

CREB AND MEMORY

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ABSTRACT

The cAMP responsive element binding protein (CREB) is a nuclear protein that modulates the transcription of genes with cAMP responsive elements in their promoters. Increases in the concentration of either calcium or cAMP can trigger the phosphorylation and activation of CREB. This transcription factor is a component of intracellular signaling events that regulate a wide range of biological functions, from spermatogenesis to circadian rhythms and memory. Here we review the key features of CREB-dependent transcription, as well as the involvement of CREB in memory formation. Evidence from *Aplysia*, *Drosophila*, mice, and rats shows that CREB-dependent transcription is required for the cellular events underlying long-term but not short-term memory. While the work in *Aplysia* and *Drosophila* only involved CREB function in very simple forms of conditioning, genetic and pharmacological studies in mice and rats demonstrate that CREB is required for a variety of complex forms of memory, including spatial and social learning, thus indicating that CREB may be a universal modulator of processes required for memory formation.

cAMP RESPONSIVE ELEMENT BINDING PROTEIN AND TRANSCRIPTION

The Multigene cAMP Responsive Element Binding Protein Family

cAMP responsive element binding protein (CREB) is a member of a large family (CREB/ATF) of structurally related transcription factors that bind to promoter cAMP responsive element (CRE) sites. Although CREB was the first to be

isolated, there are now at least 10 additional genes in the CREB family (Brindle & Montminy 1992, Sassone-Corsi 1995). This group of proteins shows many structural and functional variations, and they are expressed in a wide range of tissues and cell types.

Transcription factors from the CREB family include domains that are required for transcriptional activation and domains [basic leucine zipper (bZIP)] that mediate dimerization and DNA binding (basic region). The transcription activation domain includes glutamine-rich domains (Q domains) flanking a cluster of phosphorylation sites that regulate the activity of CREB (P-box). Not all CREB-like proteins include all of the domains described above. For example, an isoform of the cAMP response element modulator (CREM) gene called inducible cAMP early repressor (ICER) only contains the bZIP and the DNA binding domains (Molina et al 1993).

Most of the sequence homology among different members of the CREB family is restricted to the bZIP region (Hai et al 1989). Based on the extent of homology at this region, members of the CREB family can be divided into the CREB, CREM, and activating transcription factor (ATF) groups.

Besides activators of transcription, the CREB family also includes repressors. For example, the CREM gene codes for at least four different factors that block CRE-dependent transcription (Foulkes et al 1991, Molina et al 1993): the CREM α , β , and γ proteins and ICER. Most of these isoforms are generated by alternative splicing, another common feature of many CREB family genes. The CREB gene generates three main activators by alternative splicing [α (Gonzalez et al 1989), β (Blendy et al 1996), and Δ (Hoeffler et al 1989, Yamamoto et al 1990)]. These alternatively spliced isoforms have different properties, and their expression can be developmentally regulated. In addition to splicing, mechanisms responsible for the diversity of CREB isoforms include the usage of alternative start sites (e.g. CREB β) and even alternative promoters (e.g. ICER) (Molina et al 1993).

Transcriptional Activation

The crucial event in the activation of CREB is the phosphorylation of Ser133 in the P-box, or kinase-inducible domain (KID) (Gonzalez et al 1989, Gonzalez & Montminy 1989). This domain includes several consensus phosphorylation sites for a variety of kinases [e.g. protein kinase A (PKA), protein kinase C (PKC), casein kinases, calmodulin kinases (CaMKs), glycogen synthase kinase-3, p34^{cdc2}, p70^{s6k}] that can either increase or decrease the activity of CREB (Brindle & Montminy 1992, Sassone-Corsi 1995). For example, activation of adenylate cyclase by transmembrane receptors, such as D₁ dopaminergic receptors, can result in increases in cAMP. This in turn activates PKA by dissociating the regulatory from the catalytic subunits. Catalytic subunits can be translocated into the nucleus, where they can phosphorylate CREB

(Bacskai et al 1993, Hagiwara et al 1993). The phosphorylation of CREB by kinases from several signaling pathways may be a mechanism for the convergence of these signaling pathways (Figure 1) (Dash et al 1991, Sheng et al 1991). For example, increases in calcium driven by the activation of synaptic *N*-methyl-D-aspartate receptors (NMDARs) turn on CaMK (perhaps CaMKIV), which may also phosphorylate and activate CREB in neurons (Bito et al 1996, Deisseroth et al 1996). Interestingly, recent evidence suggests that increases in nuclear calcium can also activate CREB, indicating that nuclear kinases may have a direct role in the modulation of CREB activity (Hardingham et al 1997).

Transcriptional Repression

Just as phosphorylation of Ser133 seems to be a critical step in CREB activation, dephosphorylation of this residue is important for the inactivation of

