Control of Memory Formation Through Regulated Expression of a CaMKII Transgene

Mark Mayford, Mary Elizabeth Bach, Yan-You Huang, Lei Wang, Robert D. Hawkins, Eric R. Kandel*

One of the major limitations in the use of genetically modified mice for studying cognitive functions is the lack of regional and temporal control of gene function. To overcome these limitations, a forebrain-specific promoter was combined with the tetracycline transactivator system to achieve both regional and temporal control of transgene expression. Expression of an activated calcium-independent form of calcium-calmodulin–dependent kinase II (CaMKII) resulted in a loss of hippocampal long-term potentiation in response to 10-hertz stimulation and a deficit in spatial memory, a form of explicit memory. Suppression of transgene expression reversed both the physiological and the memory deficit. When the transgene was expressed at high levels in the lateral amygdala and the striatum but not other forebrain structures, there was a deficit in fear conditioning, an implicit memory task, that also was reversible. Thus, the CaMKII signaling pathway is critical for both explicit and implicit memory storage, in a manner that is independent of its potential role in development.

Explicit memory—a memory for facts, places, and events—requires the hippocampus and related medial temporal lobe structures (*1*), whereas implicit memory—a memory for perceptual and motor skills—involves a variety of anatomical systems (*2*). For example, one form of implicit memory, that for conditioned fear, involves the amygdala (*3*).

Studies with genetically modified animals have sought to relate specific genes to specific forms of explicit or implicit memory storage (*4–8*). However, current methodology does not allow one to distinguish between a direct effect on memory or its underlying synaptic mechanisms and an indirect effect on the development of the neuronal circuits in which the memory storage occurs (*4, 9*). In addition, the gene under study is typically overexpressed or ablated throughout the entire brain. As a result, the genetic modifications often affect, indiscriminately, both implicit and explicit memory as well as perceptual or motor performance. Thus, to analyze the molecular contribution of a given gene to a particular type of memory, it is essential not only to control the timing of expression but also to restrict expression to appropriate cell populations.

To address these issues and to achieve regulated transgene expression in restricted regions of the forebrain, we used a forebrain-specific promoter in combination with the tetracycline transactivator (tTA) developed by Bujard and his colleagues (*10, 11*). We examined the role of CaMKII signaling in synaptic plasticity as well as in implicit and explicit memory storage.

CaMKIIα is a serine-threonine protein kinase that is restricted to the forebrain (*12–14*). It is expressed in the neurons of the neocortex, the hippocampus, the amygdala, and the basal ganglia. After a brief exposure to Ca^{2+}, CaMKII can convert to a Ca^{2+}-independent state through an autophosphorylation at Thr^{286} (*12, 14–17*). This ability to become persistently active in response to a transient Ca^{2+} stimulus led to the suggestion that CaMKII may be a molecular substrate of memory (*18*). Targeted disruption of the CaMKIIα gene produces deficits in long-term potentiation (LTP) and severely impairs performance on hippocampal-dependent memory tasks (*5, 6*). Mutation of Thr^{286} to Asp in CaMKIIα mimics the effect of autophosphorylation at Thr^{286} and converts the enzyme to a Ca^{2+}-independent form (*15, 17*). Transgenic expression of this dominant mutation of CaMKIIα (CaMKII-Asp^{286}) results in a systematic shift in response to low-frequency stimulation such that long-term depression (LTD) is favored in the transgenic mice (*7*). Thus, although Schaffer collateral LTP in response to 100-Hz tetanus is not altered, LTP is eliminated in the range of 5 to 10 Hz, a frequency (the theta frequency) characteristic of the endogenous oscillation in neuronal activity seen in the hippocampus of animals during spatial exploration (*19*). Correlated with this selective deficit in LTP in the theta frequency range is a severe defect in spatial memory (*8*). We now have reexamined these phenomena with regulated expression of the CaMKII-Asp^{286} transgene.

Doxycycline regulation of transgene expression. The first type of mouse we generated to achieve regulated expression of CaMKII-Asp^{286} in forebrain neurons (Fig. 1A) expressed the tTA gene under the control of the CaMKIIα promoter (line B), which limits expression of the tTA transgene to neurons of the forebrain (*20*). In the second type of mouse, the tTA-responsive tet-O promoter is linked to the target gene of interest, in this case either *lacZ* or the CaMKII-Asp^{286} gene. The tTA gene expresses a eukaryotic transcription activator that binds to and activates transcription

