

CaMKII Regulates the Frequency–Response Function of Hippocampal Synapses for the Production of Both LTD and LTP

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Summary

To investigate the function of the autophosphorylated form of CaMKII in synaptic plasticity, we generated transgenic mice that express a kinase that is Ca²⁺ independent as a result of a point mutation of Thr-286 to aspartate, which mimics autophosphorylation. Mice expressing the mutant form of the kinase show an increased level of Ca²⁺-independent CaMKII activity similar to that seen following LTP. The mice nevertheless exhibit normal LTP in response to stimulation at 100 Hz. However, at lower frequencies, in the range of 1–10 Hz, there is a systematic shift in the size and direction of the resulting synaptic change in the transgenic animals that favors LTD. The regulation of this frequency-response function by Ca²⁺-independent CaMKII activity seems to account for two previously unexplained synaptic phenomena, the relative loss of LTD in adult animals compared with juveniles and the enhanced capability for depression of facilitated synapses.

Introduction

Long-lasting increases and decreases in synaptic strength in response to different patterns of synaptic activation are thought to form the basis for the neuronal changes that contribute to various forms of learning and memory. In the hippocampus, a structure that is important for the storage of explicit forms of memory, two types of long-term synaptic plasticity have been demonstrated: long-term potentiation (LTP) and long-term depression (LTD) (see Bliss and Collingridge, 1993; Bear and Malenka, 1994, for review). In the CA1 region of the hippocampus, both LTP and LTD can be induced at the same synapses by different frequencies of stimulation; low frequencies induce LTD, whereas high frequencies induce LTP (Dudek and Bear, 1992, 1993). Although the precise molecular mechanisms underlying these two forms of plasticity are unclear, both require influx of Ca²⁺ into the postsynaptic cell through N-methyl-D-aspartate (NMDA) receptors (Lynch et al., 1983; Malenka et al., 1992; Mulkey and Malenka, 1992; Artola and Singer, 1993). The different levels of postsynaptic Ca²⁺ influx achieved by different frequencies of presynaptic activity are thought to activate opposing changes in

protein phosphorylation, with the higher frequencies activating protein kinases necessary for LTP (Malenka et al., 1989; Malinow et al., 1989) and the lower frequencies activating protein phosphatases necessary for LTD (Mulkey et al., 1993, 1994; O'Dell and Kandel, 1994).

CaMKII is a multisubunit Ca²⁺/calmodulin-dependent serine–threonine protein kinase whose activity is required for LTP (see Hanson and Schulman, 1992, for review). Injection of specific peptide inhibitors of CaMKII into the postsynaptic neuron blocks the induction of LTP (Malenka et al., 1989; Malinow et al., 1989). Moreover, mice lacking the α subunit of CaMKII also lack LTP and have a deficit in spatial learning (Silva et al., 1992a, 1992b). Exactly how CaMKII contributes to LTP is not known. Purified CaMKII is inactive in the absence of Ca²⁺. However, upon exposure to Ca²⁺/calmodulin, CaMKII is activated and can phosphorylate numerous target proteins, including itself, at several sites (Miller and Kennedy, 1986). The autophosphorylation of CaMKII at one site, Thr-286, is particularly important because it is both necessary and sufficient to produce an autoactive kinase, a kinase that is active even in the absence of Ca²⁺ (Schworer et al., 1988; Miller et al., 1988; Thiel et al., 1988; Lou and Schulman, 1989). The ability to convert to a Ca²⁺-independent state following a brief exposure to Ca²⁺ has led to the suggestion that CaMKII may function as a memory molecule in LTP (Miller and Kennedy, 1986; Lisman, 1989, 1994). According to this view of LTP, the initial Ca²⁺ influx caused by high frequency stimulation activates CaMKII, which phosphorylates substrate proteins that initiate LTP. Once turned on, the kinase maintains its active state by continued autophosphorylation, even after Ca²⁺ has returned to its basal level. The CaMKII maintained in its active Ca²⁺-independent state by autophosphorylation in turn continuously phosphorylates critical substrate proteins that then maintain LTP. In this case, the kinase functions as a simple on–off switch not only for the initiation but also for the maintenance of LTP. In support of this idea, LTP-inducing stimuli or strong depolarization of the postsynaptic cell leads to an increase in the level of Ca²⁺-independent CaMKII activity in the hippocampal slice (Fukunaga et al., 1993; Bading et al., 1993; Ocorr and Schulman, 1991). Also, infection of hippocampal slices with a vaccinia virus carrying a truncated, Ca²⁺-independent CaMKII gene potentiates synaptic transmission and blocks LTP induction (Pettit et al., 1994).

To test the role of CaMKII autophosphorylation in synaptic plasticity, we took advantage of a mutation in the kinase that mimics autophosphorylation at Thr-286. In this mutant form of CaMKII, Thr-286 is replaced with an acidic aspartate group (CaMKII–Asp-286). The mutant kinase is 20- to 30-fold more active in the absence of Ca²⁺ than the wild-type enzyme (Fong et al., 1989; Waldmann et al., 1990). Using the CaMKII α promoter to limit expression to the forebrain, we generated transgenic mice that express CaMKII–Asp-286 in the hippocampus at a level of Ca²⁺ independence greater than that reached during LTP. We then examined LTP and found that, at this level of Ca²⁺-

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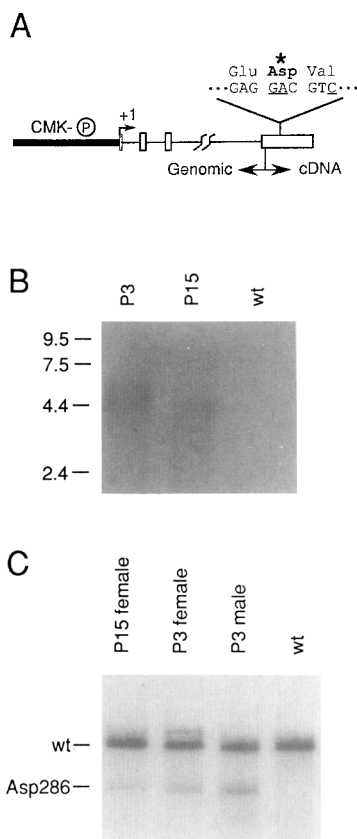


Figure 1. Expression of the CaMKII-Asp-286 Transgene
 (A) Schematic representation of the CaMKII-Asp-286 construct used to generate transgenic mice.
 (B) Northern blot of 20 μ g of total hippocampal RNA from transgenic and wild-type animals probed with an Asp-286-specific oligonucleotide.
 (C) RT-PCR quantitation of the relative level of transgene versus wild-type mRNA expression in total hippocampus.

independent activity, the kinase does not act as a simple on-off switch. Rather, the Ca^{2+} -independent kinase regulates the frequency-response function that couples pre-synaptic activity of the synapse to the production of either LTP or LTD. A high level of Ca^{2+} -independent activity favors the production of LTD even at frequencies that normally produce LTP.

Results

Generation of Mice Carrying a Transgene with a Thr-286→Asp Mutation in CaMKII

The α subunit of CaMKII is the most abundantly expressed isoform in forebrain structures such as hippocampus (McGuinness et al., 1985; Miller and Kennedy, 1985), and gene targeting experiments show that it is critical for the induction of LTP (Silva et al., 1992a). We therefore used the α subunit gene of CaMKII for the generation of transgenic mice. To limit the transgene to only specific target regions of the forebrain, we expressed the transgene under the control of its own promoter. We obtained 8.5 kb upstream of the CaMKII α transcription initiation site

