

# CREB required for the stability of new and reactivated fear memories

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Published online: 11 March 2002, DOI: 10.1038/nn819

The cAMP-responsive element binding protein (CREB) family of transcription factors is thought to be critical in memory formation. To define the role of CREB in distinct memory processes, we derived transgenic mice with an inducible and reversible CREB repressor by fusing CREB<sup>S133A</sup> to a tamoxifen (TAM)-dependent mutant of an estrogen receptor ligand-binding domain (LBD). We found that CREB is crucial for the consolidation of long-term conditioned fear memories, but not for encoding, storage or retrieval of these memories. Our studies also showed that CREB is required for the stability of reactivated or retrieved conditioned fear memories. Although the transcriptional processes necessary for the stability of initial and reactivated memories differ, CREB is required for both. The findings presented here delineate the memory processes that require CREB and demonstrate the power of LBD-inducible transgenic systems in the study of complex cognitive processes.

Several studies show a universal requirement for protein synthesis in the consolidation of long-term memory (LTM)<sup>1,2</sup>. Evidence from a variety of species, including *Aplysia*<sup>3</sup>, *Drosophila*<sup>4,5</sup>, mice<sup>6,7</sup> and rats<sup>8–10</sup>, indicates that members of the cAMP-responsive element-binding protein (CREB) family of transcription factors are key transcription factors regulating the synthesis of proteins necessary for LTM formation. However, most studies so far have not defined the precise memory processes that require CREB. To identify the role of CREB in dissociable memory processes, we used a newly identified inducible system to temporally and reversibly repress CREB function. We fused a CREB repressor ( $\alpha$ CREB isoform with a S133A mutation ( $\alpha$ CREB<sup>S133A</sup>))<sup>11,12</sup> to a ligand-binding domain (LBD) of a human estrogen receptor with a G521R mutation (LBD<sup>G521R</sup>), whose activity is regulated not by estrogen but by the synthetic ligand TAM<sup>13–15</sup>. In the absence of TAM, the LBD<sup>G521R</sup>-CREB<sup>S133A</sup> fusion protein is inactive<sup>15</sup>. However, administration of TAM activates this inducible CREB-repressor fusion protein (CREB<sup>IR</sup>), allowing it to compete with endogenous CREB and disrupt cAMP-responsive element (CRE)-mediated transcription (Supplementary Fig. 1, available on the supplementary information page of *Nature Neuroscience* online).

Here we describe the development and characterization of the CREB<sup>IR</sup> transgenic system and the use of this system to dissect the role of CREB in different memory processes. By administering TAM to activate the repressor in CREB<sup>IR</sup> transgenic mice at key time points in a fear conditioning protocol, we assessed the effects of acutely disrupting CREB function on (i) encoding or short-term memory (STM) (ii) consolidation into LTM (iii) storage or maintenance (iv) retrieval and (v) stability of memory after retrieval or reactivation.

## RESULTS

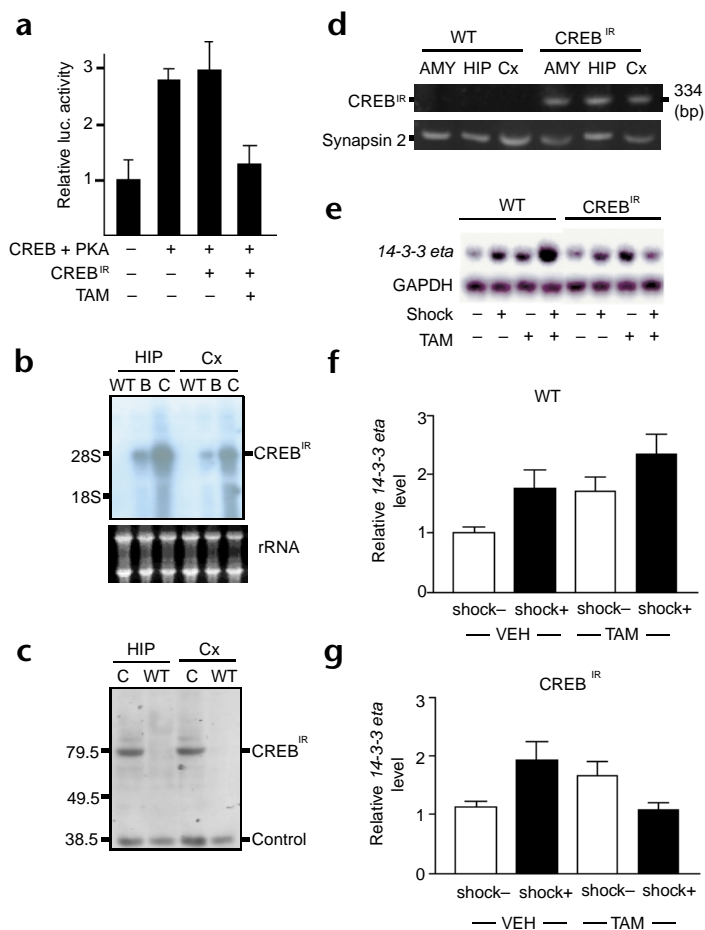
### CREB<sup>IR</sup> blocked CRE-dependent transcription

To determine whether our engineered CREB<sup>IR</sup> construct repressed CRE-mediated transcription after TAM application, we used a CRE-luciferase reporter assay in Cos-1 cells. This model system allowed us to functionally dissect the activity of the CREB<sup>IR</sup> construct. Transfection of CREB and the catalytic subunit of protein kinase A (PKA) in Cos-1 cells produced high levels of luciferase activity, indicating robust CRE-mediated transcription (Fig. 1a). Addition of the CREB<sup>IR</sup> construct in the absence of TAM did not affect these high levels of CRE-mediated transcription. However, the addition of TAM, which activates CREB<sup>IR</sup>, repressed CRE-mediated transcription to baseline levels. These results indicate that the CREB<sup>IR</sup> is capable of repressing CRE-dependent transcription only after application of TAM.

### Deriving the CREB<sup>IR</sup> transgenic mouse

We derived transgenic mice expressing CREB<sup>IR</sup> under the control of the  $\alpha$ -calmodulin kinase II ( $\alpha$ CaMKII) promoter, which is active only in excitatory neurons of forebrain areas, including the hippocampus, amygdala, cortex and striatum<sup>16</sup>. Two of the three transgenic lines that were analyzed (B and C) expressed CREB<sup>IR</sup> mRNA and protein in the hippocampus and cortex (Fig. 1b and c). RT-PCR confirmed the presence of the CREB<sup>IR</sup> construct in the hippocampus, cortex and amygdala of the transgenic mice (Fig. 1d). As line C showed threefold higher expression of CREB<sup>IR</sup>, this line was used for the studies described here.