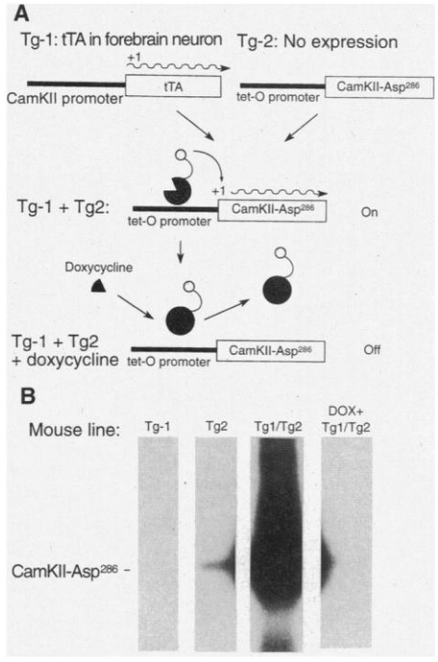

Fig. 1. Regulation of the CaMKII-Asp^{286} transgene with the tTA system. (**A**) Strategy used to obtain forebrain-specific doxycycline-regulated transgene expression. Two independent lines of transgenic mice are obtained, and the two transgenes are introduced into a single mouse through mating. (**B**) Quantitation by RT-PCR Southern blot of CaMKII-Asp^{286} expression from the tet-O promoter. RT-PCR was performed on total forebrain RNA and probed for expression of the CaMKII-Asp^{286} mutant mRNA as described (*7, 21*). Tg1, mouse carrying only the CaMKII promoter–tTA transgene (line B). Tg2, mouse carrying only the tet-O–CaMKII-Asp^{286} transgene (line 21). Tg1/Tg2, double transgenic mouse carrying both the CaMKII promoter-tTA transgene (line B) and tet-O–CaMKII-Asp^{286} (line 21) transgenes. Tg1/Tg2 + Dox, double transgenic mouse treated with doxycycline (2 mg/ml) plus 5% sucrose in the drinking water for 4 weeks.

The authors are at the Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University, and Howard Hughes Medical Institute, 722 West 168 Street, New York, NY 10032, USA.

*To whom correspondence should be addressed.

from the tet-O promoter element; this transcription is blocked by the tetracycline analog doxycycline (*10*). When both the tet-O and tTA transgenes were introduced into the same mouse, the tet-O–linked gene was activated, but only in those cells that express tTA.

We assessed the regulation of the CaMKII-Asp^{286} transgene using a reverse transcriptase–polymerase chain reaction (RT-PCR) Southern (DNA) blot (*21*) to detect only the mutant transcripts (Fig. 1B). Mice carrying either one of the transgenes alone show little or no expression of CaMKII-Asp^{286} mRNA. When both transgenes were introduced into the same mouse, there was a large activation of CaMKII-Asp^{286} expression. The expression of this transgene was completely suppressed when the mice were given doxycycline (2 mg/ml) in the drinking water for 4 weeks.

Restricted expression of the tet-O–linked transgenes. We examined the expression of β-galactosidase in two tet-O *lacZ* reporter lines of mice that also carried the CaMKIIα promoter–tTA transgene (Fig. 2A). In the first line, expression was uniform throughout the forebrain, neocortex, hippocampus, amygdala, and striatum. This pattern mimics the expression of the endogenous CaMKIIα gene (*13*). In the second *lacZ* line, expression was observed throughout the forebrain, but surprisingly, expression was absent in the CA3 pyramidal cell body layer of the hippocampus (Fig. 2B).

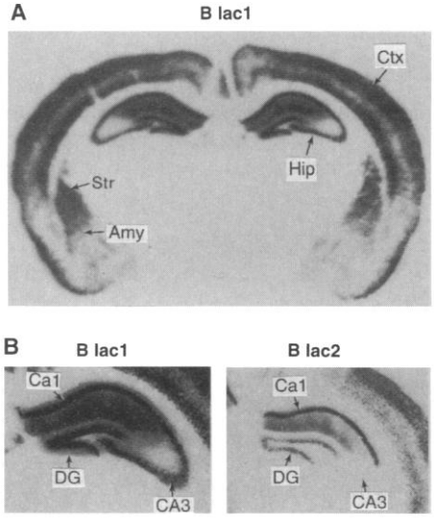

Fig. 2. Forebrain-specific activation of a tet-O–*lacZ* transgene. (**A**) Coronal section of double transgenic line B lacI stained with X-Gal as described (*42*). Ctx, cerebral cortex; Str, striatum; Hip, hippocampus; Amy, amygdala. (**B**) X-Gal–stained coronal section of the hippocampus from double transgenic lines B lac1 and B lac2. CA1, CA1 cell body layer; CA3, CA3 cell body layer; DG, dentate gyrus.

Using in situ hybridization, we next examined the pattern of expression in three lines of double transgenic mice expressing tet-O–linked CaMKII-Asp^{286} (mouse lines B13, B21, and B22) (Fig. 3). In the first line (B13), expression was evident throughout the forebrain. However, in the hippocampus, expression was strong in the dentate gyrus and CA1 region but was weak or absent in the CA3 region. In a second line of mice (B22), there was moderate expression in the hippocampus, subiculum, striatum, and amygdala, with little expression in neocortex. In the hippocampus, expression was again present in the CA1 region and absent in the CA3 region. In the third line (B21), there was little expression in the neocortex and hippocampus but strong expression in the striatum, in anterior and lateral amygdala nuclei, and in the underlying olfactory tubercle. Thus, whereas the CaMKII promoter can limit expression to forebrain neurons generally, expression of the tet-O–linked transgene is further limited to particular subsets of forebrain neurons, presumably due to integration site–dependent effects.

