Deficient Long-Term Memory in Mice with a Targeted Mutation of the cAMP-Responsive Element-Binding Protein

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Summary

The cAMP-responsive element-binding protein (CREB) has been implicated in the activation of protein synthesis required for long-term facilitation, a cellular model of memory in Aplysia. Our studies with fear conditioning and with the water maze show that mice with a targeted disruption of the α and δ isoforms of CREB are profoundly deficient in long-term memory. In contrast, short-term memory, lasting between 30 and 60 min, is normal. Consistent with models claiming a role for long-term potentiation (LTP) in memory, LTP in hippocampal slices from CREB mutants decayed to baseline 90 min after tetanic stimulation. However, paired-pulse facilitation and posttetanic potentiation are normal. These results implicate CREB-dependent transcription in mammalian long-term memory.

Introduction

The synthesis of new proteins is a pivotal requirement of long-term memory (reviewed by Davis and Squire, 1984; Matthes, 1989). A variety of inhibitors of protein and RNA synthesis have been shown to block effectively long-term, but not short-term, memory. Since these inhibitors have a general effect on all protein synthesis, they cannot be used to identify and study the specific components and mechanisms involved in memory consolidation.

Members of the cAMP-responsive element-binding protein (CREB) family of transcription factors activate the transcription of genes with CRE sequences in response to increases in the intracellular concentration of cAMP and Ca2+ (reviewed by Lee and Masson, 1993). Interestingly, trapping of CREB-like proteins by oligonucleotides with CRE sequences injected into Aplysia cultured neurons selectively blocked long-term, but not short-term, facilitation of neurotransmitter release (Dash et al., 1990), a model for a nonassociative learning response in Aplysia. Additionally, genetic studies in Drosophila have demonstrated the involvement of cAMP-dependent processes in associative learning and early memory (Tully, 1991). These data and other preliminary results (T. Tully, personal communication) suggested the involvement of cAMP-dependent transcription and CREB-like proteins in learning and memory.

To determine whether CREB was required for memory consolidation in mammals, we have studied mice with a targeted mutation of the CREB gene (CREB−/− mice, Humm ler et al., 1994). Previous molecular analysis found that these mice lack the α and the δ isoforms of CREB (Humm ler et al., 1994). Studies with fear conditioning and with the water maze reported in this manuscript show that CREB−/− mice have normal short-term memory but deficient long-term memory. Parallel electrophysiological studies in hippocampal slices revealed that LTP in CREB mutants is small and decays to baseline within 1.5 hr of induction even though two other forms of synaptic plasticity, paired-pulse facilitation (PPF) and posttetanic potentiation (PTP), are normal. Interestingly, an accompanying manuscript shows that disrupting CREB function in Drosophila blocks long-term memory (Yin et al., 1994 [this issue of Cell]).

Results

General Observations

CREB is ubiquitously expressed, but its expression is greater in some tissues, such as brain, than in others (Hummler et al., 1994). Thus, the loss of the α and δ isoforms could conceivably lead to generalized neuroanatomical and neurophysiological deficits. The CREB−/− mutants, however, appear healthy and groomed, they show no hints of ataxia or of any other motor disorders, and they show no overt abnormalities. Consistent with these observations, light microscopy analysis of thionin-stained coronal sections obtained throughout the rostral-caudal axis of the CREB−/− forebrain did not detect any gross anatomical abnormalities (Figure 1).

Fear Conditioning Studies

Fear conditioning is a form of associative learning found in many species (Dickinson and Mackintosh, 1979). We first tested whether the CREB−/− mutation altered nociceptive reactions to shock in the mutants since changes in pain sensitivity affect conditioning (Fanselow and Bolles, 1979a, 1979b). We determined the minimal amount of current required to elicit three common reactions to shock: flinch, run/jump, and vocalization. Figure 2 demonstrates that for both CREB−/− mutants and controls, each of these three stereotypical behaviors was elicited with similar current intensities (p = 0.42), indicating that pain sensitivity is normal in CREB−/− mutants. This data also provides evidence that the mutation caused neither conscious nor motor performance deficits; that is, the mutants could jump, flinch, and vocalize normally to sensory stimuli.