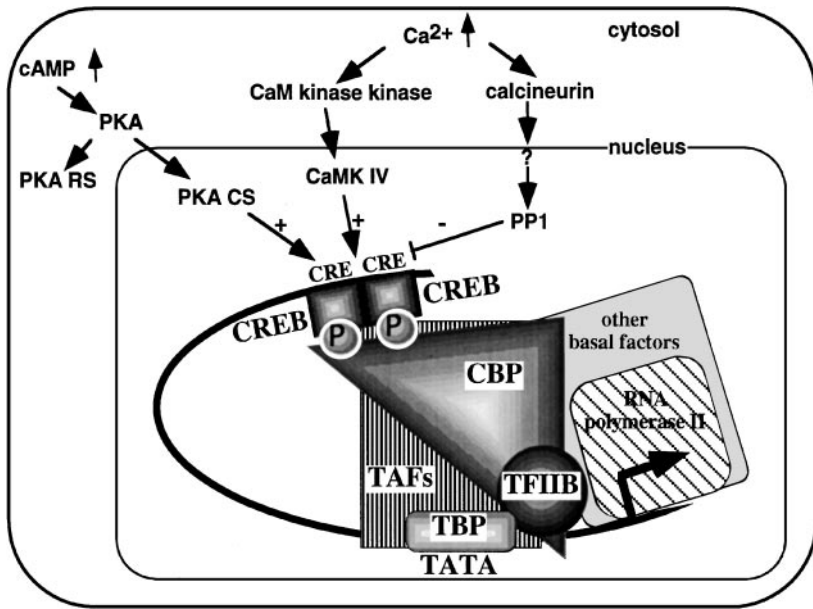


Figure 1 Scheme for transcriptional regulation by cAMP responsive element binding protein (CREB). Phosphorylation and dephosphorylation of CREB is regulated by calcium signaling and the cAMP/PKA pathway. Only phosphorylated CREB can interact with CREB binding protein (CBP). The CREB-CBP complex can recruit basal transcription factors and RNA polymerase II on the promoter and initiate transcription. Abbreviations: PKA, protein kinase A; PKA RS, PKA regulatory subunit; PKA CS, PKA catalytic subunit; PP1, protein phosphatase 1; TBP, TATA box-binding protein; TAFs, TBP-associated factors; TATA, TATA element; TFIIB, transcription factor IIB; P, phosphorylation sites.

CREB. Both protein phosphatase 1 (PP-1) and PP-2A may be involved in the dephosphorylation of CREB (Figure 1) (Hagiwara et al 1992, Bito et al 1996). Also, phosphorylation of Ser142 by CaMK appears to repress CREB activity (Ghosh & Greenberg 1995).

In addition to dephosphorylation, CREB activity can also be blocked by repressors. For example, the CREM α , β , and γ repressors lack the glutamine-rich transactivating domains but appear to bind CRE sites normally (Foulkes et al 1991, Laoide et al 1993). Thus, repressor dimers may compete for CRE sites with CREB activators. The repressors are unable to interact with the transcription machinery (they lack Q domains), thus rendering CRE-containing promoters silent.

The CREM gene has an alternative promoter that regulates the expression of ICER, a strong CREB repressor. The ICER promoter has CRE sites, and therefore it is strongly induced by activators of CREB/ATF family members (Molina et al 1993). Similarly, the main CREM promoter is also inducible, and it also has CRE sites. Activation of CREB may result in the increased expression of CREM isoforms, including ICER. The accumulation of repressors could in turn lead to the eventual repression of CREB-dependent transcription. Since ICER can repress its own transcription, the whole cycle eventually starts again. ICER does not have a P-box, so its activity is not controlled by phosphorylation. The regulatory feedback involving ICER transcription could impact several biological functions, including memory.

PROTEIN SYNTHESIS AND MEMORY

A considerable amount of evidence from a variety of model systems suggests that protein synthesis is essential for the formation of long-term memory (Barondes 1975, Davis & Squire 1984, Matthies 1989). One of the first experiments to suggest this used mice treated with puromycin (Flexner et al 1963), an antibiotic that inhibits protein synthesis. An avalanche of similar studies followed, making the effects of protein synthesis blockers an extensively studied aspect of memory research (Davis & Squire 1984).

However, there are problems that complicate the interpretation of these studies. First, the molecular mechanisms affected by the protein synthesis blockers cannot be uncovered without information concerning the exact transcriptional components and target proteins involved. Second, distinguishing between performance deficits and true amnesia is particularly problematic, since most of the agents used in these studies have a variety of side effects that could interfere with behavioral performance. For example, even anisomycin, seemingly the most selective of these agents, can result in diarrhea, decreased food intake, and lower locomotor activity. The traditional control for these effects has been to inject the drug several hours before or after training and to show that this does

not result in memory impairments. However, the complexity of the potential side effects is such that it is difficult to exclude the possibility that they affect long-term memory (Davis & Squire 1984).

SHORT VS LONG-TERM MEMORY: THE ROLE OF CREB

Electrophysiological Studies in Aplysia

The first study to suggest that CREB is required for memory formation was done in *Aplysia* cultured neurons (Dash et al 1990). This reduced neuronal preparation is used to study the synaptic modulation underlying sensitization of motor reflexes in *Aplysia*. Tactile or electrical stimulation of the animal's siphon leads to a defensive response that includes the withdrawal of the siphon and gill. Similarly, stimulation of the tail leads to the withdrawal of the siphon. Repeated stimulation can lead to short (minutes) and long-term (hours, days) sensitization of this response (Carew et al 1983, Byrne 1987). Although many neurons are involved in each of these behavioral responses, a considerable body of work indicates that facilitation of the synapses between the sensory (responding to the sensitizing stimulus) and the motor neurons (mediating the withdrawal response) is central to behavioral sensitization (Frost et al 1985, Byrne 1987).

The use of a reduced preparation with cultured sensory and motor neurons facilitated the electrophysiological and neuroanatomical analysis of the cellular events underlying long-term sensitization. These cultured neurons form synaptic connections that show long-term facilitation (LTF), a stable enhancement in synaptic function. LTF has properties that parallel behavioral long-term sensitization (LTS). Multiple spaced applications of serotonin are required for the induction of LTF, just as multiple spaced stimuli are required for the induction of LTS. Additionally, blockers of either protein synthesis or transcription inhibit LTF but not short-term facilitation (STF) (Montarolo et al 1986).

The long-term facilitatory effects of serotonin are mediated by the cAMP-PKA second-messenger pathway, even though short-term effects also involve the diacylglycerol-protein kinase C (DAG-PKC) system (Byrne et al 1993). Serotonergic stimulation transiently increases cAMP, which activates PKA. The catalytic subunit of PKA is translocated into the nucleus of sensory neurons, where it appears to activate CREB-dependent transcription of genes whose products are required for LTF (Bacskai et al 1993). Addition of cAMP alone to the media can induce 24 h of LTF in the cultured neuron preparation (Schacher et al 1988).

