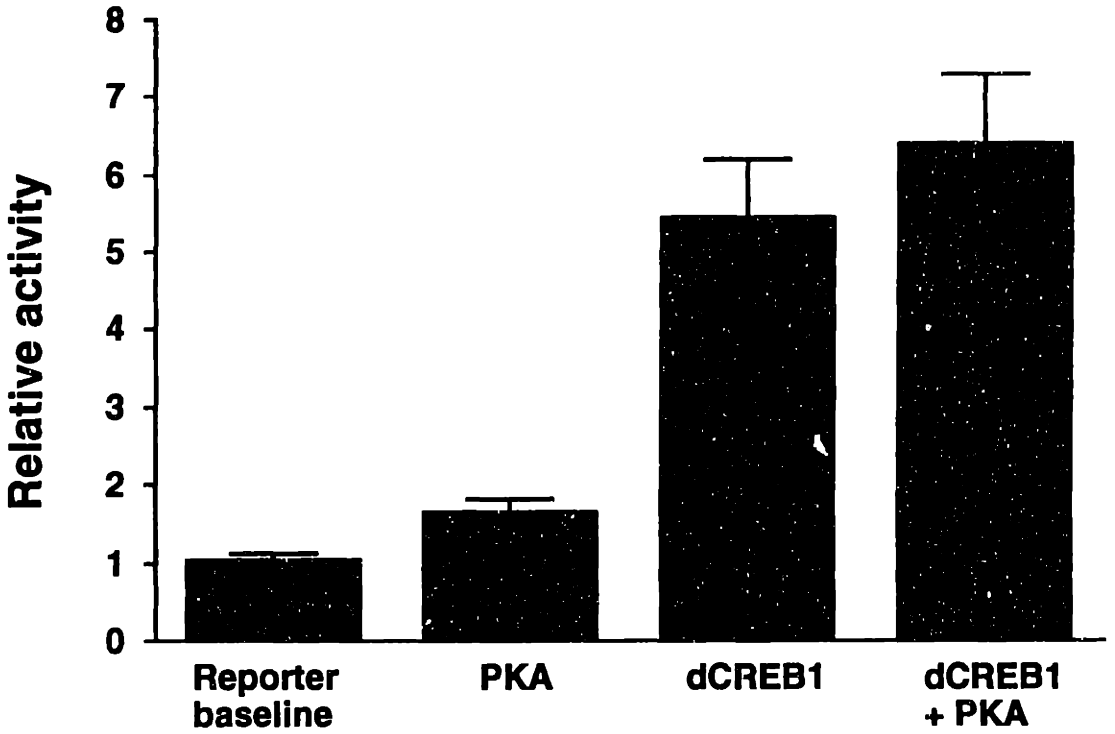
Relative activity
8
7
6
5
4
3
2
1
0
Reporter baseline
PKA
dCREB1
dCREB1 + PKA

Figure 4.2

Figure 4.3 legend. DNA sequence and predicted amino acid sequence of the dCREB1 coding region. Positively charged residues in the basic region are circled. "Zipper leucines" are boxed. Acidic residues in the putative transcriptional activation domain are underlined and prolines are indicated by diamonds.

