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An Important Role of Neural Activity-Dependent CaMKIV Signaling in the Consolidation of Long-Term Memory

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Summary

Calcium/calmodulin-dependent protein kinase IV (CaMKIV) has been implicated in the regulation of CRE-dependent transcription. To investigate the role of this kinase in neuronal plasticity and memory, we generated transgenic mice in which the expression of a dominant-negative form of CaMKIV (dnCaMKIV) is restricted to the postnatal forebrain. In these transgenic mice, activity-induced CREB phosphorylation and c-Fos expression were significantly attenuated. Hippocampal late LTP (L-LTP) was also impaired, whereas basic synaptic function and early LTP (E-LTP) were unaffected. These deficits correlated with impairments in long-term memory, specifically in its consolidation/retention phase but not in the acquisition phase. These results indicate that neural activity-dependent CaMKIV signaling in the neuronal nucleus plays an important role in the consolidation/retention of hippocampus-dependent long-term memory.

Introduction

Activity-dependent synaptic plasticity is thought to be the cellular mechanism for storing new information in the brain. Previous studies have indicated that activity-dependent increases in postsynaptic Ca^{2+} and subsequent activation of Ca^{2+}-dependent enzymes play a critical role in the relatively early phase of synaptic plasticity that may serve as a cellular cognate of memory acquisition (Bliss and Collingridge, 1993; Soderling and Derkach, 2000). The enduring phases of synaptic plasticity (e.g., L-LTP) and long-term memory seem to require cellular mechanisms that link synaptic signaling to nuclear events involving transcriptional activation and consequent protein synthesis (Frey et al., 1988; Nguyen et al., 1994). For these mechanisms, Ca^{2+} and cAMP-mediated signaling, including the cAMP-response element binding protein (CREB) and cAMP-responsive element (CRE)-dependent transcription, have been implicated in systems ranging from *Aplysia* to mammals (Bartsch et al., 1995; Bourtchuladze et al., 1994; Impey et al., 1996; Yin et al., 1994, 1995).

In mammals, the roles of Ca^{2+} and CREB in LTP and long-term memory were first addressed with a global knockout mouse in which the expression of the α and δ isoforms of CREB was disrupted (Bourtchuladze et al., 1994; Kogan et al., 1996). Under certain behavioral and electrophysiological protocols, deficits in long-term memory and synaptic plasticity were observed in these mice, however, the interpretation of these results was difficult because these mutant mice harbored compensatory increases in the level of several other CREB isoforms (Blendy et al., 1996). In addition, the confounding effects inherent to global knockout mice have made it difficult to establish a direct link between CREB function in an adult brain and the enduring form of synaptic plasticity or long-term memory.

Subsequently, a role of cAMP signaling in L-LTP and in memory consolidation was investigated by analyzing double knockout mice lacking calmodulin (CaM)-stimulated adenylyl cyclases, AC1 and AC8 (Wong et al., 1999), or transgenic mice expressing an inhibitory form (*R(AB)*) of protein kinase A (PKA) (Abel et al., 1997). Characterization of these mice revealed significant deficits in both L-LTP and long-term memory. However, L-LTP induced in hippocampal slices of these mice decayed significantly faster than that induced in control slices in the presence of a protein synthesis inhibitor, suggesting an additional role of the cAMP signaling pathway in the protein synthesis-independent, early component of L-LTP. Recent studies indeed demonstrated that PKA plays a part in the protein synthesis-independent form of LTP as well as in protein synthesis-dependent L-LTP (Blitzer et al., 1995; see discussion in Winder et al., 1998). In addition, although separate pharmacological studies implicated the cAMP signaling pathway in CREB activation (Impey et al., 1996; Yamamoto et al., 1988), it was not shown whether CREB activation was impaired in either AC1/AC8 double knockout mice or R(AB) transgenic mice. Thus, understanding the relationship between protein synthesis-dependent LTP and long-term memory, and the role of CREB in L-LTP require further studies.

The phosphorylation of CREB at Ser^{133} is essential to the transcriptional activation of CREB/CRE-mediated signaling pathway (Bito et al., 1996). Among several Ca^{2+}-dependent protein kinases that phosphorylate CREB at Ser^{133} (Sun et al., 1996), CaMKIV is the only one detected predominantly in the nuclei of neurons in adult brain (Jensen et al., 1991; Nakamura et al., 1995) and thus must be accessible to transcription factors like CREB. In cultured neurons, inhibition of endogenous CaMKIV activity by transfection with either an antisense oligonucleotide or a catalytic mutant of CaMKIV attenuates nuclear CREB phosphorylation and CRE-dependent transcription (Bito et al., 1996; Finkbeiner et al., 1997). In another study, transfection with a dominant-negative form of CaMKIV inhibited depolarization-

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induced synthesis of brain-derived neurotrophic factor (BDNF) in a CRE-dependent manner (Shieh et al., 1998). Furthermore, constitutively active CaMKIV was shown to increase the activity of CREB binding protein (CBP) and thereby stimulate CRE-dependent gene transcription. (Chawla et al., 1998; Hardingham et al., 1999; Hu et al., 1999).

Recently, two independently derived global CaMKIV knockout mice were reported and their phenotypes were very different (Ho et al., 2000; Wu et al., 2000). One of them was severely impaired in viability, fertility, and motor control and displayed poor development of cerebellar Purkinje cells (Ribar et al., 2000; Wu et al., 2000). Obviously, this mutant mouse strain is not ideal for LTP or memory studies. In contrast, the other CaMKIV knockout mouse strain exhibited no gross abnormality or motor defects, although the mice displayed some alterations in cerebellar Purkinje cell development and hippocampal physiology (Ho et al., 2000). Both basal and depolarization-induced CREB phosphorylation were reduced, and hippocampal E-LTP and L-LTP were impaired. The early onset of L-LTP deficit, namely that observed within the first 5 min after induction, suggests alterations in resident synaptic molecules in addition to those in CREB/CRE-mediated nuclear transcription events. These knockout mice showed no detectable impairment in spatial learning despite clear deficits in hippocampal LTP and cerebellar L-LTD. However, the mutant mice carried truncated transcripts and it was not clear whether the CaMKIV activity was entirely eliminated. Multiple effects that can occur in global knockout mice, including compensation during development, complicate the interpretation of these results.

To identify more specifically the role of CaMKIV in long-term memory, we generated and analyzed transgenic mice in which a dominant-negative form of CaMKIV (dnCaMKIV) inhibits Ca^{2+}-stimulated CaMKIV activity only in the postnatal forebrain.

Results

Generation of Transgenic Mice Expressing dnCaMKIV in the Postnatal Forebrain

We constructed a vector with the following components from 5′ to 3′ (Figure 1A): αCp-FLAG-*dnCaMKIV*-SV40 intron-SV40 pA, where αCp is the 8.5 kb DNA fragment derived from the 5′ flanking region of the α*CaMKII* gene and FLAG-*dnCaMKIV* encodes an epitope tag fused to the amino terminus of dnCaMKIV. We produced ten transgenic founders (C57BL/6) and chose three lines, C7, C15, and C34, carrying approximately 15, 2, and 1 copies of the transgene, respectively. Northern blot analysis of forebrain RNA, using a probe specific for the 3′ end of the transgene, revealed that a transgene transcript of about 2.4 kb was expressed in all three lines (Figure 1B). As a control, we examined the endogenous levels of α*CaMKII* transcripts and found no discernable difference between the transgenic and control mice (Figure 1B). To minimize the risk of nonspecific effects of the transgene, we selected C34, the lowest expressor among the three transgenic lines, for further characterization. The expression of the transgene in line C34 was relatively high in the cerebral cortex and hippocampal formation, lower in the striatum, amygdala, and olfactory bulb, and undetectable in the cerebellum (Figure 1C). Within area CA1 of the hippocampus, transgene expression was confined to the pyramidal cell layer (Figure 1D).

Western blot analysis carried out using anti-FLAG antibody detected the epitope tag of the transgenic dnCaMKIV protein in the forebrain of C34 mice but not in that of control littermates (Figure 1E). The anti-CaMKIV catalytic domain antibody detected the endogenous CaMKIV protein in control mice, and both the endogenous CaMKIV and the transgenic dnCaMKIV protein in transgenic mice. The total level of CaMKIV immunoreactivity in the forebrain of C34 mice was at least 2- to 3-fold higher than that in wild-type littermates. The level of the expression of other synaptic proteins such as αCaMKII and actin did not seem to be affected by the transgene (Figure 1E).