Animals learn to "fear" a previously neutral stimulus (conditioned stimulus [CS]) simply because of its temporal association with an aversive stimulus (unconditioned stimulus [US]), such as a foot shock. Conditioned animals, when exposed to the CS, tend to retrace from all but respiratory movements by freezing (Fanselow and Bolles, 1979a; Miller and Weiss, 1969). Freezing responses can be triggered with two different types of CS, each with distinct
neuroanatomical substrates (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). In cued conditioning, the CS is simply a tone, and lesions of the amygdala (but not the hippocampus) disrupt this type of conditioning (Kim et al., 1993; Phillips and LeDoux, 1992). Rodents also can be conditioned to the context in which they were exposed to the US (contextual conditioning), and this type of conditioning is known to be dependent on both hippocampal and amygdaloid function (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). These tasks are particularly appropriate for the study of memory because robust learning can be triggered with a single 3 min trial.

Figure 3A indicates that CREB-/- mutants and controls showed identical levels of freezing immediately after training (p = 0.98). A similar result was also obtained when contextual conditioning was tested 30 min after training (p = 0.58; Figure 3B). Both groups spent approximately 40% [controls (41% ± 4%), mutants (39% ± 4%)] of a 5 min testing interval without any perceptible movement. Previous studies (Fanselow, 1986; Rudy and Morledge, 1994) and our own experiments with mice (data not shown) indicate that freezing responses measured immediately after training are the result of associative processes. All together these results demonstrate that the CREB-/- mice can be contextually conditioned, and they also indicate that the CREB-/- mutation did not prevent learning or interfere with contextual memory for at least 30 min.

In striking contrast with the group tested 30 min after training, a second group of CREB-/- mutants displayed little freezing (12% ± 5%) when tested 1 hr after training (Figure 3C) even though the controls showed extensive freezing (42% ± 6%). Figure 3D shows the results of contex-

![Figure 1. Coronal Forebrain Section of a CREB Mutant and Control Mice](image)

Coronal brain sections (15 μm) from a CREB mutant (A) and control littermate (B) stained with 1% thionin. The rostral-caudal level of the mutant and control slices was matched for the hippocampus.

Abbreviations: Am, amygdala; CA1 and CA3, pyramidal cell fields of the hippocampus; DG, dentate gyrus; NC, neocortex; Th, thalamus; and CP, caudate putamen.

Figure 2. Sensitivity to Shock in CREB Mutants and Controls

To evaluate the sensitivity of the mice to shock, we determined the minimal amount of current required to elicit three stereotypical behaviors in CREB mutants (n = 9) and controls (n = 7); flinching, running/jumping, and vocalizing. ANOVA showed that there was no difference between the two genotypes [F(1,14) = 0.707, p = 0.42].
tual conditioning experiments with another group of mice tested 24 hr after training: controls once again showed significantly (p < 0.001) more freezing (48% ± 7%) than did mutants (12% ± 6%). These results indicate that the CREB− mutation resulted in an abrupt disruption of memory between 30 and 60 min posttraining.

It is possible that freezing responses measured 30 min after training are not specific to the training context and instead are the result of general indiscriminate fear of any novel context. To test this hypothesis, we trained another group of CREB− mice and controls and tested them 30 min later in a novel context (Figure 4B, Pre-CS). Unlike the training context, this novel context triggered little or no freezing (5% ± 1% and 5% ± 1% for mutants and controls, respectively).

For tests of cued conditioning, we measured freezing in response to the tone that had previously been paired with the foot shock during training (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). To avoid the confounding effects of contextual conditioning, cued conditioning was tested in a novel context. The mice were placed in a novel chamber for 3 min, and then they were exposed to the CS for another 3 min. Cued conditioning was found to be normal 30 and 60 min after training in CREB− mutants, but it was abnormally low 2 and 24 hr after training (Figure 4). These results are consistent with the hypothesis that long-term memory, but not short-term memory, is disrupted in the CREB− mutants. The fact that cued conditioning produced nearly normal freezing responses in CREB− mutants tested 1 hr after training indicates that the time course of the amnesia in CREB− mutants differs for different tasks involving different neuroanatomical structures (Kim and Fanselow, 1992; Kim et al., 1993; Phillips and LeDoux, 1992). Previous studies had shown that the time course of the amnesia caused by protein synthesis inhibitors also differed for different tasks, training protocols, and species (Davis and Squire, 1984; Flood et al., 1975). Nevertheless, our results (Figures 3 and 4) suggest that memory for contextual conditioning is more affected by the CREB mutation than by memory for cued conditioning. It is noteworthy that in our cued conditioning (2 hr and 24 hr) experiments, two mutants tested showed freezing similar to that of controls (35% and 37%). In our contextual conditioning experiments, the performance of one CREB mutant tested 24 hr after training was also similar to that of controls (39%).

