α Calcium/Calmodulin Kinase II Mutant Mice: Deficient Long-term Potentiation and Impaired Spatial Learning

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Long-term potentiation (LTP) is a type of synaptic plasticity that has been widely studied as a candidate mechanism for some types of learning and memory (Bliss and Gardner Medwin 1973; Bliss and Lomo 1973; Schwartzkroin and Webster 1975; McNaughton et al. 1978). In some hippocampal synapses where LTP has been studied, the voltage-sensitive and glutamate-gated ion channel, the *N*-methyl-D-aspartate receptor (NMDAR) plays a critical role in the induction of LTP by regulating a calcium (Ca^{++}) current (Collingridge and Singer 1990). The Ca^{++} influx facilitated by an opening of NMDARs leads to an increase in the synaptic connection via a series of biochemical events that include activation of Ca^{++} and/or calmodulin-dependent kinases.

To date, the evidence supporting the linkage between LTP and learning and memory primarily comes from the analysis of rats in which the NMDAR is blocked by an antagonist, aminophosphonovaleric acid (APV) (Morris et al. 1986; Staubli Thibault et al. 1989; Davis et al. 1992). The interpretation of those results is difficult, however, because inhibiting NMDAR function also disrupts synaptic function (Bekkers and Stevens 1990b) and might therefore alter the character of information processing in the hippocampus. Thus, the deficits in learning might be due to this alteration in hippocampal synaptic function and not to the deficits in LTP. A second line of evidence linking LTP to learning and memory comes from the observation that induction of saturating levels of LTP in the hippocampus impairs the ability of rats to acquire new spatial information (Castro et al. 1989). However, these findings have also been given an alternative interpretation (Keith and Rudy 1990; Sutherland et al. 1991).

We have adopted a new strategy for the study of the mechanisms of mammalian memory, suitable for addressing the problem of LTP and learning: production of mice with mutations in individual enzymes likely to be involved in the regulation of candidate memory mechanisms, such as LTP. Such specific mutations can be made by using gene targeting (Capecchi 1989). As a first step in this program, we report here studies on a strain of mutant mice that do not express the α isoform of Ca^{++}/calmodulin-dependent protein kinase type II (α CaMKII). This enzyme is neural specific and is present presynaptically, as well as being richly represented adjacent to the postsynaptic membrane at synapses that express LTP (Erondu and Kennedy 1985; Schulman and Lou 1989). Calmodulin (CaM) loaded with Ca^{++} activates this enzyme and induces its autophosphorylation. Once autophosphorylated, the CaMKII holoenzyme no longer requires Ca^{++} or CaM for activity. This switch-like mechanism can maintain the enzyme in an active state beyond the duration of the activating Ca^{++} signal (Miller et al. 1988; Hanson et al. 1989) and has been invoked in learning models (Miller and Kennedy 1986; Lisman and Goldring 1988). Furthermore, pharmacological experiments have implicated this holoenzyme in the induction of LTP (Malenka et al. 1989; Malinow et al. 1989).

We find that although postsynaptic mechanisms seem normal in the CA1 hippocampal region of these mutant mice, they exhibit little or no LTP. These mice are therefore an ideal model to examine the association of hippocampal α CaMKII activity, LTP, and learning and memory processes. We examined whether α CaMKII mutant mice can learn to solve a complex spatial learning task, the Morris water tasks (Morris 1981), and found that these mutant mice have a pronounced deficit in spatial learning performance compared to normal wild-type littermates. Our results demonstrate that α CaMKII is important for spatial learning and support the hypothesis that LTP is the electrophysiological basis for certain types of learning processes.

EXPERIMENTAL PROCEDURES

Production of α CaMKII mutant mice. We transfected the linealized p23 plasmid into E14 embryonic stem (ES) cells (Thompson et al. 1989) by electroporation and isolated 150 (*neo*) colonies, of which two (E14-20 and E14-84) were shown by Southern blotting analysis to harbor a homologously integrated plasmid. We injected the E14-20 ES cells into C57BL/6J blastocysts and transferred the blastocysts into pseudo-pregnant mothers. Twelve male chimeric mice were born, and we bred them with BALB/c females. Southern blot analysis of tail DNA from the offspring of the chimeric males revealed that 11 of the 12 males transmitted the α CaMKII mutation to 20–40% of their offspring. Southern blot analysis of a litter from a cross between two mice heterozygous for the α CaMKII mutation iden-

tified mice with two mutant copies of the α CaMKII locus (mutation homozygous), as well as mice with one mutant copy (mutation heterozygous) and mice with normal α CaMKII loci (wild type).