There was no difference in basal CaMKIV activity in the hippocampal homogenates of wild-type and C34 transgenic mice (Figure 1F). However, when the hippocampal slice was treated with KCl, CaMKIV activity was significantly lower (~80% reduction) in the homogenates derived from transgenic mice compared to those from the wild-type littermates. We also examined the activity of CaMKII in the hippocampal homogenates. As shown in Figure 1F, neither basal activity nor the activity induced by KCl treatment was significantly affected by the presence of the *dnCaMKIV* transgene in C34 mice. The transgenic animals appear healthy, are fertile, and exhibit no obvious behavioral abnormalities. Furthermore, terminal deoxynucleotidyl transferase-mediated biotinylated-dUTP nick-end labeling (TUNEL) assay revealed that the expression of the *dnCaMKIV* transgene did not alter the viability of cells in the adult hippocampus (see Supplmental Data available online at http://www.cell.com/cgi/content/full/106/6/771/DC1).

Suppression of CREB Phosphorylation and CRE-Dependent Gene Expression

We first tested the ability of dnCaMKIV to prevent CREB/CRE-mediated transcription using COS7 cells. Constitutively active forms of CaMKIV (caCaMKIV) and CaMK kinase (caCaMKK) were used to assess CRE-mediated transcription to avoid the complex effects of elevated intracellular Ca^{2+} on other endogenous Ca^{2+}-activated enzymes (Enslen et al., 1996). Cells were transfected with plasmids as indicated in Figure 2A and CRE-mediated transcription was assayed using a CRE-luciferase reporter construct (Grewal et al., 2000). Transfection with caCaMKIV alone had no effect, whereas cotransfection with caCaMKK gave a 5- to 6-fold increase in luciferase activity (Figure 2A). This increase in CRE-mediated transcription was almost completely blocked by cotransfection with dnCaMKIV or a CREB mutant in which Ser^{133} was mutated to Ala (Figure 2A).

We next examined the ability of dnCaMKIV to inhibit activity-induced CREB phosphorylation in hippocampal slices of C34 transgenic mice. For this purpose, we perfused C34 or wild-type hippocampal slices with saline containing 90 mM KCl or 100 μM glutamate, and 30 min later determined the levels of Ser^{133}-phosphorylated CREB (pCREB) by Western blot analysis. In wild-type slices, both KCl and glutamate treatments resulted in robust increases in CREB phosphorylation, whereas

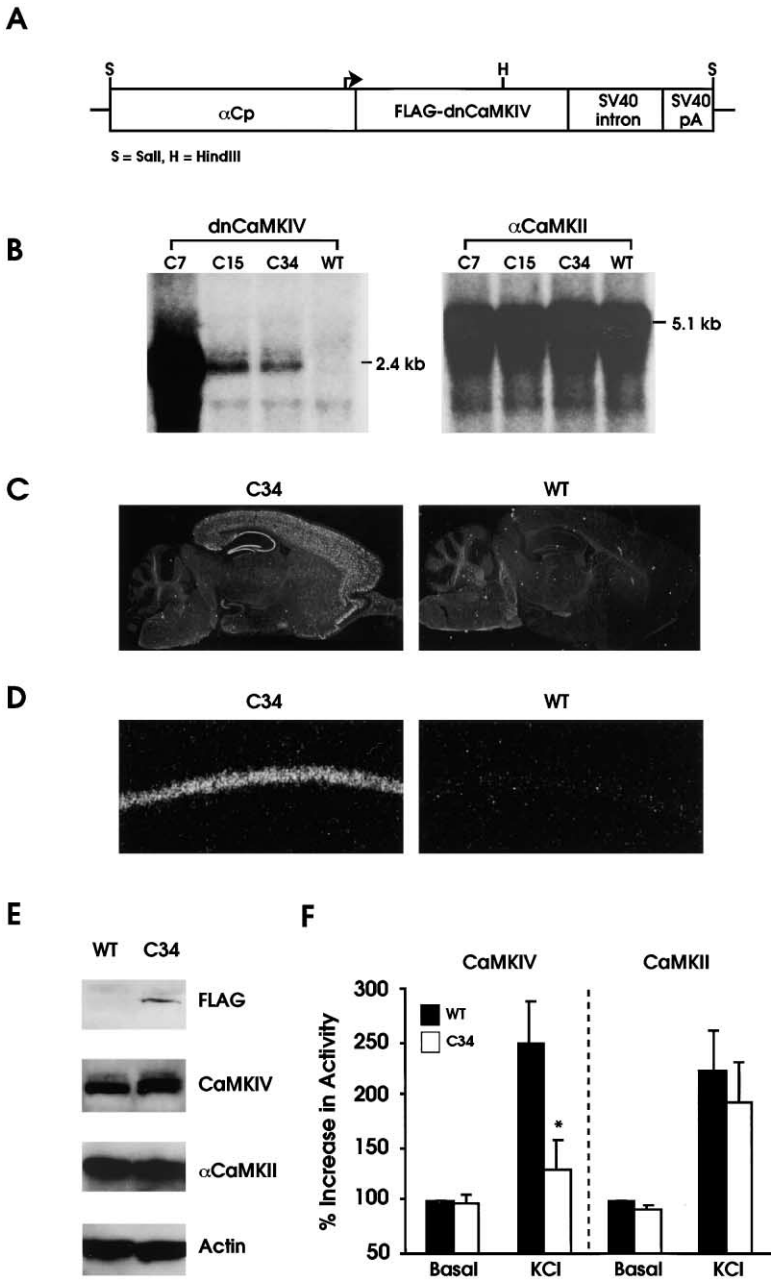

Figure 1. Expression of the *dnCaMKIV* Transgene Inhibits Depolarization-Induced Activation of Endogenous CaMKIV

(A) Schematic representation of the *dnCaMKIV* transgenic construct.

(B) Northern blot analysis of total RNA from the forebrain of three transgenic lines, C7, C15, and C34. The SV40 probe detects the 2.4 kb transgenic mRNA. The α*CaMKII* probe reveals the normal expression of 5.1 kb α*CaMKII* mRNA in the transgenic mice.

(C) Regional distribution of the *dnCaMKIV* transgene in line C34 revealed by in situ hybridization. Sagittal sections from the brains of transgenic (C34, left) and wild-type (WT, right) mice were hybridized with the SV40 probe specific to the transgene.

(D) Expression of the transgene in CA1 region of the hippocampus.

(E) Western blot analysis of forebrain extracts from wild-type and C34 mice. Both anti-FLAG and anti-CaMKIV antibodies detect ~66 kDa transgenic protein, which is slightly bigger than the endogenous form of CaMKIV. The *dnCaMKIV* transgene does not alter the expression of other synaptic proteins.

(F) Enzyme activity determined in hippocampal extracts from wild-type and C34 mice. The activity of immunoprecipitated CaMKIV (left) or CaMKII (right) was measured by the phosphorylation of a specific peptide substrate. Depolarization-induced CaMKIV activity is significantly inhibited in the hippocampus of transgenic mice (wild-type [WT]: 249.6 $\pm$ 41%, n = 8; C34: 129.5 $\pm$ 27.8%, n = 5, $p < 0.05$). The activity of CaMKII appears normal in C34 transgenic mice (WT: 223.4 $\pm$ 39%, n = 7; C34: 193.7 $\pm$ 37.5%, n = 4, ns). *$p < 0.05$ for C34 versus control group.

only modest increases were observed in C34 slices (Figures 2B and 2C). The effects of the transgenic dnCaMKIV were specific for stimulation-induced CREB phosphorylation and no difference was observed in the basal levels of CREB phosphorylation. Furthermore, the total amount of CREB was not affected either by the presence of dnCaMKIV or by the stimulation treatments.

It is known that activation of some of the immediate early genes such as *c-fos* depends on CREB phosphorylation (Sheng and Greenberg, 1990). We therefore examined whether transgenic dnCaMKIV inhibits activity-dependent c-Fos expression. We found that the levels of KCl- and glutamate-induced c-Fos were significantly lower in C34 slices compared to wild-type slices 3 hr after KCl or glutamate treatments (Figures 2B and 2C). The effect of dnCaMKIV was specific to the inhibition of CaMKIV-mediated CREB phosphorylation and c-Fos expression. We did not observe any effect of transgenic dnCaMKIV on the level of pCREB or c-Fos induced by an activator of PKA signaling pathway, forskolin (50 μM; Figures 2B and 2C). In addition, KCl-induced phosphorylation of mitogen-activated protein kinase (MAPK) was not affected (Figure 2D). Taken together, these data indicate that CaMKIV signaling is crucially involved in Ca^{2+}-stimulated CREB phosphorylation and activation of CRE-dependent transcription in the adult hippocampus, and that this signaling is significantly impaired in *dnCaMKIV* transgenic mice.