We also tested whether additional training might compensate for the memory deficits of the CREB− mutants. Thus, we gave CREB− mice and controls three additional CS/US pairings 3 days after training with one CS/US. Indeed, CREB− mutants trained with a total of four foot shocks showed contextual conditioning (42% ± 4%) 24 hr after training. However, even with four shocks, the CREB− mice were still worse than controls (66% ± 9%; F(1,21) = 27.36, p < 0.001; n = 13 and n = 10, respectively) trained the same way, under the impact of the CREB mutation on memory. Nevertheless, these results show that the mutation of CREB did not block all long-term memory.
Our results are consistent with the model (Kim et al., 1992; Rudy and Morledge, 1994) that fear conditioning is dependent on at least two temporally distinct memory processes since we show that the CREB mutation did not affect an early memory phase lasting up to 30 min; it only affected a later phase.

Water Maze Studies
We also tested the CREB− mutants in the Morris water maze tasks (Morris, 1981; reviewed by Brandeis et al., 1989). In this test, animals are placed in a round pool, and they escape the water by learning to swim to a platform. In the "spatial" version of the water maze, a test known to require hippocampal function (Morris et al., 1982; Sutherland et al., 1982), mice learn to navigate to a submerged platform using distal cues in the room. A disruption of memory, as was found in the fear conditioning studies, should affect performance in this task.

We started by testing mice on the spatial version of the water maze with a single training trial each day. Figure 5A shows that the overall performance of control mice was significantly better than that of CREB− mutants during training in this task (p < 0.01). It is noteworthy that the performance of the mutants did not improve during training (p = 0.13). Time to reach the platform, however, is only one of a number of different measures of spatial learning in the water maze (Brandeis et al., 1989). Therefore, we also tested the mice in a probe trial in which the platform was removed, and the trained mice were allowed to search for it for 60 s. Figure 5D shows that, while controls searched selectively for the absent platform (spending 48% ± 3% of their time swimming in the quadrant in which the platform had been during training [p < 0.001]), CREB− mice searched randomly in all four quadrants (p = 0.33). Controls also crossed the position of the platform more frequently than did CREB− mutants (Figure 5C). Taken to-
Deficient Long-Term Memory in CREB Mice

As in our fear conditioning experiments, we next determined whether more intensive training could partially alleviate the performance deficit of CREB mice in the water maze. We tested a new group of mutants and controls with three blocks of four trials per day (total of twelve trials per day; with a 1 min intertrial interval) for three days. Figure 6A shows that both mutants and controls improved (p < 0.05) and that with intensive training, the performance of the CREB mice in acquisition trials was indistinguishable from that of controls (p = 0.45). After 3 days of training, the mutants spent 40% ± 6% of the 60 s probe trial searching for the missing platform in the training quadrant. Nevertheless, analysis of the probe trials indicate that the searching of controls were significantly (p < 0.01) more selective than were those of CREB mutants (Figure 6B and 6C). Therefore, with this training schedule, the deficit in the CREB mutants was too subtle to be detected during acquisition but not too subtle to be detected during probe trials. It is noteworthy that the performances of CREB mice and controls were identical in the first day of training both in the one trial per day (p = 0.92) and in the twelve trials per day experiments (p = 0.38), demonstrating that initial differences in motivation, perception, swimming speed, or coordination could not account for the impairment of the CREB mutants.