In double transgenic mice, a high level of expression of the CaMKII-Asp^{286} mRNA was obtained (Figs. 1B and 3). To determine the effect of this expression on enzyme activity, we measured CaMKII activity in the striatum of the B21 line of mice (Table 1). In these mice, Ca^{2+}-independent CaMKII activity was increased sevenfold relative to that of the wild type. However, when the mice were treated with doxycycline (1 mg/ml), CaMKII activity was suppressed to wild-type values. When the doxycycline treatment was discontinued, Ca^{2+}-independent CaMKII activity returned to those of the untreated transgenic mice. Thus, the CaMKII-Asp^{286} transgene is functionally expressed and can be regulated with doxycycline.

Effects on LTP of CaMKII-Asp^{286} ex-

Table 1. Effect of CaMKII-Asp^{286} mRNA expression on enzyme activity. Brains were removed and the striatum was dissected and immediately homogenized in 20 mM tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 2 mM leupeptin, 0.4 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.4 mM molybdate, and 10 mM sodium pyrophosphate. CaMKII enzyme activity was determined as described (*7*). B21 + Dox animals received doxycycline (1 mg/ml) plus 5% sucrose in the drinking water for 3 to 5 weeks. B21 + Dox withdrawal animals received doxycycline (1 mg/ml) for 3 to 5 weeks and were then switched to normal water for 6 weeks. The number of mice is given in parentheses.

Mouse line	CaMKII activity		
	Without Ca^{2+} (pmol min^{-1} μg^{-1})	With Ca^{2+} (pmol min^{-1} μg^{-1})	Ca^{2+}-independent (%)
Wild type	0.13 ± 0.01 (5)	10.4 ± 1.2	1.33 ± 0.21
B21	0.90 ± 0.14 (5)	20.9 ± 2.9	4.62 ± 1.02
B21 + Dox	0.16 ± 0.04 (5)	12.9 ± 1.5	1.22 ± 0.30
B21 + Dox withdrawal	0.80 ± 0.03 (3)	14.2 ± 0.7	5.70 ± 0.43

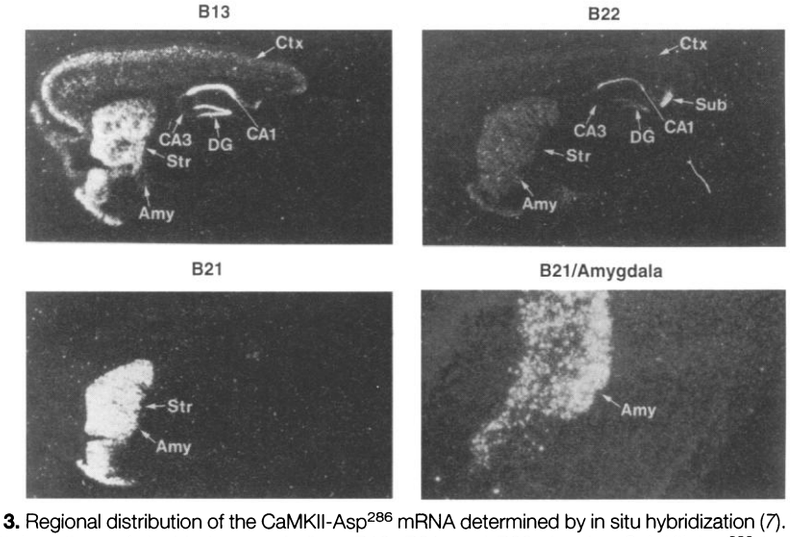

Fig. 3. Regional distribution of the CaMKII-Asp^{286} mRNA determined by in situ hybridization (*7*). Medial sagittal sections of double transgenic lines B13, B21, and B22 showing CaMKII-Asp^{286} transgene expression. B21/Amygdala shows a close-up view of a coronal section from the B21 double transgenic line of mouse.

pression in the hippocampus. Constitutive expression of the CaMKII-Asp^{286} transgene in the mouse forebrain shifts the stimulation frequency required for the production of LTP and LTD in the Schaffer collateral pathway of the hippocampus (*7*). In wild-type mice, stimulation at 1 Hz produced LTD, whereas stimulation at 5, 10, or 100 Hz produced LTP. In transgenic mice, stimulation at 100 Hz still produced LTP. However, stimulation in the 5- to 10-Hz range no longer produced LTP, but rather produced LTD or no change in synaptic strength.

We investigated whether the transgene was acting presynaptically or postsynaptically by asking whether expression of the transgene specifically in the postsynaptic CA1 neurons would produce a shift in the frequency threshold for LTP and LTD. We examined the B13 line of mice, which showed a uniformly high level of expression in the CA1 region, with little or no expression in CA3 (*22*). Thus, when Schaffer collateral LTP is measured in the B13 mice, the transgene will be expressed only in the postsynaptic neurons. Stimulation of slices from wild-type mice at 10 Hz resulted in a long-lasting potentiation of 123 ± 9% (n = 12 slices, 6 mice) (Fig. 4). By contrast, 10-Hz stimulation in B13 transgenic mice produced a slight depression to 89 ± 6% of baseline (n = 9 slices, 3 mice), which was significantly different from wild-type mice [$t(19) = 3.148$; $P < 0.01$, Student's t test].