In vitro phosphorylation assays. The brain was isolated quickly, dissected, weighed, and homogenized gently in 4 volumes of cold extraction buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 0.5 mM DTT, 0.1 mM PMSF, 5 mg/l leupeptin, 20 mg/l soybean trypsin inhibitor. This homogenate was kept on ice and was typically diluted 1/10 to 1/200 in the same cold buffer. The assays were carried out after 2- to 20-fold dilution of the homogenate in cold water and were immediately followed by the addition of cold assay buffer to a final concentration of 50 μM Syntide 2, 25 mM HEPES (pH 7.4), 10 mM MgCl, 0.5 mM DTT, 15 μM ATP, and 50 μCi/ml of [γ-^{32}P]ATP. For the Ca^{++}/CaM-induced assays, we also added to this buffer 1.5 μM calmodulin and 2.0 mM $CaCl_2$. For the Ca^{++}/CaM-independent reactions, we added 0.5 mM EGTA to the assay buffer. After adding the assay, buffer reactions were briefly mixed and quickly placed at 30°C for 45 seconds. The reaction was terminated by spotting half of the reaction volume (25 μl) in perforated disks of phosphocellulose. These disks were then washed of nonincorporated [γ-^{32}P]ATP with 1% phosphoric acid and water. The radioactivity bound to the disks was counted, and the values were plotted. The phosphorylation results were derived from four independent experiments, each with at least three different concentrations of homogenates to check that substrate was not limiting, and with duplicates at each concentration point.

Electrophysiological procedures. All animal handling and tissue preparation were in accordance with a protocol approved by the Salk Institute and MIT Animal Use and Care Committee. Transverse hippocampal slices (~350 μm) were prepared from normal (wild type) or mutant mice (male or female, 1–4.5 months old, mostly 1.5–3 months old). Slices were then maintained in an incubation chamber for at least 1 hour at room temperature (24° ± 1°C). An individual slice was transferred to a submerged recording chamber, where it was held by a net made with flattened platinum wire and nylon threads and continuously perfused with artificial cerebrospinal fluid (ACSF) at a rate of ~2 ml/min. The temperature in the recording chamber was 30.5° ± 0.5°C. The ACSF, equilibrated with 95% O_2/5% CO_2, is composed of (in mM): NaCl 120, KCl 3.5, NaH_2PO_4 1.25, $NaHCO_3$ 26, $MgCl_2$ 1.3, $CaCl_2$ 2.5, picrotoxin (PCTX) 0.05. The solution for dissection has the same composition as regular ACSF except there is no PCTX and NaCl is replaced with equimolar sucrose (Aghajanian and Rasmussen 1989). CA3 region was usually removed to prevent epileptiform activity. The cell layer was visualized under an inverted microscope with phase contrast (Zeiss, Thornwood, New York). Extracellular field excitatory postsynaptic potentials (f-EPSPs) were recorded in the stratum radiatum of CA1 with electrodes (1–2 Mohms) filled with ACSF. Excitatory postsynaptic currents (EPSCs) were recorded in CA1 pyramidal neurons with the whole-cell patch-clamp mode, using electrode 3–4 Mohms; no fire polishing; soft glass (Drummond Sci. Co., Broomal, Pennsylvania), filled with (in mM) cesium gluconate 130, $CsCl_2$ 5, EGTA 0.5, $MgCl_2$ 1, Mg-ATP 2, GTP 0.2, NaCl 5, HEPES 10 (pH 7.25). The seal formed on cell bodies was typically 2–3 gigaohms, and the input resistance of cells was typically around 100 Mohms. Bipolar tungsten stimulating electrodes (Frederick Haer & Co., Brunswick, Maine) were positioned in Schaffer collateral-commissural afferents to evoke f-EPSPs (150–200 μm away) or to evoke EPSCs (50–100 μm away). The stimulus intensity was adjusted to evoke pretetanic responses of similar sizes for all the neurons or slices. The stimulus duration was 100 μsec. Recordings were performed with an Axopatch-1A (Axon Instruments, Inc., Foster City, California), filtered at 1–2 kHz, and sampled at 5–10 kHz. Data were collected and analyzed with programs written by C.F. Stevens in AxoBASIC/QuickBASIC. The data collected from normal other strains of mice were combined with the data from normal littermates, since they were indistinguishable. CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) and D-APV (D-2-amino-5-phosphonovaleric acid) were from Cambridge Research Biochemicals (Valley Stream, New York), and PCTX was from Sigma (St. Louis, Missouri).

RESULTS

α CaMKII Mutant Mice

To produce mice with a mutation in the α CaMKII locus, we constructed the plasmid p23 (Fig.1A) which contains a 6.1-kb mouse genomic α CaMKII sequence that is disrupted by insertion of a neomycin-resistance gene (*neo*) from the plasmid pgkneo (Adra et al. 1987). The insertion is within the α CaMKII exon encoding most of the regulatory domain, and the inserted sequence replaced a 130-bp mouse genomic sequence flanked by a pair of *Sph*I sites, which includes the entire inhibitory domain and five amino acids in the amino end of the calmodulin-binding domain (Colbran et al. 1989). We have mutation homozygous mice that were born in June of 1990: The α CaMKII mutation appears not to affect long-term survival under laboratory conditions. In the experiments described in this paper, unless otherwise noted, we used as controls the wild-type littermates of the mutant mice. Northern blot analysis of total brain RNA confirmed that the α CaMKII mutant mice lacked α CaMKII mRNA or any truncated form of it, whereas the β CaMKII mRNA was present at the normal level (data not shown). Western blot analysis of forebrain tissue from the mutants and their normal littermates with the monoclonal antibody 6J9 (Erondu and Kennedy 1985), which recognizes α CaMKII, confirmed that α CaMKII is absent in the mutants but present in the mutation heterozygous and wild-type mice (Fig.1C). A similar analysis with a poly-