**Fig. 1.** Inducible regulation of CRE-mediated transcription and expression of CREB<sup>IR</sup> in transgenic mice. **(a)** Activating CREB<sup>IR</sup> blocked CRE-mediated transcription in a model system (Cos-1 cells). Co-transfection of CREB and catalytic subunit of protein kinase A (PKA) increased expression of a CRE-reporter (CRE-luciferase; relative luciferase activity). Expression of CREB<sup>IR</sup> produced no effect on its own, but CRE-regulated luciferase activity was blocked upon addition of TAM. **(b)** Northern blot analysis showing expression of CREB<sup>IR</sup> mRNA in the hippocampus (HIP) and cortex (CX) of transgenic mice (lines B and C), but not in WT control mice. **(c)** Western blot analysis showing expression of CREB<sup>IR</sup> protein in the HIP and CX of CREB<sup>IR</sup>, but not in WT, mice. **(d)** RT-PCR showing expression of the CREB<sup>IR</sup> transgene in CREB<sup>IR</sup> mice in the amygdala (AMY), HIP and CX of CREB<sup>IR</sup> transgenic mice but not WT littermate control mice. The lower panel shows the RT-PCR product corresponding to the Synapsin 2 control in these regions. **(e)** Activating CREB<sup>IR</sup> repressed the expression of a CRE-containing gene (*14-3-3 eta*) that is normally increased after contextual fear conditioning. The upper panel shows the expression of RNA encoding *14-3-3 eta* in CREB<sup>IR</sup> and WT mice that were pretreated with TAM or VEH after contextual fear training (*Shock*<sup>+</sup>) or similar training in which no shock occurred (*Shock*<sup>-</sup>). The lower panel shows the expression level of GAPDH mRNA, which served as an internal control. **(f, g)** Quantification of the expression of *14-3-3 eta* from the northern blot of **(f)** in WT and **(g)** CREB<sup>IR</sup> mice. The error bars represent the s.e.m. (*Shock*<sup>-</sup> groups: CREB<sup>IR</sup>/TAM, *n* = 6; WT/TAM, *n* = 6; CREB<sup>IR</sup>/VEH, *n* = 8 and WT/VEH, *n* = 8; *Shock*<sup>+</sup> groups: CREB<sup>IR</sup>/TAM, *n* = 7; WT/TAM, *n* = 6; CREB<sup>IR</sup>/VEH, *n* = 6 and WT/VEH, *n* = 10).

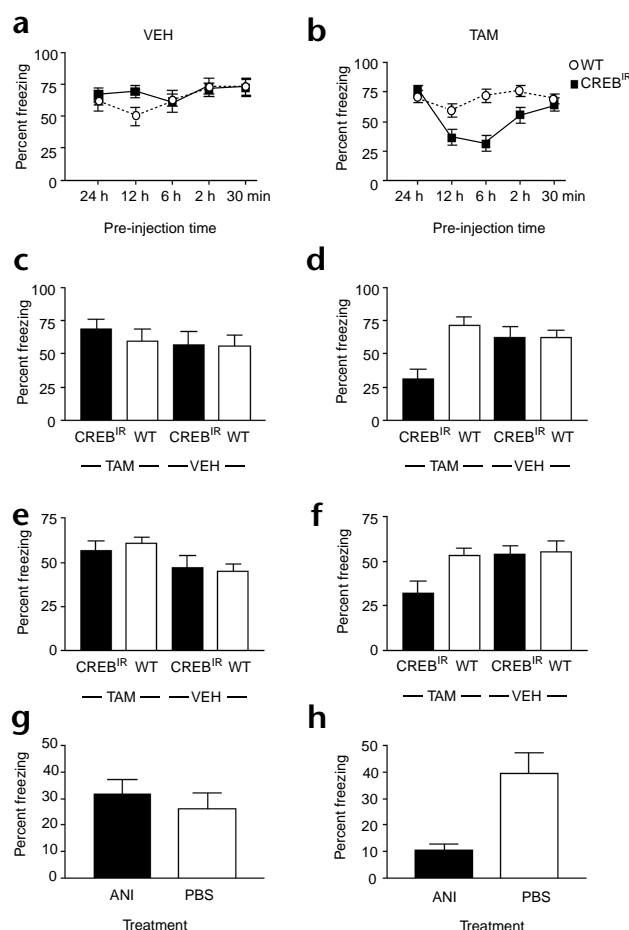
**CREB<sup>IR</sup> repressed CRE-dependent transcription in mice**

To extend the results observed in Cos-1 cells, we investigated whether activation of CREB<sup>IR</sup> inhibited expression of a CREB target gene in the transgenic mice. First, we used cDNA microarray hybridization assays to identify a gene that both (i) contained CRE sites in the promoter region and (ii) showed increased expression after behavioral training. The behavioral training task we chose was contextual fear conditioning, a form of Pavlovian conditioning in which animals learn to recognize a training context (conditioned stimulus, CS) that had been previously paired with an aversive stimulus (footshock, the unconditioned stimulus or US). Groups of CREB<sup>IR</sup> and wild-type (WT) mice were pretreated with the activator TAM or vehicle (VEH, peanut oil) and placed in a training context in which they received either shock (*Shock*<sup>+</sup>) or no shock (*Shock*<sup>-</sup>). Contextual fear conditioning (*Shock*<sup>+</sup> versus *Shock*<sup>-</sup>) increased the expression of *14-3-3 eta*, a gene that contains consensus CRE elements in its promoter region<sup>17</sup>. Mutation of a homologous gene in *Drosophila* blocks learning and memory<sup>18</sup>. To assess whether the increased levels of *14-3-3 eta* expression were decreased by activating CREB<sup>IR</sup>, northern blots were performed on whole-brain RNA samples isolated from independent groups of mice (WT and CREB<sup>IR</sup>) from the following four treatment groups: (i) VEH/*Shock*<sup>-</sup> (ii) VEH/*Shock*<sup>+</sup> (iii) TAM/*Shock*<sup>-</sup> and (iv) TAM/*Shock*<sup>+</sup> (Fig. 1e). The results of an analysis of variance (ANOVA) performed on the relative levels of *14-3-3 eta* from the four groups of WT mice (VEH/*Shock*<sup>-</sup>, VEH/*Shock*<sup>+</sup>, TAM/*Shock*<sup>-</sup> and TAM/*Shock*<sup>+</sup>) showed a significant effect of