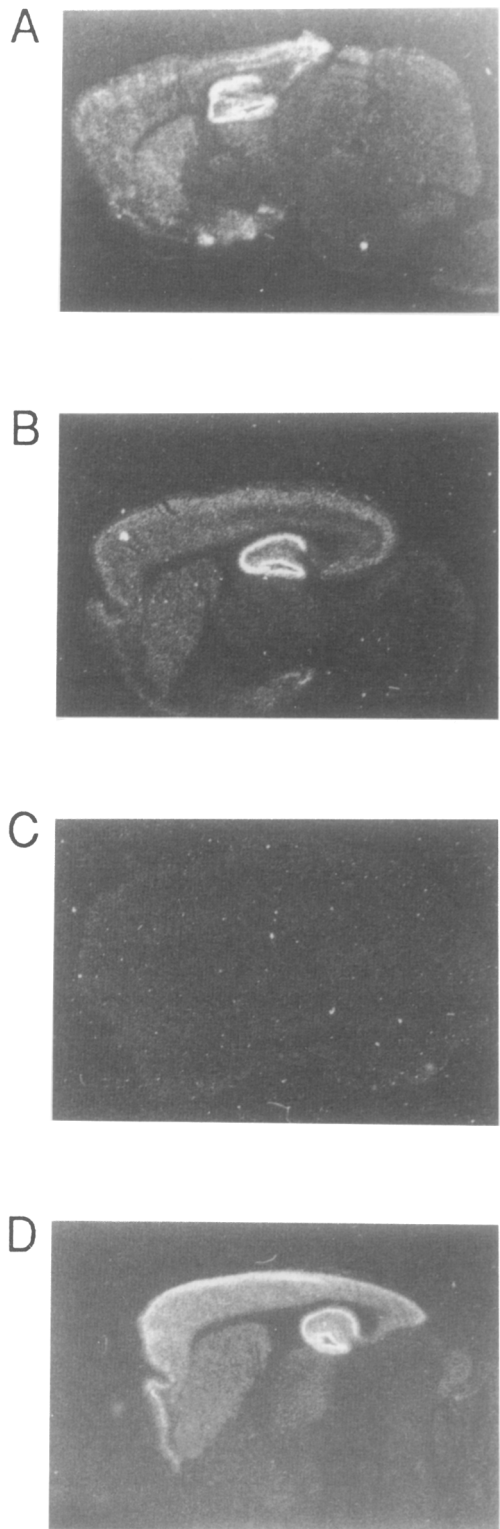


Figure 2. Regional Distribution of CaMKII-Asp-286 Transgene Expression by In Situ Hybridization
 Sagittal sections from transgenic and wild-type animals hybridized with probes specific for the CaMKII-Asp-286 transgene or wild-type CaMKII α . (A) P3 mouse, Asp-286 probe; (B) P15 mouse, Asp-286 probe; (C) wild-type mouse, Asp-286 probe; (D) wild-type mouse, wild-type probe.

Table 1. CaMKII Enzyme Assays

	Without Ca ²⁺ (pmol/min/μg)	With Ca ²⁺ (pmol/min/μg)	Ca ²⁺ -Independent (%)
Adult (+PO ₄)			
P3	1.25 ± 0.29 (n = 6) ^a	8.75 ± 1.27	13.4 ± 1.5 ^c
P15	1.00 ± 0.19 (n = 6) ^a	7.08 ± 0.94	14.6 ± 2.7 ^a
Wild type	0.60 ± 0.09 (n = 10)	7.90 ± 0.72	7.4 ± 0.6
Young (+PO ₄)			
P3	2.72 ± 0.55 (n = 4) ^b	10.23 ± 2.63	28.2 ± 2.6 ^c
P15	1.28 ± 0.27 (n = 4)	6.82 ± 2.04	20.1 ± 1.9 ^a
Wild type	1.05 ± 0.22 (n = 9)	8.70 ± 0.87	11.7 ± 1.7
Adult (-PO ₄)			
P3	0.351 ± 0.039 (n = 6) ^c	6.74 ± 1.02	6.06 ± 1.41 ^b
P15	0.406 ± 0.023 (n = 6) ^c	9.53 ± 0.67 ^b	4.39 ± 0.41 ^c
Wild type	0.076 ± 0.008 (n = 6)	5.77 ± 0.71	1.47 ± 0.30
Young (-PO ₄)			
P3	0.654 ± 0.059 (n = 3) ^c	13.95 ± 1.23	4.73 ± 0.44 ^c
P15	0.325 ± 0.048 (n = 4) ^b	10.10 ± 1.27	3.19 ± 1.9 ^a
Wild type	0.058 ± 0.013 (n = 3)	8.95 ± 1.70	0.64 ± 0.03

Enzyme activity determined from total hippocampal extracts from adult (2- to 8-month-old) or young (3- to 4-week-old) mice. All values are mean ± SEM. For statistical comparisons, transgenic (P3 or P15) values were compared with wild-type values within the same group using a Student's *t* test. +PO₄, assayed in the presence of protein phosphatase inhibitors; -PO₄, assayed in the absence of protein phosphatase inhibitors.

^a *p* < .05.

^b *p* < .01.

^c *p* < .001.

(Sunyer and Sahyoun, 1990) as well as a 5' portion of the coding region from a mouse genomic library. We then isolated a mouse cDNA clone that contained the entire 3' untranslated region, which we have found to be necessary for appropriate targeting of the CaMKII α mRNA to dendrites (M. M. and E. R. K., unpublished data). Using oligonucleotide-directed mutagenesis, we introduced an aspartate into the cDNA at codon 286. The CaMKII α cDNA containing the Thr-286→Asp mutation was then fused in phase to the corresponding exon of the genomic clone (Figure 1A). Transgenic mice were generated using this construct, and 7 independent founder animals were obtained, of which 3 reproduced successfully and transmitted the transgene. Two of these lines, referred to as P3 and P15, were analyzed in detail for the present study.

The Transgene Is Expressed in a Restricted Manner

By Southern blot analysis, we found a copy number of 1 for the P15 line and about 4 for the P3 line (data not shown). Breeding indicated that the transgene was X-linked in the P3 line. Northern blot analysis of hippocampal RNA, using an oligonucleotide probe specific for the Asp-286 mutant form of CaMKII, revealed the expression in the transgenic mice of a transcript of about 4.8 kb (Figure 1B). We used the reverse transcription-polymerase chain reaction (RT-PCR) to examine the relative level of CaMKII-Asp-268 mRNA as compared with the endogenous wild-type CaMKII mRNA (Figure 1C). In P15 hippocampus the transgene was expressed at 10% of wild-type levels, whereas in the P3 line expression was 16% (females) or 21% (males) of wild-type levels.

We examined the distribution of neuronal expression of the transgene by *in situ* hybridization using an oligonucleotide probe specific for the Asp-286 mutation. In both lines, the transgene was expressed selectively in forebrain re-

gions, in a pattern that was indistinguishable from that of the endogenous CaMKII α gene, except that the transgene appeared to be reduced or absent from a medial layer of the cortex that normally expresses endogenous CaMKII α (Figure 2). The transgene was found throughout the dendritic layers of the hippocampus, suggesting that the mRNA was properly targeted to dendrites.

To determine whether the CaMKII-Asp-286 protein was functionally expressed, we compared CaMKII activity in hippocampal extracts from transgenic and nontransgenic litter mates. We measured the kinase activity in crude homogenates against autocamtide-2, a peptide substrate specific for CaMKII (Ocorr and Schulman, 1991). The transgenics showed a significant elevation in the Ca²⁺-independent kinase activity without a corresponding elevation in the Ca²⁺-dependent kinase activity, leading to an increase in the fraction of kinase in the Ca²⁺-independent state (Table 1). The level of Ca²⁺-independent activity measured in both transgenic lines was similar and nearly double that of wild-type animals (13.4% ± 1.5% [P3] and 14.6% ± 2.7% [P15] versus 7.4% ± 0.6% [wild type]). This level of kinase activation is greater than that observed following LTP induced by tetanic stimulation (Fukunaga et al., 1993) and equal to that produced by global depolarizing stimuli (Ocorr and Schulman, 1991; Bading et al., 1993).

The basal Ca²⁺-independent CaMKII activity in the hippocampus is normally maintained by autophosphorylation of the endogenous enzyme. To determine whether the elevated kinase activity in the transgenic mice was due to expression of the mutant form of the enzyme and to provide an estimate of the total amount of transgene expression, we measured the activity in the absence of protein phosphatase inhibitors (Table 1). Under these conditions, phosphorylation of the endogenous CaMKII is

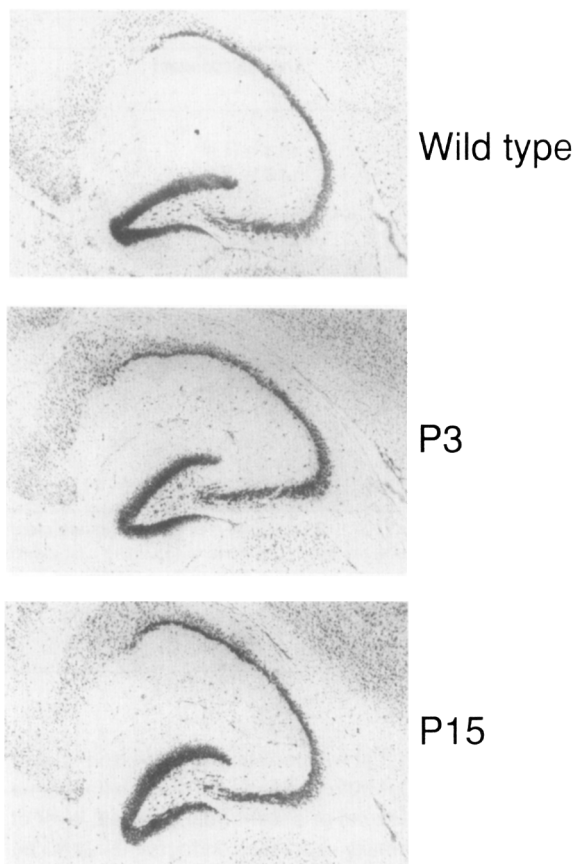


Figure 3. Nissl-Stained Sections from Wild-Type and CaMKII-Asp-286 Mice

Horizontal sections (20 μ m) were stained as described by Grant et al. (1992).

eliminated, and the level of Ca^{2+} -independent activity is dramatically reduced. The remaining difference between wild-type and transgenic animals reflects the presence of the CaMKII-Asp-286 protein, whose activity is not affected by phosphorylation state. These data allow us to estimate the relative amount of mutant enzyme in the hippocampal extracts. Assuming that the CaMKII-Asp-286 enzyme has 35% Ca^{2+} -independent activity (Waldmann et al., 1990), the percentage of total enzyme in the hippocampal extract that derives from the mutant protein is 9% for the P15 line and 14% for the females of the P3 line. This is consistent with the estimate of mRNA expression in the two lines. Since the level of expression of the transgene is only 10%–15% of the wild-type gene, we expect that the phenotypic effects we observe result from the Ca^{2+} -independent nature of the enzyme rather than from simple overexpression.