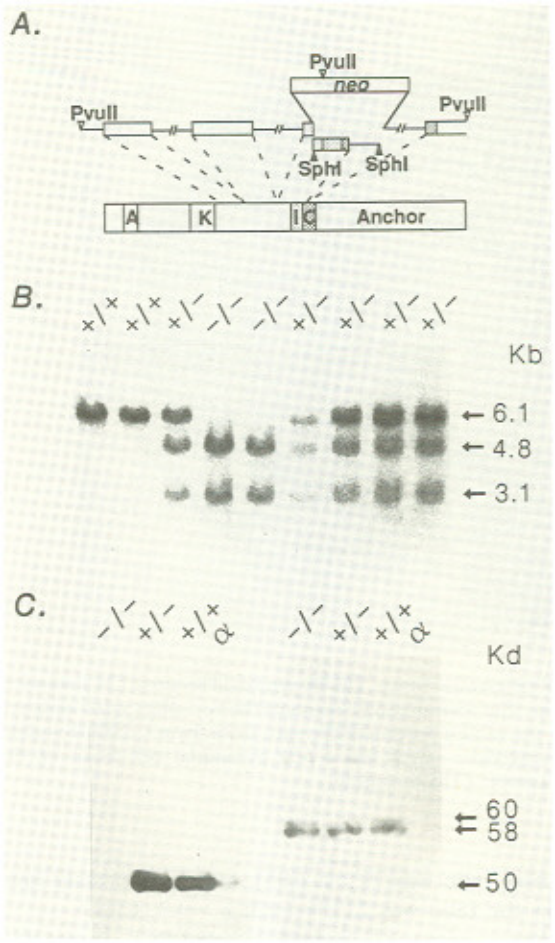

Figure 1. The α CaMKII mutant mice lack α CaMKII. (*A*) Schematic representation of the p23 construct used for the targeting experiment. The 6.1-kb *Pvu*II genomic fragment used in the construction of p23, as well as the *neo* and its insertion site, are shown on the upper part of this figure. We also show the deleted *Sph*I fragment and the functional domains of the α CaMKII enzyme in the lower tracings of this figure. The intron/exon boundaries and their correspondence to the functional domains of the protein are shown by dashed lines. (A) ATP-binding domain; (K) catalytic domain; (I) inhibitory domain; (C) CaM-binding domain; (Anchor) association/anchor domain. (*B*) Western analysis with a monoclonal antibody that recognizes α CaMKII (6g9; left panel), and with a polyclonal antibody that recognizes the β CaMKII, and β′ CaMKII ("Darcy"; right panel) of forebrain homogenates (5 mg) from a mutation homozygous (+ / −), mutation heterozygote (+ / −), and wild-type (+ / +). As a control we included purified rat α CaMKII protein (70 ng) next to the other samples in the Western procedure. Parallel to the autoradiographs, we show the m.w. of the protein bands detected (α CaMKII, β CaMKII, and β′ CaMKII, respectively, 50, 58, and 60 kD). An ANOVA with repeated measures confirms this observation. There was main effect of Strain ($F[1,23] = 22.778$, $p = .0001$) showing that overall, wild-type controls were better than the mutant mice. There was also a main effect of Trial Block ($F[5,115] = 43.371$, $p < .00001$) showing that overall, animals' latency to swim to the platform decreased during training. Finally, there was a significant Strain × Trial Block interaction ($F[5,115] = 4.528$, $p = 008$). Post hoc analysis of the interactions (Neuman-Keuls, $p < .05$) showed that the wild-type animals had significantly lower escape latencies compared to the mutant mice on trial block numbers 1, 2, 3, and 4, but were not different on trial blocks 5 and 6.

clonal antibody that recognized β CaMKII detected the kinase in all three types of mice (Fig.1C). Consistent with the complete absence of α CaMKII in the mutant mice is the reduced (45 ±4%) level of in vitro phosphorylation of α CaMKII-specific peptide substrate, synthide2, in the forebrain homogenates of these mice as compared to the control mice. In contrast, such difference in the in vitro phosphorylation levels was not observed with the homogenates of cerebellum, where the α isoform is known to be a minor CaMKII component (Erondu and Kennedy 1985; Burgin et al. 1990). We confirmed the specificity of our CaMKII assays by adding a peptide inhibitor of CaMKII activity and showing that it reduces phosphorylation levels by 95%.

Ca^{++} calmodulin-independent kinase II activity of the forebrain of mutant mice was also measured and shown not to be increased compared to control mice ($n = 4$). This observation, as well as the lack of any truncated RNA (see above), strongly suggests that the mutant mice do not harbor truncated α CaMKII enzyme, which retains the catalytic domain without the inhibitory domain.