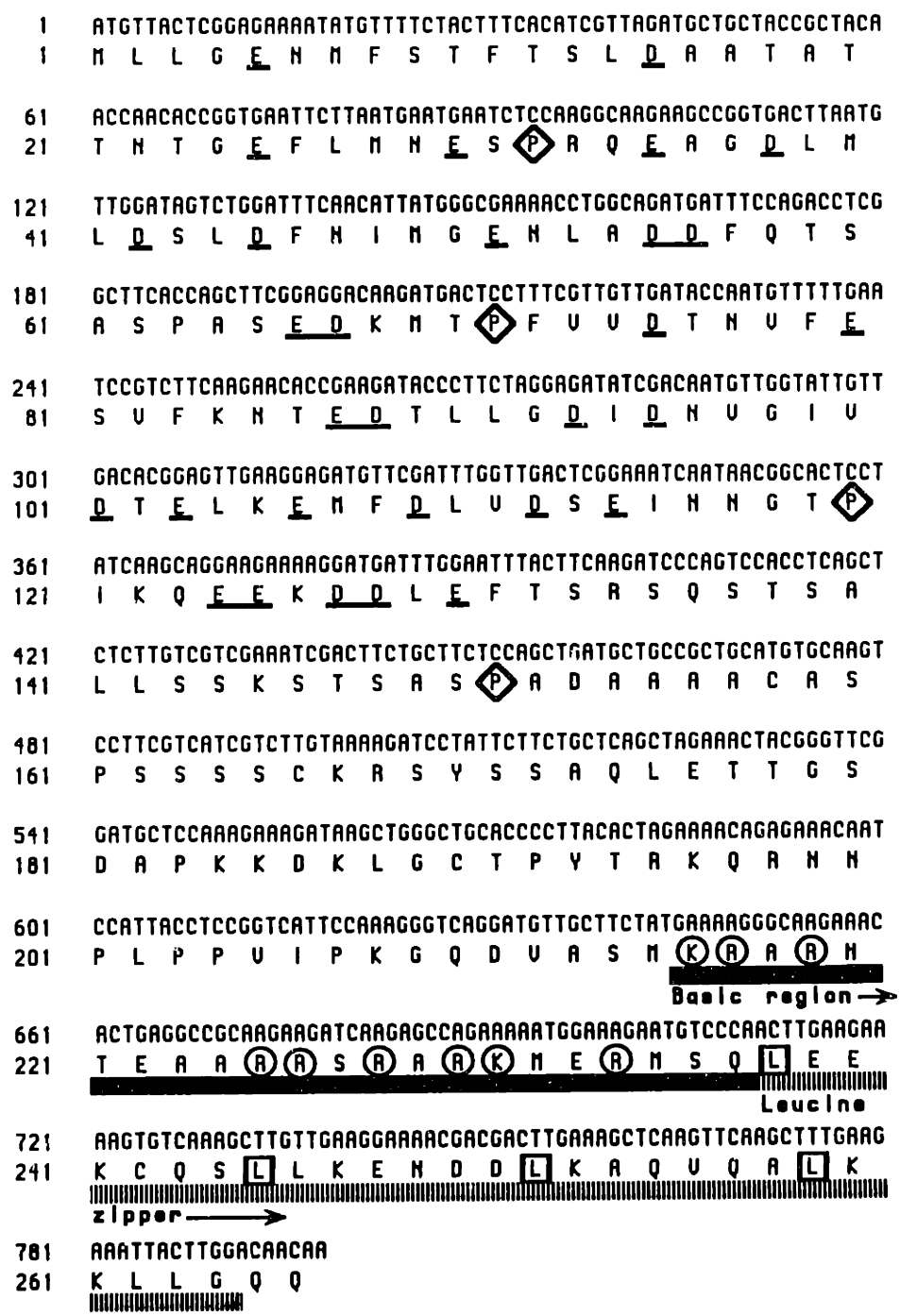

Figure 4.3

Chapter 5

Conclusions

The experimental findings presented here indicate that *Drosophila* has a system for CREB-mediated cAMP-responsive gene expression similar to that characterized in mammals, and that this system is required for long-term memory after olfactory classical conditioning.

***dCREB2* is closely related to the subfamily of cAMP-responsive CREBs in mammals, and plausibly represents an ancestor of those mammalian genes.** Predicted dCREB2 proteins have a strong structural resemblance to the three known mammalian cAMP-responsive CREBs - CREB, CREM and ATF-1. The dCREB2 bZIP is nearly identical to the highly conserved bZIPs of this subfamily. The glutamine-rich activation domain of the dCREB2-a activator is the same type found as mammalian cAMP-responsive transcription activators. The dCREB2 P-box occurs in a similar position in the molecule to those of CREB, CREM and ATF-1. It has a consensus PKA phosphorylation site, and other kinase phosphorylation sites that are similar to those in CREB, CREM and ATF-1. *dCREB2* is also functionally homologous to the mammalian PKA-responsive CREB genes, as discussed below.

The structural organization of *dCREB2* is similar to that of the mammalian *CREB* and *CREM* genes. Interestingly, *CREB* has alternatively-spliced mRNAs that are markedly different from those of *CREM*. The *Drosophila* *dCREB2* gene generates alternative splice forms that are similar to members of each of these mutually exclusive sets. This observation, together with strong sequence and structural conservation between *dCREB2* and mammalian PKA-responsive CREB gene products, indicates that*dCREB2*

combines the function of the mammalian genes. From our data, the simplest explanation for the presence of a subfamily of genes encoding highly conserved PKA-responsive CREBs in mammals is that they are the result of gene duplication and diversification from a single ancestral gene like *dCREB2*.