Gross Neuroanatomy and Behavior

In the P3 line, about 50% of the males died by 8 weeks of age and in many cases were observed to have what appeared to be severe seizures. Based on its transmission pattern, the transgene in this line seemed to be X-linked, suggesting that the seizures could be the result of inser-

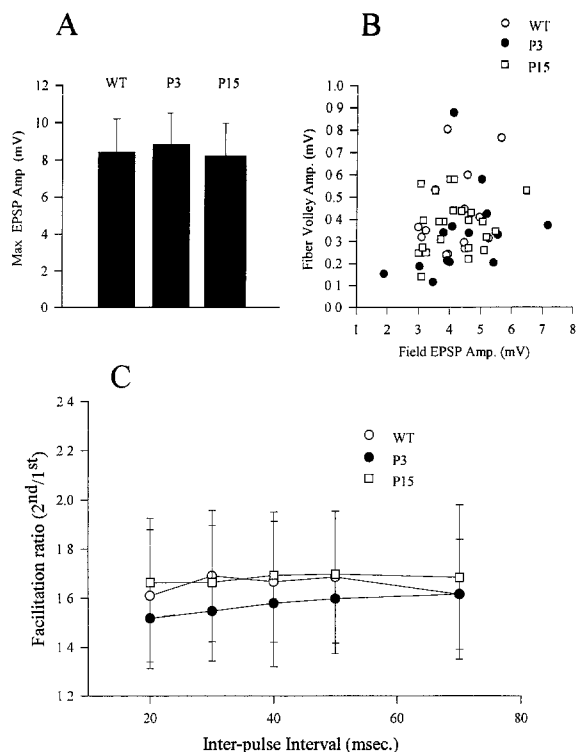


Figure 4. Basic Synaptic Physiology

(A) The maximal EPSPs determinable from slices from wild-type (WT) and transgenic (P3 and P15) animals were not significantly different. Values are as follows (mean \pm SD): 8.39 ± 1.79 mV for wild type ($n = 43$ animals, 164 slices), 8.79 ± 1.69 mV for P3 ($n = 32$ animals, 115 slices), 8.18 ± 1.75 mV for P15 ($n = 30$ animals, 93 slices). Wild type versus P3: $t(73) = 1.09$, not significant; wild type versus P15: $t(71) = 0.40$, not significant.

(B) The EPSP versus fiber volley in millivolts measured at a stimulating intensity that produces 50% of the maximal EPSP. No significant difference was observed in the EPSP size or fiber volley between wild types and transgenics. Fiber volleys were 0.427 ± 0.19 mV for wild type ($n = 7$ animals, 14 slices), 0.337 ± 0.20 mV for P3 ($n = 14$ animals, 67 slices), and 0.378 ± 0.12 mV for P15 ($n = 12$ animals, 23 slices). Wild type versus P3: $t(26) = 1.24$, not significant; wild type versus P15: $t(35) = 0.78$, not significant. EPSPs were 4.13 ± 0.8 mV (wild type), 4.38 ± 1.27 mV (P3), and 4.16 ± 0.91 mV (P15). Wild type versus P3: $t(26) = 0.62$, not significant; wild type versus P15: $t(35) = 0.12$, not significant.

(C) Paired stimulation pulses were delivered at five inter-pulse intervals, and the ratio of the EPSP amplitude of the second to the first pulse was measured. No significant differences between wild-type and transgenic mice were observed at any of the inter-pulse intervals measured.

tional inactivation of a critical gene on the X chromosome. The males from the P3 line were therefore not analyzed further in the present study. The P3 females showed normal viability rates, and only 1 out of about 100 animals was observed to have seizures. The P15 line displayed normal viability, and we never observed seizures in this line. The P3 females as well as P15 males and females were indistinguishable from wild-type animals when observed during routine handling. However, the females from both lines failed, in most cases, to rear their young.

Since expression of the transgene might alter neuronal development, we examined Nissl-stained horizontal sec-

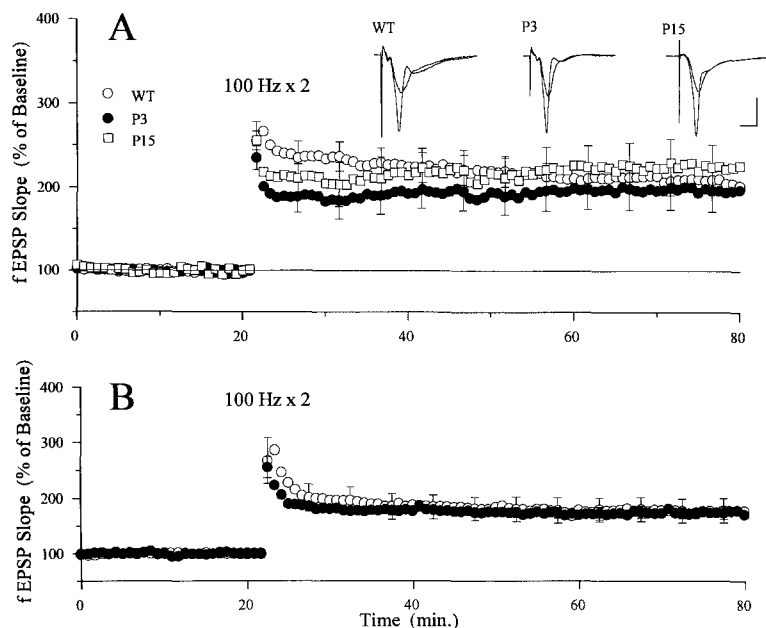


Figure 5. Induction of CA1 LTP by High Frequency Stimulation

(A) LTP induced by two 100 Hz, 1 s trains produced by shocks delivered at an intensity that produces 50% of the maximal EPSP level. The two trains were delivered 10 s apart. Recording was also at 50% of the maximal EPSP. Values are the percentage of prestimulation baseline measured 1 hr after stimulation (mean ± SEM; n = number of animals): 205% ± 18% (wild type; n = 7); 189% ± 20% (P3; n = 8), and 203% ± 28% (P15; n = 6). Wild type versus P3: $t(13) = 0.59$, not significant; wild type versus P15: $t(11) = 0.74$, not significant. Traces show individual field EPSPs evoked just prior to (smaller response) and 60 min after (larger response) the induction of LTP. Bars, 4 mV, 4 ms.

(B) LTP produced by two 100 Hz, 1 s trains delivered at 75% of the maximal EPSP level. The two trains were delivered 20 s apart. Recording was at 25% of maximal EPSP (wild type: 173% ± 20% [n = 4]; P3: 172% ± 13% [n = 3]; $t(5) = 0.031$, not significant).

tions. We found no difference in the arrangement of cell bodies within the neuronal layers of the hippocampus between transgenics from either line and control littermates (Figure 3), suggesting that transgene expression does not produce any gross developmental abnormalities.

LTP Is Normal in CaMKII-Asp-286 Mice

We next investigated the effect of CaMKII-Asp-286 expression on synaptic transmission and synaptic plasticity in the hippocampus, by recording extracellular field potentials produced in the stratum radiatum of hippocampal slices from P3 and P15 animals following Schaffer collateral stimulation.

We first compared basal synaptic transmission in hippocampal slices of wild-type and CaMKII-Asp-286 transgenic animals and found no difference in either the size of the field excitatory postsynaptic potential (EPSP; Figure 4A) or the fiber volley required to produce this EPSP (Figure 4B) in the transgenic animals as compared with wild types. This suggests that synaptic transmission is not substantially altered by the introduction of the CaMKII-Asp-286 transgene. We also examined paired-pulse facilitation in the transgenic mice. Paired-pulse facilitation is a short-lasting (<1 s) enhancement in synaptic strength that is thought to be mediated by a presynaptic mechanism (Kamiya and Zucker, 1994). There were no significant differences in paired-pulse facilitation from transgenic and wild-type animals (Figure 4C). Thus, we did not detect any change in the presynaptic processes that are thought to be regulated by CaMKII (Llinás et al., 1985; Lin et al., 1990; Nichols et al., 1990; Silva et al., 1992a).

We next asked whether CaMKII serves as a simple on-off switch for the generation of LTP. If so, the elevated kinase present in the transgenic animals in the basal state, which is equal to or greater than that present in wild-type animals following strong depolarizing stimuli that induce

LTP (Ocorr and Schulman, 1991; Fukunaga et al., 1993; Bading et al., 1993), should switch LTP on in the basal state and thereby occlude further attempts to elicit LTP. To test this idea, we used a standard 100 Hz tetanus protocol that produces saturating amounts of LTP in adult wild-type animals. We found no difference in either the amplitude or duration of LTP between wild-type and CaMKII-Asp-286 mice (Figure 5A). In the P3 line, which expressed the highest level of CaMKII-Asp-286, we used a second high intensity LTP induction protocol and again found no difference between the transgenic and wild-type mice. These results argue against CaMKII functioning as a simple switch in a pathway for the production of LTP.