Oligonucleotides with CRE sequences injected into cultured sensory neurons selectively block LTF but not STF (Dash et al 1990). Presumably, the CRE

oligos trap the CREB proteins needed for the transcriptional activation of genes involved in LTF (Kaang et al 1993, Alberini et al 1994). Indeed, by injecting a reporter gene with a CRE-containing promoter into sensory neurons, Kaang et al (1993) were able to show that induction of LTF also triggers CREB activation. Recent cloning studies have identified CREB-like proteins in *Aplysia* (Bartsch et al 1995).

Multiple applications of serotonin result in both a long-lasting increase in neurotransmitter release and a long-term increase in the excitability of the cultured sensory neurons (Dale et al 1987, Mercer et al 1991). Treatment with transforming growth factor- β (TGF- β) triggers LTF without inducing long-term increases in neuronal excitability or any STF (Zhang et al 1997). Additionally, an inhibitor of TGF- β blocks LTF produced by electrical stimulation (Zhang et al 1997). Long-term sensitization training increases the expression of an *Aplysia* tolloid/BMP-1 (bone morphogenic protein)-like protein (apTBL-1), which may function as a secreted protease that activates TGF- β (Zhang et al 1997). Hence, LTF may involve the expression of apTBL-1 (CREB dependent?), which then activates TGF- β , which in turn triggers a cascade of events that results in increased neurotransmitter release. This cascade may resemble that triggered by neurotrophins in hippocampal neurons (Kang & Schuman 1996). TGF- β triggers LTF without STF, confirming the finding that these two phases of sensitization are independent of each other (Emptage & Carew 1993, Mauelshagen et al 1996).

Olfactory Memory Studies in Drosophila

Just as the cAMP-PKA pathway has been implicated in long-term sensitization in *Aplysia* (Byrne et al 1993), *Drosophila* genetic screens for learning and memory mutants showed the involvement of this intracellular signaling pathway in memory formation (Tully 1991). Two of the mutants isolated turned out to be disruptions in adenylate cyclase (Dunce) and in phosphodiesterase (Rutabaga), which are both key enzymes in the regulation of intracellular levels of cAMP (Tully 1991).

The involvement of the cAMP-PKA pathway in *Drosophila* learning, as well as the findings implicating CREB in LTF in *Aplysia*, led naturally to the idea that CREB may be required for memory formation in *Drosophila*. Flies show robust olfactory learning and memory: After exposure to two odors, one of which is paired with electric shock, flies learn to avoid the paired odor in a T-maze (Tully 1991). Importantly, when training is spaced over time, memory for the odor paired with electric shock lasts for many days and is sensitive to blockers of protein synthesis (Tully et al 1994). Targeted mutagenesis is not possible in *Drosophila*, so disruption of CREB function was achieved by the transgenic expression of a dominant-negative CREB protein (Yin et al 1994).

This repressor of CREB activity binds CRE sites but lacks two exons required for transcriptional activation. To control the time of the induction, the transgene (dCREB2-b) was placed under the control of a promoter that could be activated by increases in temperature (heat-shock promoter). These experiments showed that induction of the CREB repressor prior to training disrupted long-term memory without affecting either short-term memory or a middle-term memory phase called anesthesia-resistant memory. Importantly, developmental complications, a potential confound of the mouse CREB experiments (Bourtchuladze et al 1994), could not affect the results with *Drosophila*, since the expression of the repressor was only triggered 3 h prior to behavioral training (Yin et al 1994).

Memory Studies in CREB Mutant Mice

To mutate the CREB gene in mice, a neomycin resistance (neo) gene was inserted into exon 2, which was thought to contain the translation initiation site for all CREB isoforms (Hummler et al 1994). Indeed, the neo insertion resulted in the loss of the two main CREB isoforms α and Δ in the CREB $^{\alpha\Delta-}$ mice (Hummler et al 1994). However, the translation of a previously unknown CREB isoform (CREB β) starts from exon 4, and consequently the neo gene insertion into exon 2 did not disrupt the expression of this isoform. Instead, the expression level of CREB β , which is usually very low, is strikingly up regulated in the CREB $^{\alpha\Delta-}$ mutants (Blendy et al 1996). Interestingly, the levels of CREM activator (τ) and repressor isoforms (α and β) are also increased in these mutants (Hummler et al 1994). Despite the presence of CRE sites in the promoters of the CREB and CREM genes, the loss of the CREB α and Δ activators did not seem to change the transcription rates of the upregulated genes (Blendy et al 1996). Therefore, the higher levels of CREB β and CREM isoforms in the CREB $^{\alpha\Delta-}$ mutants are probably due to some other mechanism, such as an increase in the stability of their mRNAs.

The CREB $^{\alpha\Delta-}$ mutation was expected to cause developmental deficits, since the CREB gene is expressed during development and in all adult tissues (Brindle & Montminy 1992). Additionally, the transgenic expression in the pituitary of a CREB dominant-negative mutant resulted in severe growth retardation (Struthers et al 1991). Surprisingly, the CREB $^{\alpha\Delta-}$ mutants appeared healthy and groomed, they showed no hints of developmental deficits, they were not ataxic, and they showed no overt abnormalities. Neuroanatomical analysis also did not detect any gross deficits, suggesting that the CREB $^{\alpha\Delta-}$ mutation does not have a generalized impact on central nervous system development (Bourtchuladze et al 1994). It seems that the up-regulation of CREB β and CREM isoforms may have compensated in part for the loss of the α and Δ CREB isoforms. The extent of this compensation differs in different genetic

backgrounds: In the C57Bl/6 background the CREB $^{\alpha\Delta-}$ mutation is almost completely lethal, while in hybrid backgrounds the lethality is drastically reduced (Kogan et al 1996).