Normal Synaptic Transmission and Impaired L-LTP

We examined basal synaptic transmission at Schaffer collateral-CA1 synapses of hippocampal slices derived

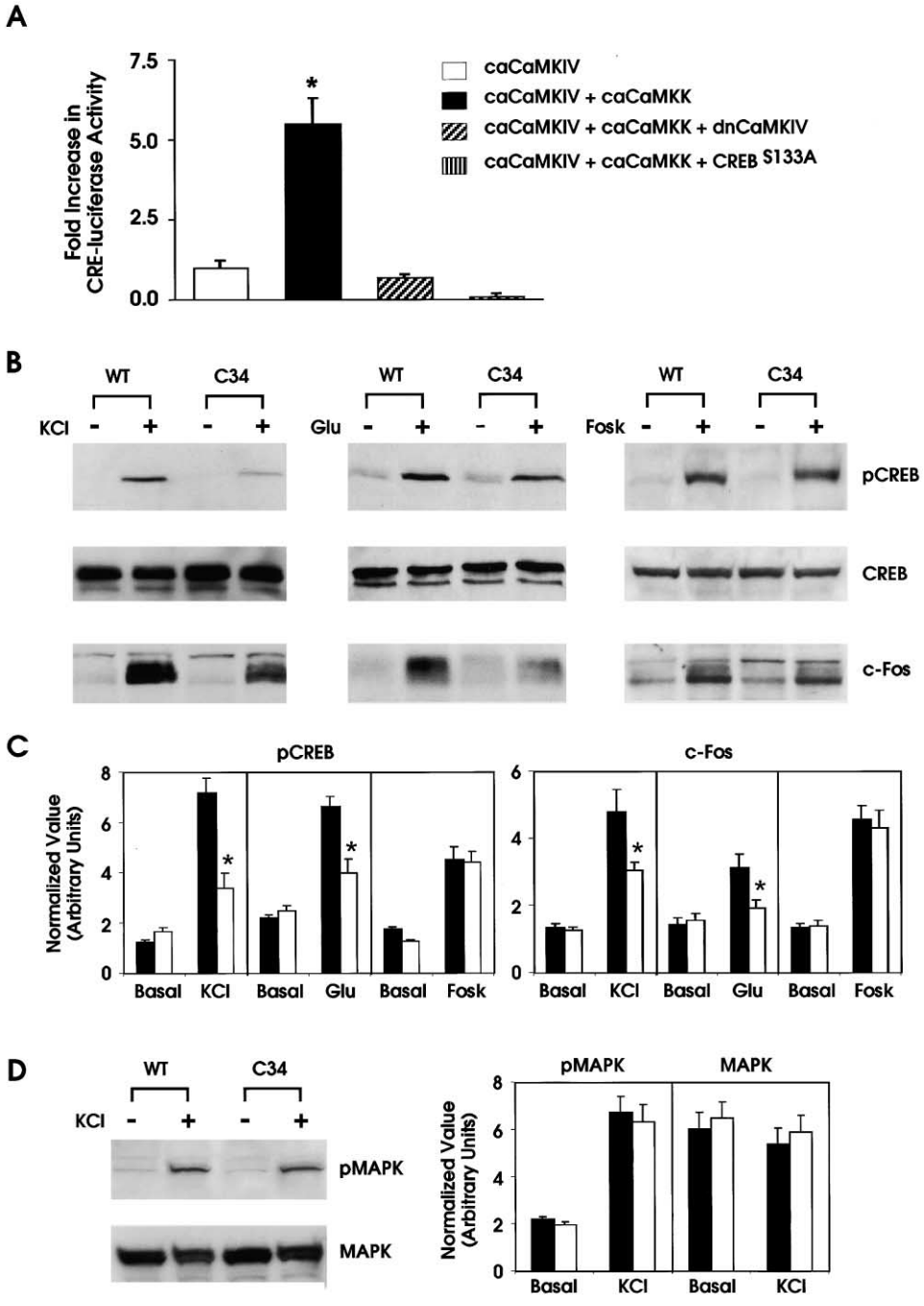

Figure 2. Expression of dnCaMKIV Suppresses Activity-Induced CREB Phosphorylation and CRE-Dependent Gene Expression

(A) Effects of dnCaMKIV on CRE-mediated transcription. COS7 cells were transfected with plasmids expressing the reporter gene CRE-luciferase and caCaMKIV alone (open bar) or with (all others) caCaMKK. DnCaMKIV or CREB^{S133A} was transfected as indicated. Luciferase activity was measured in the cell extracts: caCaMKIV alone (1.0 $\pm$ 0.20, n = 12) or with caCaMKK (5.5 $\pm$ 0.79, n = 12, $p < 0.001$). This CaMKIV/CaMKK stimulation of luciferase expression was completely inhibited when cells were transfected with either dnCaMKIV (0.67 $\pm$ 0.11, n = 6) or CREB^{S133A} (0.08 $\pm$ 0.10, n = 6).

(B) Immunoblot analysis of hippocampal extracts from wild-type and C34 transgenic slices stimulated with KCl, glutamate (Glu), or forskolin (Fosk). The blots were probed with anti-pCREB, anti-CREB, or anti-c-Fos antibodies, respectively.

(C) Quantification of average pCREB and c-Fos immunoreactivity obtained from three independent experiments. *$p < 0.05$ for C34 versus control group.

(D) Immunoblot analysis of hippocampal extracts from either unstimulated or KCl-stimulated wild-type and C34 transgenic slices. The blots were probed with anti-pMAPK or anti-MAPK antibodies. Right panel, quantification of average pMAPK and MAPK immunoreactivity obtained from two independent experiments.

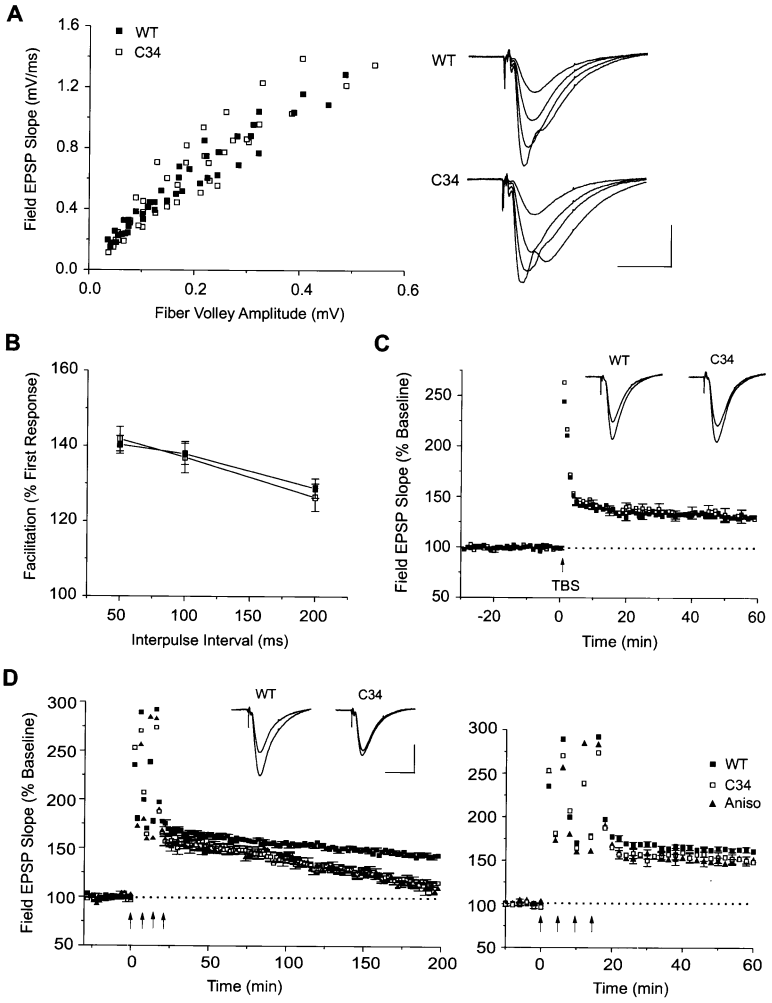

Figure 3. L-LTP Is Disrupted in *dnCaMKIV* Transgenic Mice

(A) Input-output curves plotting the fEPSP slopes and their corresponding presynaptic fiber volley amplitudes in wild-type (filled squares) and C34 transgenic (open squares) slices (n = 8 mice, 41 slices). Superimposed representative EPSPs shown were obtained at stimulation strength 15, 20, 30, and 50 μA. Calibration bars, 1 mV and 20 ms.