A Frequency-Dependent Shift in Synaptic Plasticity

Although not sufficient to induce LTP by itself, CaMKII might instead serve as a regulator of LTP. If this were so, we might expect a shift in the stimulation threshold for the induction of LTP. To test this idea, we stimulated the Schaffer collaterals at three additional frequencies (1, 5, and 10 Hz) and examined the consequent changes in synaptic strength.

As first predicted on theoretical grounds (Bienenstock et al., 1982; Bear et al., 1987) and later confirmed experimentally (Dudek and Bear, 1992), high frequency stimulation leads to LTP, intermediate frequencies produce either no change or a low level of LTP, and low frequencies produce LTD. How is this relationship affected by the expression of the active form of CaMKII? As is the case with rats and guinea pigs (Dudek and Bear, 1993; O'Dell and Kandel, 1994), wild-type mice receiving low frequency (1 Hz) stimulation (900 pulses) show substantial LTD only in the immature animal. With 1 Hz stimulation, hippocampal slices from adult wild-type mice show only a very slight depression (95% ± 2%; n = 7; Figure 6C; all values are percentage of prestimulation synaptic strength ± SEM,

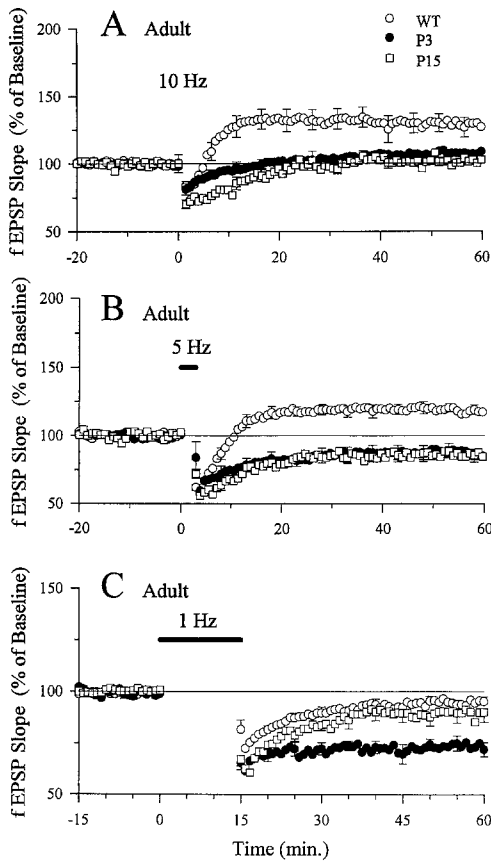


Figure 6. LTP/LTD Produced by Stimulation at 1, 5, and 10 Hz in Adult Mice

Mice ranged in age from 2 to 8 months.

(A) Stimulation at 10 Hz for 1.5 min. Wild type: $129\% \pm 6\%$ ($n = 8$); P3: $109\% \pm 3\%$ ($n = 3$); P15: $102\% \pm 2\%$ ($n = 4$). Wild type versus P3: $t(9) = 1.854$, $p < .05$; wild type versus P15: $t(7) = 2.88$, $p < .01$. (B) Stimulation at 5 Hz for 3 min. Wild type: $117\% \pm 3\%$ ($n = 6$); P3: $85\% \pm 7\%$ ($n = 4$); P15: $87\% \pm 5\%$ ($n = 3$). Wild type versus P3: $t(8) = 4.85$, $p < 0.01$; wild type versus P15: $t(7) = 5.41$, $p < .01$. (C) Stimulation at 1 Hz for 15 min. Wild type: $95\% \pm 2\%$ ($n = 7$); P3: $72\% \pm 3\%$ ($n = 3$); P15: $90\% \pm 5\%$ ($n = 10$). Wild type versus P3: $t(8) = 5.59$, $p < .01$; wild type versus P15: $t(15) = 0.83$, not significant.

measured 1 hr after the onset of stimulation). Stimulation at 5 and 10 Hz (900 pulses) produced LTP to $117\% \pm 3\%$ ($n = 6$) and $129\% \pm 6\%$ ($n = 8$) of baseline, respectively (Figures 6A and 6B).

In the CaMKII-Asp-286 mice, the same frequencies of stimulation yielded a systematic shift in size and direction of the synaptic change produced by the same presynaptic stimulation. Thus, 10 Hz stimulation, which produced a potentiation in the wild-type mice, produced little change in CaMKII-Asp-286 mice (P3: $109\% \pm 3\%$ [$n = 3$]; P15: $102\% \pm 2\%$ [$n = 4$]; Figure 6A), and 5 Hz stimulation, which produced a small amount of LTP in wild-type animals, produced a small amount of LTD in the transgenic animals (P3: $85\% \pm 7\%$ [$n = 4$]; P15: $87\% \pm 5\%$ [$n = 3$]; Figure 6B). Finally, 1 Hz, which produced slight LTD in wild-type animals ($95\% \pm 2\%$; $n = 7$), produced substantial LTD in the P3 line ($72\% \pm 3\%$; $n = 3$; P3 versus wild type: $t(8) = 5.59$, $p < .01$). In the P15 line, only a slight enhancement in LTD was observed, and this was not

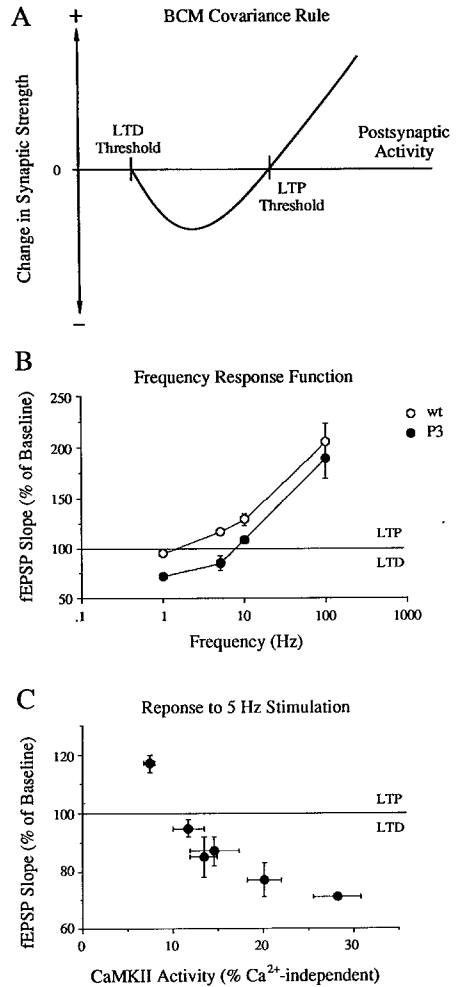


Figure 7. The Frequency-Response Function Shifts with Higher Ca²⁺-Independent CaMKII Activity

(A) Predicted theoretical relationship between postsynaptic activity and synaptic change (adapted from Bear et al., 1987).

(B) Frequency-response function in transgenic and wild-type mice. The percentage change in synaptic strength from baseline in transgenic and wild-type animals at 1 hr following stimulation at the indicated frequency. Values are mean \pm SEM (data from Figure 5A and Figures 6A-6C).

(C) Correlation between 5 Hz plasticity and Ca²⁺-independent CaMKII activity. Data are for both young and adult wild-type and transgenic animals measured at 1 hr after 5 Hz stimulation (see Figure 6B; Figure 8A). Values are mean \pm SEM for both synaptic strength change and percentage of Ca²⁺-independent CaMKII activity.

statistically different from that in wild-type animals ($90\% \pm 5\%$; $n = 10$; P15 versus wild type: $t(15) = 0.83$, not significant; Figure 6C). This was the only difference found between the two lines and may be due to the greater level of transgene expression in the P3 line.

That CaMKII could affect LTD, and particularly that it affects it systematically by changing the frequency-response relationship, was unexpected. Since activation of CaMKII is thought to be necessary in the induction of LTP, the transgenic mice in which CaMKII is already partly activated would be expected to undergo LTP more readily. However, at the level of Ca²⁺-independent CaMKII activity achieved

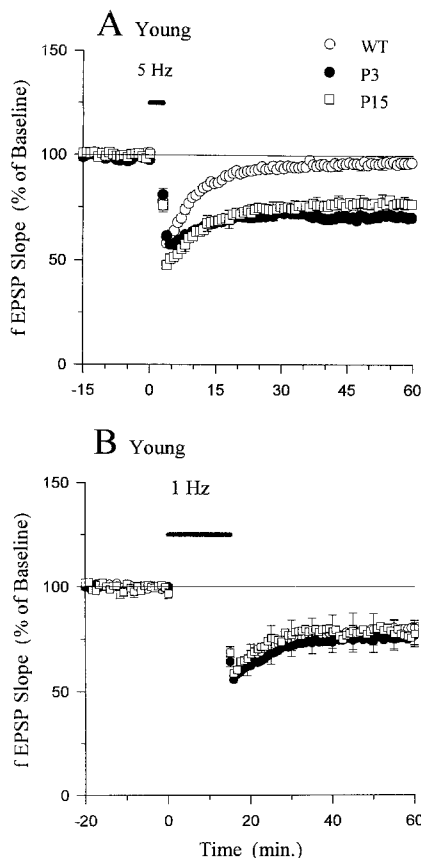


Figure 8. LTD Produced by Stimulation at 1 and 5 Hz in Young Mice Mice ranged in age from 3 to 4 weeks.