General Observations on Brain Anatomy and Behavior

Observations at the light microscopic level reveal no obvious differences between the mutants and wild-type mice. We concentrated our survey on the hippocampus and neocortex, two structures with high expression of α CaMKII (Burgin et al. 1990). A comparison between coronal brain sections, stained with thionin, from mutants and wild-type mice ($n = 3$; Fig. 2) showed that the arrangement of cells in the major layers of the neocortex and hippocampus appear normal. Tangential sections of the somatosensory cortex stained with cytochrome oxidase or with cresyl violet revealed the same topographical organization of the barrel fields as described for normal mice (data not shown).

The behavior of the mutant mice appears remarkably normal: The mutant pups gain weight at the same rate as their normal littermates, which suggests that their suckling behavior is not deficient. The mutants whisk, sniff, mate, and are not ataxic, indicating that the absence of the α CaMKII does not result in generalized loss of neuronal function. However, they are clearly more jumpy than their wild-type littermates. For instance, unlike wild-type littermates, mutant mice try to avoid human touch long after weaning. Interestingly, this "nervousness" was previously reported for animals with hippocampal lesions (Douglas 1967).

Synaptic Function

Using whole-cell recording in hippocampal slices, we have studied synaptic currents evoked in CA1 pyramidal cells by stimulating Schaeffer collaterals. Our conclusions are based on studies of 37 neurons or slices from 14 homozygotes, 2 slices from 1 heterozygote, and 35 neurons or slices from 20 littermates or other strains.

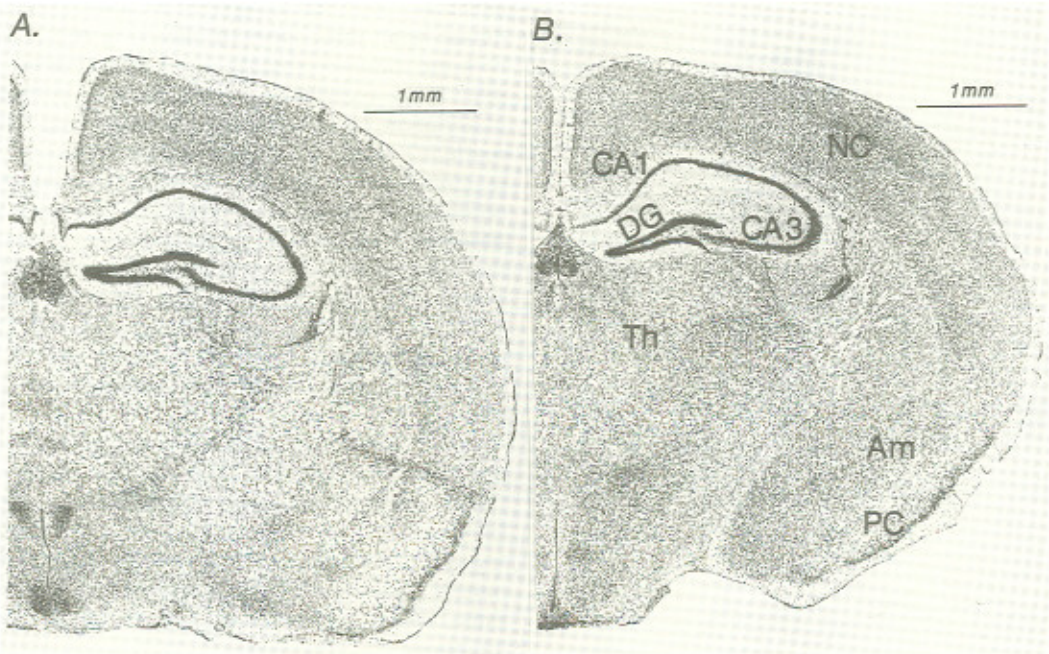

Figure 2. Coronal sections from a wild-type (*A*) and α CaMKII mutant (*B*) mouse brain. The frozen brain sections (14 μm) were thaw-mounted on slides coated with gelatin and stained with 1% thionin. The rostral/caudal levels of the two slices were matched for the hippocampus, but due to slightly different angles of sectioning, the ventral portion of the slice from the α CaMKII mutant mouse is posterior to the one from the wild-type mouse. (Am) Amygdala; (CA1 and CA3) pyramidal cell fields of the hippocampus; (DG) dentate gyrus; (NC) neocortex; (PC) pyriform cortex; (Th) thalamus.