Group ( $F_{3,23} = 4.17, p < 0.05$ ) (Fig. 1f). A Newman Keuls *post-hoc* test revealed that *14-3-3 eta* levels were higher in VEH/*Shock*<sup>+</sup>, TAM/*Shock*<sup>-</sup> and TAM/*Shock*<sup>+</sup> groups than in the VEH/*Shock*<sup>-</sup> control group. Therefore, the northern blot data confirmed the cDNA microarray results showing that contextual fear conditioning increased *14-3-3 eta* levels in WT mice. A similar analysis performed on the relative levels of *14-3-3 eta* in the four groups of CREB<sup>IR</sup> mice also showed a significant effect of group ( $F_{3,27} = 9.99, p < 0.05$ ) (Fig. 1g). The Newman Keuls *post-hoc* test showed that the levels of *14-3-3 eta* were higher in the VEH/*Shock*<sup>+</sup> and TAM/*Shock*<sup>-</sup> groups than in the VEH/*Shock*<sup>-</sup> control group. However, there was no difference between the levels of *14-3-3 eta* in the TAM/*Shock*<sup>+</sup> and the VEH/*Shock*<sup>-</sup> control group. Thus, contextual fear conditioning increased *14-3-3 eta* expression in WT mice (administered VEH or TAM) and CREB<sup>IR</sup> mice administered VEH; however, this increase was blocked by inducibly repressing CREB function (CREB<sup>IR</sup> mice administered TAM). Together with the findings from Cos-1 cells, these results demonstrate that the CREB<sup>IR</sup> system decreased CRE-mediated transcription only after application of the activator (TAM) both *in vitro* and *in vivo*.

Our results also showed that TAM alone increased *14-3-3 eta* expression to levels similar to those triggered by training (*Shock*<sup>+</sup>). Despite this, the activation of CREB<sup>IR</sup> blocked the increase in *14-3-3 eta* expression produced by training, demonstrating the efficacy of this repressor. Furthermore, administration of TAM without shock (*Shock*<sup>-</sup>) increased *14-3-3 eta* expression in the CREB<sup>IR</sup> mice. This is consistent with the idea that other transcriptional regulatory elements, such as estrogen-response elements (ERE), modulate the expression of the *14-3-3 eta* gene. Also, not all genes with CRE sites showed increased expression after fear conditioning or decreased





**Fig. 2.** Activating the CREB repressor disrupted long (LTM) but not short-term (STM) memory for contextual fear conditioning. **(a)** Effects of varying the time of pre-injection of **(a)** VEH or **(b)** TAM on LTM after contextual fear conditioning. **(a)** VEH administered to WT or CREB<sup>IR</sup> mice at different time points before contextual fear conditioning does not disrupt LTM. **(b)** Activating the CREB<sup>IR</sup> with TAM 12 or 6 h, but not 24 or 2 h or 30 min, before training disrupted subsequent LTM. The number of mice in the four groups (WT/VEH, CREB<sup>IR</sup>/VEH, WT/TAM, CREB<sup>IR</sup>/TAM) was as follows: for 24 h,  $n = 11, 10, 8, 9$ ; for 12 h,  $n = 12, 16, 15, 14$ ; for 6 h,  $n = 21, 10, 5, 14$ ; for 2 h,  $n = 9, 11, 8, 11$ ; for the 30 minutes pre-injection time,  $n = 9, 10, 14, 12$ , respectively). All data points represent the mean ( $\pm$ s.e.m.) percent time spent freezing during context replacement. **(c)** Activating CREB<sup>IR</sup> does not affect 2-h memory (STM) for contextual fear conditioning (CREB<sup>IR</sup>/TAM,  $n = 9$ ; WT/TAM,  $n = 6$ ; CREB<sup>IR</sup>/VEH,  $n = 4$ ; WT/VEH,  $n = 6$ ). **(d)** Activating CREB<sup>IR</sup> disrupted 24-h memory (LTM) for contextual fear conditioning (CREB<sup>IR</sup>/TAM,  $n = 14$ ; WT/TAM,  $n = 5$ ; CREB<sup>IR</sup>/VEH,  $n = 10$ ; WT/VEH,  $n = 21$ ). **(e)** Activating CREB<sup>IR</sup> does not affect 2 h memory (STM) for tone fear conditioning (CREB<sup>IR</sup>/TAM,  $n = 9$ ; WT/TAM,  $n = 11$ ; CREB<sup>IR</sup>/VEH,  $n = 6$ ; WT/VEH,  $n = 9$ ). **(f)** Activating CREB<sup>IR</sup> disrupted 24 h memory (LTM) for tone fear conditioning (CREB<sup>IR</sup>/TAM,  $n = 10$ ; WT/TAM,  $n = 10$ ; CREB<sup>IR</sup>/VEH,  $n = 10$ ; WT/VEH,  $n = 11$ ). **(g)** Anisomycin (ANI) does not affect 2 h memory (STM) for contextual conditioning (ANI,  $n = 8$ ; PBS,  $n = 8$ ). **(h)** ANI disrupted 24 h memory (LTM) for contextual conditioning (ANI,  $n = 8$ ; PBS,  $n = 8$ ).

by *Genotype*,  $F_{4,108} = 0.80$ ,  $p > 0.05$ ; *Time*,  $F_{4,108} = 1.47$ ,  $p > 0.05$ ; and *Genotype*,  $F_{1,108} = 0.82$ ,  $p > 0.05$ ) (Fig. 2a). In contrast, administration of TAM impaired memory in CREB<sup>IR</sup> transgenic mice when injected at a subset of times before training, but did not impair LTM in WT mice regardless of the time of injection (*Time* by *Genotype* interaction,  $F_{4,101} = 6.80$ ,  $p < 0.05$ ; *Time*,  $F_{4,101} = 3.18$ ,  $p < 0.05$ ; and *Genotype*,  $F_{1,101} = 6.32$ ,  $p < 0.05$ ) (Fig. 2b). A *post-hoc* Newman Keuls test revealed that the CREB<sup>IR</sup> mice treated with TAM 6 or 12 hours before training froze significantly less than each of the other groups, which did not differ from each other. Therefore, administration of TAM 12 or 6 hours before training disrupted memory, demonstrating the temporal and inducible control of the CREB<sup>IR</sup> system. In addition, CREB<sup>IR</sup> mice administered TAM 24 hours before training showed no deficit in subsequent freezing, demonstrating that the impairment produced by TAM in CREB<sup>IR</sup> mice is behaviorally reversible. Because the 6-hour time point produced the largest deficit in freezing, this pre-injection time point was used in all further studies.