(A) Stimulation at 5 Hz for 3 min. Wild type: 95% ± 3% (n = 6); P3: 71% ± 1% (n = 3); P15: 77% ± 6% (n = 3). Wild type versus P3: $t(7) = 5.75, p < .01$; wild type versus P15: $t(7) = 3.13, p < .01$. (B) Stimulation at 1 Hz for 15 min.

in the transgenic animals, we find that the capacity for LTP induction is reduced, while that for LTD is enhanced. Thus, the size and direction of synaptic change seems to be determined by the degree of Ca^{2+} -independent CaMKII activity. When CaMKII is already partially activated, as in the transgenic mice, the frequency-response curve is shifted so as to favor depression (Figure 7B). To explore further the role of Ca^{2+} -independent CaMKII in LTD, we have examined this phenomenon in other contexts.

The Normal Loss of LTD with Age in Wild-Type Animals Parallels Loss of Ca^{2+} -Independent CaMKII Activity

In rats, LTD is robust only in young animals and diminishes with age (Dudek and Bear, 1993). If the ability to produce LTD were related to the degree of autophosphorylation of CaMKII, we might expect that the maturation effect on LTD would be paralleled by changes in CaMKII activity. In fact, decreases in Ca^{2+} -independent CaMKII activity with age have been seen in the rat hippocampus (Molloy and Kennedy, 1991). There is a similar decrease in Ca^{2+} -independent CaMKII activity in the hippocampus as a function of maturity in both wild-type and CaMKII-Asp-286 transgenic mice (see Table 1). Thus, young wild-type mice

show an elevated level of Ca^{2+} -independent activity compared with adult wild-type animals (adult, 2–8 months: 7.4 ± 0.6 [n = 10]; young, 3–4 weeks: 11.7 ± 1.7 [n = 9]; $t(17) = 2.5, p < 0.025$), and this level is similar to that achieved by the adult transgenics. Young transgenics show the highest levels of Ca^{2+} -independent activity.

If the level of Ca^{2+} -independent CaMKII activity were an important regulator of LTD, we would expect to find enhanced LTD in young wild-type animals as compared with adults, and we would expect the largest LTD in young transgenics. This indeed proved to be the case. LTD produced by 1 Hz stimulation in young wild-type mice is more robust than that produced by the same stimulation in the adult (adult wild type: $95\% \pm 2\%$ [n = 7]; young wild type: $80\% \pm 3\%$ [n = 8]; $t(13) = 3.5, p < 0.005$). Interestingly, the LTD produced by 1 Hz stimulation in young wild-type animals is similar to that produced in adults from the P3 line of CaMKII-Asp-286 mice ($72\% \pm 3\%$; n = 3). The young transgenic animals show normal levels of LTD at 1 Hz (Figure 8A). However, the frequency-response curve is again shifted in favor of LTD, so that now, in the young transgenics, 5 Hz stimulation produces robust LTD (P3: $71\% \pm 1\%$ [n = 3]; P15: $77\% \pm 6\%$ [n = 3]), whereas in the young wild-type animals, 5 Hz produces only a slight depression in synaptic strength ($95\% \pm 3\%$; n = 6; Figure 8B).

When the data for young and old, wild-type and transgenic animals are combined, we find that the size and direction of synaptic plasticity produced following 5 Hz stimulation are strongly correlated with the level of Ca^{2+} -independent kinase activity (see Figure 7C). At higher levels of Ca^{2+} -independent CaMKII activity, LTD is enhanced. This suggests it is the developmental decrease in Ca^{2+} -independent CaMKII activity that results in the apparent loss of LTD in the adult wild-type animal. As a corollary, the adult transgenic mice, which show elevated levels of Ca^{2+} -independent CaMKII into adulthood, also maintain some of the phenotypic properties of the immature wild-type animal.

LTP Occludes the Effect of the CaMKII-Asp-286 Mutation

In adult wild-type mice, 5 Hz stimulation of baseline synapses leads to a slight LTP. By contrast, if LTP is first produced by a high frequency train that is known to cause a long-lasting increase in Ca^{2+} -independent CaMKII activity (Fukunaga et al., 1993), then 5 Hz stimulation now produces a depression or depotentiation of the facilitated synapse (Staubli and Lynch, 1990; Fujii et al., 1991; O'Dell and Kandel, 1994). This initially paradoxical finding is consistent with the idea that increased levels of autophosphorylated kinase, in this case produced by synaptic activation, shift the frequency-response function to favor LTD.

To test this idea further, we compared the ability of 5 Hz stimulation to produce synaptic depression following a high frequency train in both transgenic and wild-type animals. Stimulation (5 Hz) of naive synapses revealed a difference between wild-type and transgenic animals, with wild-types showing a potentiation to 117% of baseline and transgenics showing a depression to 86% of baseline (see

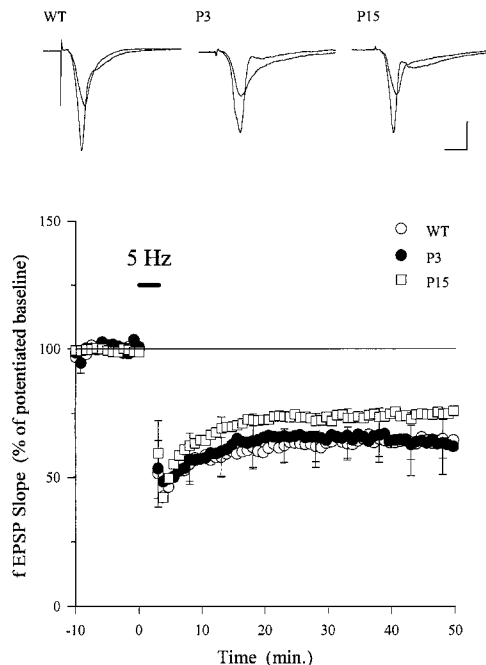


Figure 9. Prior High Frequency Stimulation Occludes the Effect of the CaMKII-Asp-286 Transgene

Slices from transgenic and wild-type animals were first stimulated at 100 Hz, two times for 1 s each, with an interstimulus interval of 10 s. This produced similar levels of LTP in both transgenic and wild-type animals ($242.11\% \pm 19.87\%$ [wild type], $214.58\% \pm 24.78$ [P3], $201.60\% \pm 19.83\%$ [P15]; wild type versus P3: $t(5) = 0.785$, not significant; wild type versus P15: $t(5) = 1.449$, not significant). The baseline was then set at the new potentiated level, and 20 min following the 100 Hz trains, a 5 Hz, 3 min train was delivered. This produced the same level of depression in both transgenic and wild-type animals. The percentage of potentiated baseline 45 min after 5 Hz stimulation was as follows (mean \pm SEM): $64\% \pm 8\%$ (wild type; $n = 4$ animals, 7 slices), $62\% \pm 11\%$ (P3; $n = 4$ animals, 7 slices), $74\% \pm 3\%$ (P15; $n = 3$ animals, 7 slices). Wild type versus P3: $t(6) = 0.11$, not significant; wild type versus P15: $t(5) = 1.1$, not significant. Traces at the top show field EPSPs recorded in slices from wild-type and transgenic mice just prior to (larger response) and 45 min after (smaller responses) 5 Hz stimulation. Compare with Figure 6B. Bars, 2.5 mV, 4 ms.

Figure 6B). By contrast, following two 100 Hz trains that produced LTP, this difference between wild-type and transgenic animals was occluded so that subsequent 5 Hz stimulation now produced a similar level of depression in both cases (Figure 9). This suggests, as discussed below, that the shift in 5 Hz response following 100 Hz stimulation is due to the elevation in Ca^{2+} -independent CaMKII activity.