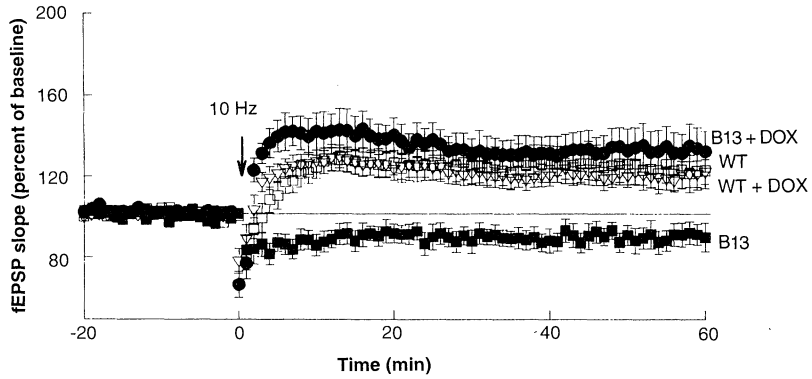

Fig. 4. Reversal of 10-Hz LTP deficit in CA1 of hippocampal slices. Field EPSP slopes before and after 10-Hz tetanic stimulation were recorded and expressed as the percentage of pre-tetanus baseline (*22*). Stimulation at 10 Hz for 1.5 min induced a transient depression followed by potentiation in wild-type mice (123 ± 9% at 60 min after tetanus; n = 12 slices, 6 mice) (□). Tetanus (10 Hz) induced a slight depression in B13 double transgenic mice (89 ± 6% at 60 min after tetanus; n = 9 slices, 3 mice) (■). Doxycycline treatment reversed the defect in B13 mice (132 ± 10%; n = 8 slices, 4 mice) (●). Doxycycline treatment had no effect on synaptic potentiation in wild-type mice (122 ± 6%; n = 16 slices, 6 mice) (▽).

To determine whether this effect was reversible, we suppressed transgene expression by administering doxycycline (1 mg/ml) for 2 to 3 weeks. Ten-hertz stimulation then produced potentiation similar to that in wild-type mice (132 ± 10%; n = 8 slices, 4 mice) (Fig. 4). Thus, suppression of transgene expression in adult mice reversed the electrophysiological phenotype [$t(15) = 3.675$, $P < 0.005$]. These results suggest that the selective expression of the CaMKII-Asp^{286} transgene in the postsynaptic CA1 neurons of the Schaffer collateral synapse is sufficient to alter the frequency threshold for LTP. Moreover, the shift in the frequency threshold is due to the acute expression of the transgene rather than to an irreversible developmental defect (*23*).

Effect on explicit memory storage of CaMKII-Asp^{286} expression in the hippocampus. Expression of the CaMKII-Asp^{286} transgene in the forebrain interferes with spatial memory, a form of explicit memory, as measured in the Barnes circular maze (*8*). The Barnes circular maze is a brightly lit open disk with 40 holes in the perimeter (Fig. 5A). Mice have an aversion for brightly lit open areas and hence are motivated to escape from the maze. This can be achieved by finding the 1 hole in 40 that leads to a darkened escape tunnel. In the spatial version of this task, the mouse must use distal cues in the room to locate the hole that leads to the escape tunnel (*24*).

Expression of the CaMKII-Asp^{286} transgene throughout the forebrain as seen in the B13 mice results in an impairment in the spatial but not the cued version of the Barnes maze task (*8*). To investigate those areas in the forebrain that are critical for this type of defect in spatial memory, we examined the B22 transgenic mice that show expression in the hippocampus, subiculum, striatum, and amygdala, but relatively little expression in the neocortex (Fig. 3). These mice exhibited significant impairment in spatial memory on the Barnes circular maze. None of the transgenic mice was able to acquire the task by using the spatial strategy, despite the fact that they were trained for 40 consecutive days (Fig. 5, B to D). Nevertheless, this profound memory impairment was reversed by suppression of transgene expression.

Effect on implicit memory of CaMKII-Asp^{286} expression in the amygdala and striatum. Fear conditioning is a simple associative form of learning, in which both a novel environment and a tone are paired