At least three laboratories have screened cDNA libraries looking for *Drosophila* CREBs, and this has resulted in the cloning of three CRE-binding proteins (Abel et al., 1992; Smolik et al., 1992; Usui et al., 1993; and work presented here). So far, *dCREB2* is the only known *Drosophila* gene with clear homology to the mammalian PKA-responsive CREB subfamily, and it is also the only one known to produce a PKA responsive activator. The dCREB1 protein described here is able to bind to CREs, but has not acted as a cAMP-responsive activator. (It is not a particularly good candidate since it lacks a recognizable PKA phosphorylation site.) dCREB1 amino acid sequence and structure much more closely resemble the yeast GCN4 protein than mammalian cAMP-responsive CREBs. It is possible that undiscovered *Drosophila CREB* genes exist, but based on the the extensive screening done by us and by others, and indications that *dCREB2* combines the function of at least two mammalian CREB genes, it is likely that *dCREB2* is the sole gene coding for cAMP-responsive CREBs in *Drosophila*.

dCREB2-a is a cAMP-responsive transcriptional activator

Transient transfection experiments in F9 cells done by me showed that dCREB2-a is a PKA-responsive activator from a CRE-containing reporter. The dCREB2-a isoform is the most complete *dCREB2* product, containing all of the known coding exons present in the structural gene. It retains two alternatively-spliced exons (exons 2 and 6) that appear to be analogous to the alternatively-spliced Q1 and Q2 exons of the mammalian *CREM* gene. In *CREM*, these exons are required in transcriptional activator isoforms.

dCREB2-b antagonizes PKA-responsive activation by dCREB2-a

The alternatively-spliced dCREB2-b isoform lacks exons 2 and 6 (and also the 12-bp exon 4). This molecule is not a PKA-responsive transcriptional activator in F9 cell culture, and my experiments showed that dCREB2-b is in fact an antagonist of PKA-responsive transcriptional activation by dCREB2-a.

If dCREB2-a and dCREB2-b expression constructs were cotransfected in equimolar amounts, along with an expression vector for PKA, then PKA-responsive dCREB2-a activation was blocked. A construct expressing a mutant dCREB2-b, with two of its zipper leucines changed to valines, was not able to block PKA-responsive transcriptional activation by dCREB2-a. This indicated that the conventional dimerization and/or DNA-binding functions of dCREB2-b are required for blocking, but this expriment cannot distinguish between them, since dimerization is required for DNA-binding.

***dCREB2* is involved in long-term memory in *Drosophila*.**

Experimental evidence from a variety of systems indicates that the cAMP second-messenger system is involved in learning and memory, and that the formation of long-term memory requires *de novo* gene expression around the time of training. Work in *Aplysia* from Kandel's laboratory first put these two strains of evidence together experimentally, in work that broadly implicated the operation of the CREB system as necessary in their model system for gene expression-dependent, long-lasting neuromodulation.

Work presented here indicates that a specific CREB gene, *dCREB2*, is involved in long-term memory of olfactory classical conditioning in *Drosophila*, as tested in the whole, behaving animal. My work cloning and functionally testing *dCREB2* products from the *Drosophila* head shows that a system for PKA-responsive transcriptional activation exists there, raising the possibility of its involvement in *Drosophila* long-term memory. My co-workers and I employed dCREB2-b as a tool to interfere with long-term memory (LTM) after olfactory classical conditioning that lasts at least a week in wild-type flies. Our experiments indicated that we achieved a complete blockade of long-term memory by inducing a dCREB2-b transgene. This block was fully equivalent to the blockade produced by treatment with the general protein synthesis inhibitor, cycloheximide. Induction of a mutant dCREB2-b construct where two codons for zipper leucines were changed to valines had no effect on LTM. This same zipper mutant dCREB2-b molecule was unable to block PKA-responsive dCREB2-a activation in my cell culture experiments. A reasonable interpretation of our results is that the overexpression of dCREB2-b blocks cAMP-responsive transcriptional activation of genes required for the formation of LTM, by interfering with dCREB2-a by a mechanism requiring

leucine zipper interaction.