These results show that CREB mutants are impaired in the water maze tasks, which corroborates and extends our fear conditioning experiments. Interestingly, intensive training partially compensates for the deficits shown in both cases. Other cAMP-responsive transcription factors might have compensated partially for the loss of the α and δ isoforms of CREB in the brain (Hummler et al., 1994), cAMP-independent mechanisms (Bliss and Collingridge, 1993) might contribute to long-term memory, or both (Matthijs, 1999). Interestingly, mice with a targeted mutation of a Ca2+-calmodulin-dependent kinase II (αCaMKII) revealed a more dramatic deficit in the water maze even when trained with 12 trials per day for 5 days (Silva et al., 1992). However, analysis of that mutation implicated the

Figure 5. One-Trial-a-Day Training in the Morris Water Maze

(A) CREB mutants (n = 11) and controls (n = 8) were trained with one trial per day for 15 days. The average time (in seconds) to reach the submerged platform was plotted versus trial number. ANOVA with repeated measures showed that the overall performance of controls was significantly better than that of CREB mutants (F[1,16] = 11.14, p < 0.01). However, planned comparisons between controls and CREB mutants for each of the 15 trials revealed no significant differences.

(B) The graph shows the results of a probe trial given after 15 days of training. ANOVA with dependent measures showed that controls searched selectively in the quadrant in which the platform was located during training (F[1,64] = 68.56, p < 0.001), while CREB mutants did not (F[1,64] = 0.06, p = 0.33). Planned comparisons between the time that controls spent in the training quadrant and the time spent in all other quadrants confirmed significant differences (T versus AL, p < 0.001; T versus AR, p < 0.001; and T versus OP, p < 0.001).

(C) The control mice (F[3, 30] = 90.638, p < 0.001), but not the CREB mutants (F[3,38] = 2.044, p = 0.13), crossed the exact site where the platform was located during training more often than they crossed equivalent sites in the other three quadrants. A planned comparison between control and mutants revealed a significant difference in the number of platform crossings in the training quadrant (F[1,64] = 84.59, p < 0.001).

Abbreviations: T, training quadrant; AR, the adjacent quadrant to the right of T; AL, the adjacent quadrant to the left of T; and OP, the quadrant opposite to T.
\(\alpha\text{CaMKII}\) in the induction of LTP and in learning (Silva et al., 1992), while the CREB mutation does not seem to affect learning but only memory. Additionally, intensive training is capable of partially compensating for the memory deficit in the CREB mutants but not for the learning deficit in the \(\alpha\text{CaMKII}\) mutants tested with the water maze (Silva et al., 1992) or with fear conditioning tasks (R. B. and A. J. S., unpublished data).

**Hippocampal Electrophysiological Analysis**

Since current models suggest that hippocampal LTP may be a mechanism underlying learning and memory (Bliss and Collingridge, 1993; Eichenbaum and Otto, 1993) and since our behavioral analysis of the CREB-\(\alpha\) mice focused primarily on hippocampal-dependent tasks, we tested hippocampal LTP in CREB-\(\alpha\) mutants. LTP was measured in hippocampal slices of adult mice (~3–4 months) by stimulating Schaffer collaterals and recording field excitatory postsynaptic potentials (fEPSPs) in stratum radiatum of CA1. After collecting stable baseline responses for at least 30 min, LTP was induced by a train of 100 pulses at 100 Hz. We followed LTP for at least 2 hr after tetanic stimulation since the amnesia of CREB-\(\alpha\) mutants was detected within the first hour after conditioning. The experiments with mutant mice were interleaved with the experiments with normal controls.

Figure 7A shows data from wild-type and mutant slices in which LTP was followed for 2 hr. Wild-type slices (6 slices from 5 animals) showed stable LTP at 2 hr, whereas the LTP of mutant slices (10 slices from 9 animals) decayed to baseline 90 min after the tetanus and remained at that level until the end of the experiment (Figure 7A). Interestingly, previous studies showed that a single tetanus similar to the one used in this study does not result in a measurable increase in cAMP in hippocampal slices (Frey et al., 1993). This suggests that if there is an increase in cAMP triggered by this tetanus, it is limited perhaps only to the tetanized synapses and might therefore be difficult to measure in slice homogenates. Although in the CREB-\(\alpha\) mutants both LTP and memory decay within the first hour, it is unclear whether the decay in LTP is related to the decay

\[F[1,48] = 53.99, p < 0.001\) and mutants \(F[1,48] = 11.41, p < 0.01\) searched selectively for the platform in the training quadrant. Planned comparisons between the time that controls spent in the training quadrant and the time spent in all other quadrants showed significant differences (T versus AL, \(p < 0.001\); T versus AR, \(p < 0.001\); and T versus OP, \(p < 0.001\)). Similarly, planned comparison analysis between controls and mutants revealed that mutants spent significantly less time in the training quadrant than did controls \(F[1,48] = 7.88, p < 0.01\).