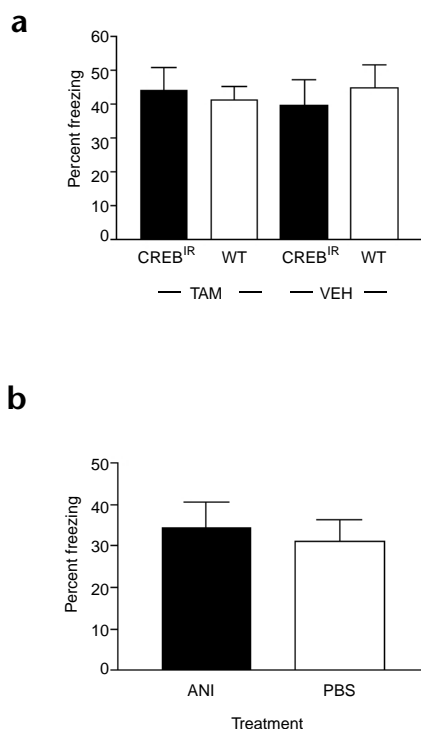
### CREB, memory encoding and consolidation into LTM

To dissect the role of CREB in potentially dissociable memory processes, we activated the CREB repressor to acutely disrupt CREB function at key points in the fear conditioning procedure. First, we injected separate groups of CREB<sup>IR</sup> and WT littermate control mice with TAM or VEH 6 hours before contextual fear training, and examined memory either 2 hours (STM) or 24 hours (LTM) after training. All groups of mice (CREB<sup>IR</sup>/TAM, WT/TAM, CREB<sup>IR</sup>/VEH and WT/VEH) showed similar levels of STM, as measured by the percentage of time spent freezing when replaced in the training context (Fig. 2c). An ANOVA showed no difference in freezing levels between the groups ( $F_{3,21} = 0.52$ ,  $p > 0.05$ ), indicating that activating the CREB repressor did not impair either initial encoding or STM. In contrast, CREB<sup>IR</sup> mice administered TAM before contextual fear training showed impaired LTM (Fig. 2d). The results of an ANOVA performed on the freezing levels of the four independent groups of mice showed a significant effect

expression in CREB<sup>IR</sup> mice treated with TAM, suggesting that other transcription factors have a dominant effect on the expression of those other genes (data not shown).

### Time course of effects of CREB<sup>IR</sup> activation

Previous studies using a similar LBD-inducible system in cell culture show that the mutant LBD fusion protein translocates to the nucleus 2–8 hours after application of TAM<sup>19</sup>. To investigate the behavioral time course of CREB<sup>IR</sup> activation in the transgenic mice, we pretreated groups of mice with TAM at different time points before contextual fear training and assessed memory 24 hours later. We chose to examine memory at this 24-hour time point because mice with a (chronic) targeted disruption of the two main CREB activators ( $\alpha$  and  $\delta$ ; CREB <sup>$\alpha\delta$ -/-</sup> mice) show robust contextual fear memory deficits measured 24 hours after training<sup>6</sup>. Our index of memory was the conditioned freezing response. Animals show a range of species-typical conditioned fear responses, including freezing (the cessation of all movement except for respiration), after being placed back in a training context in which they were previously shocked<sup>20–22</sup>. We used an automated procedure to assess the percentage of time mice spent freezing when replaced back in the training context<sup>23</sup>. Separate groups of CREB<sup>IR</sup> and WT mice were pretreated with TAM or VEH either 30 minutes, 2, 6, 12 or 24 hours before contextual fear conditioning; memory was assessed 24 hours later. WT and CREB<sup>IR</sup> mice administered VEH before training showed equally high levels of freezing during the test, regardless of the time of VEH administration (*Time*



**Fig. 3** Activating the CREB repressor or pharmacologically disrupting protein synthesis does not affect memory retrieval or expression. **(a)** CREB<sup>IR</sup> mice that were trained drug free but administered TAM or VEH before testing showed no deficit in memory retrieval or expression (CREB<sup>IR</sup>/TAM,  $n = 9$ ; WT/TAM,  $n = 9$ ; CREB<sup>IR</sup>/VEH,  $n = 11$ ; and WT/VEH,  $n = 5$ ). **(b)** WT mice that were trained drug free but administered ANI or PBS before testing showed no deficit in memory retrieval (ANI,  $n = 12$  and PBS,  $n = 12$ ).

### CREB and memory retrieval

To examine the role of CREB in the retrieval or the expression of conditioned fear, we injected separate groups of mice with TAM or VEH before memory testing rather than training. CREB<sup>IR</sup> and WT mice that were trained drug free but administered TAM or VEH 6 hours before testing froze at equal levels ( $F_{3,30} = 0.12$ ,  $p > 0.05$ ) (Fig. 3a). Similarly, WT mice administered ANI or PBS before the memory retrieval test (but trained drug free) showed equivalent levels of freezing ( $F_{1,22} = 0.16$ ,  $p > 0.05$ ) (Fig. 3b). Together, these results indicate that CRE-mediated transcription and *de novo* protein synthesis are not required for the retrieval or expression of conditioned fear memory.

### CREB and memory stability after memory retrieval

The results presented above indicate that conditioned fear memories are retrieved independent of protein synthesis or CRE-mediated transcription. Previous studies suggest, however, that memory retrieval may induce a state of plasticity in which memories become labile before becoming stable again. The stabilization of memory after retrieval (or reactivation) may involve a second wave of consolidation ('reconsolidation')<sup>24–27</sup>. Furthermore, disruption of some processes required for initial memory consolidation may also disrupt the stabilization of a reactivated memory<sup>24,28</sup>. We therefore assessed whether stabilization of a reactivated or retrieved fear memory is dependent on CRE-mediated transcription, similar to initial memory consolidation. To this end, CREB<sup>IR</sup> and WT mice were trained drug free but administered TAM or VEH 6 hours before replacement in the training context (CS), which was previously paired with shock. During this 90-second re-exposure to the training context, mice did not receive a shock. LTM was assessed 24 hours later. Therefore, mice were trained and tested with normal CREB function, but CREB function was acutely disrupted during re-exposure to the CS previously paired with shock. CREB<sup>IR</sup> mice that were administered TAM before context re-exposure froze less than the other groups of mice in the subsequent LTM test ( $F_{3,46} = 5.82$ ,  $p < 0.05$ ) (Fig. 4a). A *post-hoc* Newman-Keuls test showed that only the CREB<sup>IR</sup>/TAM group froze significantly less than the other groups, which did not differ from each other. All groups of mice showed normal and equal levels of freezing during the 90-second re-exposure to the training context ( $F_{3,46} = 1.69$ ,  $p > 0.05$ ; CREB<sup>IR</sup>/TAM =  $54.6 \pm 7.2$ , WT/TAM =  $61.9 \pm 8.4$ , CREB<sup>IR</sup>/VEH  $65.3 \pm 5.9$  and WT/VEH =  $46.5 \pm 5.7$ ), a finding that is consistent with the lack of effect of CREB disruption on memory retrieval. These results indicate that activating the CREB repressor before re-exposure to the training context impaired memory for contextual fear conditioning.