To determine whether the CREB $^{\alpha\Delta-}$ mutation affected memory, the mutants were tested in three distinct tasks: contextual fear conditioning, the Morris water maze, and the social transmission of food preferences (Bourtchuladze et al 1994, Kogan et al 1996). Each of these tasks is believed to be dependent upon hippocampal function. Contextual conditioning is a form of associative learning in which animals learn to fear the context in which they receive a foot shock (Dickinson & Mackintosh 1978). Control mice can be trained with a single 3 min trial and tested immediately or 24 h later, making it possible to distinguish learning from long-term memory deficits in this task. CREB $^{\alpha\Delta-}$ mutants show profound contextual memory deficits when tested 24 h but not 30 min after training, suggesting that learning and short-term memory are intact but that long-term memory is disrupted by the mutation (Bourtchuladze et al 1994). Consistent with the idea that synthesis of new proteins is critical for long-term memory formation, inhibition of protein synthesis with anisomycin blocks 24-h memory in this task, without affecting performance 60 min after training (Abel et al 1997).

Consistent with the contextual conditioning results, analysis of the CREB $^{\alpha\Delta-}$ mice in the social transmission of food preferences task (Galef & Wigmore 1983, Strupp & Levitsky 1984) also revealed a selective deficit in long-term memory. Rodents develop a natural preference for foods that they have recently smelled on the breath of other rodents (Galef et al 1988). Memory for this preference can be triggered by a single 5-min interaction with another mouse, and it lasts many days. This task is hippocampal dependent, and unlike fear conditioning, it does not involve aversive stimuli (Winocur 1990, Bunsey & Eichenbaum 1995). CREB $^{\alpha\Delta-}$ mutants show normal immediate memory, but no 24-h memory for socially transmitted food preferences (Kogan et al 1996). Thus, in two very different tasks, CREB $^{\alpha\Delta-}$ mutants showed normal short-term but abnormal long-term memory.

In the Morris water maze, mice learn to find a submerged platform in a pool of opaque water (Morris 1981). Hippocampal lesions can block spatial learning in the water maze (Morris et al 1982, Sutherland et al 1982). Training in this task takes several days, and therefore it is difficult to distinguish learning from memory deficits. The results of the analysis of the CREB $^{\alpha\Delta-}$ mice in the water maze demonstrated that these mice have profound spatial learning or memory impairments. In contrast, learning in the visible platform version of the water maze is intact in the CREB $^{\alpha\Delta-}$ mice. In this task mice have to learn to swim towards a marked escape platform. Lesion studies showed that learning in the visible platform version of the maze is not dependent

on hippocampal or neocortical function (Morris et al 1982, Sutherland et al 1982). These behavioral analyses demonstrated that the CREB ^{$\alpha\Delta$ -} mutation affects three distinct learning tasks, indicating that the mutation of CREB has widespread effects on memory. The behavioral results described also suggest that the loss of the α and Δ CREB isoforms preferentially disrupts hippocampal function.

Analysis of the CREB ^{$\alpha\Delta$ -} mutants in cued conditioning suggests that the CREB lesion also affects amygdala-dependent learning. In this task animals learn to fear a tone (conditioned stimulus) paired with electric shock (unconditioned stimulus) (Kim & Fanselow 1992, Phillips & LeDoux 1992). Just as with contextual conditioning, memory was found to be intact 30 min after training, but not 24 h later. One hour after training, memory for cued conditioning was still intact, even though memory for contextual conditioning was not (Bourtchuladze et al 1994). This temporal dissociation between the effects of the CREB ^{$\alpha\Delta$ -} mutation on cued and contextual conditioning indicates that these two tasks may be mediated by distinct neural processes.

Rat Studies with CREB Antisense Oligonucleotides

CREB is expressed during development, so structural deficits caused by deregulation of these early processes could lead to memory abnormalities. Therefore, it was important to test whether acute modulations of CREB function could also affect memory. Recently, oligonucleotides designed to bind and trap CREB mRNA have been used to address this issue. Intrahippocampal infusions of these oligonucleotides prior to training do not disrupt short-term spatial memory in rats but affect memory tested two days after training. In contrast, infusions of these oligonucleotides one day after training do not affect spatial memory tested two days after training (Guzowski & McGaugh 1997). This finding is consistent with the previously discussed results from *Drosophila* and *Aplysia*, which suggested that the critical period for CREB function is shortly after training.

Injection of the CREB antisense oligonucleotides initially resulted in a decrease in the α and Δ levels of CREB, but 14 h after injection, CREB levels were actually higher than in control rats. This rebound effect may reflect mechanisms similar to those responsible for increases in the levels of CREB β and of CREM isoforms in the CREB ^{$\alpha\Delta$ -} mutants. Surprisingly, training while CREB levels were lower (within 6 h of injection) or during the period of elevated CREB (within 20 h of injection) resulted in similar spatial memory deficits (Guzowski & McGaugh 1997). This indicates that unlike olfactory memory in *Drosophila*, either decreases or increases in CREB levels can lead to spatial memory deficits in rats.

PKA/CREB PATHWAY IN LONG-TERM POTENTIATION AND MEMORY

The most extensively studied candidate memory mechanism is long-term potentiation (LTP) (Madison & Schuman 1991, Bliss & Collingridge 1993, Malenka 1994, Malinow 1994). LTP refers to a class of long-lasting enhancements in synaptic efficacy that have the properties expected of a memory mechanism (e.g. long-lasting, associativity, reversibility). A variety of different studies have suggested that LTP is involved in memory formation (Barnes 1995, Maren & Baudry 1995, Mayford et al 1995).