Typical synaptic currents from mutant and normal hippocampal pyramidal neurons are illustrated in Figure 3, A and B. These experiments were carried out in normal magnesium (1.3 mM), and at the neuron's membrane potential (−50 mV) that reveals the NMDA as well as the non-NMDA components of the synaptic currents. Although the absolute amplitude of the synaptic currents depends on stimulus intensity, we used comparable stimuli in all experiments and found no apparent difference in the peak size and time course of synaptic currents from normal and mutant mice. In the presence of CNQX (10 μM; antagonist of non-NMDA receptor) and PCTX (50 μM; antagonist of $GABA_A$ receptor) and at holding potential −30 mV, the rise time of the NMDA component (average 4–10 traces) was 6.1 msec (±0.5 S.E.M.) for the normal mice ($n = 4$) and 6.1 msec (±0.4) for the mutant mice ($n = 4$); the decay time constant was 31.2 msec (±4.9) for the normal mice and 33.7 msec (±4.9) for the mutant mice. The rise time of the non-NMDA component (average 4–15 traces) was 1.5 msec (±0.2) for the normal mice ($n = 4$) and 1.6 msec (±0.1) for the mutant mice ($n = 6$); the decay time constant was 8.9 msec (±0.8) for the normal mice and 7.1 msec (±0.9) for the mutant mice at holding potential −80 mV. All above corresponding values were not significantly different (p values ranged from 0.2 to 0.9, t-test). Furthermore, the ratio of NMDA to non-NMDA components does not differ between normal and mutant neurons (Fig. 3C): For 8 normal neurons the NMDA/non-NMDA component ratio was 29.1% (±5), and for 8 mutant neurons the ratio was 23.8% (±3; $p > 0.3$, t-test).

The previous results demonstrated that, at one particular voltage, the NMDA component of synaptic currents is the same size at normal and mutant synapses. The Mg^{++} block of NMDAR channels that endows these channels with the voltage dependence so important for making LTP associative could, however, differ between normal and mutant synapses. For example, the action of protein kinase C has recently been shown to modify the Mg^{++} dissociate constant at the NMDAR regulatory site (Chen and Huang 1990), and some direct or indirect action of α CaMKII could possibly have an analogous effect. Were this the case, an essential difference in normal and mutant synaptic function would not have been revealed in the preceding experiments. We have investigated this point by directly measuring the voltage dependence of NMDAR in normal and mutant mice. As illustrated in Figure 4, the dependence of NMDAR channel conductance on voltage is not distinguishably different between normal and mutant mice. The activation curves from both normal mice ($n = 4$) and mutant mice ($n = 3$) are well fitted by the equation developed by Jahr and Stevens (1990a,b) (see legend to Fig. 4). In summary, we could detect no differences between NMDAR function in normal and mutant mice.

Historic effects, as revealed by the response to the second of a stimulus pulse pair, did appear to be one aspect of synaptic function that differs between normal

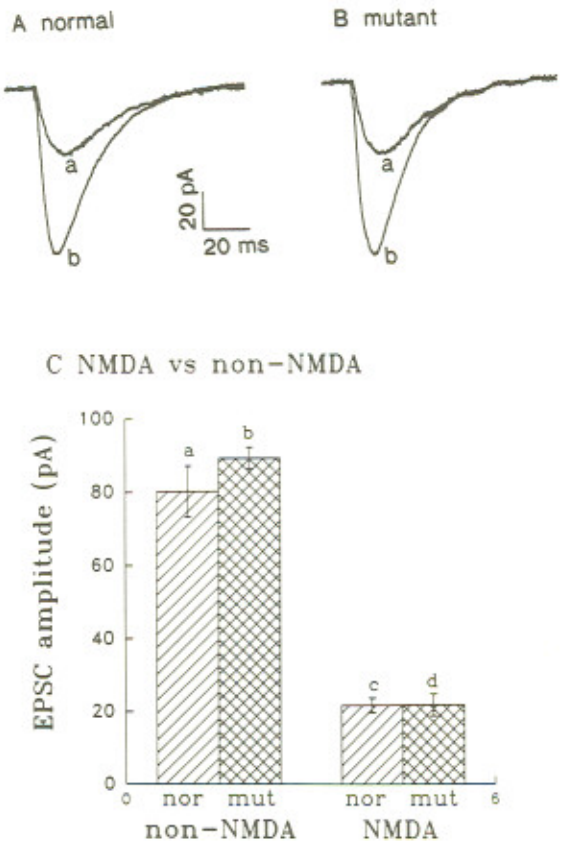

Figure 3. Synaptic currents from whole-cell recordings as a function of time for normal (*A*) and mutant (*B*) mice. The superimposed pair of traces in *A* and *B* show the total synaptic current that includes both the NMDA and non-NMDA components (*b*) and the NMDA component alone (*a*). The NMDA component was obtained by subtracting the non-NMDA component remaining after application of 50 μM D-APV (an antagonist of NMDA receptor; traces not shown) from the total current (shown in *b*). (*C*) Averages of EPSC amplitudes for non-NMDA and NMDA components from eight normal (nor) and eight mutant (mut) mice. The holding potential was −50 mV and the stimulus intensity was increased to reveal the NMDA (in addition to non-NMDA) components of the synaptic currents. The NMDA components were measured at 20–35 msec after the peak current (always judged by the traces mostly after D-APV or at −80 mV). There are no significant differences between *a* and *b* ($p > 0.2$, t-test) and between *c* and *d* ($p > 0.9$).