(B) Paired-pulse facilitation is not altered. The facilitation ratio (slope of second EPSP/slope of first EPSP) was plotted as a function of interpulse interval, 50, 100, and 200 ms (n = 9 mice, 16 slices). For each group, the mean ± SEM is indicated.

(C) LTP induced by theta-burst stimulation (TBS). Significant LTP was elicited in both groups after TBS. Superimposed representative EPSPs shown were recorded 5 min before and 1 hr after LTP induction.

(D) Late-phase LTP (L-LTP) induced by four trains of tetanic stimulation spaced by 5 min intervals. Right panel, magnified view of the first 60 min of L-LTP. Filled triangles indicate L-LTP obtained with wild-type slices in the presence of anisomycin (Aniso). Superimposed representative EPSPs shown were recorded 5 min before and 3 hr after L-LTP induction. Calibration bars, 1 mV and 20 ms.

from wild-type and transgenic animals. The synaptic input-output relationship, namely field EPSP (fEPSP) slopes versus their corresponding presynaptic fiber volley amplitudes elicited at different stimulus strengths, was not different between wild-type and C34 slices (Figure 3A). The mean ratios of fEPSP slope to fiber volley sizes for wild-type (3.45 ± 0.11, n = 41) and C34 transgenic mice (3.43 ± 0.12, n = 41) were comparable, indicating that the transgenic dnCaMKIV did not perturb basic synaptic response properties.

Because the transgene is expressed in both CA1 and CA3 pyramidal neurons in C34 transgenic line (see Figure 1C), we examined the integrity of the presynaptic machinery by analyzing two forms of short-term synaptic plasticity, paired-pulse facilitation (PPF) and posttetanic potentiation (PTP). At all interpulse intervals, PPF was indistinguishable in wild-type and mutant slices (Figure 3B). The peak PTP elicited by 100 Hz stimulation was also normal in C34 transgenic mice (WT: 256.1 ± 8.7%, n = 9; C34: 263.3 ± 12.1%, n = 9, ns). These data suggest that presynaptic neurotransmitter release processes are not affected by the expression of *dnCaMKIV* transgene.

We next explored the role of CaMKIV in LTP induction. Administration of theta burst stimulation (TBS) at 100 Hz produced LTP that was similar in wild-type and transgenic mice (Figure 3C). The magnitude of potentiation measured at 1 hr after stimulation was 129.9 ± 3.0% (n = 10) for wild-type and 131.5 ± 5.9% (n = 9) for C34 transgenic mice. We then addressed the role of CaMKIV in L-LTP, especially its protein synthesis-dependent late phase, by applying repeated tetanization to the Schaffer collateral pathway. Wild-type slices showed stable L-LTP for at least 200 min, whereas the same stimulation protocol produced a continually decaying potentiation in C34 mutant slices (Figure 3D). The potentiation observed in mutant slices was significantly less than that observed in wild-type at 3 hr (WT: 145.5 ± 1.5%, n = 9; C34: 117.9 ± 2.7%, n = 9, $p < 0.05$), and this significance appeared from 1 hr posttetanization (WT: 162.0 ± 1.9%, n = 9; C34: 147.9 ± 5.5%, n = 9, $p < 0.05$). The decay kinetics of L-LTP in C34 transgenic mice was similar to that observed with L-LTP in wild-type slices in the presence of a protein synthesis inhibitor, anisomycin (Aniso, 40 μM, at 1 hr: 150.4 ± 8.1%; at 3 hrs: 115.5 ± 7.9%, n = 7, ns for C34 versus Aniso) (Figure 3D). Anisomycin (40 μM) did not exert any effect on a nonpotentiated baseline synaptic transmission (at 1 hr: 101.6 ±

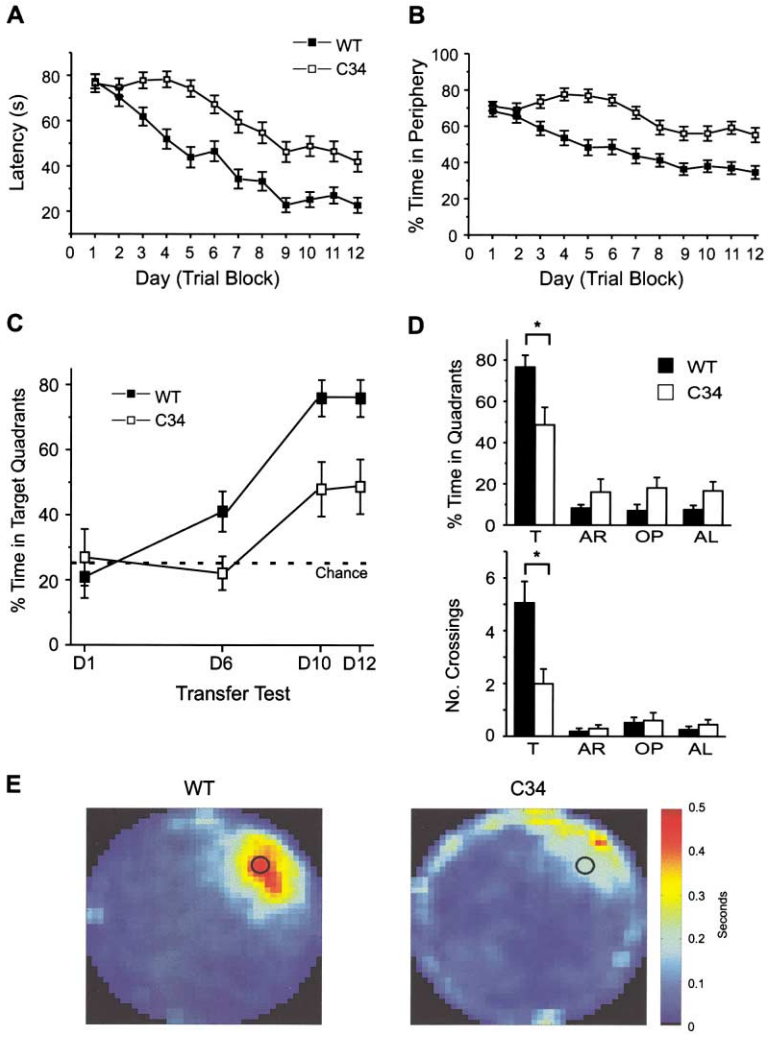

Figure 4. The Performance of *dnCaMKIV* Transgenic Mice in a Spatial Memory Task—Morris Water Maze

The transgenic mice are impaired in the hidden platform version of the Morris water maze. Mean ± SEM values are plotted against the training day for the wild-type (filled squares, n = 15) and C34 transgenic (open squares, n = 13) mice.

(A) Mean escape latencies for each group to reach the hidden platform. The transgenic mice display a longer latency in every block (4 trials per day) than the wild-type mice. On the last day of training (12th day), the transgenic mice took 42 ± 4.5 s to find the platform whereas the wild-type controls required only 22.8 ± 3.4 s ($p < 0.05$).

(B) Mean percentage of swim time spent in the peripheral portion (outer 40% of the total volume) of the pool for each group. The transgenic mice show a higher tendency of thigmotaxis than the wild-type controls from the 3rd day of training.

(C) Comparison of mean percentage of time (± SEM) spent in target quadrant by wild-type and C34 mice in probe trials on day 1 (WT: 20.9 ± 6.5%; C34: 26.9 ± 8.7%, $p > 0.1$), day 6 (WT: 41.1 ± 6.2%; C34: 22.1 ± 5.2%, $p < 0.05$), day 10 (WT: 76.6 ± 4.4%; C34: 47.1 ± 8.5%, $p < 0.05$), and day 12 (see below).

(D) Results of the probe trial given on day 12. Upper panel, mean percentage of time (± SEM) spent searching in each quadrant for the wild-type (76.7 ± 6.5%) and C34 transgenic mice (48.7 ± 8.4%). Lower panel, mean number of target platform crossings performed by wild-type mice compared to the transgenic mice (WT: 5.1 ± 0.8 crossings; C34: 2.0 ± 0.6 crossings). There is a significant group difference in both measures. *$p < 0.05$ for C34 versus control group. T, target quadrant; AR, adjacent right quadrant; OP, opposite quadrant; and AL, adjacent left quadrant.