(C) The control mice \(F[3,26] = 20.81, p < 0.01\) as well as the CREB mutants \(F[3,26] = 10.97, p < 0.01\) crossed the exact site where the platform was located during training more often than they crossed equivalent sites in the other three quadrants. However, a planned comparison between genotypes revealed that mutants crossed the platform site in the training quadrant significantly fewer times than did controls \(F[1,48] = 9.44, p < 0.01\).

**Figure 6. Intensive Training in the Morris Water Maze**

(A) CREB mutants \(n = 7\) and controls \(n = 7\) were trained with three blocks of four trials per day (for 3 days). The average time to reach the submerged platform was plotted versus trial block. ANOVA with repeated measures revealed that there was no significant difference between the overall performance of the controls and mutant mice \(F[1,12] = 0.6046, p = 0.45\). Post-hoc analysis indicated that both improved from day 1 to day 3 \(p < 0.05\).

(B) The graph shows the results of a probe trial given after 3 days of training. ANOVA with dependent measures revealed that both controls
Deficient Long-Term Memory in CREB+ Mice

We also found that even as early as 10 min after tetanic conditioning, the level of synaptic potentiation in CREB+ mutants (124% ± 3%) was significantly lower (p < 0.05) than that in controls (144% ± 6%). However, peak PTP, measured during the first 2 min after the tetanus, was no different (p > 0.05) in mutants and controls (238% ± 6% and 253% ± 10%, respectively; Figure 7A).

An analysis of input-output curves in untetanized slices revealed an approximately 1.6-fold increase in the ratio of fEPSP slope to fiber volley amplitude (fEPSP/fv) in the mutants (Figure 7B). This indicates that for a given fiber volley (i.e., presynaptic axonal activation), there is a larger postsynaptic response in untetanized mutant neurons as if some of the synapses were already in a potentiated state in the mutants. Alternatively, the disruption of the CREB transcription factor might have prevented the expression of a protein required for the down-regulation of neurotransmitter release.

To establish a possible locus for the fEPSP/fv increase in CREB mutants, PPF was analyzed. PPF is an enhancement of synaptic responses believed to be the result of presynaptic mechanisms (McNaughton, 1982; Muller and Lynch, 1989; Hess et al., 1987; Zaletsky and Nicoll, 1990). Figure 7C shows that, at all interpulse intervals measured, PPF was indistinguishable in mutant (n = 9 slices from eight mice) and wild-type slices (n = 10 slices from 15 mice). This argues against a possible increased probability of release in mutants accounting for their greater basal fEPSP/fv ratio. If the initial probability of neurotransmitter release was higher, PPF would be expected to be lower in the mutants. Alternative explanations include differences in the number of release sites for transmitter, in the postsynaptic regulation of membrane potential, or in postsynaptic receptor responsiveness. Future studies with single-cell recordings such as whole-cell patch clamping might distinguish among these possibilities. Nevertheless, finding normal PPF and PTP indicates that the CREB mutation does not affect all aspects of synaptic function.

Discussion

Studies with Aplysia cultured neurons suggest the involvement of CAMP and CREB in the expression of genes required for long-term synaptic plasticity (Dash et al., 1990; Kaang et al., 1993; Alberini et al., 1994). In remarkable parallel with these findings, a late stage of hippocampal LTP appears to be dependent both on protein synthesis and on an increase in PKA activity triggered by CAMP (Stanton and Survev, 1984; Frey et al., 1988, 1983; Reymann, 1993; Huang and Kandel, 1994). Importantly, forward and reverse genetic studies in Drosophila showed that mutations in genes involved in CAMP signaling pathways (cAMP phosphodiesterase, adenylyl cyclase, and protein kinase A) affected olfactory learning (reviewed by Tully, 1991). All of these observations suggest the involvement of CAMP-dependent processes in memory formation. In this manuscript, we evaluate the role of CREB-dependent
mechanisms in memory by studying mice with a genetic disruption of the α and δ isoforms of CREB.