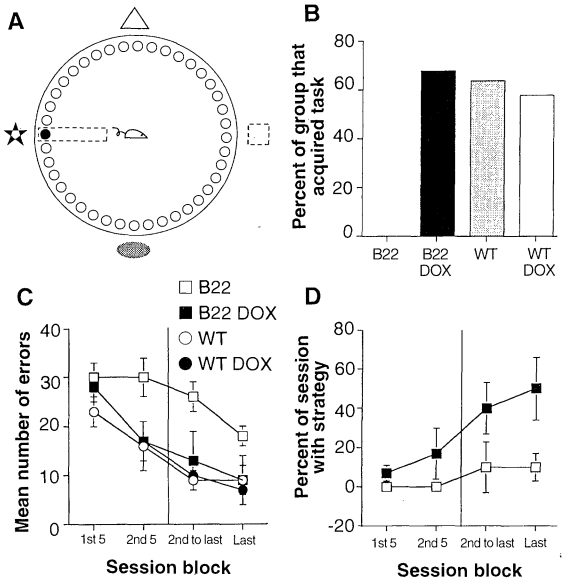

Fig. 5. Reversible deficits in explicit learning and memory in mice expressing the CaMKIIα transgene. (**A**) The Barnes circular maze. (**B**) Percentage of B22 transgenic and wild-type mice that met the learning criterion on the Barnes circular maze (*24*). A chi-square analysis revealed that the percentage of B22 transgenics acquiring the Barnes maze (0%) was significantly different from B22 transgenics on doxycycline and both wild-type groups ($X^2 = 53.05$, $P < 0.0001$). Four groups of mice were tested: B22 transgenics (n = 6), B22 transgenics on doxycycline (1 mg/ml) for 4 weeks (n = 6), wild types (n = 8), and wild types on doxycycline (1 mg/ml) for 4 weeks (n = 7). (**C**) Mean number of errors across session blocks composed of five sessions. Values represent group means ± SEM. A three-way ANOVA revealed a main effect of genotype [$F(1,23) = 4.28$, $P = 0.04$). (**D**) The percentage of sessions in which the spatial search strategy was used across session blocks by B22 transgenic mice. Values represent group means ± SEM. A two-way ANOVA revealed a significant main effect of doxycycline [$F(1,10) = 7.313$, $P = 0.02$).

with a foot shock on the training day (25). Memory is assessed 24 hours later by measurement of the amount of freezing (the fear response) elicited by either the novel environment (context conditioning) or the tone (cued conditioning). Fear conditioning shows components of both implicit and explicit forms of learning. The contextual version of the task is selectively impaired by lesions of the hippocampus (26) and thus can be viewed as an explicit form of learning, whereas both the cued and contextual versions of the task are impaired by lesion of the amygdala and are therefore viewed as implicit. In contrast to their spatial memory deficit, the B22 line of mice showed normal fear conditioning to both the cue and the context (Fig. 6, A and B). Thus, even though the B22 mice are impaired in spatial memory on the Barnes maze, they are not impaired in a second hippocampal-dependent task (contextual fear conditioning). This dissociation has been observed previously with constitutive expression of the CaMKII-Asp^{286} transgene and may reflect the use of different synaptic mechanisms for the storage of memory in the two tasks (7). In addition, these results demonstrate that the moderate level of transgene expression in the amygdala and striatum seen in the B22 mice (Fig. 3) is insufficient to interfere with the implicit component of fear conditioning.

Does a higher level of expression of the CaMKII-Asp^{286} transgene in the striatum and amygdala affect implicit memory storage? To explore this question, we studied the B21 mice that showed strong expression in the lateral amygdala and striatum but little transgene expression in the hippocampus or neocortex (Fig. 3). The B21 transgenic mice exhibited a severe impairment in both context and cued conditioning (Fig. 6, A and B). This learning impairment was again reversed by administration of doxycycline for 4 weeks before training.

This deficit in fear conditioning most likely arises from expression in the lateral amygdala, a structure that has been implicated in this form of learning by lesion studies (27). However, because there are many reciprocal connections between the striatum and the amygdala (28), we cannot rule out the possibility that the deficit results from a functional disruption in the striatum that secondarily alters the amygdala.

Effect on memory retrieval of CaMKII-Asp^{286} expression in the amygdala and striatum. Withdrawal of doxycycline after an initial period of transgene suppression resulted in a reactivation of gene expression (Table 1). We examined whether reexpression of the transgene, after normal learning has occurred, interferes with later stages of memory storage such as consolidation or retrieval. We trained B21 mice with the transgene expression suppressed and observed robust fear conditioning. Once the animals had learned the task, we reactivated transgene expression by withdrawing doxycycline (Fig. 6C). After a 6-week period, the expression of the CaMKII-Asp^{286} transgene returned to the same levels found in animals that had not received the drug (Table 1). We then examined these mice for retention of both context and cued conditioning and found a significant reduction in freezing compared to B21 mice in which we maintained suppression of the transgene (Fig. 6D).

This reduction in freezing reflects either an impairment in memory consolidation or recall, or a deficit in performance. The evaluation of performance deficits is critical to the study of memory because one can only infer that memory storage is defective once all possible defects in perception, motor performance, and cognitive understanding of the task have been excluded. Although it is difficult to control for all consequences of a genetic manipulation on various components of performance, we have examined the two most likely classes of performance variables: (i) the ability to perceive the unconditioned stimulus, and (ii) the ability to attend to and freeze in response to fearful stimuli (the conditioned response). To rule out an impairment in perception of the unconditioned stimulus (foot shock), we examined sensitivity to shock and found no difference between B21 transgenic and wild-type mice, suggesting that the observed fear-conditioning deficit did not result from a difference in the perception of the unconditioned stimulus (29). We next examined the possibility of a defect in performance of the conditioned response (freezing) by measuring unconditioned freezing in response to an intruder (30). We again found no difference in the ability of B21 mice to freeze to an intruder (a rat) when the transgene was expressed (Fig. 6E). Thus, the B21 transgenic mice were able to attend to fearful stimuli and to express a normal freezing response. Although some occult defect in performance might have been present that we have not detected, these control experiments argue that the transgene does not produce its effect on the perception of the unconditioned stimulus or on performance of the conditioned response. Rather, the results suggest that the CaMKII signaling pathway is important for some later aspects of memory storage such as the ability to consolidate or to recall the learned information.