(E) The activity histogram representing the total occupancy by wild-type and transgenic mice during the last probe test. Each pixel represents 4 × 4 cm^2 space. The wild-type mice are more accurate than the transgenic mice in searching the previous platform location (black circle).

2.5%; at 3 hrs: 98.4 ± 4.2%, n = 7, ns). This suggests that CaMKIV signaling may contribute to the protein synthesis-dependence of L-LTP.

Impaired Spatial Memory in Morris Water Maze Task

To investigate whether *dnCaMKIV* transgenic mice display a deficit in hippocampus-dependent learning and memory that may correlate with the synaptic plasticity impairments, we subjected these mice to the hidden platform version of the Morris water maze (Morris et al., 1982). The C34 transgenic mice consistently showed longer escape latencies (Figure 4A), although they displayed some improvement in their performance over prolonged training. There were no differences between the two types of mice during the first two days of training, suggesting that the learning impairments of the transgenic mice were not associated with initial performance deficits such as motor impairment. However, the C34 mice showed a greater tendency than the wild-type animals to swim in the vicinity of the pool wall (Figure 4B, see below). As shown in Figures 4C and 4D, with the control mice, the preference for the target quadrant increased steadily from a random level on day 1 to over 75% on day 10 and day 12. By contrast, with C34 transgenic mice, the preference for the target quadrant remained at a random level until day 6 and then increased to a moderate level (~47%) on day 10. Two more days of training did not further improve their quadrant preference (~48% on day 12). Even when both groups reached the plateau in their performance by day 12, the spatial memory attained by C34 transgenic mice seemed to be less accurate, as indicated by a larger difference in the number of platform crossing between the two genotypes (Figure 4D). This last point is further demonstrated by the analysis of the navigational paths: while wild-type mice exhibited a high total occupancy peak at the expected location of the platform, the transgenic mice navigated more diffusely around it (Figure 4E).

We next tested the wild-type and C34 transgenic mice in the fixed location/visible platform version of the Morris water maze (McDonald and White, 1994; Morris, 1981).

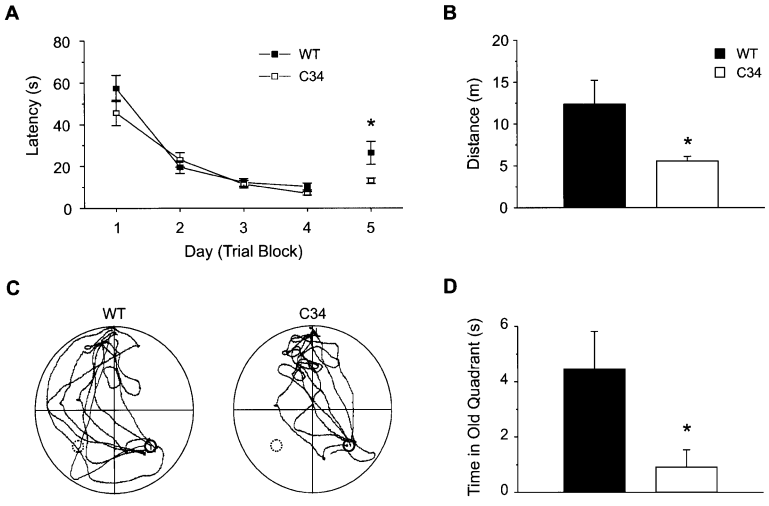

Figure 5. The Performance of *dnCaMKIV* Transgenic Mice in a Fixed Location/Visible Platform Task

(A) Mean escape latencies for each group across 4 days of acquisition trials and on a single competition test on the 5th day. The C34 transgenic mice (open squares, n = 8) are comparable to the wild-type controls (filled squares, n = 8) during the acquisition trials, but escape faster than the wild-type mice to the new platform location on the competition test (WT: 26.4 ± 5.5 s; C34: 13.0 ± 1.3 s, $p < 0.05$).

(B) Mean path lengths for each group to reach the new platform location during the competition test. The wild-type mice (black, WT: 12.4 ± 2.8 min) take longer than C34 mice (white, C34: 5.6 ± 0.5 min, $p < 0.05$) to find the platform in a different quadrant.

(C) Distribution of the swim paths of wild-type and C34 transgenic mice on the competition test. The visible platform is symbolized as a circle with solid line, and the location of the platform during the acquisition trials is shown as a circle with dotted line.

(D) Average time spent in old quadrant for wild-type (black, n = 8) and C34 transgenic (white, n = 8) mice. The C34 mice (4.46 ± 1.4 s) spent significantly longer time in old quadrant than WT mice (0.9 ± 0.6 s, $p < 0.05$). *$p < 0.05$ for C34 versus control group.

The transgenic and wild-type littermates displayed similar latency curves (Figure 5A). The average swimming speed and fractional periphery occupancy were also indistinguishable between the two groups (data not shown). However, on the 5th day when the visible platform was moved to a new location (competition test) (Devan and White, 1999), the wild-type mice exhibited significantly longer latencies than the transgenic mice (Figure 5A) and the distance they traveled to reach the new platform location was also greater than C34 transgenic mice (Figure 5B). Most of the wild-type mice (5 out of 8) swam to the area within 15 cm of the perimeter of the previous platform location, prior to directing themselves toward the visible platform which was placed in the adjacent quadrant. In contrast, 6 out of 8 transgenic mice swam directly to the new platform location (Figure 5C). The average time spent in the old quadrant (Figure 5D) also indicates that the wild-type mice preferentially employed a spatial strategy even in this visible platform version of the Morris water maze. The data suggest that the transgenic mice are impaired in the ability to use a spatial strategy and rely more on a cue-platform association strategy.

We subjected C34 transgenic mice and wild-type littermates to open-field and elevated plus maze tests. The percent dwell time in the open arms, percent number of entries into the open arms (Figure 6A), and the total number of entries into the closed and open arms (Figure 6B) were all comparable between the two groups of mice. We also found that the two types of mice were indistinguishable in both total and marginal activity in the open-field test (Figures 6C and 6D). These findings suggest that the observed higher thigmotactic tendency of C34 transgenic mice (Figure 4B) may be caused by an impairment in learning the spatial location of the platform rather than the general locomotor or emotional defects such as reduced activity or increased anxiety.

Selective Deficits in Long-Term Memory of Contextual Fear Conditioning

Because the Morris water maze task requires repeated training over several days, it is likely that the spatial memory evaluated by probe trials at day 6 and day 12 is a composite measure of acquisition and consolidation/retention of spatial memory. To assess the role of CaMKIV in different temporal phases of the mnemonic process, we turned to contextual fear conditioning in which the acquisition and consolidation phases are expected to overlap less. Thus, if CaMKIV is solely involved in the latter phase, the transgenic mice may show an enhanced decay of an initially normal freezing response over time. To address this issue, we tested mice in the same context at 24 hr and 7 days after 1 tone (CS)/shock (US) training. The *dnCaMKIV* transgenic and wild-type mice showed similar levels of freezing at 24 hr after training (Figure 7A). By contrast, transgenic mice exhibited a significant reduction (~40%) in context-dependent freezing compared to wild-type controls 7 days after training (one way ANOVA: $p < 0.05$). As Figure 7B shows, the *dnCaMKIV* mice displayed normal memory for cued conditioning at 24 hr or 7 days after training. These data suggest that *dnCaMKIV* transgenic mice have a selective deficit in the consolidation/retention phase of context-dependent fear memory.

Discussion

Importance of CaMKIV Function in CREB Phosphorylation and CRE-Dependent Gene Transcription

Using conditional transgenic mice in which the dominant-negative transgene inhibited the activation of endogenous CaMKIV only in the postnatal forebrain, we have shown that CaMKIV is crucially involved in Ca^{2+}-induced CREB phosphorylation and c-Fos expression.