Discussion

CaMKII as a Molecular Regulator of the Frequency-Response Function for Synaptic Plasticity

Much current thinking about the mechanisms of associative learning and about parallel associative changes during late stages of development has been guided by a synaptic learning rule postulated by Hebb (1949). Hebb's rule

states that the synapse will be strengthened when activity in the pre- and postsynaptic components of a synapse is synchronous. Research over the last several years has made clear that, as stated, this rule is inadequate to account for synaptic modification in the hippocampus and requires modification. A theoretical extension of the Hebb rule called the covariance rule, first proposed by Sejnowski (1977) and later extended by Bienenstock et al. (1982), adds the capacity for synaptic depression to that for facilitation (see Figure 7A). The covariance rule holds that there is a continuum of associative synaptic change that is determined by the relationship between the specific level of postsynaptic depolarization paired with presynaptic activity. Strong postsynaptic depolarization leads to synaptic potentiation, weak depolarization to depression. Artola and Singer (1993) have elaborated this idea further by suggesting that different levels of depolarization lead to different levels of Ca^{2+} influx into the postsynaptic cell (Artola et al., 1990), and that the Ca^{2+} influx is critical for both depression and potentiation. Our data and those of Dudek and Bear (1992) provide independent support for the continuity between LTP and LTD and suggest specifically that a key determinant of postsynaptic depolarization and consequent Ca^{2+} influx is the frequency of presynaptic stimulation. Different frequencies produce different patterns of postsynaptic depolarization and, presumably, different patterns of NMDA receptor-mediated Ca^{2+} influx, resulting in a systematic relationship between presynaptic firing frequency, postsynaptic depolarization, Ca^{2+} influx, and synaptic strength. For the same number of presynaptic pulses, a high frequency of presynaptic firing leads to greater temporal summation of the individual EPSPs, and thus a greater overall postsynaptic depolarization. As a result, there will be greater Ca^{2+} influx, which favors the generation of LTP. Lower frequencies produce more modest postsynaptic depolarization and a lower level of Ca^{2+} influx. This seems to favor the generation of LTD.

Our data indicate that the frequency-response function for the production of LTP and LTD is closely regulated by development and prior synaptic activity. Moreover, our results show that CaMKII is an important molecular regulator of this function. Thus, in mice expressing elevated levels of Ca^{2+} -independent CaMKII, the entire frequency-response curve is shifted systematically to favor LTD. This yields the result that, at moderate frequencies of stimulation, LTP is lost in the transgenics and LTD is enhanced.

How Does the Ca^{2+} -Independent CaMKII Produce Its Action?

According to the simple switch hypothesis of LTP, CaMKII activation alone is sufficient to induce and maintain synaptic potentiation, perhaps through the phosphorylation of postsynaptic glutamate receptors (McGlade-McCulloh et al., 1993; Tan et al., 1994). Our results provide a view of the function of CaMKII that is somewhat different from the conventional switch hypothesis.

In the CaMKII-Asp-286 mice, there is no alteration in the total amount of LTP obtained at high frequencies, but there is a systematic shift to favor LTD at medium to low frequencies. Thus, our results suggest a direct role for

CaMKII in the production of LTD. The observation that mice with a targeted disruption of CaMKII α lack LTD supports this interpretation (Stevens et al., 1994). How can the apparent importance of CaMKII for LTD be reconciled with the genetic and pharmacological evidence suggesting a critical role for CaMKII in LTP? Our data are consistent with the two possibilities outlined in Figure 10.

In the first model, it is not the enzymatic activity of the autophosphorylated form of CaMKII that is critical but the 100-fold increased affinity of the autophosphorylated CaMKII subunit for Ca²⁺/calmodulin (Meyer et al., 1992; Hanson et al., 1994). Here, CaMKII would function as a regulatable Ca²⁺/calmodulin sink that is sensitive to the prior history of Ca²⁺ influx into the dendritic spine in which it is located (Figure 10A). According to this view, both LTP and LTD require the action of Ca²⁺/calmodulin, with low levels of free Ca²⁺/calmodulin leading to LTD and high Ca²⁺/calmodulin leading to LTP. In this case, CaMKII enzyme activity is not necessary for the induction of either LTP or LTD. However, the amount of autophosphorylated CaMKII regulates the balance between the two processes by regulating the levels of free Ca²⁺/calmodulin in the dendritic spine. The dissociation constant for calmodulin from CaMKII increases from about 0.2 s in the unphosphorylated state to about 20 s for CaMKII that is autophosphorylated at Thr-286. Thus, at higher levels of autophosphorylated CaMKII, the same size Ca²⁺ signal would produce a smaller increase of free Ca²⁺/calmodulin, owing to the sequestration of a larger fraction of the Ca²⁺/calmodulin by the high affinity autophosphorylated form of the kinase. This would in turn lead to a systematic shift in favor of LTD at all submaximal levels of Ca²⁺ influx (i.e., at low frequencies of stimulation). This effect of the autophosphorylated kinase should be overcome at saturating levels of Ca²⁺/calmodulin, as might be achieved by high frequency stimulation.

This model depends on the ability of CaMKII phosphorylated at Thr-286 to bind tightly to Ca²⁺/calmodulin. It is clear that Thr-286 phosphorylation is sufficient to convert the enzyme to the high affinity form (Hanson et al., 1994); however, the ability of the CaMKII-Asp-286 mutant form of the kinase to mimic autophosphorylation in this case, and thereby trap Ca²⁺/calmodulin, has not been tested. The model in Figure 10A is directly testable by expression of a form of CaMKII containing the Thr-286→Asp mutation as well as a second site mutation (Lys-42→Met), which knocks out enzymatic activity. This should allow the dissociation of the effects of the Thr-286→Asp mutation on enzyme activity from the effect on Ca²⁺/calmodulin trapping.

According to the second view, CaMKII activation is necessary for both LTP and LTD, but is the rate limiting step only for LTD (Figure 10B). While strong evidence exists for the involvement of CaMKII in LTP, there is equally strong evidence for the involvement, in the induction process, of other kinases such as tyrosine kinases and protein kinase C (O'Dell et al., 1991; Grant et al., 1992; Abeliovich et al., 1993). If CaMKII activation were necessary but not sufficient for the generation of LTP, then expression of the activated form of the enzyme should not produce LTP and therefore should not alter the level of LTP obtained

at high frequencies. If CaMKII activation were not the rate-limiting step in LTP induction, then no enhancement in the ability to induce LTP should be observed in the transgenic mice. However, CaMKII activation appears also to be necessary for LTD induction, perhaps along with the activation of other molecules such as protein phosphatases. If CaMKII activation were the rate-limiting step in LTD induction, then the CaMKII-Asp-286 mice, in which CaMKII is already partially active, would show an enhanced capacity to undergo LTD.

The two models we present postulate that the effect of the CaMKII-Asp-286 transgene is postsynaptic. However, since the transgene is also expressed in the presynaptic CA3 neurons, it may exert its effects on the frequency-response function presynaptically. In fact, although the induction of both LTP and LTD occurs postsynaptically and requires Ca²⁺ influx into the postsynaptic neuron, both

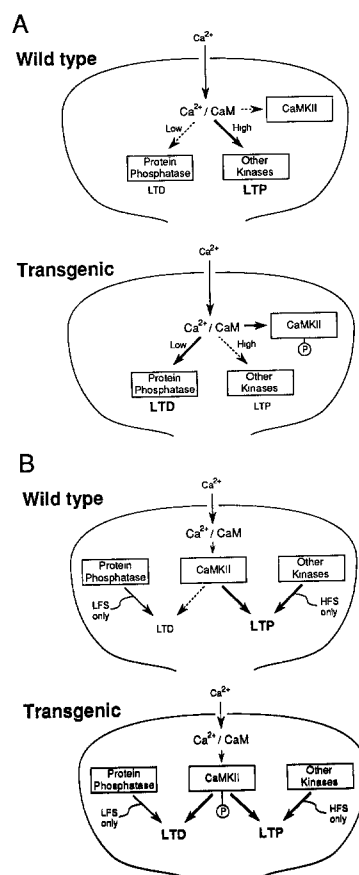


Figure 10. Possible Mechanisms for CaMKII Regulation of LTP and LTD Both models assume that the level of Ca²⁺ influx increases with increasing frequency of stimulation.

(A) Calmodulin trapping model. In the transgenic animals, the greater level of autophosphorylated CaMKII binds a greater amount of Ca²⁺/calmodulin, shifting the balance toward LTD at all but the highest levels of Ca²⁺ influx.

(B) Enzyme activity model. In the transgenic animals, the enzyme is already partially activated such that, at low levels of Ca²⁺ influx, greater enzyme activity, which is necessary for LTD, is achieved.

CaM, calmodulin; HFS, high frequency stimulation; LFS, low frequency stimulation; P, phosphorylation at Thr-286.

processes appear to be maintained presynaptically (Bolshakov and Siegelbaum, 1994; Stevens and Wang, 1994). Moreover, during prolonged stimulation, significant Ca^{2+} elevation occurs in the presynaptic terminal, and it has been suggested that presynaptic kinase activity is important for the maintenance of LTP. The two alternative biochemical mechanisms outlined in Figure 10 could therefore also apply to the presynaptic terminal.