Discussion. High levels of Ca^{2+}-inde-

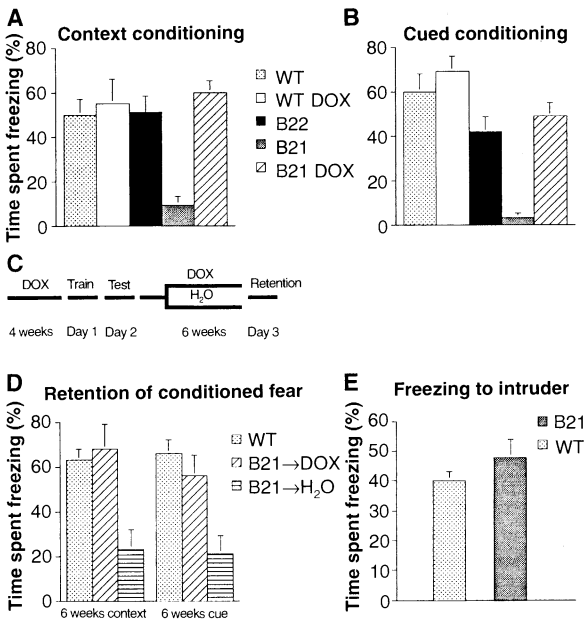

Fig. 6. Reversible deficits in implicit learning and memory in mice expressing the CaMKIIα transgene. Percentage of time spent freezing to context (**A**) and to cue (**B**) 24 hours after training in the B22 and B21 lines. Values represent group means ± SEM. A three-way ANOVA revealed a significant three-way interaction for context (genotype by line by doxycycline) ($F[1,55] = 9.177$, $P = 0.0037$) and a significant two-way interaction for cue (line by genotype) ($F[1,55] = 5.087$, $P = 0.0281$). Six groups of mice were tested: B22 transgenics ($n = 6$), B22 transgenics on doxycycline for 4 weeks ($n = 11$), B21 transgenics ($n = 8$), B21 transgenics on doxycycline for 4 weeks ($n = 19$), wild types (from both B22 and B21 lines) ($n = 11$), and wild types (from both B22 and B21 lines) on doxycycline for 4 weeks ($n = 8$). (**C**) Time line illustrating administration of doxycycline and behavioral training and testing. (**D**) Retention of context and cued conditioning. Percentage of time spent freezing to context and cue 6 weeks after training. Values represent group means ± SEM. Post hoc analysis by the Scheffe test revealed that B21 transgenic mice that were switched to water froze significantly less to context than B21 transgenic mice on doxycycline ($P = 0.01$) and wild types ($P = 0.008$) and significantly less to cue than B21 transgenic mice on doxycycline ($P = 0.02$) and wild types ($P = 0.0088$). Three groups of mice were tested: B21 transgenics on doxycycline for 4 weeks before training and 6 weeks after training ($n = 8$), B21 transgenics on doxycycline for 4 weeks before training that were switched to water for the 6 weeks after training ($n = 8$), and wild-type mice (from both B22 and B21 lines, $n = 19$). (**E**) The percentage of time spent freezing to an intruder during the first 120 s after the mouse was exposed to a rat. Values represent group means ± SEM.

pendent CaMKII activity shifted the stimulation-frequency threshold for hippocampal LTP and LTD to favor LTD (*7*). This shift in threshold is associated with an impairment in explicit, but not implicit, memory (*8*). To obtain regulated expression of this transgene in restricted regions of the forebrain so that we could study the underlying cellular and behavioral functions more effectively, we used the tTA system for regulated gene expression (*13*, *14*).

We found that expression of the CaMKII-Asp^{286} transgene altered adult synaptic plasticity and memory formation directly, and not by effects on neuronal development. In addition, expression of the transgene postsynaptically was sufficient to alter the frequency threshold for LTP induction, at least at 10 Hz. Finally, high-level activation of CaMKII in the striatum and lateral amygdala also interfered with implicit forms of memory.

How might an increase in Ca^{2+}-independent CaMKII activity alter the stimulation frequency required to produce LTP and LTD, and how might this in turn alter learning and memory storage? Our results demonstrate that the effect of the CaMKII-Asp^{286} transgene is likely mediated by changes in the postsynaptic CA1 neurons of the Schaffer collateral pathway. A simple mechanism for systematically shifting the frequency threshold for LTP and LTD to favor LTD would be to reduce the size of the postsynaptic Ca^{2+} signal produced during the stimulation [(*31*); however, see (*32*)]. This could occur either through the increased phosphorylation of particular substrate proteins of CaMKII or by increased binding of Ca^{2+}-calmodulin by autophosphorylated CaMKII (*33*). Independent of its detailed mechanisms, however, our data indicate that CaMKII activation alone may not be sufficient to produce the increase in synaptic strength associated with LTP, as has been suggested (*18*, *34*). Rather, the level of CaMKII activation regulates the stimulation conditions under which LTP and LTD are produced.