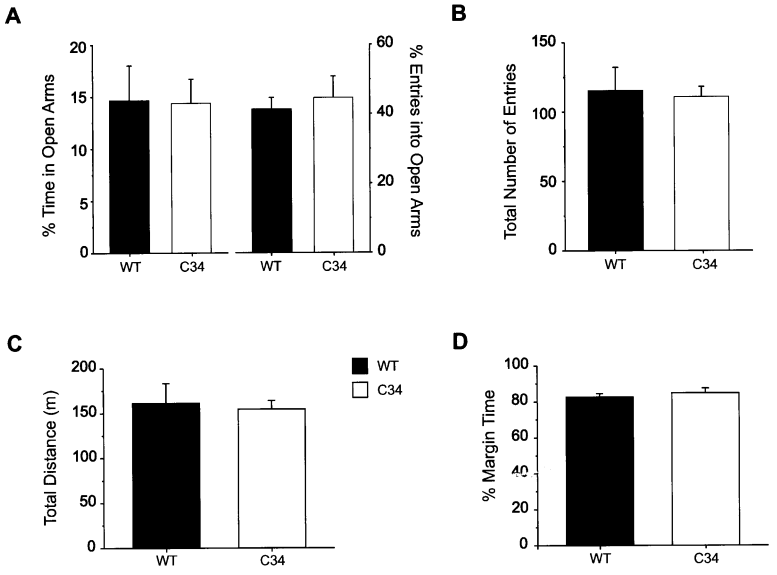

Figure 6. Normal Performance of *dnCaMKIV* Transgenic Mice in Elevated Plus Maze and Open Field Test

Mean ± SEM values are shown for the wild-type (black, n = 8) and C34 transgenic (white, n = 8) mice. There are no significant differences between groups in any of these test variables.

(A and B) Elevated plus maze. The wild-type and transgenic mice did not reveal significant difference in percent of time spent on the open arms or percent number of entries into the open arms during the 10 min testing (A). Total number of entries into the open or closed arms was also similar in wild-type and transgenic mice (B).

(C and D) Open field activity. The results show that the two groups of mice explore the activity chamber similarly. There were no group differences in activity indices such as total distance traveled (C) and percent of time spent within a margin of 1 cm from the wall (D) during the 10 min testing.

The modest yet still significant stimulation-induced increases in pCREB and c-Fos signals detected in slices from the C34 transgenic mice may be due to the incomplete inhibition of endogenous CaMKIV by dnCaMKIV (Figure 1F) and/or the involvement of other kinases in CREB/CRE signaling pathway. Ser[133] phosphorylation of CREB has been shown to depend on multiple Ca^{2+}-activated kinases including PKA and MAPK (Impey et al., 1998; Wu et al., 2001). In fact, both PKA and MAPK signaling pathways are intact in C34 transgenic mice (Figures 2B, 2C, and 2D). A recent study suggests that the fast CaMKIV-dependent pathway and the slower MAPK-dependent pathway contribute to pCREB formation at different times after stimulation, and convey differential information to the nucleus with respect to the precise timing and stimulus amplitude, respectively (Wu et al., 2001).

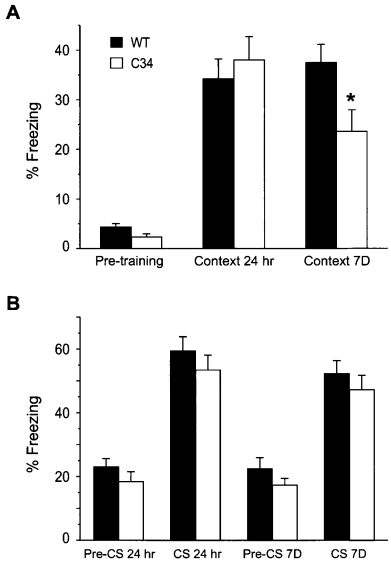

Figure 7. Fear Conditioning in *dnCaMKIV* Transgenic Mice

The transgenic mice show a selective impairment in long-term memory for contextual fear conditioning. Mean ± SEM values are plotted against the test day for the wild-type (black, n = 16) and C34 transgenic (white, n = 16) mice.

(A) Freezing responses for each group at 24 hr and 7 days after conditioning in the same context. The transgenic mice display significantly less freezing compared to wild-type mice after 7 days (WT: 37.5 ± 3.6%; C34: 22.9 ± 1.3%, $p < 0.05$) but not after 24 hr (WT: 34.3 ± 3.9%, C34: 38.0 ± 4.7%, ns). *$p < 0.05$ for C34 versus control group.

(B) Cued fear conditioning revealed no significant difference between wild-type and C34 mice at 24 hr (WT: 59.4 ± 4.4%; C34: 53.5 ± 4.6%, ns) or 7 days after conditioning (WT: 52.3 ± 3.9%; C34: 47.4 ± 4.6%, ns).

In the global CaMKIV knockout mice, the basal level of pCREB was also reduced (Ho et al., 2000; Ribar et al., 2000). In contrast, we did not observe any difference in basal levels of CREB phosphorylation or c-Fos expression in our transgenic mice (Figures 2C, 2D, and 2E). Thus, the basal level phosphorylation of CREB and activation of the *c-fos* gene seem to be achieved by the activity of CaMKIV in its basal state, which is not affected by the transgenic dnCaMKIV. A previous study also reported similarly selective action of this dominant-negative form of CaMKIV on depolarization-induced activity of CaMKIV (Impey et al., 1998). Although further work is needed to determine precise mechanisms for the dominant-negative effect of transgenic dnCaMKIV, one possibility is that the catalytically inactive form of CaMKIV with a mutation in the ATP binding site (Lys[71]→Ala) binds to CREB and inhibits the interaction between CREB and potential CREB kinases. This mechanism, however, would not result in the specific inhibition of stimulus-induced CREB phosphorylation, which was shown in this study (Figures 2B and 2C). Moreover, our experiments showing that dnCaMKIV is without effect on PKA-mediated CREB phosphorylation do not support this possibility (Figures 2B and 2C). Another possibility is that dnCaMKIV competes with endogenous CaMKIV for the phosphorylation by CaMKK. It is known that the large increases in CaMKIV activity induced by massive depolarization require the phosphorylation of Thr[196] by CaMKK (Tokumitsu and Soderling, 1996). The trans-

genic dnCaMKIV with Thr^{196}→Ala mutation may inhibit the stimulus-induced activation of endogenous CaMKIV by competitively blocking this critical phosphorylation event. In that case, dnCaMKIV would not interfere with the basal activity of CaMKIV but would prevent further increase of its activity mediated by CaMKK. This possibility is further supported by our experiments showing that dnCaMKIV prevents the transcriptional activation mediated by CaMKK-CaMKIV signaling in COS7 cells (Figure 2A). It should be noted that the inhibition of CaMKK function by dnCaMKIV may also hamper the function of CaM kinase I (CaMKI), which is also known to be activated by CaMKK (Sun et al., 1996). However, it is unlikely that the inhibition of CaMKI contributes to the observed effects of dnCaMKIV on CREB phosphorylation since CaMKI is not expressed in the neuronal nucleus (Picciotto et al., 1995). Regardless of the actual mechanism, the selective effects of dnCaMKIV on stimulus-induced CaMKIV activation and CREB phosphorylation allowed us to assess the consequence of these stimulus-induced events in synaptic plasticity and memory.

L-LTP Is Disrupted in *dnCaMKIV* Transgenic Mice

Activation of CREB and CRE-dependent transcription have been proposed to contribute to L-LTP but not to E-LTP (Impey et al., 1996; Silva et al., 1998). In support of these notions suggested by earlier studies, both the CREB-dependent signaling pathway in the hippocampus and the L-LTP at Schaffer collateral-CA1 synapses were impaired in our forebrain-specific *dnCaMKIV* transgenic mice. Furthermore, as shown in Figure 3D, the decay kinetics of L-LTP in C34 slices parallels that observed in anisomycin-treated wild-type slices, suggesting a specific inhibition of protein synthesis-dependent component of L-LTP by transgenic dnCaMKIV. Since CaMKIV has been shown to contribute to Ca^{2+}-induced activation of CREB (Bito et al., 1996; Finkbeiner et al., 1997; and Figures 2B and 2C), which has been in turn implicated in L-LTP, it is tempting to conclude that the L-LTP impairment observed in C34 transgenic mice is due to the dominant-negative effect of the transgene product on CREB activation. There is, however, no direct evidence for this causal relationship. For instance, the requirement of CaMKIV for L-LTP could result from the action of CaMKIV on the components of other transcriptional machinery, such as serum-response factor (SRF), and CREB binding protein CBP (Hu et al., 1999; Sun et al., 1996).

DnCaMKIV Transgenic Mice Are Impaired in the Consolidation/Retention Phase of Hippocampus-Dependent Memory

The C34 transgenic mice are clearly impaired in learning the hippocampus-dependent, hidden platform version of the Morris water maze task. Nevertheless, the transgenic mice seem to overcome the learning deficit to some extent as the training becomes more extensive. We do not know how these mice achieved this partial and/or slower learning. One explanation would be the incomplete inhibition of CaMKIV by the transgenic dnCaMKIV (Figure 1F). Another possibility would be that the requirement for CaMKIV is not absolute and other kinases are recruited as the training becomes more extensive. One can speculate that as the training is extended, kinases such as PKA (Yamamoto et al., 1988) or MAP kinase (Wu et al., 2001) known to be capable of activating CREB may be recruited to compensate for the inhibition of CaMKIV.