We made a fairly strong inference from our behavioral results with transgenic flies: *dCREB2* is involved in long-term memory. Extrapolating from our cell culture results, we asserted that dCREB2-b interferes with PKA-responsive transcriptional activation by dCREB2-a in the fly. Cell culture results showing that dCREB2-b does not interfere with transcriptional activation by a Rel family member (see Appendix 2) suggest that it is not a promiscuous blocker. Northern blot analysis of constitutively expressed mRNAs in induced dCREB2-b transgenic flies indicates that the blocker does not impair operation of the general transcriptional apparatus.

A recent result reported by Yin et al. (1995) argues strongly for direct and critical involvement of *dCREB2* in LTM. As described in earlier sections, obtaining LTM in wild-type *Drosophila* requires ten rounds of spaced training in the olfactory classical conditioning paradigm. A single round of training or even ten rounds of massed training are insufficient to produce LTM. Yin et al. constructed transgenic lines carrying a heat shock-inducible construct expressing the *dCREB2* activator isoform, dCREB2-a. When this transgene is induced prior to training, a single round of olfactory classical conditioning is sufficient to produce LTM. A control transgene, in which the consensus PKA phosphoacceptor serine is mutated to an alanine, has no effect on LTM formation. In combination with the blocker results presented here, this experiment indicates that long-term memory can be positively and negatively manipulated by expression of *dCREB2* products with opposite effects on cAMP-responsive transcriptional activation. This is powerful evidence for the direct involvement of the *dCREB2* cAMP-responsive transcriptional activation system in LTM.

Based on our behavioral data, our current working hypothesis is that the switch for LTM formation lies in changes in the functional balance between cAMP-responsive transcriptional activator and blocker molecules. We believe that in the normal situation before training, blockers predominate, preventing LTM-related induction of gene expression by cAMP-responsive activators. Spaced training changes this, by unknown mechanisms, to favor activation and this allows the cAMP-responsive transcriptional activation

necessary for LTM to proceed.

This theory fits our results if induction of the dCREB2-b transgene before training overbalances the scale in the direction of blocking to a degree that cannot be overcome by 10-cycle spaced training. This would prevent LTM formation. Similarly, induction of the dCREB2-a transgene could make activator already predominant when training begins, and cAMP-responsive transcriptional activation necessary for LTM can occur with the first training trial. (This interpretation, in combination with Yin et al.'s experimental results showing that a non-phosphorylable dCREB2-a mutant has no effect, also suggests that sufficient PKA activity develops with the first training trial. Thus nuclear catalytic subunit availability is not likely to be the rate-limiting step in the LTM switch in our system.)

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Appendix 1

Continuing work

As with most projects, there are any number of experiments that we haven't done that might have been undertaken. I will be continuing to investigate the role of CREBs in *Drosophila* LTM, and thus will be in a position to do some of this work.

Cell culture

Several isoforms of *dCREB2* have not yet been tested for activity. The molecular and functional characterization of dCREB2-a and dCREB2-b, a PKA-responsive activator and a blocker form, respectively, were sufficient to give a biological footing to our hypotheses and to perform some telling behavioral experiments. In light of the proven involvement of *dCREB2* in LTM, it becomes even more important to functionally characterize the remainder of the *dCREB2* products. Any isoform (and perhaps other non-dCREB2 molecules) could potentially contribute to the balance of PKA-responsive activators and antagonists which appears to be the switch for LTM.

With regard to the testing of *dCREB2* isoforms, we have been unable to visualize dCREB2 proteins expressed in F9 cell culture on Western blots. One reason for this could be that our affinity-purified antipeptide antiserum was not sufficiently sensitive (see chapter 3). On Western blots, it could detect bacterially-expressed dCREB2 protein and dCREB2 protein in extracts from induced transgenic flies, but not in extracts from wild-type or uninduced *dCREB2* transgenic fly extracts. Our inability to detect dCREB protein in cell culture extracts may also be linked to the level of efficiency of our transfections. *In situ* staining for β-galactosidase indicated that approximately 5% of cells were transfected. This was adequate for measuring transcriptional effects using enzymatic assays, but may have been insufficient for detection of the expressed protein in cell extracts, at least with our reagents. We are raising new antisera and exploring new transfection

protocols (e.g., electroporation). We also have expression constructs based on promoters other than the RSV-LTR, and future constructs may be cloned with an epitope tag, allowing us to use commercially-available immune reagents of known quality for detection of expressed protein. We also have the option of using GAL4-dCREB2 fusion proteins in cell lines where expression of transfected constructs might be more robust, although the F9 system is considered the "gold standard" for testing PKA-responsive transcription factors.