In contrast to our result, Pettit et al. (1994) have shown that infection of hippocampal slices with a vaccinia virus expressing a truncated Ca^{2+} -independent form of CaMKII enhances synaptic responses and blocks LTP. It is unclear why we did not see an enhanced synaptic response or block of LTP in the CaMKII-Asp-286 transgenic mice, since we achieved a 5-fold greater increase in Ca^{2+} -independent CaMKII activity than did Pettit et al. One difference was that they expressed a truncated monomeric form of CaMKII in hippocampal slices using a vaccinia virus vector. The truncated form of the enzyme does not contain the domains necessary for proper assembly of the enzyme into multimers or for the dendritic localization of mRNA. This form of the enzyme may therefore be aberrantly localized within the neuron. Also, it is possible that some synergism between the CaMKII expression and the normal progression of the vaccinia virus life cycle, which will eventually kill the neuron, leads to the LTP block. Alternatively, it may be that in the transgenic animals some compensatory change occurs during development that restores the capacity for LTP even at high levels of Ca^{2+} -independent CaMKII activity.

A Role for CaMKII in Depotentiation

Prior theoretical and experimental work has suggested that the frequency-response function is not fixed, but can be modified by prior synaptic input (Bienenstock et al., 1982; Bear et al., 1987; Staubli and Lynch, 1990; Fujii et al., 1991; O'Dell and Kandel, 1994). Bienenstock et al. specifically suggested that the frequency-response function is regulated by the time-averaged activity of the neuron. Our data suggest that CaMKII serves as the sensor of the level of neuronal activity and in turn controls the frequency-response function. When a synapse is activated at medium to high frequencies, three changes occur: Ca^{2+} -independent CaMKII activity is increased, some LTP is produced, and the frequency-response function is shifted to favor LTD. Our data suggest that the elevated Ca^{2+} -independent kinase causes the shift in the frequency-response function to favor LTD. Thus, at a previously stimulated synapse, future stimuli will be less likely to produce LTP and more likely to produce LTD. In this way, CaMKII can serve as a molecular regulator of the dynamic range of synaptic strength.

An example of the role of CaMKII as a sensor of neuronal activity is illustrated in our study of depotentiation. In mature wild-type animals, stimulation of naive synapses at 5 Hz in hippocampal area CA1 leads to a small potentiation above baseline. If LTP is first induced by high frequency stimulation, subsequent 5 Hz stimulation produces a strong depression. The CaMKII-Asp-286 mice differ from wild types in that stimulation of the naive synapse at 5 Hz

produces a depression. However, following high frequency stimulation, the responses of transgenic and wild-type slices to 5 Hz are indistinguishable. Thus, prior synaptic stimulation can occlude the effect of the CaMKII-Asp-286 transgene. These data, in addition to providing a test of the importance of the Ca^{2+} -independent CaMKII in shifting the frequency-response function, also serve as important controls for the secondary consequences of the transgene.

In the generation of transgenic mice, it is always possible that the observed phenotype results from a secondary effect of transgene expression. For example, the observed difference in response to 5 Hz stimulation in the CaMKII-Asp-286 mice as compared with wild types could result from a subtle change in development or from effects on neuronal processes not directly related to LTP. However, the occlusion of the phenotypic difference between wild-type and transgenic animals by LTP-inducing stimuli suggests that this is not the case. The high frequency stimulation would not be expected to reverse a developmental difference in the transgenic animals or to alter signaling pathways unrelated to LTP. However, this result would be expected if the high frequency 100 Hz stimulation produced a saturating level of Ca^{2+} -independent CaMKII activity, even in the wild-type animal. In this case, the CaMKII in the transgenic and wild-type animals would be stimulated to the same level, or beyond some threshold level of Ca^{2+} -independent activity, and thus show the same frequency-response function. Alternatively, if the shift in 5 Hz response caused by prior 100 Hz stimulation were mediated by something other than changes in CaMKII activity, the effect of 100 Hz stimulation should be additive with the effect of CaMKII-Asp-286 expression, and the transgenic mice should continue to show a larger 5 Hz depression than wild-type animals, even following 100 Hz stimulation.

Changes in CaMKII Activity in Maturation May Contribute to the Closure of the Critical Period

The amount of Ca^{2+} -independent CaMKII activity in hippocampal extracts normally decreases during development between 3 and 8 weeks of age (see Table 1) (Molloy and Kennedy, 1991). During this time, there is also a corresponding decrease in the ability of low frequency stimulation to produce LTD. In the CaMKII-Asp-286 mice, where the Ca^{2+} -independent kinase activity in adults is maintained at an elevated level similar to that of a 3-week-old wild-type animal, LTD produced by low frequency stimulation is enhanced. These results suggest that hippocampal synapses normally undergo a developmental shift in the frequency-response function that is mediated, at least in part, by a corresponding change in CaMKII activity. In the CaMKII-Asp-286 mice, the CaMKII activity due to the expression of the transgene maintains the synapses at the juvenile setting.

While the amount of LTD obtained at 5 Hz is clearly correlated with Ca^{2+} -independent CaMKII levels (see Figure 7B), it is unclear whether the quantitative level of LTD attainable is regulated by CaMKII. At 1 Hz, only the P3 line of transgenics shows quantitatively enhanced LTD in

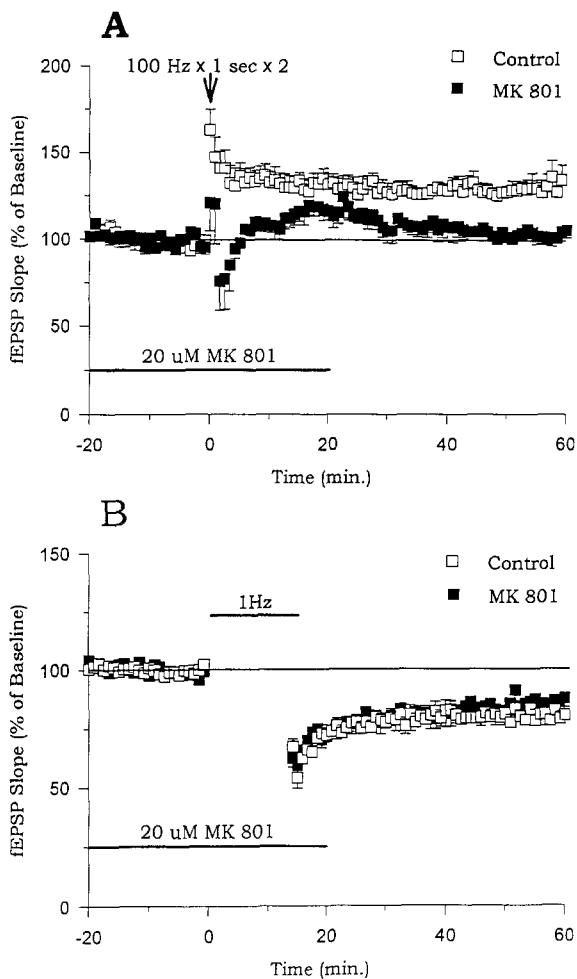


Figure 11. MK801 Blocks LTP but Not LTD

(A) Hippocampal slices from 2- to 3-week-old wild-type mice were stimulated at 100 Hz two times, 1 s each, with an interstimulus interval of 20 s. Stimulation was at 50% of the maximal EPSP. Application of 20 μ M MK801 as indicated completely blocked LTP (control: 129% \pm 5% [n = 5]; MK801: 103% \pm 3% [n = 3]; $t(6) = 3.68$, $p < .02$). (B) Stimulation at 1 Hz for 15 min produced LTD that was not blocked by 20 μ M MK801 (control: 80% \pm 4% [n = 7]; MK801: 83% \pm 5% [n = 8]; $t(13) = .377$, not significant). Slices in 20 μ M MK801 showed significant depression at 1 hr poststimulus onset compared with baseline ($t(7) = 3.44$, $p < .02$).

the adult animal. This line expresses a greater level of mutant transgene than the P15 line, even though the Ca^{2+} -independent activity measured in adult hippocampal extracts is equal in the P3 and P15 lines. This suggests that there may be some compensatory change in a protein phosphatase activity in the P3 line. It may be that the absolute level of LTD obtainable is mediated by the level of phosphatase expressed, while the frequency threshold is mediated by the level of Ca^{2+} -independent CaMKII activity.

In young animals the capacity for LTD is enhanced and is in fact present in the hippocampus prior to the appearance of LTP (Müller et al., 1989). What might be the function of the enhanced ability to depress synapses through activity in young animals? During neuronal development the major projections between brain regions are set up

using extracellular cues for axonal growth and guidance. In a particular region, such as the lateral geniculate or striate cortex, the initial axonal arborization is diffuse and only later becomes segregated through an activity-dependent process thought to involve a mechanism similar to that of LTP (see Goodman and Shatz, 1993, for review). When this normal developmental fine tuning is blocked with 2-amino-5-phosphonovaleric acid (APV) or tetrodotoxin, synaptic connections that would normally be lost during maturation to adulthood are maintained (Stryker and Harris, 1986; Shatz and Stryker, 1988; Bear et al., 1990). This suggests that a mechanism of selective activity-dependent synaptic depression such as LTD may be an important mechanism by which the inappropriate connections in young animals are lost. Both LTD and LTP require NMDA receptor activation, and both are blocked by APV, which also blocks synapse segregation. However, Cline and Constantine-Paton (1990) have found that segregation of axonal arbors in the frog tectum is not blocked by MK801, an open-channel blocker of the NMDA receptor. We therefore examined the effect of MK801 on hippocampal plasticity and found that, though it blocks LTP, it does not affect LTD produced at low frequencies such as 1 Hz (Figure 11). This supports the idea that a LTD-like phenomenon may be the predominant mechanism for activity-dependent synaptic segregation. If this interpretation is correct, the decrease of Ca^{2+} -independent CaMKII activity may signal the end of a critical period of development by reducing the capacity for LTD. One prediction of this model is that in the CaMKII-Asp-286 mice the critical period for developmental fine tuning should be extended into adulthood.