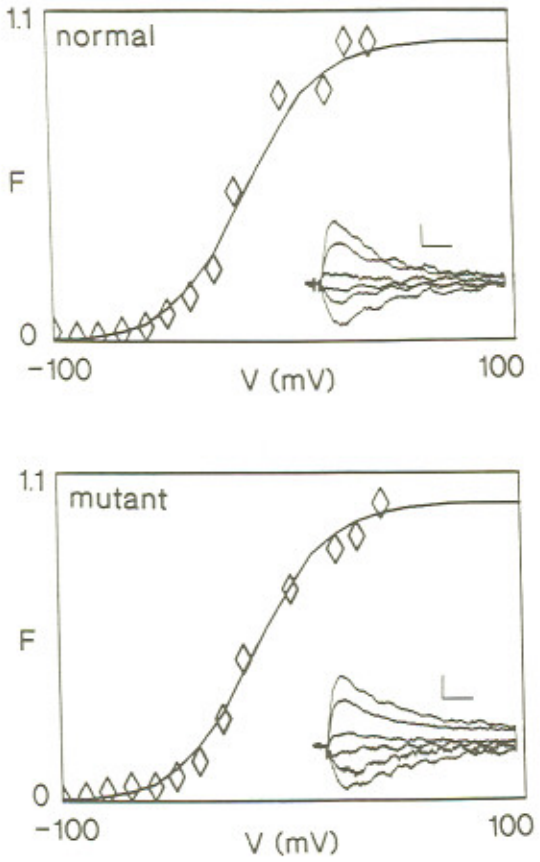

Figure 4. Voltage dependence of NMDA receptor channels for typical normal (*top*) and mutant (*bottom*) neurons in the presence of CNQX (10 μM) and PCTX (50 μM). The fraction (F) of the maximum conductance through the NMDA receptor channels is given as a function of membrane potential. The insets show sample traces of synaptic current as a function of times. The holding potentials (mV) for the sample traces are (from top to bottom) +40, +30, +20, −90, −30, −20 for normal neurons and +40, +30, +20, −70, −40, −20 for mutant neurons. The vertical calibration bars indicate 25 pA current. The horizontal bars indicate 10 and 15 msec for normal and mutant neurons, respectively. The smooth curves were fitted with the equation: $g(V) = 1/[1 + \exp(-0.062V)(C/3.57)]$, where g is conductance in pS, V is membrane potential in mV, and C is extracellular magnesium concentration in mM (Jahr and Stevens 1990a,b). Note that no free parameters are used in fitting the theoretical curve to the data.

and mutant mice. Figure 5 presents the average paired pulse results. Although the slices from both normal and mutant mice showed paired pulse facilitation, the degree of facilitation was less in mutant mouse slices at all interpulse intervals tested. The impaired pulse facilitation in the mutant mice may not be surprising in view of the fact that synapsin I, a molecule believed to be involved in making vesicles available for release, is regulated by CaMKII (Lin et al. 1990).

Deficient Long-term Potentiation

We have used two approaches to evaluate LTP in the mutant mice: field potential recording to survey properties of large populations of neurons, and whole-cell recording to investigate LTP under conditions that are most sensitive for detecting the presence of LTP (the effect is typically larger with whole-cell recordings than with field potentials) and to permit examination of the factors, such as the release probability, that participate in LTP.

Figure 6 shows the typical time course of synaptic strength in a normal and a mutant mouse following tetanic stimulation that typically induces LTP. As is apparent from the figure, the normal mouse exhibited an obvious increase in synaptic strength, whereas synaptic transmission in the mutant mouse was unaffected by the tetanic stimulation except for a usual

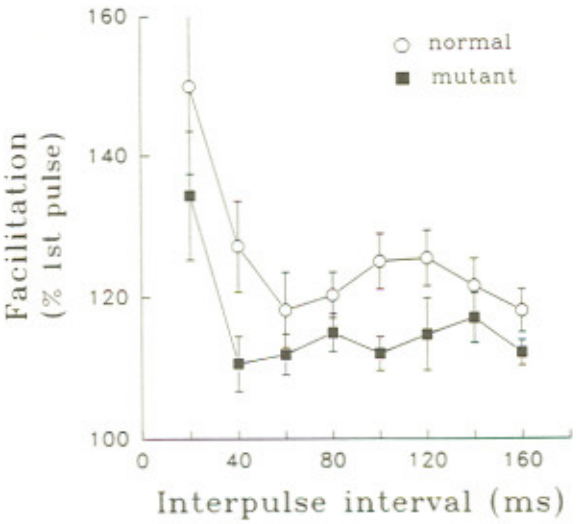

Figure 5. Paired pulse facilitation in normal and mutant mice. The size of the peak field potential for the second pulse of a pair (expressed as the percent of the first pulse size) as a function of the time interval between the pulses. Data are from 10 normal and 10 mutant slices. Error bars show the S.E.M.