The idea that the observed behavioral deficits are due to the animal's inability or difficulty in adopting a spatial strategy is strongly suggested by the results of the competition test in the fixed location/visible platform version of the Morris water maze. Furthermore, these results indicate that *dnCaMKIV* transgenic mice have intact vision, escape motivation, and coordinated motor skills required for learning the water maze task. The specificity of the behavioral deficits of *dnCaMKIV* transgenic mice was further supported by the results of the open field test and the elevated plus maze, which revealed no discernable abnormalities in general locomotor or emotional aspects of behavior.

The *dnCaMKIV* transgenic mice displayed a deficit in the neural activity-dependent activation of CREB/CRE signaling pathway and a relatively specific impairment in synaptic plasticity, namely the late component of L-LTP. Since these protein synthesis-related events have been suggested as a molecular or cellular basis for memory consolidation, it was of great interest to determine whether the memory deficit displayed by these mice in the Morris water maze task was primarily in its consolidation phase rather than in the acquisition phase. Our fear conditioning study demonstrates that in C34 transgenic mice, the contextual memory is intact one day after training but is attenuated significantly 7 days after training. These data support the view that the effect of dnCaMKIV on context-dependent fear conditioning is not on the acquisition phase of the memory but on its consolidation or retention phase. The in vivo process of memory consolidation is likely to require a cascade of gene activation, protein synthesis, and protein alterations in a series of different types of synapses within a complex neural circuitry, which may take days or weeks. On the other hand, the readout of in vitro L-LTP at one particular type of synapse (e.g., Schaffer collateral-CA1 synapses) is an enhancement of synaptic transmission that can be detected within hours. Therefore, the deficit of L-LTP evident within hours may well underlie the impairment in memory consolidation detected 7 days after conditioning. Similar to our results, in AC1/AC8 double knockout mice, while hippocampal L-LTP was impaired within 1 hr after induction, the impairment of the contextual fear conditioning was detectable only after 8 days (Wong et al., 1999). It was also previously shown that rats subjected to hippocampal lesions one day after training exhibited a profound deficit in context-dependent fear conditioning, whereas those lesioned one month after training maintained the contextual fear memory. This suggests that the consolidation of hippocampus-dependent memory may occur sometime between one to 30 days after training (Anagnostaras et al., 1999; Kim and Fanselow, 1992).

Although the most robust site of the transgenic dnCaMKIV expression is the hippocampus, it is also expressed in other forebrain areas, albeit at lower levels (Figure 1C). Therefore, we cannot conclude that the consolidation-dampening effect of the transgenic dnCaMKIV

is exerted within the hippocampus. Nonetheless, it is interesting to note that such an effect was not observed in cue-dependent fear conditioning (Figure 7B), known to be independent of the integrity of the hippocampus (Phillips and LeDoux, 1992). These results can be explained either by the lower levels of dnCaMKIV expression outside of the hippocampus or by the possible differential role of CaMKIV in the consolidation of hippocampus-independent implicit memory. In either case, our data are consistent with the notion that hippocampal CaMKIV plays a crucial role in the consolidation and/or retention of hippocampus-dependent explicit memory.

Possible Links among CaMKIV and CREB Activation, L-LTP, and Memory Consolidation

We have shown that the postnatal forebrain-specific expression of a *dnCaMKIV* transgene results in deficits at multiple levels of complexity. At the molecular level, neural activity-induced activation of CaMKIV, CREB, and CRE-dependent transcription was impaired in the hippocampus. At the level of synaptic physiology, the L-LTP in hippocampal CA1 area was impaired while basic synaptic function and protein synthesis-independent E-LTP were intact. At the level of behavior, the *dnCaMKIV* transgenic mice were impaired in the consolidation and/or retention phase of hippocampus-dependent memory but not in its acquisition phase, or in any of these phases of hippocampus-independent memory. While one cannot conclude that these deficits observed at different levels of complexity are causally related from the lower to higher levels, our data are not only consistent with this notion but also add information to that in the literature and thereby strengthen the hypothesis. First, unlike the L-LTP deficits observed earlier in dnPKA (*R(AB)*) mice or adenylyl cyclase knockout mice, the LTP deficit observed in *dnCaMKIV* transgenic mice seems to be targeted to the protein synthesis-dependent component and hence correlates better with the deficit in the protein synthesis-dependent memory consolidation and/or retention (Davis and Squire, 1984). Second, in *dnCaMKIV* transgenic mouse, the perturbation at the molecular level is restricted to a neural activity-induced activation, be it of CaMKIV, CREB, or CRE-dependent transcription. Since L-LTP and memory consolidation are also neural activity-induced phenomena, the correlation of the deficits observed at the molecular level with those at the higher levels can be regarded as more stringent. Finally, unlike the global knockout mice (Ho et al., 2000; Wu et al., 2000), the *dnCaMKIV* transgenic mice, along with the *dnPKA* transgenic mice, are perturbed only in the postnatal forebrain. This certainly helps when one evaluates the relationship between deficits observed at multiple levels of complexity. For instance, we could dissociate the role of CaMKIV in the forebrain from that in many other brain areas of the adult animal as well as from its developmental function. Indeed, all tests we performed for detection of motor or emotional impairments gave negative results and we could detect no gross developmental abnormality.

How do the roles of CaMKIV in CREB/CRE-mediated signaling, L-LTP, and long-term memory relate to those of cAMP signaling pathway or MAP kinase pathway that have also been implicated in similar molecular, physiological, and behavioral processes? In view of the findings that the duration of CREB phosphorylation is a key factor in determining transcriptional initiation (Bito et al., 1996), it is conceivable that these three kinase pathways act in concert to accomplish optimal levels of sustained CREB phosphorylation and memory consolidation events. Alternatively, CaMKIV may play a dominant role in CREB phosphorylation by modulating other kinase pathways. Studies in cell culture system have demonstrated the regulatory role of CaMKIV in both MAP kinase and cAMP pathway (Enslen et al., 1996; Wayman et al., 1996). Regardless, perturbations in any one of these pathways would disrupt the balanced control of CREB-mediated gene expression and consequently result in L-LTP and long-term memory impairments.

In summary, our study demonstrates that interfering with neural activity-dependent CaMKIV signaling in the nucleus of neurons in the postnatal forebrain impairs the consolidation/retention but not the acquisition of hippocampus-dependent long-term memory. Deficits in the neural activity-dependent activation of the CREB/CRE signaling pathway and in the late component of L-LTP in the hippocampus may contribute to this memory impairment.

Experimental Procedures

Generation of Transgenic Mice and Genotyping

A cDNA encoding a dominant-negative form of murine CaMKIV has the following mutations: (1) Lys^{71} in the ATP binding site is mutated to Ala; (2) the phosphorylation site for CaMK kinase, Thr^{196}, is mutated to Ala; and (3) the autoinhibitory domain is inactivated by the triple mutation, $HMDT^{308–311}$ to DEDD. Transgenic mice were generated following the standard procedures using C57BL/6 embryos and kept in this pure genetic background. All analysis was carried out on 8- to 12-week-old mice unless specified otherwise.

Northern Blot Analysis

Approximately 15 μg of total forebrain RNA was subjected to electrophoresis through 1% agarose-formaldehyde gels and blotted onto Zeta-Probe membranes (Bio-Rad). RNA blots were then hybridized with ^{32}P-labeled DNA probes containing either SV40 poly(A) (from pMSG) or α*CaMKII* cDNA. The blots were stripped and reprobed with a human *G3PDH* cDNA (Clonetech) to confirm that each lane contained similar amounts of RNA.

In Situ Hybridization

The mouse brains were dissected and rapidly frozen in mounting medium. Cryostat sections (20 μm) were taken and post-fixed for 15 min in 4% paraformaldehyde in PBS (pH 7.3). The sections were hybridized to a SV40 poly(A) probe specific for the transgene. Hybridization was performed at 60°C overnight in a solution containing 50% formamide, 10 mM Tris-HCl (pH 8.0), 10% dextran sulfate, 1× Denhardt's, 600 mM NaCl, 0.25% SDS, 1 mM EDTA, 200 μg/ml tRNA, and [α-^{33}P]-labeled antisense probe. The hybridized sections were exposed to films, dipped in Kodak NTB3 nuclear emulsion, and developed after 12 days.