LTP is not a single phenomenon; rather, there are various forms of LTP with distinct time courses and with different underlying biochemical mechanisms (Madison & Schuman 1991, Malenka 1994, Malinow 1994, Huang et al 1996). The best studied form of LTP is NMDA receptor dependent. This LTP tends to dissipate between 1 to 2 h post-induction [early LTP (E-LTP)], and it is sensitive to blockers of CaMKs but insensitive to inhibitors of protein synthesis (Frey et al 1988, Huang & Kandel 1994, Frey et al 1993). E-LTP is normal in the CA1 region of hippocampal slices prepared from CREB $^{\alpha\Delta-}$ mutant mice (J Kogan & A Silva, unpublished results).

Late-LTP (L-LTP) lasts much longer (>7 h) than E-LTP and is blocked by PKA, protein synthesis, and transcription inhibitors (Frey et al 1993, Huang & Kandel 1994, Nguyen et al 1994). This form of LTP appears to be impaired in the CA1 region of CREB $^{\alpha\Delta-}$ mice (A Silva, unpublished results). Perhaps the loss of α and Δ CREB isoforms results in lower levels of a synaptic molecule(s) that may be involved in the induction and maintenance of L-LTP. Several studies have shown clear evidence of synthesis of new proteins as early as 30 min after tetanic stimulation, suggesting that the loss of some of these proteins may contribute to the instability of L-LTP in the mutants (Silva & Giese 1994).

In parallel with the findings from the CREB $^{\alpha\Delta-}$ mice, transgenic mice that express an inhibitory form of the regulatory subunit of PKA [R(AB)], show deficits in long-term but not in short-term memory for contextual conditioning (Abel et al 1997). Like the CREB $^{\alpha\Delta-}$ mice, these transgenics with a reduction in PKA activity also show spatial learning impairments. The studies of the R(AB) transgenics also showed that the late phase of L-LTP is compromised in these mutants. Thus, two genetic manipulations that affect the PKA/CREB pathway affect LTP and memory in similar ways, suggesting that this second-messenger pathway is required for L-LTP and that this form of synaptic plasticity is required for memory formation.

cAMP inhibitors block the induction of L-LTP, while sparing the expression of E-LTP. Conversely, addition of cAMP agonists, in the absence of tetanization, induces a long-lasting potentiation that peaks within 90 min (Frey et al 1993,

Huang & Kandel 1994). It appears that under certain conditions, even E-LTP may be sensitive to manipulations of the cAMP second-messenger pathway. Studies with hippocampal slices suggest that the cAMP system, together with protein phosphatases, may form an intricate gating mechanism that determines when synapses can express LTP (Blitzer et al 1995).

CREB LEVELS AND MEMORY

Photographic Memory for Olfactory Cues

If an odor is paired with electric shocks, flies may learn to associate the two stimuli. For effective memory formation, flies require multiple training sessions that are spaced over time (Tully et al 1994). Long intervals (spaced training) but not short intervals (massed training) between the trials result in long-term memory. For example, 10 spaced training sessions, but not 48 massed training sessions given over the same total time period, resulted in long-term memory (Yin et al 1995). These findings are similar to results from other species—including *Aplysia* (Carew et al 1972), mice (Kogan et al 1996), rats (Fanselow & Tighe 1988, Mandel et al 1989), and humans (Ebbinghaus 1885)—where it has been shown that spaced training is more effective at inducing long-term memory.

In contrast, transgenic flies over-expressing a CREB activator show robust 7-day memory with only one training session (Yin et al 1995). This result suggests that CREB may be a limiting component during memory formation in *Drosophila*. Transgenic flies with a mutant activator, where Ser231 (similar to Ser133 of the mammalian CREB) was substituted for alanine, did not show 7-day memory after one training session, indicating that phosphorylation is required for the memory enhancement (Yin et al 1995).

CREB and Inter-Trial Intervals in Mice

After only a single trial in which they receive a foot shock, mice with an intact CREB gene can remember the cage in which they were shocked for weeks. Despite normal sensory perception, the same single trial can only trigger a transient (<60 min) memory in CREB^{αΔ-} mice (Bourtchuladze et al 1994). Not even training that produces maximal long-term memory in WT mice [5 trials with 1-min inter-trial intervals (ITIs)], can compensate for the profound contextual amnesia of these mutants. In contrast, two spaced trials with a 1-h ITI, which in control mice do not trigger higher levels of conditioning than a single trial, can induce robust 24-h memory in CREB^{αΔ-} mutants (Kogan et al 1996).

Similarly, a single 5-min social interaction with another mouse is sufficient to trigger a long-term memory for food preference in normal mice but not

in the mutants. In CREB $^{\alpha\Delta-}$ mice, spaced (two trials; 60-min ITIs) but not massed (two trials; 1-min ITIs) training is required for long-term memory of socially transmitted food preference. In both contextual conditioning and socially transmitted food preferences, the CREB $^{\alpha\Delta-}$ mutation had a selective effect on long-term memory, which can be compensated for by multiple spaced training trials.

In contrast to fear conditioning and social learning, mice need several days to learn the Morris water maze, most likely because of the greater difficulty of this test. Learning in the water maze is gradual, and to master the task, mice must remember what they learn from day to day. While normal mice can learn the maze in 10 days of one trial/day training, CREB $^{\alpha\Delta-}$ mutant mice are profoundly deficient when trained under the same conditions (Bourtchuladze et al 1994). As with the two other tasks, two trials/day with a 1-min ITI (massed training) does not overcome the deficits of the mutants. Rather, two trials/day with a 60-min ITI (spaced training) elicits normal spatial learning in the CREB $^{\alpha\Delta-}$ mutants (Kogan et al 1996).