To determine whether the re-exposure to the training context was critical to the amnesic effects of CREB repression on memory after reactivation, separate groups of mice were treated as described above, except that context re-exposure was omitted. Thus, TAM and VEH were administered at the same time-point, but in the homecage. During the subsequent LTM test, WT and CREB<sup>IR</sup> mice,

of group ( $F_{3,46} = 6.27$ ,  $p < 0.05$ ). A Newman Keuls *post-hoc* test revealed that the CREB<sup>IR</sup>/TAM group froze significantly less than each of the other groups (WT/TAM, CREB<sup>IR</sup>/VEH and WT/VEH) and that the other groups did not differ from each other. WT mice injected with TAM and CREB<sup>IR</sup> mice injected with VEH froze at normal levels, indicating that neither the presence of the transgene nor the administration of TAM alone was sufficient to disrupt consolidation of LTM.

Similar results were found using discrete cue (tone) fear conditioning. In this version of the task, a tone was paired with a footshock, and the index of memory we used was the percentage of time animals spent freezing when the tone was replayed in a different chamber. All groups of mice (CREB<sup>IR</sup>/TAM, WT/TAM, CREB<sup>IR</sup>/VEH and WT/VEH) showed similar levels of STM, as measured by the percentage of time spent freezing to the tone (Fig. 2e). An ANOVA showed no difference in freezing levels between the groups ( $F_{3,31} = 2.41$ ,  $p > 0.05$ ). However, separate groups of mice that were tested 24 hours after training differed in their freezing levels ( $F_{3,37} = 3.91$ ,  $p < 0.05$ ) (Fig. 2f). A *post-hoc* Newman Keuls test showed that only the CREB<sup>IR</sup>/TAM group froze less than the other groups, which did not differ from each other. Therefore, activating the CREB repressor (by administering TAM to CREB<sup>IR</sup> mice) did not impair either initial encoding or STM, but disrupted LTM for tone fear conditioning.

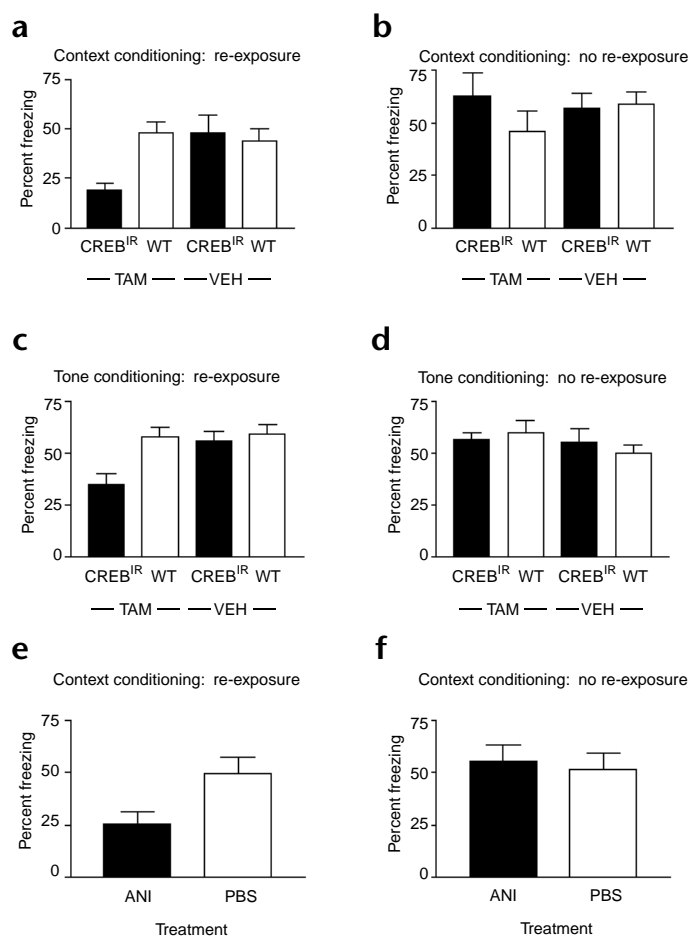
Because CREB may be a key transcription factor regulating *de novo* protein synthesis, we performed parallel experiments using a pharmacological agent to block protein synthesis. Groups of WT mice were systemically administered anisomycin (ANI), a protein synthesis inhibitor, or VEH (PBS) 30 minutes before contextual fear conditioning, and memory was assessed 2 or 24 hours later. ANI disrupted memory measured 24 hours after training ( $F_{1,14} = 13.42$ ,  $p < 0.05$ ), but not 2 hours ( $F_{1,14} = 0.45$ ,  $p > 0.05$ ), after training (Fig. 2g and h). Therefore, LTM but not STM for conditioned fear is dependent on both CRE-mediated transcription and protein synthesis.

**Fig. 4.** Activating the CREB repressor or inhibiting protein synthesis impaired the stability of a reactivated memory. (a) Activating CREB<sup>IR</sup> before re-exposure to the training context associated with shock impaired memory stability (CREB<sup>IR</sup>/TAM,  $n = 14$ ; WT/TAM,  $n = 15$ ; CREB<sup>IR</sup>/VEH,  $n = 10$ ; and WT/VEH,  $n = 11$ ). (b) Activating CREB<sup>IR</sup> in the homecage, without re-exposure to the training context, did not impair memory storage (CREB<sup>IR</sup>/TAM,  $n = 9$ ; WT/TAM,  $n = 8$ ; CREB<sup>IR</sup>/VEH,  $n = 5$ ; and WT/VEH,  $n = 9$ ). (c) Activating CREB<sup>IR</sup> before re-exposure to a tone that was previously paired with shock impaired memory stability (CREB<sup>IR</sup>/TAM,  $n = 12$ ; WT/TAM,  $n = 12$ ; CREB<sup>IR</sup>/VEH,  $n = 11$ ; WT/VEH,  $n = 12$ ). (d) Activating CREB<sup>IR</sup> without re-exposure to the tone previously paired with footshock did not impair memory storage (ANI,  $n = 12$ ; PBS,  $n = 12$ ). (e) ANI, administered before re-exposure to the training context previously paired with shock, impaired memory stability in WT mice (ANI,  $n = 12$ ; PBS,  $n = 12$ ). (f) ANI, administered in the homecage, did not block memory storage (ANI,  $n = 16$ ; PBS,  $n = 12$ ).

regardless of treatment (TAM or VEH), froze at similar levels ( $F_{3,34} = 0.64$ ,  $p > 0.05$ ) (Fig. 4b). These results indicate that simply activating CREB<sup>IR</sup> 1 day after training does not disrupt contextual fear memory. Therefore, CREB does not play a critical role in the storage or maintenance of conditioned fear memories that are not retrieved or reactivated.