In this study, we did not measure synaptic physiology and behavior in the same group of animals (*35*). Nevertheless, the effects of CaMKII activation on behavior are likely a consequence of its effect on the frequency threshold for LTP and LTD induction. That CaMKII activation interferes with synaptic plasticity in the 5- to 10-Hz range is particularly relevant for the explicit hippocampal-based spatial memory paradigm. Animals exploring the space of a novel environment show a rhythmic oscillation in hippocampal activity in the 5- to 10-Hz range (the theta rhythm) (*19*). Changes in synaptic strength can be produced by this endogenous activity and are thought to be necessary for storing information about space. Synaptic plasticity in the theta frequency range may regulate hippocampal place cells, the pyramidal neurons (in the CA3 and CA1 subfields) whose activity is correlated with the animals' location in the environment (*36*).

Several lines of evidence implicate the lateral amygdala as the site of plasticity for fear conditioning. First, the lateral amygdala is the first site of convergence of somatosensory (unconditioned stimulus) and auditory (conditioned stimulus) information in the fear-conditioning pathway (*26*). Second, fear conditioning enhances the auditory-evoked responses of neurons in the lateral amygdala (*37*). Third, these neurons exhibit robust LTP that can contribute to enhanced auditory-evoked responses (*38*). Finally, lesions of the lateral amygdala block fear conditioning (*26*). How might the expression of CaMKII-Asp^{286} affect fear conditioning? Expression of this transgene in the hippocampus increases the stimulation frequency required to produce LTP (Fig. 4). Were a similar increase in the frequency threshold to occur at excitatory synapses in the lateral amygdala, this increase in threshold could form the physiological basis for the observed impairment in implicit memory storage.

Expression of the transgene in striatum and amygdala also affected memory consolidation or recall. Models of learning generally invoke changes in synaptic strength only during the initial learning process (*39*). Once formed, the changes in synaptic strength are thought to remain stable and to carry the actual memory trace. However, for some memories such as hippocampal-based explicit memories, the anatomical locus of the memory changes with time during a several-week period after the initial learning (*26*). Moreover, the recall of memory typically is reconstructive—it requires a new recapitulation of the learned experience. Both transfer and reconstruction of memory might require an activity-dependent change in synaptic strength. If a similar process occurs for fear conditioning in the amygdala, the defect in retrieval observed in the transgenic mice could reflect a defect in synaptic plasticity caused by CaMKII-Asp^{286} expression during this memory transfer or reconstruction phase.

The methods for regional and regulated transgene expression that we describe here represent initial steps toward the development of an optimal technology for the genetic study of cognitive processes. To carry the molecular dissection of behavior further, it will be necessary to use promoters that are even more restricted in their pattern of expression and to adapt this technology to the regulation of targeted gene disruption. With further modifications, the methods we describe here should prove generally useful and should help in elucidating the cellular and molecular signaling pathways important for higher cognitive processes.

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20. The CaMKIIα promoter consisted of 8.5 kb of genomic DNA upstream of the transcription initiation site of the mouse CaMKIIα gene, as well as 84 base pairs of the 5′ noncoding exon. Genomic DNA was isolated from a C57 B16/J mouse spleen cosmid library with a rat genomic probe consisting of a 0.4-kb Ava I fragment comprising the transcription-initiation region of rat CaMKIIα (*40*). The tTA gene from plasmid pUHD 15-1 (*10*) was flanked by an artificial intron and splice sites at the 5′ end (*41*) and by a polyadenylation signal from SV40 at the 3′ end. The cDNA with intron and polyadenylation signal was placed downstream of the 8.5-kb CaMKII promoter fragment. The cDNAs for *Escherichia coli lacZ* and mouse CaMKIIα were similarly flanked by the hybrid intron and polyadenylation signal and placed downstream of the tet-O promoter element of plasmid pUHD 10-3 (*10*). The CaMKIIα gene was a full-length cDNA (4.8 kb) isolated from a C57B16/J mouse brain cDNA library. The *lacZ* gene carried an SV40 large T antigen nuclear localization signal as well as the 3′ untranslated region (UTR) of CaMKIIα, which targets the mRNA to dendrites (*42*).
21. RT-PCR was performed essentially as described (*7*). Total forebrain RNA (100 ng) was used in each reaction with oligonucleotide primers to amplify a region of the transcript that includes the Thr^{286}→Asp mutation. Equal amounts of amplified cDNA (both wild-type and mutant sequences) were separated on a 3% agarose gel, transferred to nylon membranes, and hybridized with a ^{32}P-labeled oligonucleotide probe specific for the Asp^{286} mutation (oligonucleotide sequence: 5′-CTTCAGGCAGTCGACGTC-CTCCTGTCTGTG-3′). Blots were washed under conditions in which only the Asp^{286} mutant cDNA

was detected (2′ 15 min, 60°C, 0.2′ standard saline citrate). A Northern (RNA) blot of total forebrain mRNA revealed expression of a shorter-than-expected CaMKII-Asp^{286} transcript (~3.4 kb). As shown in Fig. 3, this shorter CaMKII-Asp^{286} transcript did not localize to dendrites, presumably as a result of the loss of a sequence element in the 3′ UTR that is necessary for mRNA targeting to dendrites (*42*).