A key aspect of our results is that the loss of CREB function disrupts long-term memory for cued and contextual conditioning without affecting initial memory. Consequently, it is unlikely that either motor, sensory, motivational, or attention abnormalities could explain the memory deficits of the CREB−/− mutants. These results are consistent with the hypothesis that CREB-dependent transcription is required for memory consolidation (Dash et al., 1990). An accompanying manuscript (Yin et al., 1994) also reports compelling evidence for the involvement of CREB in Drosophila memory formation. Induction of a dominant negative CREB transgene, which blocks cAMP-responsive transcriptional activation, disrupts long-term memory but not learning, early memory, or several performance measures (Yin et al., 1994). Other behavioral studies in different species have also demonstrated that formation of long-term, but not short-term, memories can be blocked by agents that interfere with transcription or translation (Davis and Squire, 1984).

It is possible that the CREB−/− mutation in mice has subtle effects on developmental processes in brain structure(s), such as the hippocampus, that are involved in memory formation (Elkenbaum and Otto, 1992). Hippocampal lesions, however, are probably not the cause for the memory deficits in CREB mutants since CREB mutant mice showed normal retention of hippocampal-dependent memories for 30–60 min, while animals with hippocampal lesions showed normal recall only for a few seconds to minutes after training in a variety of hippocampal-dependent tasks (Squire, 1992).

Our studies with the water maze suggest that the memory deficits observed in the mutant mice are not restricted to Pavlovian tasks. The water maze studies also confirmed that extended training can partly overcome the profound memory deficits of the mutants, suggesting that other mechanisms can compensate for the mutation of CREB. Consistent with the hypothesis that the CREB mutation has a selective effect on memory, we found that the CREB−/− mutants were normal in most other aspects studied. They appear groomed and healthy, and they gain weight normally. We found no evidence of ataxia or of any other movement and posture disorders. We did not observe any tremors, snakes, head nods, unusual sudden bursts of activity, or any other hints of possible seizure activity. Their nociceptive reactions to a range of noxious stimuli were normal, and they showed normal freezing responses. Our cued conditioning studies demonstrate that they can hear the tone used for cued conditioning and that they show normal memory 60 min after training in this task. Contextual conditioning studies indicate that the CREB−/− mutants can distinguish between contexts, suggesting that the mutation did not disrupt perception. That hippocampal- and amygdaloid-dependent learning are normal in CREB−/− mutants was suggested by observing normal memory for both cued (90 min) and contextual (30 min) conditioning. Additionally, our water maze studies indicated that they also have the required motivation and motor coordination to search for the hidden platform in the Morris water maze.

Despite the ubiquitous expression of the CREB gene, the CREB−/− mutation has a surprisingly restricted impact on developmental and behavioral processes. Previous studies of the CREB−/− mutants found a 2- to 3-fold increase in the expression of the cAMP-responsive modulator, another CREB-like transcription factor (Hummier et al., 1994). The expression of both the activator (α and δ) and the repressor (γ) isoforms of cAMP-responsive modulator was increased. Disruption of the α and δ isoforms of CREB, however, did not affect another closely related transcription factor, activating factor 1 (Hummier et al., 1994). Furthermore, a new CREB splice variant has recently been identified. This isoform lacks part of the transactivation domain but is still capable of transcriptional activation. This isoform is present in wild-type mice and in the CREB−/− mutants (J. B. and G. S., unpublished data). The presence of a CREB isoform as well as the up-regulation of the cAMP-responsive modulator could explain the unexpected restricted impact of the disruption of the CREB mutation.

To address the physiological basis of the long-term memory deficits of the mutants, we studied LTP, a candidate memory mechanism (Bliss and Collingridge, 1993; Elkenbaum and Otto, 1993). Consistent with Hebbian models of learning and memory, our electrophysiological studies found abnormal LTP in CREB−/− mutants. The LTP of these mutants is smaller and decays to baseline within 90 min of induction. In contrast, other forms of synaptic plasticity, such as PPF and LTP, are normal. Future studies will determine whether the decay in LTP is correlated with the decay in memory or whether these two phenomena are unrelated consequences of the CREB−/− mutation.