Whereas training with 1-h ITIs can overcome the amnesia of the mutants in all three tasks discussed above, 10-min intervals are only partially effective, suggesting that 10 min may be just below the threshold for optimal memory induction in the mice mutants (Kogan et al 1996). However, a 10-min ITI is optimal for *Drosophila* olfactory learning (Yin et al 1995). Recent findings suggest that it may take 3–8 min for synaptic activation to trigger maximal CREB activation (phosphorylation) (Moore et al 1996). After committing the general transcriptional machinery to transcribing genes with CRE promoters, it may take a few more minutes before another round of transcription can be started. Additionally, the longer intervals between training trials may result in optimal inactivation of phosphatases (e.g. calcineurin), which may allow greater phosphorylation of CREB (Bito et al 1996, Liu & Graybiel 1996).

All together these results indicate that CREB-mediated transcription has an impact on both the number of trials and the inter-trial intervals required for the formation of long-term memories in flies and mice. Flies and mice with presumably more active CREB require less training than flies and mice with less active CREB (Yin et al 1994, 1995; Kogan et al 1996).

Massed vs Spaced Stimulation of Synapses

Five applications of serotonin, given at 20-min intervals, can trigger LTF that is blocked by inhibitors of transcription and translation. This facilitation also stimulates the growth of new synaptic connections. In contrast, a single serotonin application induces a facilitation that lasts only minutes. However, a single application of serotonin given together with antiserum against a presumed CREB repressor (ApCREB2) can trigger a long-lasting facilitation that mimics and occludes LTF that is induced with 5 spaced applications of serotonin (Bartsch

et al 1995). Therefore, in flies, *Aplysia*, and mice manipulations that appear to increase CREB activity facilitate memory processes.

Electrophysiological experiments with hippocampal slices also indicate that the interval between tetani is crucial for the induction of L-LTP. One-minute intervals between periods of high-frequency stimulation (e.g. 100 Hz) lead to unstable LTP that lasts only a couple of hours. The same tetani, however, delivered at 10-min intervals trigger a stable, long-lasting LTP (>4 h) that is sensitive to blockers of protein synthesis (Huang et al 1996). Recent results from our laboratory suggest that increasing the interval between tetani can overcome the L-LTP deficits observed in the CREB^{αΔ-} mutants (A Silva & J Kogan, unpublished observations). This parallel between the properties of L-LTP and long-term memory in the CREB^{αΔ-} mice suggests a functional connection between the two phenomena.

CREB: Repressors Which May Be Activators and Vice Versa

Researchers do not yet know how the CREB manipulations described above impair long-term memory. For example, the CREB^{αΔ-} mice lack two activator isoforms, but they have a compensatory increase in other activators and in repressors (Hummler et al 1994, Blendy et al 1996). Therefore, it is unclear whether the physiological and behavioral effects of the mutation are due to a decrease in the CREB activators or an increase in the CREM repressors. Since there is widely documented cross talk between families of transcription factors (Sassone-Corsi 1995), the increase in CREM repressors could be affecting other transcription systems.

Results in *Aplysia* suggest that the ApCREB2 antiserum facilitates the induction of LTF by inhibiting the repression of CREB-mediated transcription by ApCREB2 (Bartsch et al 1995). Indeed, transfection studies in F9 cells showed that ApCREB2 can repress the function of a CREB activator (ApCREB1) from inducing a reporter with one CRE site. However, other experiments in F9 cells have shown that ApCREB2 can be just as active as ApCREB1 at inducing the transcription of a lacZ reporter gene under the regulation of a promoter with five CRE sites (Bartsch et al 1995). Similarly, ATF4, which has about a 50% identity to ApCREB2 in the bZIP domain (Hai et al 1989, Bartsch et al 1995), can act as either a repressor or an activator of CREB-mediated transcription (Karpinski et al 1992; T Hai, personal communication). In conclusion, it is unclear whether ApCREB2 is acting as an activator or a repressor and whether LTF in *Aplysia* is due to an increase or to a decrease in CREB function.

The *Drosophila* experiments that resulted in flies with “photographic” olfactory memories used a genetic procedure that yielded high levels of CREB activator prior to training (Yin et al 1995). But is this activator really working to promote CREB-dependent transcription? For example, higher levels of

phosphorylated CREB activator could trap CREB binding protein (CBP), resulting in the down-regulation of other transcriptional pathways that require this factor (Kamei et al 1996). Alternatively, an excess of activator may overwhelm the upstream kinases, which could result in abundant nonphosphorylated CREB. Unphosphorylated CREB can act as a repressor of C/EBP(CCAAT enhancer binding protein)-induced transcription (Vallejo et al 1995).

CREB as a Gain Control Memory Device

The idea that single proteins have the capacity to function as computational devices is not new. For example, the NMDA receptor is thought to function as a coincidence detector crucial for associative learning. To be active, this ion channel requires both glutamate (presynaptic activity) and postsynaptic depolarization to induce a cascade of events that could ultimately result in synaptic changes underlying learning and memory (Bliss & Collingridge 1993).

Similarly, the CREB family of transcriptional factors may have a defined computational role as general gain control devices during memory formation. High levels of CREB activity may allow neuronal circuits to acquire memory rapidly, while low activity levels would result in slow acquisition. Accordingly, this gain control device could be critical in setting the number of trials and the ITIs required to lay down memories in a given circuit.

Evolution may have resulted in the tuning of the net levels of active CREB in any given memory system according to its storage and computational capacity. One-trial learning may be common for emotionally charged memories because the neural systems involved have adapted to rapidly process and store information that is critical for survival (McGaugh 1989, LeDoux 1995). There is a clear evolutionary advantage in learning to identify a predator. High ratios of CREB activators to repressors may allow circuits to encode this type of information after a single brief encounter.