22. Transverse slices (400 μm thick) of mouse hippocampus were prepared and placed in an interface slice chamber perfused with artificial cerebrospinal fluid as described (*7*). Field excitatory postsynaptic potentials (EPSPs) were elicited once per minute with fine tungsten bipolar stimulation electrodes (0.05-ms pulse duration). Stainless steel recording electrodes were placed in striatum radiatum. The stimulation strength was set to produce 50% of the maximum obtainable EPSP in each slice. Baseline synaptic response was collected for 20 min before the tetanus. The 10-Hz tetanus was delivered for 1.5 min at the same intensity as used in the baseline recording. For doxycycline treatment, animals were administered doxycycline (1 mg/ml) plus 5% sucrose in the drinking water for 2 to 3 weeks, and the slices were the exposed to doxycycline (1 ng/ml) in the perfusate. All animals were 2.5 to 6 months of age at the time of recording.
23. It would also be useful to suppress transgene expression during development and then activate the gene only in the adult animal. However, we found that treatment of wild-type mice with doxycycline (1 mg/ml) during development impaired adult spatial memory and memory for fear conditioning. This result suggests that doxycycline itself produces a defect in neuronal development. We therefore used transgene suppression only in the adult animal in which the doxycycline treatment did not affect memory. Given the activation of the transgene throughout development, it is possible that the LTP and memory phenotypes observed with the transgene active in the adult animal result from a synergistic interaction between developmental and adult expression rather than a direct acute effect of transgene expression in the adult animal.
24. On the Barnes circular maze (*8*), the mice (2.5 to 6 months of age) were tested once a day until they met the criterion (five out of six sessions with three or fewer errors) or until 40 days had elapsed. The order of holes searched was recorded by an observer who was blind to genotype and doxycycline condition, and from these data the number of errors was determined. Errors were defined as searches of any hole that did not have the tunnel beneath it. Searches included nose pokes and head deflections over the hole. At the end of each session the search strategy used was recorded by the observer. The spatial search strategy was operationally defined as reaching the escape tunnel with both error and distance scores ≤3. Distance was calculated by counting the number of holes between the first hole searched within a session and the escape tunnel. A one-factor analysis of variance (ANOVA) (gender) revealed no significant effect of gender for either transgenic or wild-type mice, so the data were collapsed across this variable. For the error data, a three-factor ANOVA (genotype, doxycycline, and session block) with one repeated measure was used. For the spatial search strategy data, the two groups of B22 transgenic mice were compared with a two-way ANOVA (doxycycline and session block) with one repeated measure.
25. In the conditioned fear task (*8*), freezing was defined as a total lack of movement with the exception of respiration and was measured by an experimenter who was blind to genotype and doxycycline condition. The percentage of time spent freezing to context and cue was calculated. No significant effect of gender was observed in the B22 or B21 transgenic mice or the wild-type mice, so the data were collapsed across this variable. Freezing to context and cue on testing day was analyzed by two three-factor ANOVAs (genotype, line, and doxycycline) that were used to compare the B22 and B21 transgenic and wild-type mice. Two one-way ANOVAs were used to compare the amount of freezing 6 weeks later to cue and context in B21 transgenics on doxycycline, B21 transgenics switched to water, and wild-type mice.
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30. Unconditioned freezing in the presence of an intruder was measured in B21 transgenic (n = 8) and wild-type mice (n = 10) in a Nalgene plastic metabolism cage. The mice and intruder were placed in the upper and lower chambers, respectively. The chambers were separated by a metal grid floor. A seven-week-old male Sprague-Dawley rat served as the intruder and was placed in the lower chamber 10 min before introduction of the mouse. The amount of unconditioned freezing occurring during the first 120 s after the mouse was introduced was measured by an experimenter who was blind to genotype. A t test revealed no significant effect of genotype.
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35. The expression of the CaMKII-Asp^{286} transgene in the CA1 region of the B22 mice was patchy; that is, some neurons expressed the transgene well, whereas in other neurons expression was absent. This patchy expression precluded an assessment of LTP in this line of mice by means of field recordings, which sample many synapses from different neurons in a region. However, it is assumed that in those neurons where the transgene was strongly expressed in these mice, a shift in the LTP/LTD frequency threshold would occur.
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43. We thank R. Axel and T. Jessell for critically reading this manuscript; J. Finkelstein for maintaining and genotyping the mice; R. Shih, V. Winder, and L. Varshavsky for help with behavioral experiments; C. Lam for help with figures; H. Ayers and I. Trumpet for typing the manuscript; and M. Osman for animal care. This research was supported by the Howard Hughes Medical Institute and the National Institute of Mental Health.

8 August 1996; accepted 23 October 1996