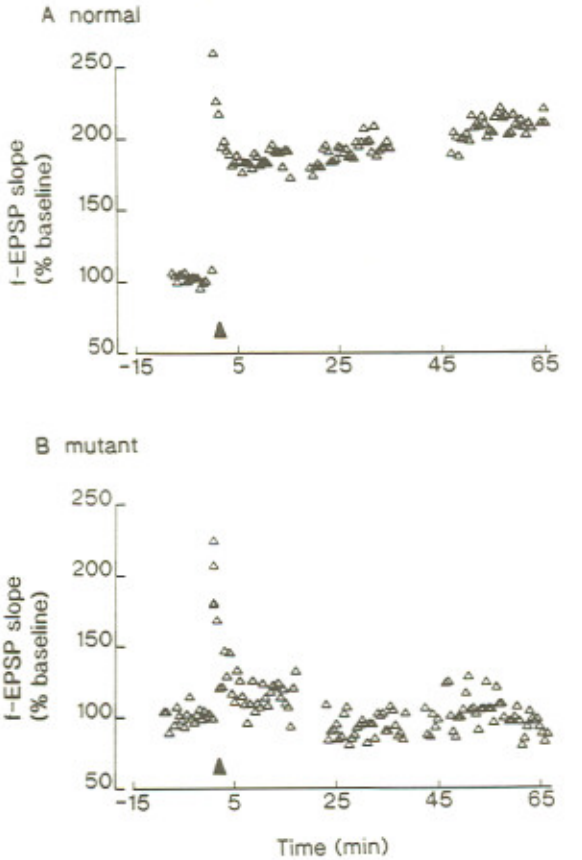

Figure 6. Synaptic strength, expressed as a percentage of the average pretetanus initial field potential rate of change (f-EPSP), as a function of time in minutes during the experiments for a typical normal (*A*) and mutant (*B*) slice. Testing stimuli were given once each 30 sec. Our standard tetanus, given at time = 0 (indicated by arrowhead), was 100 Hz, 3 trains, and 20 pulses for each train with an intertrain interval of 11 sec. In some mutant slices, a second tetanus (100 Hz, 200-msec duration, 5 trains, and 6-sec interval) was given.

amount of posttetanic potentiation. Of 11 slices from normal mice, 9 exhibited LTP (defined as an increase in synaptic strength of 20% or greater sustained for at least 30 minutes), whereas only 2 of 16 slices from mutant mice showed LTP (Table 1). These two slices were from different mutant mice. The distribution of synaptic strength 30–60 minutes after a tetanus for 11 normal and 16 mutant neurons is shown in Figure 7. Many mutant neurons actually showed some decrease in synaptic posttetanus period. The effect was not just an increased threshold for LTP because, in slices from mutant mice, a second tetanus with increased trains (group of pulses, see legend to Fig. 8) still failed to produce potentiation. Based on field potential recordings that report the characteristics of large neuronal populations, we conclude that most hippocampal synapses do not exhibit significant LTP in mice lacking α CaMKII.

With whole-cell recording, 9 of 10 neurons from normal mice exhibited LTP with an average increase in synaptic strength of 286% (±48) of baseline at 30–60 minutes following a tetanic stimulus. For the mutant mice, 2 of 12 cells exhibited LTP, and the average synaptic strength after tetanic stimulation for these two cells was 221% (±34); for the neurons from mutant mice that did not show LTP (less than 20% increase), the average was 98% (±8, $n = 10$) (Table 1; Fig. 8). These two cells were from two different mutant mice.

For normal cells, LTP is accompanied by a decrease in the number of "failures"—those stimuli that produce no postsynaptic response—and an increase in the quantal content as revealed by a standard quantal anal-

Table 1. Summary of the Changes of Synaptic Strength in Normal and Mutant Mice

Mouse	LTP *n*/ Total *n*	Potentiation (% baseline) mean ± S.E.M.	No LTP *n*/ total *n*	Change (% baseline) mean ± S.E.M.
Field EPSP				
normal	9/11	183 ± 18	2/11	114 ± 1
homozygote	2/16	168 ± 32	14/16	99 ± 3
heterozygote	1/2	141	1/2	115
EPSC				
normal	9/10	286 ± 48	1/10	96
homozygote	2/12	221 ± 39	10/12	98 ± 8

n represents the number of slices (field EPSP slope) or neurons (EPSC amplitude).

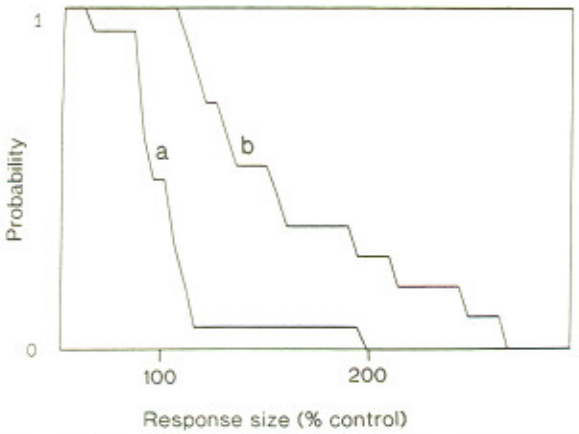

Figure 7. Cumulative probability as a function of initial field EPSP slope, expressed as percentage of the pretetanus average, and valued for neurons 30–60 min after tetanic stimulation. The 16 mutant slices are represented by graph a and 11 normal slices by graph b. The ordinate gives the fraction of the slices that exhibited a posttetanic field EPSP slope of the corresponding abscissa size or smaller.

ysis (Bekkers and Stevens 1990a; Malinow and Tsien 1990). The synapses from one mutant animal that did exhibit LTP exhibit the same properties: a decrease in failures following tetanic stimulation (from a pretetanic failure rate of 2.3% to a posttetanic rate of less than 0.07%) and an increase in quantal content that can account for the entire amount of potentiation.