In contrast, there may be memory systems that function best under conditions of slow acquisition. For example, the neocortex is probably the final repository of many different types of memories. Modeling studies have proposed that neocortical memories must be laid down slowly, so as not to disrupt previously stored memories (McClelland et al 1995). In the same way, low ratios of CREB activators to repressors may guard against the consolidation of spurious memories and the disruption of previously stored long-term memories.

CREB AND MEMORY: MECHANISMS

CREB Activation in Aplysia

Studies in *Aplysia* show that sustained, intensive activation of synapses leads to activation of CREB. Although during synaptic activity the catalytic subunit

of PKA can diffuse into the nucleus, the holoenzyme cannot. Imaging studies in *Aplysia* neurons have shown that 2 h following repeated applications of serotonin, the concentration of PKA catalytic subunits is increased in the nucleus, where they can activate CREB (Bacskai et al 1993). In contrast, nuclear injection of the PKA inhibitor (PKI) results in the rapid exit of the catalytic subunits from the nucleus of *Aplysia* neurons (Fantozzi et al 1994, Wen et al 1994). Thus, the regulated distribution of catalytic subunits by cAMP and PKI may have a pronounced impact on CREB function (Fantozzi et al 1994).

A single application of serotonin leads to a rapid elevation of cAMP levels in *Aplysia* neurites. In contrast, the increase observed in the soma of the neurons is much smaller (Bacskai et al 1993). This steep gradient in cAMP concentration would favor cAMP-dependent processes at synapses (e.g. short-term plasticity), while filtering out somatic processes dependent on cAMP (e.g. CREB activation and LTF) (Bacskai et al 1993).

CREB Activation in Hippocampal Neurons

Electrophysiological studies with hippocampal slices suggest that cAMP-dependent transcription is required for the maintenance of LTP (Frey et al 1990, 1993; Huang & Kandel 1994; Impey et al 1996). CREB activation can be detected in cultured hippocampal neurons using an antibody (Ginty et al 1993) specific to phosphorylated CREB proteins (Ser133 of CREB) (Deisseroth et al 1996). Studies using this approach found that while NMDAR-dependent synaptic stimulation results in CREB phosphorylation, action potential firing alone does not (Deisseroth et al 1996). This finding indicates that CREB phosphorylation is not a general marker for neuronal activity, but instead responds to specific synaptic signals engaging both NMDARs and L-type calcium channels (Deisseroth et al 1996).

However, phosphorylation of CREB at Ser133 does not strictly correlate with CREB-dependent gene expression (Bito et al 1996). Only longer synaptic stimulation (5 Hz for 180 s rather than for 18 s) results in both long-lasting CREB phosphorylation and CREB-dependent gene expression (Bito et al 1996).

What is the nature of the synaptic signal that activates CREB in the nucleus? Synaptic increases in calcium may diffuse to the nucleus and activate kinases that phosphorylate CREB. However, increases in calcium in the nucleus do not seem to be necessary for CREB phosphorylation in response to synaptic signals (Deisseroth et al 1996). Instead, calcium increases within 1–2 μm of the cell membrane appear to be crucial, suggesting that a calcium sensor (e.g. calmodulin) associated with synaptic membranes triggers events leading to the activation of CREB in the nucleus of hippocampal neurons (Deisseroth et al 1996).

In contrast to findings in *Aplysia*, PKA inhibitors fail to block CREB phosphorylation driven by synaptic stimulation in cultured hippocampal neurons

(Deisseroth et al 1996). CaMK inhibitors, however, block synaptically activated CREB phosphorylation (Bito et al 1996, Deisseroth et al 1996). Notably, CaMKIV is expressed in the nucleus at a time consistent with the appearance of phospho-CREB in hippocampal cultures (Bito et al 1996). Furthermore, antisense oligonucleotides against the α and β splice variants of CaMKIV disrupt CREB phosphorylation. Consistent with the idea that a CaMK cascade is involved in the activation of CaMKIV and CREB, an inhibitor of CaMK activity (KN-93) blocks both CaMKIV and CREB phosphorylation (Bito et al 1996). These results indicate that signaling pathways dependent on this class of kinases may be crucial for the modulation of CREB function in hippocampal neurons.

The activity of protein phosphatases may be primarily responsible for the stability of CREB phosphorylation. Okadaic acid, a substance that blocks the activity of certain protein phosphatases, slows down the time course of CREB dephosphorylation. Additionally, treatment with FK506, an inhibitor of the phosphatase calcineurin, also decreases CREB dephosphorylation. After treatment with phosphatase inhibitors, even a brief stimulation protocol (18 s) can trigger long-lasting CREB phosphorylation and the expression of *c-fos* and somatostatin. These results suggest that the duration of CREB phosphorylation and related gene expression is dependent on phosphatase activity (Bito et al 1996).

Transgenic mice, with a β -galactosidase reporter construct under the regulation of a CRE-containing promoter (CRE-LacZ), have been used to determine the type of stimuli that activate CREB-dependent transcription in hippocampal slices (Impey et al 1996). Consistent with the findings in hippocampal cultures (Deisseroth et al 1996), a brief stimulus (100-Hz, 1-s tetanus) fails to activate CREB-dependent transcription in hippocampal slices from the CRE-LacZ mice. In contrast, a more extended stimulation paradigm (three tetani given with a 5-min ITI), which induces protein synthesis-dependent LTP lasting many hours, can also trigger an increase in LacZ expression in the transgenic hippocampal slices. Both types of LTP stimuli used (a single tetanus or three tetani) increase the levels of CREB phosphorylation, although only the repeated tetanic stimulation results in increases in LacZ expression (Impey et al 1996). These results are consistent with the hypothesis that CREB-mediated transcription is required for L-LTP, and they are in agreement with results obtained in hippocampal cultures.