Isoform-specific detection

Tissue *in situ* experiments indicated that *dCREB2* mRNA is present throughout the fly brain. I plan to pursue isoform-specific detection of *dCREB2* mRNA and protein. This is likely to require the use of *in situ* PCR for exon-specific detection of transcripts, and we will probably jave to raise better anti-dCREB2 reagents than we now have for immunohistochemistry. This is likely to be effort well spent, since these experiments present possibilities beyond anatomizing the static distribution of *dCREB2* isoforms. We might be able to observe the induction or disappearance of CREB isoforms after training. Information at that level would help us to further evaluate our LTM switch hypothesis, and also might be a way to find brain regions, or even individual neurons, that are candidates for involvement in the long-term memory process.

PKA-responsive reporter activation *in vivo*

The conclusion that the blockade of LTM in the whole fly by dCREB2-b overexpression is probably due to antagonism of dCREB2-a PKA-responsive activation is, in essence, a direct extrapolation from cell culture results. The information we have gathered about *dCREB2* persuades us that this explanation is justified, but we currently have no way of detecting PKA-responsive transcriptional activation in flies. I am planning to characterize reporter expression in transgenic flies carrying presumptive cAMP-responsive constructs, looking for changes in pattern or intensity of expression that occur with drug, genetic and behavioral manipulations. Treatment with forskolin, a powerful activator of adenylyl cyclase, is a reasonable first step, both to demonstrate that cAMP-responsive transcription

can occur in*Drosophila*, and as a preliminary screen for identifying promising reporter lines. To look at the interaction between heat shock-induced *dCREB2* transgene products and cAMP-responsive reporters, I intend to set up crosses to make transgenic flies that carry both.

The ultimate goal of any effort with cAMP-responsive reporters *in vivo* will be to find training-specific changes in reporter expression, which might directly identify cells involved in learning and memory. Molecular investigation of those cells could open the door to obtaining hard biological evidence concerning our LTM switch hypothesis.

Appendix 2

Supporting experiment

The ability of dCREB2-b to block transcriptional activation by p65-Rel, a member of the Rel transcription factor family, was tested in transient transfection experiments in F9 cells. Our results indicate that co-expressed dCREB2-b does not significantly interfere with activation of a reporter containing the DNA-binding site for p65-Rel, suggesting that dCREB2-b does not indiscriminately block transcriptional activation.

Methods

Preparation of plasmid DNAs, gel mobility shift assays and F9 cell transfections and enzymatic assays were as described in chapter 2. DNA constructs used in transfections that have not been described in other chapters were: RSV-p65-Rel, an expression construct containing the coding region from a human p65-Rel cDNA driven by the RSV promoter, and 3xκB-CAT, a reporter construct containing three kappa B enhancer sites inserted upstream of a *c-fos* (-56 to +109)-CAT fusion gene. These constructs were obtained courtesy of M.Z. Gilman's lab.

Results and discussion

Figure A2.1 indicates that undifferentiated F9 cells can support transcriptional activation by p65-Rel, as assayed in transient transfection experiments using a reporter plamid containing three tandem NF-κB enhancer elements. Cotransfection of an expression construct for dCREB2-b does not significantly affect this activity of p65-Rel.

In experiments described elsewhere, dCREB2-b was shown to block transcriptional activation by dCREB2-a from a CRE-containing reporter in F9 cells. It was possible that dCREB2-b would promiscuously interfere with activation by any given transcription factor. To examine this, we chose to test

dCREB2-b against a member of a non-CREB transcription factor family which binds to an enhancer site that is substantially different from the CRE. p65-Rel is a member of the Rel transcription factor family, a group defined by the presence of a conserved segment of about 300 amino acids, called the Rel homology domain (Gilmore, 1990). There are known members of this gene family in *Drosophila,* namely *dorsal* and *Dif* (Ip et al., 1993; Steward, 1987).