Activating the CREB repressor before re-exposure to a discrete cue (CS, a tone) similarly impaired the stability of a reactivated tone fear memory. CREB<sup>IR</sup> and WT mice were given tone fear conditioning drug free but administered TAM or VEH 6 hours before re-exposure to the tone CS in a novel chamber. CREB<sup>IR</sup> mice administered TAM before the tone re-exposure froze less than the other groups in the subsequent LTM test ( $F_{3,43} = 5.41$ ,  $p < 0.05$ ) (Fig. 4c). A *post-hoc* Newman-Keuls test showed that only the CREB<sup>IR</sup>/TAM group froze significantly less than the other groups, which did not differ from each other. All groups of mice froze at equal levels to the 30-second tone during re-exposure ( $F_{3,43} = 0.66$ ,  $p > 0.05$ ; CREB<sup>IR</sup>/TAM =  $59.7 \pm 8.1$ , WT/TAM =  $61.1 \pm 8.5$ , CREB<sup>IR</sup>/VEH =  $69.7 \pm 8.0$  and WT/VEH =  $54.2 \pm 6.2$ ). As in contextual fear conditioning, the memory impairment produced by repressing CREB function was contingent on re-exposure to the CS. CREB<sup>IR</sup> and WT mice that were given tone fear conditioning and administered TAM or VEH before placement in the novel chamber, but not re-exposed to the tone CS, showed normal and equal freezing levels to the tone when tested 24 hours later ( $F_{3,32} = 0.66$ ,  $p > 0.05$ ) (Fig. 4d). Furthermore, CREB repression did not affect memory measured 2 hours after re-exposure to the tone. Separate groups of CREB<sup>IR</sup> and WT mice were given tone fear conditioning and administered TAM or VEH 6 hours before re-exposure to the tone (as above). However, these mice were tested 2 hours, rather than 24 hours, later. The levels of freezing in this STM test of a reactivated memory did not differ between groups ( $F_{3,24} = 0.14$ ,  $p > 0.05$ ; CREB<sup>IR</sup>/TAM =  $63.9 \pm 5.9$ , WT/TAM =  $58.7 \pm 9.6$ , CREB<sup>IR</sup>/VEH =  $65.5 \pm 9.2$  and WT/VEH =  $65.5 \pm 8.8$ ;  $n = 7$  for each group). Disrupting CREB function during reactivation of memory produced a disruption of memory 24 but not 2 hours later, suggesting that memory was maintained by a CREB-independent mechanism during this time. The dynamics of amnesia produced by CREB after initial memory consolidation and memory reactivation are similar.

We also performed similar experiments using ANI to pharmacologically block protein synthesis before memory reactivation.



WT mice were trained drug free but administered ANI or PBS 30 minutes before re-exposure to the training context previously paired with shock. Mice administered ANI froze less than mice administered PBS in the subsequent LTM test ( $F_{1,22} = 6.35$ ,  $p < 0.05$ ) (Fig. 4e). However, the levels of freezing measured during the 90-second re-exposure to the context did not differ between the groups ( $F_{1,22} = 0.43$ ,  $p > 0.05$ ; ANI =  $46.1 \pm 6.8$  and PBS =  $52.0 \pm 5.9$ ), again showing that ANI did not affect memory retrieval. To examine the requirement of context re-exposure on the amnesic effects of ANI, separate groups of WT mice were treated similarly except that ANI or PBS was administered in the homecage. The levels of freezing during the subsequent test did not differ between groups of mice that received ANI or PBS in the homecage ( $F_{1,26} = 0.11$ ,  $p > 0.05$ ) (Fig. 4f). Therefore, re-exposure to the training cage — that is, memory retrieval or reactivation — is essential for both the amnesic effects of CREB repression and ANI on memory stability after retrieval.

Together, these data indicate that disrupting either CREB function or protein synthesis during re-exposure to a CS (tone or context) that was previously paired with shock impairs subsequent memory. However, these same treatments have no effect when given without re-exposure to the CS, suggesting that memory retrieval triggers the CREB-dependent transcription and protein synthesis required for memory stability after retrieval.

## DISCUSSION

Here we directly assessed the effects of acutely decreasing CREB function on different memory processes of conditioned fear. Mem-

ory consolidation involves several stages<sup>29–31</sup>. The studies described here addressed only the earliest stages of protein synthesis-dependent memory consolidation. By gaining temporal and reversible control of CREB function in a transgenic mouse, we showed that this transcription factor is not only required for the initial consolidation of LTM, but also for consolidation of reactivated conditioned fear memory. Consistent with previous data<sup>3,5–9,32</sup>, our results confirmed that CREB is necessary for the initial consolidation of LTM but not for memory encoding or STM, as measured by either discrete cue (tone) or contextual fear conditioning. In addition, we identified a gene downstream from CREB (*14-3-3 eta*) that may be important in memory processes<sup>18</sup>.

Our finding, along with others, shows that some treatments that impair initial memory consolidation also impair the stability of memory after retrieval. Thus, protein synthesis inhibitors<sup>24,27,33</sup>, electroconvulsive shock<sup>25,34,35</sup>, hypothermia<sup>36,37</sup>, AP-5 (ref. 38) and  $\beta$ -adrenergic antagonists<sup>39</sup> administered around the time of memory reactivation disrupt the stabilization of reactivated memories. These findings suggest that memory retrieval may induce a state of plasticity in which memories become labile before being stabilized.

There are several reports, however, of failure to disrupt reactivated memory with treatments known to disrupt memory consolidation<sup>40–44</sup>. These experiments suggest that the mechanisms underlying memory stability after reactivation may be both brain region and task specific. For example, infusion of ANI into the insular cortex of rats around the time of training blocks initial consolidation for conditioned taste aversion, whereas similar infusion around the time of retrieval strengthens the memory<sup>45</sup>. In addition, systemic, but not intrahippocampal, administration of ANI disrupts memory stability after memory reactivation of an inhibitory avoidance task<sup>33</sup>.