PKA inhibitors, however, block CREB-dependent transcription in hippocampal slices from the CRE-lacZ mice (Impey et al 1996), but not in hippocampal cultures (Bito et al 1996). Agents that increase cAMP are able to trigger a protein synthesis-dependent form of LTP in hippocampal slices that is blocked by PKA inhibitors (Frey et al 1993, Huang & Kandel 1994). In addition,

NMDAR inhibitors (APV and MK801) block CREB-dependent transcription in hippocampal cultures (Deisseroth et al 1996), but they do not block it in the transgenic hippocampal slices (Impey et al 1996). Differences between the two preparations (cell cultures and hippocampal slices), the cell types analyzed (CA3/CA1 vs CA1 only), the developmental age of the preparations, and the reporters used (CRE-LacZ vs native somatostatin and c-fos gene) could account for the differences in results obtained.

Mosaic CREB Activation

An elegant example of how CREB can respond differently in two cell populations was provided by studies with organotypic cultures of neonatal striatum (Liu & Graybiel 1996). These cultures comprise at least two distinct cell populations: striosome and matrix. After D1/D5 receptor activation (by SKF-81297) only DARPP-32 staining cells in striosomes, but not in matrix, show stable CREB phosphorylation (30 min after induction). In contrast, stimulation with an L-type calcium channel agonist (BAY 8644) increases CREB phosphorylation in matrix neurons, while driving only moderate increases in striosomes (Liu & Graybiel 1996).

Similar to hippocampal neurons, inhibition of phosphatases in neonatal striatal cultures prolong the phosphorylation of CREB and increased c-Fos expression (Liu & Graybiel 1996). These results demonstrate that the time course of CREB phosphorylation following induction is a critical parameter regulating CREB-dependent transcription. The advantage of spaced training may be partly due to this (Kogan et al 1996). Spaced training may allow the inactivation of phosphatases and the consequent potentiation of CREB activation.

CREB and Synaptic Remodeling

There is a considerable amount of data indicating that behavioral long-term facilitation in *Aplysia* triggers not only stable increases in neurotransmitter release, but also results in synaptic structural changes that can even include the growth of new synapses. There are many parallels between the actions of agents affecting LTF and those affecting these structural changes, a finding which strengthens the connection between synaptic function, structural changes, and memory formation in *Aplysia* (Bailey & Kandel 1994). A single pulse of serotonin paired with an injection of an antibody against a CREB blocker (ApCREB2) is sufficient to induce the growth of new synapses, just as it is sufficient to induce LTF (Bartsch et al 1995). Similar to LTF, the growth of new synapses normally requires multiple spaced serotonin applications. These results indicate that CREB-dependent protein synthesis is involved in the growth of new synapses during long-term memory formation in *Aplysia*.

Studies in the neuromuscular junction of *Drosophila* larvae have also implicated CREB in developmental changes in synaptic function (Davis et al 1996). These studies dissociated developmental changes in synaptic structure from those in neurotransmitter release, suggesting that CREB is required for the latter but not for the former (Davis et al 1996). The same activator and blocker constructs used in the fly behavioral experiments described above were also used in these synaptic studies. Presumably, the increase in bouton number and quantal content in the larvae neuromuscular junction of *dnc* mutants (*Dunce*; cAMP phosphodiesterase mutants) is due to their increased cAMP levels (Schuster et al 1996a,b). The dCREB2-b (CREB repressor) decreased the neurotransmitter quantal content of *dnc* mutants without affecting the increased bouton number. These results suggest that the increase in bouton number, presumably driven by the cAMP increases in *dnc* mutants, is not CREB-dependent (Davis et al 1996).

Expression of dCREB2-a (CREB activator) does not increase transmitter release or bouton number at the larvae neuromuscular junction of normal flies. However, in conjunction with a fasciculin II mutation, which increases bouton number without affecting neurotransmitter release, expression of the CREB activator results in greater neurotransmitter release. This result suggests that CREB triggers the expression of factors required for neurotransmitter release and that an increase in these factor(s) alone is not sufficient for driving an increase in release (Davis et al 1996). CREB may regulate the expression of a critical component of the synaptic release machinery, which is normally present in limiting amounts. Therefore, under certain conditions (e.g. after sprouting), increasing CREB activity could lead to increased synthesis of this limiting release factor and to greater neurotransmitter release (Davis et al 1996). Interestingly, CREB is thought to regulate the expression of the synapsin I gene, an important component of the neurotransmitter release machinery.

CONCLUSION

The findings reviewed here represent a significant advance in our understanding of memory formation. In *Aplysia*, *Drosophila*, mice, and rats, CREB-dependent transcription appears to be a crucial component of long-term memory formation. The levels of active CREB may also be a determinant of the amount and schedule of training required for long-term memory. Studies in *Aplysia*, *Drosophila*, and mice all suggest that deficits in CREB can be compensated for with extended spaced training, while increases in CREB can circumvent the requirement for extended spaced training.

CREB's involvement in memory formation is not restricted to certain forms of memory, such as aversive conditioning, but appears to have a much

broader impact on memory formation. Tests as diverse as olfactory conditioning in flies and fear conditioning, spatial learning, and social learning in mice revealed the involvement of CREB in memory formation. Studies of CREB function are starting to identify not only the mechanisms responsible for its activation in neurons, but also the molecular processes that its transcriptional products trigger. For example, synaptic restructuring, believed to be crucial for memory formation, seems to be dependent on CREB transcription. The importance of CREB in memory likely also extends to humans, where the mutation of CBP results in the Rubinstein-Taybi Syndrome (Petrij et al 1995), a condition characterized by mental retardation. Thus, the findings from these studies not only further our understanding of memory, they may also influence the development of treatments for cognitive disorders associated with memory loss.

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