The behavioral and electrophysiological findings reported in this manuscript identify CREB as an important component of memory consolidation in mammals. These findings, together with studies in Aplysia and Drosophila, strongly suggest that CREB is an evolutionary conserved component of the molecular cascade of events leading to memory consolidation.

**Experimental Procedures**

**Raising and Genotyping the Mice**

In the experiments described, controls always included littermates of the mutants. At 4–5 weeks postnatally, the animals were weaned, and their genotypes were determined with Southern blot analysis of tail DNA samples. All experiments were done with mice that were 3–6 months old. The mice were kept on a 12:12 light-dark cycle, and the experiments were always conducted during the light phase of the cycle. With the exception of testing times, the mice had ad lib access to food and water. The Cold Spring Harbor Laboratory animal facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care, and animals are maintained in accordance with the Animal Welfare Act and the Department of Health and Human Services guide.

**Nociception Tests**

We measured the sensitivity of mutants and controls to foot shock. In this test, the mice were placed in the conditioning chamber (described below) and were then given 1 s shocks of increasing intensity. The interval between shocks was 10 s, and the sequence of currents used was as follows: .075 mA, .1 mA, .25 mA, .35 mA, .45 mA, .55 mA, .65 mA, and .75 mA. We determined the level of current required to elicit the following behaviors: flinching, running/jumping, and vocalization. These experiments were performed blindly.
Deficient Long-Term Memory in CREB Mice

Fear Conditioning
Our conditioning chamber was a soundproof box (72 cm x 51 cm x 28 cm). With surrounding noise measuring 75 dBA, a white noise box registered 68 dB. A clear Plexiglas window (2 cm thick, 12 cm x 20 cm) allowed the experimenter to observe the mice. To provide background noise (72 dB), a single computer fan was installed in one of the sides of the isolation chamber. The conditioning chamber (33 cm x 20 cm x 22 cm) is made of transparent Plexiglas on two sides and metal on the other two. Each of the metal sides has a speaker and a 24 V light. The chamber has a 36-bar insulated shock grid floor. The floor is removable, and after each experimental subject, we cleaned it with 75% ethanol and then with water. Each bar (1.5 cm in diameter) is connected through a harness to a Master Shocker (Model 604039S), a device that delivers scrambled shocks. The Sonalert speaker used to deliver the conditioned stimulus is connected to a power source with an adjustable current output that was kept constant throughout the experiments. Only one subject at a time was present in the experimental room. The other subjects remained in their home cages. Each subject was carried to the behavioral room (5 ft away) in a cage with shavings from its home cage, and the proceedings were filmed.

For the conditioned and contextual conditioning experiments, mice were placed in the conditioning chamber for 2 min before the onset of the discrete CS (lasted 30 s at 2800 Hz and 65 dB of sound). In the last 2 s of the CS, they were exposed to the US (at 0.75 mA for 2 s of continuous foot shock). After the CS/US pairing, the mice were left in the conditioning chamber for another 30 s and were then placed back in their home cages. Conditioning was assessed by measuring freezing: the animals were judged as either completely immobile or not completely immobile (respiratory movement). Motor activity was measured at 5 s intervals. If the animal did not move in that interval, we scored it positively. For contextual conditioning, freezing was measured for 5 min (cumulative) in the chamber in which the mice were trained. Groups 1, 2, and 3 were tested at 0.5 hr, 1 hr, and 24 hr after training, respectively. In cued conditioning, the mice were placed in a novel context (triangular cage with smooth flat floor and with lid odorant) for 3 min (pre-CS test), after which they were exposed to the CS for 2 s (CS/US test). Groups 1, 2, and 3 were tested at 0.5 hr, 1 hr, 2 hr, and 24 hr after training, respectively. For both the cued and contextual conditioning experiments, the mice were trained in three sessions. Mice from each session were tested at each of the different time points tested. Since the experiments were done in a balance manner and since the training procedure was identical, we pooled all of the training data for each of the cued and contextual conditioning experiments.