The similarities and differences between memory consolidation and the stabilization of a reactivated memory have not been precisely determined. Both processes are enhanced by strychnine sulfate or glucose<sup>46,47</sup> and produce comparable interfering effects on later retention<sup>48</sup>. However, the vulnerability and permanence of the disruption produced by amnesic agents may be different<sup>27,36,49</sup>. Furthermore, there may be differences in the molecular mechanisms underlying memory consolidation and memory stabilization after memory retrieval. The transcription factor C/EBP $\beta$  (CCAAT enhancer binding protein  $\beta$ ) is required for initial memory consolidation, but not for memory stability after retrieval. Injecting antisense oligonucleotides specific for C/EBP $\beta$  into the hippocampus after training blocks initial memory consolidation for inhibitory avoidance. In contrast, similar infusion after retrieval did not disrupt memory<sup>33</sup>. Unlike C/EBP $\beta$ , CREB is critically involved in both initial memory consolidation and stability after retrieval. Thus, our finding is in agreement with recent results showing that re-exposure to a tone previously paired with shock increases phosphoCREB levels in the amygdala<sup>50</sup>.

Little is known about the molecular events underlying the retrieval of conditioned fear memories. Previous studies have shown that protein synthesis is necessary for these stabilizing reactivated memories. Here, we find that CREB is also crucial for the stability of conditioned fear memories after retrieval. Although the transcriptional mechanisms necessary for initial memory consolidation and stability after retrieval differ, CREB is required for both. The findings presented here characterize the memory processes that require CREB and, furthermore, demonstrate the power of LBD-inducible transgenic systems for the temporal dissection of complex cognitive processes.

## METHODS

**CREB<sup>IR</sup> construct and CRE-mediated transcription in a model cell culture system.** Cos-1 cells were transiently transfected with expression vectors for LBD<sup>G521R</sup>-CREB<sup>S133A</sup> (pRSV-HA-LBD<sup>G521R</sup>-CREB<sup>S133A</sup>), CREB (RSV-CREB; kindly provided by M. Montminy), the catalytic subunit of protein kinase A (PKA; pFc-PKA; Stratagene, La Jolla, California) and a luciferase reporter plasmid (pCRE-luc). After incubation in the presence or absence of TAM (1  $\mu$ M) for 48 h, luciferase activity was examined using a luminometer. Transfection efficiencies were normalized using a  $\beta$ -galactosidase assay and transfections were repeated twice, each time in triplicate.

**Transgene construction and generation of CREB<sup>IR</sup> mice.** The transgene used in these studies contains an  $\alpha$ CaMKII promoter, a hybrid intron in the 5' untranslated leader, the coding region of CREB<sup>IR</sup> and a polyadenylation signal. The LBD<sup>G521R</sup> cDNA (kindly provided by F. Steward) was fused 5' to the CREB<sup>S133A</sup> cDNA (kindly provided by M. Montminy) by PCR, and restriction enzyme sites, a Kozak and an hemagglutinin virus (HA)-tag sequence were added by PCR. The resulting cDNA was subcloned into the pNN265 plasmid (resulting in the pNN-LBD<sup>G521R</sup>-CREB<sup>S133A</sup> plasmid). A 3.5 kb *NotI* fragment from pNN-LBD<sup>G521R</sup>-CREB<sup>S133A</sup> was subcloned downstream of the  $\alpha$ CaMKII promoter in the pMM403 vector (kindly provided by M. Mayford). pMM-LBD<sup>G521R</sup>-CREB<sup>S133A</sup> was digested with *SfiI*, and transgenic mice were generated by injecting the purified insert into pronuclei of C3H zygotes. Founders were backcrossed into C3H mice (Taconic Farms, Germantown, New York). All behavioral analyses using TAM were carried out in F1 mice (aged 3–12 months) that were offspring of a cross between the founders and C57Bl/6 mice (Taconic Farms). All procedures used were approved by Animal Research Committee of UCLA. Animals were maintained in accordance with the applicable portions of the Animal Welfare Act and the Department of Health and Human Services *Guide to the Care and Use of Laboratory Animals*.

**RT-PCR.** Mouse brains were sliced, and regions of the hippocampus, cortex and amygdala were isolated. Total RNA (1  $\mu$ g) was isolated using an acid guanidinium thiocyanate/phenol/chloroform (AGPC) method and reverse-transcribed with an oligo dT primer. The PCR reactions were carried out for 35 cycles (a 1-min cycle at each of the following temperatures: 94°, 56° and 72°C). PCR products were amplified using two primers for LBD-CREB<sup>S133A</sup> mRNA (LBD-CREB<sup>S133A</sup> 5'-ATT CGC TGT CTG CGA GGG CC and LBD-CREB<sup>S133A</sup> 3'-TCA TGG AGG GTC AAA TCC AC), or synapsin2 mRNA (synapsin2 5'-ATC ACT ACG ACT TCC AGG AC, synapsin2 3'-CCG GTT CCT TAA GTG TTC CA) as internal control. The transgene for LBD-CREB<sup>S133A</sup> contains an intron (235 bp) in the region between sequences corresponding to 5' and 3' such that the PCR product from LBD-CREB<sup>S133A</sup> mRNA is distinct from other PCR products. The PCR products were analyzed by agarose gel electrophoresis and then stained by ethidium bromide.

**DNA microarray.** CREB<sup>IR</sup> and WT mice were administered 16 mg/kg intraperitoneal (i.p.) TAM (4-hydroxytamoxifen; Sigma, St. Louis, Missouri) or vehicle (VEH: a similar volume of peanut oil) ( $n = 2–4$  per group) 6 h before contextual fear conditioning training (*Shock*<sup>+</sup> or *Shock*<sup>-</sup>) and decapitated 1 h later. The microarray and protocols used were according to instructions of the Atlas Mouse cDNA Expression Array system (Clontech, Palo Alto, California). Total RNA was extracted from pooled brains to produce labeled cDNA probes for hybridization with the microarray membranes. Washed nylon arrays were exposed to phosphorimager screens (Bio-Rad Laboratories, Hercules, California) for 12–14 d. Multianalyst software (Bio-Rad) extracted the phosphorimaging counts of duplicate spots from each of the 588 cDNAs on the arrays. The data were normalized with custom software to determine the differences in gene expression between all experimental groups. In addition to *14-3-3 eta*, the cDNA library identified further putative CREB-regulated genes that we are now investigating.