The CaMKII-Asp-286 mice provide a useful model for the study of the role of synaptic change produced at different frequencies of neuronal firing. The shift in the transgenics is pronounced in the range of the θ rhythm (4–12 Hz), at which learning-related synaptic plasticity in the hippocampus is thought to be produced in freely behaving animals. In the accompanying paper (Bach et al., 1995 [this issue of Cell]), we explore the effects of this shift in θ frequency synaptic plasticity on hippocampally mediated learning and memory tasks.

Experimental Procedures

Transgene Construction

The full-length mouse CaMKII α cDNA and a genomic clone containing the 5' portion of the CaMKII α coding region, as well as 8.5 kb of upstream DNA, were isolated from a mouse brain (C57BL6) cDNA library and a mouse spleen (C57BL6) cosmid library, respectively, using a rat cDNA probe (kindly provided by N. Sahyoun; Sunyer and Sahyoun, 1990). The Thr-286 \rightarrow Asp mutation was introduced into the cDNA clone between an XmnI and SpeI site using a synthetic fragment composed of the following two oligonucleotides: 5'-CACAGACAGGAGGACGTC-GACTGCCTGAAGA-3' and 5'-TCTTCAGGAGTCGACGTCCTCCT-GTCTGTGCATG-3'. The 3 bp change from the wild-type sequence introduces an AatII site into the Thr-286 \rightarrow Asp mutant cDNA. The poly(A) signal from bovine growth hormone (obtained from plasmid pRc/CMV, Invitrogen) was placed 3' to the cDNA. The cDNA was fused in phase to the corresponding exon in the genomic clone using an EagI site present in both. Cloning junctions were verified by DNA sequencing. The 45 kb insert (see Figure 1A) was removed from the vector by NotI digestion and used for the generation of transgenic mice.

Transgenic Mice

Transgenic mice were generated by DNX, Inc., using C57BL6/SJL F2 hybrid embryos for microinjection. Founders were backcrossed onto a C57BL6 background. Mice were screened for the transgene by Southern blot or PCR for routine typing. Animals were maintained and bred under standard conditions.

Northern Blot and PCR

The hippocampus was removed from transgenic or wild-type mice, and RNA was purified using the guanidium thiocyanate method. For Northern blots, 20 µg of total hippocampal RNA was used. Filters were hybridized to an [α - 32 P]dATP-tailed oligonucleotide probe specific for the Thr-286→Asp mutation (5'-CTTCAGGCAGTCGACGTCCTCTGTCTGTG-3'). Hybridization was at 42°C overnight in a solution containing 50% formamide, 10% dextran sulfate, 25 mM HEPES, 1 mM EDTA, 6× Denhardt's, 240 mM NaCl, 100 µg/ml denatured salmon sperm DNA, and 100 µg/ml poly(dA). Filter was washed once for 10 min in 2× SSC at room temperature and three times for 10 min each in 0.2× SSC at 60°C and exposed to film for 1 week.

For RT-PCR, 100 ng of total hippocampal RNA was amplified in a 100 µl reaction with rTth DNA polymerase (Perkin Elmer) as recommended by the manufacturer. Amplification was for 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The following oligonucleotides were used as primers: 5'-AGATGCTGACCATCAACCCA-3' and 5'-CTCTGGTTCAAAGGCTGTCA-3'. These produce a 401 bp fragment containing the site of the Thr-286→Asp mutation. The PCR reaction was done in the presence of 1 µCi of [α - 32 P]dCTP, and the amplified fragment was digested with AatII. The AatII site is present only in the mutant transcripts, and digestion leads to the production of 294 and 107 bp fragments. The digested sample was run on a 2% agarose gel. The gel was fixed, dried, and exposed to film. The expression of the mutant transgene relative to the wild type was estimated by densitometric comparison of the respective bands on the autoradiogram.

In Situ Hybridization

Mice were killed by cervical dislocation, and the brains were dissected and rapidly frozen in mounting medium. Cryostat sections (20 µm) were taken, postfixed for 10 min in 4% paraformaldehyde in PBS (pH 7.3), dehydrated, and stored frozen at -70°C until use. The slices were hybridized to the Asp-286-specific oligonucleotide probe or to a probe specific for the wild-type mouse CaMKII α gene (5'-CTCAGGCAGTCCACGGTCTCTGTCTGTG-3'). The probes were labeled by 3' poly(A) tailing using [α - 35 S]thio-dATP and terminal transferase to a specific activity of 0.5×10^9 to 1×10^9 cpm/µg. Hybridization was overnight at 42°C in a solution containing 50% formamide, 10% dextran sulfate, 25 mM HEPES (pH 7.0), 600 mM NaCl, 100 mM DTT, 1× Denhardt's, 200 µg/ml denatured salmon sperm DNA, 200 µg/ml poly(dA), and 10^7 cpm/ml oligonucleotide probe. Slides were washed two times for 10 min each in 2× SSC at room temperature and two times for 60 min each in 0.2× SSC at 65°C, dried, and exposed to film for 3 weeks (mutant probe) or 4 days (wild-type probe).

Electrophysiology

Transverse slices (400 µm thick) of mouse hippocampus were prepared using standard techniques (see Grant et al., 1992) and maintained in an interface-type recording chamber perfused (1–3 ml/min) with a mouse artificial cerebrospinal fluid consisting of 124 mM NaCl, 4.4 mM KCl, 12.0 mM Na₂PO₄, 25 mM Na₂HCO₃, 2.0 mM CaCl₂, 2.0 mM MgSO₄, and 10 mM glucose, gassed with 95% O₂ and 5% CO₂. All experiments were done at 30°C and began after allowing the tissue to recover for at least 60 min following slice preparation.

EPSPs were elicited once every 50 s (0.02 Hz) using nichrome or tungsten wire bipolar stimulation electrodes (0.01–0.02 ms duration pulses) placed in stratum radiatum of the CA1 region. The resulting potentials were monitored using low resistance glass microelectrodes (5–10 M Ω , filled with artificial cerebrospinal fluid) also placed in stratum radiatum of CA1. In each experiment, we first determined the maximal field EPSP amplitude by gradually increasing the stimulation intensity until the EPSP amplitude reached a saturating level. The stimulation intensity was then decreased so as to evoke a response that was ~50% of the maximal EPSP amplitude. In all experiments baseline synaptic transmission was monitored for 15–20 min before delivering

either high or low frequency stimulation. In general, LTP was induced using two trains of 100 Hz stimulation (each 1 s in duration; intertrain interval = 10 s). However, in one series of experiments (see Figure 5B), baseline EPSPs were evoked using a stimulation intensity sufficient to evoke EPSPs that were 25% of the maximum, and LTP was induced using two trains of 100 Hz stimulation (1 s duration) separated by 20 s and delivered at an intensity sufficient to evoke EPSPs that were 75% of the maximum. In the majority of the electrophysiological experiments, the experimenter was blind to the genotype of the animal. For all statistical comparisons, the *n* used was the number of animals rather than number of slices.

CaMKII Enzyme Assays

Hippocampal slices were prepared in the same manner as those used for electrophysiology and then incubated for 1 hr at 30°C in an oxygenated chamber. Extracts were prepared from the entire slice and enzyme assayed essentially as described by Ocorr and Schulman (1991). Slices were frozen rapidly in liquid nitrogen and homogenized in a buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM DTA, 2 mM leupeptin, 0.4 mM DTT, and 0.1 mM PMSF, either with or without phosphatase inhibitors (0.4 mM molybdate, 10 mM sodium pyrophosphate). Protein levels were assayed using the micro BCA Kit (Pierce) with BSA as standard. Samples without phosphatase inhibitors were incubated for 15 min at room temperature before enzyme assay. Enzyme reaction mix consisted of 50 mM PIPES (pH 7.0), 10 mM MgCl₂, 100 µg/ml BSA, 200 µg/ml leupeptin, 0.4 mM DTT, 0.6 mM EGTA, 0.2 mM EDTA, 200 µM ATP, 100 µCi/ml [α - 32 P]ATP, and 20 µM autocamtide-2, with either 1 mM CaCl₂ and 5 µg/ml calmodulin (+Ca²⁺) or 2 mM EGTA (-Ca²⁺). Enzyme reactions were carried out at 30°C for 1 min in a final volume of 50 µl. The reaction was initiated by the addition of 0.5–2 µg of hippocampal extract and terminated by the addition of an equal volume of 10% ice-cold TCA. Protein was pelleted and the supernatant was spotted onto Whatman P81 filter paper disks and washed three times for 10 min each with water. The level of incorporated 32 P was determined by scintillation counting.

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