Stein et al. (Stein et al., 1993a; Stein et al., 1993b) have reported direct *in vitro* interactions between mammalian Rel proteins and specific bZIP transcription factors of the AP-1 and C/EBP families. Their transient cotransfection experiments have shown effects of these same factors on Rel-dependent transcriptional activation in cell culture. Our experiment, therefore, posed a useful and relevant test of dCREB2-b specificity. The results suggest that blocking of transcriptional activation by dCREB2-b is not promiscuous or indiscriminate. It may not interfere with any transcription factor besides dCREB2-a, or perhaps a subset of bZIP proteins in*Drosophila.*

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Figure A2.1 legend. Co-expression of dCREB2-b and p65-Rel in transiently transfected cells does not affect reporter gene activation by p65-Rel. DNA co-transfection experiments were performed in undifferentiated F9 cells. All dishes received 8 μg of the 3xκB-CAT reporter construct and 5 μg of RSV-βgal reporter. **Baseline** dishes received no other active constructs. **p65-Rel** dishes additionally received 2 μg of RSV-p65 expression construct. **p65-Rel + dCREB2-b** dishes received 2 μg of RSV-p65-Rel and 6 μg of RSV-dCREB2-b DNA. All transfections were supplemented with RSV-0 to a total mass of 25 μg.

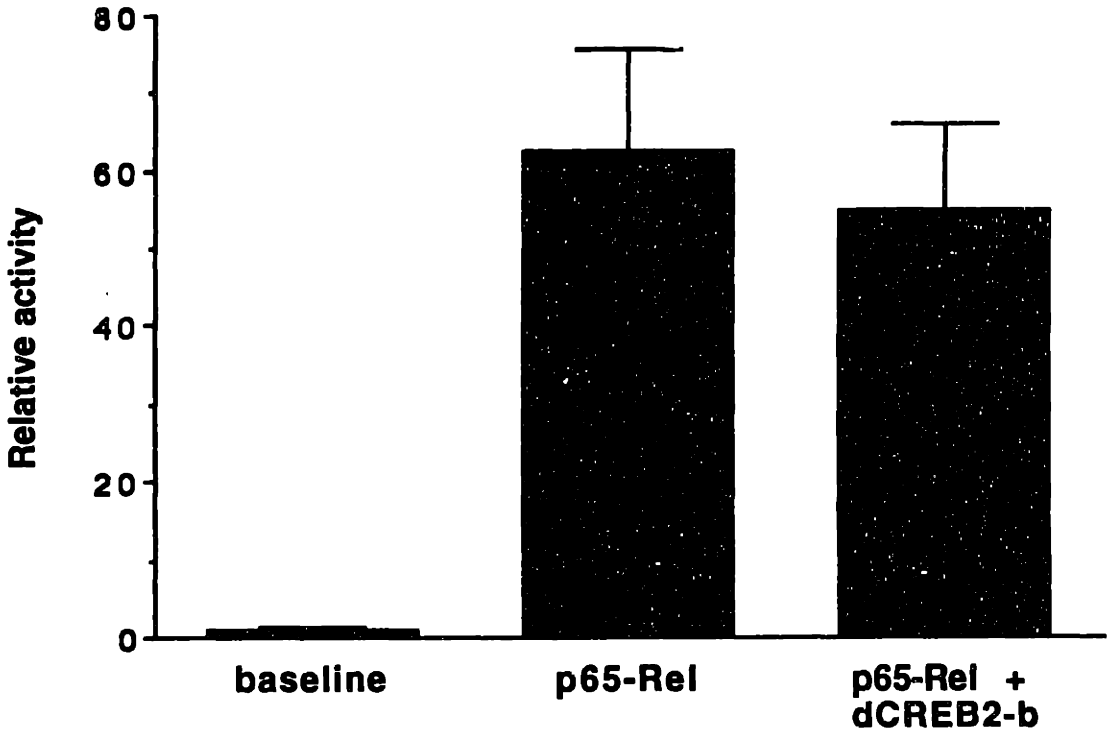
80
60
40
20
0
Relative activity
baseline
p65-Rel
p65-Rel +
dCREB2-b

Figure A2.1