Western Blot Analysis and Kinase Assay

Fifty micrograms of forebrain homogenates were resolved by SDS-PAGE and then transferred onto PVDF membranes for immunoblotting. After a blocking reaction (5% nonfat milk in PBS [pH 7.3] and 0.05% Tween-20), the blots were incubated for 1 hr at room temperature with one of the following primary antibodies: mouse monoclonal anti-FLAG M2 antibody (Sigma), anti-CaMKIV antibody (Transduction Laboratories), anti-CaMKIIα antibody (GibcoBRL), or anti-Actin antibody (Boehringer Mannheim). After incubation with a secondary antibody (HRP-conjugated goat anti-mouse antibody), the blots

were developed using an ECL chemiluminescence procedure (Amersham).

To monitor activity-dependent changes in pCREB and c-Fos immunoreactivity, the hippocampal slices were prepared and perfused with 90 mM KCl for 10 min or with either 100 μM glutamate or 50 μM forskolin for 20 min in a submerged chamber. After certain periods of time specified in the text, the slices were lysed in RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, Complete protease inhibitor tablet [Boehringer Mannheim], 1 mM DTT, 1 mM Na_3VO_4, and 1 mM NaF). Western Blot analysis was conducted as described above using rabbit polyclonal anti-phospho CREB antibody (NEB), anti-CREB antibody (NEB), anti-phospho MAPK antibody (NEB), anti-MAPK antibody (NEB), or anti-c-Fos (Ab-2) antibody (Oncogene Research Products). Quantification was done using NIH Image software. The mean pixel density for pCREB or c-Fos signal was measured and normalized to the background.

For kinase activity assays, hippocampal slices were stimulated with 90 mM KCl for 10 min and then immediately homogenized in RIPA buffer. The lysates were immunoprecipitated overnight with 5 μg of anti-CaMKIV antibody (Transduction Laboratories) or goat polyclonal CaMKIIβ antibody (Santa Cruz Biotechnology). The final immunoprecipitates were resuspended in 30 μl CaMK assay buffer and assayed for CaMKIV or CaMKII activity as previously described (Gringhuis et al., 1997). Peptide-γ and autocamtide-2 (Biomol) were used as peptide substrates for CaMKIV and CaMKII, respectively.

Transient Transfection and Luciferase Assay

COS7 cells were transfected when 80% confluent with LipofectAMINE (Gibco BRL) according to the manufacturer's instructions. The pRL-TK vector containing *Renilla* luciferase (Promega) was included in each transfection to normalize for protein levels and transfection efficiency. The following plasmids were transfected with the amounts indicated per 10 cm dish: caCaMKIV (0.02 μg), caCaMKK (0.4 μg), dnCaMKIV (0.2 μg), $CREB^{S133A}$ (0.2 μg), *Renilla* luciferase (0.3 μg), and 5XCRE-luciferase (0.5 μg). Empty vector was used to compensate amounts of plasmids. The caCaMKIV was generated by the triple mutation in the autoinhibitory domain of CaMKIV ($HMDT^{308–311}$ → DEDD) and has been shown to be active in the absence of Ca^{2+}/CaM (Enslen et al., 1996). The caCaMKK was a truncated form (residues 1–413) of CaMKK. Forty-eight hours after transfection, COS7 cells were lysed and luciferase levels were measured using the dual-luciferase reporter assay system (Promega).

Electrophysiology

Transverse hippocampal slices (400 μm thick) were prepared and submerged in a stream (2 ml/min) of ACSF (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11.0 mM glucose) oxygenated with 95% O_2/5% CO_2. Bath temperature was maintained at 22–24°C. Extracellular fEPSPs were evoked by stimulation of the Schaffer collateral afferents at a frequency of 0.033 Hz and at a stimulus intensity that produces fEPSP slopes approximately 35% of the maximum. TBS consisted of 10 bursts of stimuli with four pulses each (100 Hz, 100 μs duration) and 200 ms interburst interval; this TBS was repeated two times with an ISI of 30 s. Tetanic stimulation was delivered at the test intensity in 1 s trains at 100 Hz, with 4 trains 5 min apart to induce late LTP (L-LTP). Anisomycin (Sigma) was added to the perfusate at a final concentration of 40 μM 30 min prior to the application of the first tetanus. We analyzed the initial slope of the fEPSP and used it as a measure of synaptic strength. Paired pulse facilitation (PPF) was tested with 50, 100, and 200 ms interpulse interval. To assess statistical significance, paired t tests, comparing the average slope size for 10 min before LTP induction to either 50–60 min or 170–180 min after LTP induction, were performed on nonnormalized data. Between group comparisons were made of percent of baseline values using unpaired t tests; p values greater than 0.05 are designated as not significant (ns). The genotype of all animals was only revealed after the experiments.

Behavioral Tests

Behavioral tests were conducted in a blind fashion during the light phase at approximately the same time each day. Data were analyzed with one-way analysis of variance (ANOVA) and Student's t test; p values greater than 0.05 are designated as not significant (ns).

The Morris water maze tests were conducted as previously described (Tsien et al., 1997). The apparatus consisted of a circular pool (160 cm diameter, 60 cm deep at the side) filled with water at 21–22°C that was completely covered with floating polypropylene beads (Hanna resin distribution). Black curtains were drawn around the pool (90 cm from the pool periphery) and contained a number of illuminated objects that served as distal cues. Using a computerized tracking system (Dragon), we analyzed the escape latency (platform search time), path length (the distance traveled to reach the platform), the time spent in the periphery (a rim of 18 cm from the wall), and swimming velocity for each trial. For the hidden platform task, the mice were trained to find the hidden platform (10 cm × 10 cm, 1.5 cm below the surface of water) that was placed in the middle of the radial quadrant. The training was carried out in blocks of 4 trials per day (intertrial interval of about 30 min) for 12 days. During each block of trials, the mice were released from four pseudorandomly assigned start locations (N, S, E, and W). If a mouse did not find the platform within 90 s, it was manually guided to the platform and allowed to rest on the platform for 15 s. A probe test was given at the end of days 1, 6, 10, and 12 in the absence of the platform. We measured the time they spent in the radial quadrant in which the platform had been located during training (quadrant occupancy) and the number of times they crossed over the exact platform location (platform crossing).

The fixed location/visible platform task was adapted from Kim et al. (2001). In this task, the location of the platform was made apparent by attachment of an orange flag to the platform, which protruded 15 cm above the surface of water. During the acquisition trials (4 days, total of 16 trials), the mice were trained to swim to the visible platform in a fixed location, NE or SW. On day 5, the visible platform was moved to the center of the adjacent quadrant (NW For NE group, SE for SW group) and the mice were released from start positions equidistant to the old and new platform location. Analysis was performed to determine whether the mice swam to within 15 cm of the perimeter of the previous platform location before escaping to the visible platform, in the adjacent quadrant.

The general activity and anxiety level of the mice was evaluated using the two tests conducted according to the previously published methods (Churchill et al., 1998; Cruz et al., 1994). Accuscan Digiscan (Columbus, OH) automated activity monitors were used to quantify total distance and margin time. Total distance traveled was quantified by measuring the consecutive breaking of adjacent photocell beams. The percent of margin time was obtained by dividing the total testing period (10 min) by the period of time the mouse spent in close proximity (within 1 cm) to the walls of the chamber. For the elevated plus maze, the mice were placed on the central platform of the maze facing one of the walled arms and were observed for 10 min, during which the number of entries into and time spent in the open or enclosed arms were measured. The digitized image of the path taken by each animal was stored and analyzed with a semiautomated analysis system (PolyTrack, San Diego Instruments, Inc.). For contextual fear conditioning, we generally followed the procedure described in previous publication (Bourtchuladze et al., 1994). On training day, mice were placed in a shocking chamber (Coulbourn instruments) and a 30 s white noise tone (CS) was presented 2 min later. A mild foot shock (0.75 mA intensity, 2 s duration) was delivered at the end of the tone and coterminated with it. Mice were left in the training chamber for another 30 s and returned to their home cage. During testing, mice were placed back in the conditioning chamber for 3 min. Live 8 bit grayscale video was digitized at 2 Hz using computerized NIH Image software (Anagnostaras et al., 2000; Miyakawa et al., 2001). Freezing was defined as immobility for a consecutive one-second period. Cued fear conditioning tests were conducted in a similar manner to contextual tests except that on test days, mice were placed in a white plexiglass chamber for a total of 5 min. Baseline freezing in the novel context (pre-CS) was monitored for 2 min prior to delivering the continuous 3 min tone.

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