**Northern blotting.** Northern blot analysis was performed with probes derived from (i) the 1.1 kb *EcoRV*-*NotI* fragment from pNN-LBD<sup>G521R</sup>-CREB<sup>S133A</sup> (Fig. 1b) and (ii) the 300 bp of *14-3-3 eta* cDNA fragment corresponding to the 3' untranslated region of mouse *14-3-3 eta* (Fig. 1e). The filters hybridized with *14-3-3 eta* cDNA were rehybridized

with GAPDH cDNA as an internal control. To measure the intensity of *14-3-3 eta* or GAPDH, hybridization densitometry was performed using a Fuji-Bio image analyzer BAS2000 (Fuji Photo Film, Kanagawa, Japan). Optical density measures for *14-3-3 eta* expression were normalized against the corresponding optical density for GAPDH expression. A ratio for the relative level of *14-3-3 eta* induced by fear conditioning was calculated for WT and CREB<sup>IR</sup> mice that were administered VEH or TAM, as a ratio of the normalized optical density measures from other groups versus WT mice pretreated with VEH that were not shocked (Fig. 1f and g). The value for this group (WT VEH/*Shock*<sup>-</sup>) was set to 1. Additional groups of mice (CREB<sup>IR</sup> and WT) were treated similarly except that they were housed in the training context 12 h before receiving the shocks. As expected, the levels of *14-3-3 eta* expression in these 'latent inhibition' control groups were not higher than the levels of *14-3-3 eta* expression in the *Shock*<sup>-</sup> control group (data not shown).

**Western blotting.** Samples of the hippocampus and cortex of CREB<sup>IR</sup> and WT littermate control mice were isolated, homogenized in SDS buffer (0.1 M dithiothreitol (DTT), 2% SDS and 0.05 M Tris, pH 6.8), heated at 95°C for 10 min and centrifuged at 4°C for 10 min. The supernatants (50 µg of total protein) were analyzed by western blotting. The blotting filter was probed with an HA-antibody (Santa Cruz, Santa Cruz, California) and specific protein was detected by ECLplus (Amersham, Arlington Heights, Illinois). The CREB<sup>IR</sup> protein containing an HA tag is shown at 79.5 kDa, whereas an HA-labeled nonspecific band (Control) is shown at 34 kDa.

**Assessment of STM and LTM.** Groups of naïve mice (CREB<sup>IR</sup> and WT littermate controls) were administered TAM (16 mg/kg i.p.) or VEH (similar volume of peanut oil, i.p.) 6 h before fear conditioning training. Mice were trained and tested in conditioning chambers (32 × 25 × 25 cm<sup>3</sup>) (Med-Associates, St. Albans, Vermont) that had stainless steel rod floors through which footshocks could be delivered. Contextual fear training consisted of placing the mice in the chamber and delivering footshocks (0.75 mA for 2 s) 30 and 90 s later. Mice were returned to the homecage 30 s after the final footshock. Memory was assessed either 2 or 24 h after training as the percentage of time mice spent freezing when replaced in the training context (5 min) using an automated technique to score freezing<sup>23</sup>.

A similar procedure was used for cued fear conditioning; CREB<sup>IR</sup> and WT mice were administered VEH or TAM 6 h before training. Mice were placed in the training context, and 2 min later, a 30-s tone (2,800 Hz at 85 dB) co-terminated with a 2-s shock (0.75 mA). Mice were given a similar tone-shock pairing 1 min later and returned to the homecage 1 min after the final shock. Two or 24 h later, mice were placed in a different chamber for 2 min and the tone was replayed for 3 min. Memory for cued (tone) fear conditioning was assessed as the percentage of time mice spent freezing during the 3-min tone.

The procedure for the ANI experiments was similar except that WT mice (C57Bl/6; Taconic Farms) were injected with ANI (150 mg/kg i.p.; dissolved in PBS and the pH adjusted to 7.0–7.4; Sigma) 30 min before contextual fear training. At this dose, ANI inhibits cerebral protein synthesis by ~96% (ref. 1). We found no effects of this dose of ANI on the percentage of time spent freezing before the first shock was delivered in the training session ( $F_{1,30} = 0.39$ ,  $p > 0.05$ , ANI ( $n = 16$ , both 2 and 24 h test groups included) =  $8.7 \pm 2.5$ , PBS ( $n = 16$ ) =  $7.9 \pm 3.3$ ).

**Assessment of retrieval.** Mice were given contextual fear training drug-free (with no pretreatment). Testing occurred 24 h later and consisted of placing the mice back in the context in which they were previously shocked and measuring freezing for the 5-min test session. However, mice were injected with either (i) TAM or VEH 6 h before testing in CREB<sup>IR</sup> and WT littermate control mice or (ii) ANI or PBS 30 min before testing in WT mice (C57Bl/6).

**Assessment of stability after retrieval.** CREB<sup>IR</sup> and WT mice were trained (drug-free) for contextual fear conditioning as described, except three footshocks were delivered at minutes 2, 3 and 4 of the training session. After training (18 h), mice were administered TAM or VEH (i) 6 h before a 90-s re-exposure to the training context (re-exposure group) or (ii) in the homecage (no re-exposure group). WT mice (C57Bl/6) were treated sim-

ilarly, except ANI (or PBS) was administered 30 min before re-exposure to the training context (re-exposure group) or at the same time in the homecage (no re-exposure group). No shock was delivered during the re-exposure to the context. Memory was assessed 24 h later as the percentage of time spent freezing during a 5-min memory test in the training context.

For the assessment of memory stability after retrieval study using cued fear conditioning, the timing of the re-exposure and drug injection protocol were similar. CREB<sup>IR</sup> and WT mice (drug-free) were given cued (tone) fear conditioning (with two tone-shock pairings). After training (18 h), mice were injected with TAM or VEH 6 h before (i) placement in a different chamber and re-exposed to the tone for 30 s (re-exposure group) or (ii) placement in a different chamber but not re-exposed to the tone (no re-exposure group). Memory was assessed 2 or 24 h later in this second chamber as the percentage of time spent freezing to a 3-min tone 2 min after placement in this chamber.

Note: Supplementary Figure 1 is available on the Nature Neuroscience web site.

## Acknowledgements

This work was supported by an SNRP-NIH, NARSAD and McKnight grant to A.J.S., and an SNRP-NIH grant to S.P.O. S.A.J. was supported by a NARSAD Young Investigator Fellowship. S.K. and S.M. were supported by a Grant-in Aid for High Technology Research from the ministry of Education and by a Grant-in Aid for Scientific Research from the ministry of Education, Science and Culture, Japan. We would like to thank P. Frankland and K. Nader for suggestions and discussions that helped to shape the work described in this manuscript, R. Costa and S. Kushner for comments on a previous version of this manuscript, and Y. Elgersma, Y. I. Robles, H. G. Ortiz-Zuazaga, J. Coblenz and M. Lacuesta for technical advice and assistance.

## Competing interests statement

The authors declare that they have no competing financial interests.

RECEIVED 14 JANUARY; ACCEPTED 30 JANUARY 2002

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