In the experiments with additional training trials, three CS/US pairings were given, with a 1 min interval between shocks. The experimenter was blind to the genotype of the subjects.

Water Maze Tests
Mice tested in the water maze were extensively handled (2 min every day for 10 days). We tested approximately equal numbers of male and female mice 12–15 weeks old. Only mice housed in groups of 3 (those heavier than 25 g per mouse) or 4 (those lighter than 25 g per mouse) per standard mouse cage were used. Each experiment (2 hr), the mice were brought to the water maze room to allow them to aclimatize to it. Our pool is 1.2 m in diameter and is made of polypropylene white plastic. The test subjects were kept on shavings underneath the pool to eliminate directional affinity and auditory cues. Underneath the pool, a thermoregulated spiral coil keeps the water temperature at 28°C ± 1°C. The temperature of the room is also kept at 28°C.

The water in the pool is made opaque with nonoxic white paint to hide the escape platform. The Plexiglas platform is 10 cm x 10 cm, and its borders are slanted and scarred to help the mice climb onto it. The water surface is 10 cm from the rim of the pool, and the white plastic inner wall is always carefully wiped to eliminate any local cues. The rim of the pool is 1.5 m from the nearest visual cue. The walls of the room are painted white, and each wall has a single salient cluster of cues (e.g., a dark soccer ball). The room has adjustable indirect illumination. A camera is fixed to the ceiling of the room, 1.5 m from the water surface. The camera is connected to a digital tracking device (VP118, HVS Image). The tracking information is then processed by an IBM computer with the HVS Water Maze software.

On the first day of training, the mice are placed on the platform for 30 s. Then they were allowed one 30 s practice swim as well as three platform climbs. They are allowed to rest for another 30 s on the platform, and then testing was initiated. During training in the four-plate platform test of the Morris maze, the platform was not marked by any cut, and it was kept in the same place throughout training. The mice were divided into groups of two or three animals. Each of these groups was trained in a different platform site to avoid quadrant biases. In a block of trials, the starting position of the mice was varied randomly so as to ensure that all four starting positions were used. The mice were either trained with twelve trials per day for 3 days or with one trial per day for 15 days. Each trial started with the mice facing the wall of the pool and ended when they climbed the platform. The mice were not allowed to search for it for more than 60 s, after which they were placed on the platform. At the end of each trial, they always stayed on the platform for 60 s. The mice were tested at approximately the same time every day.

In the probe test used, we removed the platform and measured the time the mice spent in the quadrant in which the platform was located during training, and we counted the number of times the mice crossed the platform site during searching. All experiments were done blind to the genotype of the subjects.

Studies of LTP
Hippocampal slices (400 µm thick) were prepared by standard methods. The mice tested were used in behavioral studies 2–3 weeks before. Experiments were conducted in medium containing 125 mM NaCl, 2 mM KCl, 1.25 mM NaHPO4, 26 mM NaHCO3, 10 mM D-glucose, 1.5 mM MgSO4, and 2.5 mM CaCl2. Stimulating bipolar concentric and recording (1–5 MΩ filled with cerebrospinal fluid) electrodes were placed in stratum radiatum in CA1 to record LTP. Stimulus intensity was determined by first using a range of stimulus strengths to define a synaptic input-output curve and then by choosing an intensity that produced a response that was 20–50% of the maximum evoked response with a 250 ms pulse width (stimulation rate, 0.033 Hz or 0.067 Hz). This intensity was then used for the baseline, PPF, tetanic stimulation, PTP, and LTP. Initial slope of EPSPs was used in all analysis. In the LTP studies, only those slices that had a stable baseline for 20 min were included in the analysis. Experiments were carried out in a submerged slice chamber at room temperature (20°C–23°C). In the majority of the studies, the experimenter was blind to the genotype of the animals.

Data Analysis
Results from male and female mice were combined since we did not find any significant differences between them. For the analysis of experiments, we used analysis of variance (ANOVA) with repeated measures and ANOVA with dependent measures. Planned comparisons were used for post-hoc analysis. All values in the text and figure legends are expressed as mean ± SEM.

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