We conclude, then, that mice lacking expression of α CaMKII are markedly deficient in LTP, but in those cases where LTP does occur, it appears to be indistinguishable from what is observed in normal animals.

The Morris Water Task

In the Morris water task, mice are placed in a round pool filled with opaque water. To escape the water, they must swim to a submerged platform. In the "visible-platform" version of the Morris task, a visually conspicuous white flag is placed on top of the submerged platform which is positioned in random locations during each trial. To solve this task and swim directly to the platform, it is sufficient for an animal to learn that the flag indicates the location of the platform. Hence, distal extramaze cues are irrelevant in this task. In the "hidden-platform" version of the Morris task, the escape platform is located in a fixed location within the pool. Since there are no immediate proximal cues indicating the platform's location, and an animal cannot see the platform through the water, it must learn the multiple spatial relationships between distal objects in the room surrounding the pool and the platform in order to locate and swim directly to it. There are five phases to the behavioral study. All animals were subjected to each phase in the order presented below.

Phase 1: Mutant mice can solve the visible-platform task. In the visible-platform task, the platform location alternated among four possible places within each block of trials (see legend to Fig. 9). Animals were

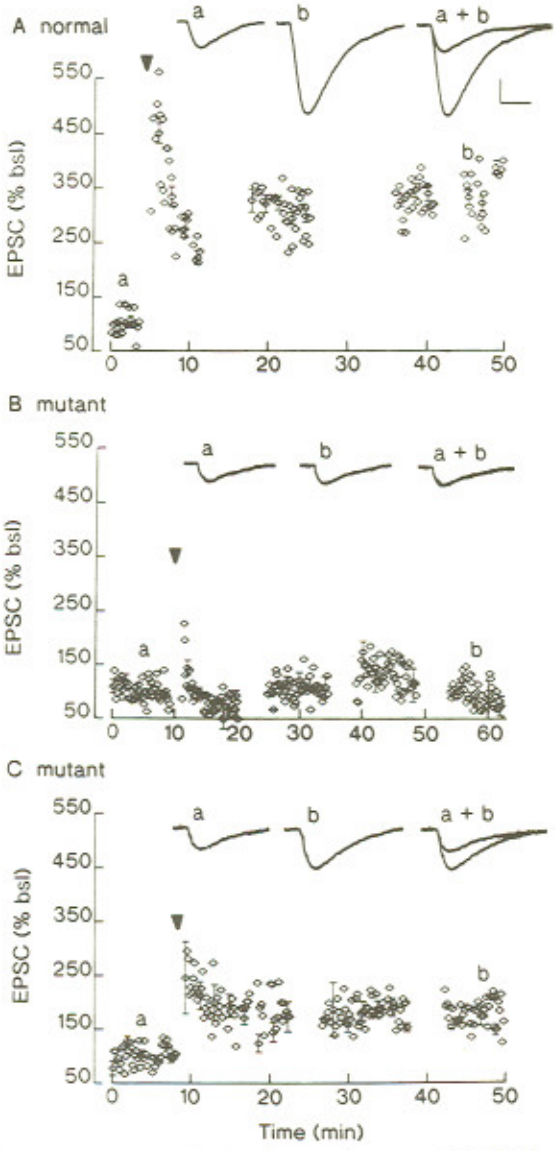

Figure 8. Peak amplitude of synaptic current (EPSC) from whole-cell recordings as a function of time. Amplitudes are expressed as percentage of mean amplitude before tetanic stimulation. (*A*) Typical normal cell, (*B*) typical mutant cell, and (*C*) one of two mutant cells that exhibited LTP. Traces a and b show typical recordings of synaptic current made at the times indicated by the same letters in the amplitude graphs immediately below. The last trace is the preceding pair superimposed. Testing stimuli were given once each 2 sec; the points plotted here are averages of 5 consecutive samples. Holding potential was either −60 or −70 mV (−60 mV for this figure). The tetanus was given at the time point indicated by arrowhead. The tetanus protocol was typically 50 Hz, 2–3 trains, 30 pulses for each train, and 10–11 sec between trains while holding membrane potential at −30 to −40 mV. (With higher frequency tetani, such as 100 Hz, the synaptic current was often depressed, particularly in the mutant neurons.) The amplitude calibration bar (vertical) is 20 pA and the time calibration bar (horizontal) is 10 msec. In many cases (including field potential recording), we tested different things between the times we collected data for synaptic strength; this accounts for the gaps in the graphs. In addition, two to seven different tetanus protocols were tested in some mutant neurons if the typical tetanus protocol did not induce LTP. These procedures made it difficult to average plots among neurons or slices precisely, so we preferred to show the representative plots.

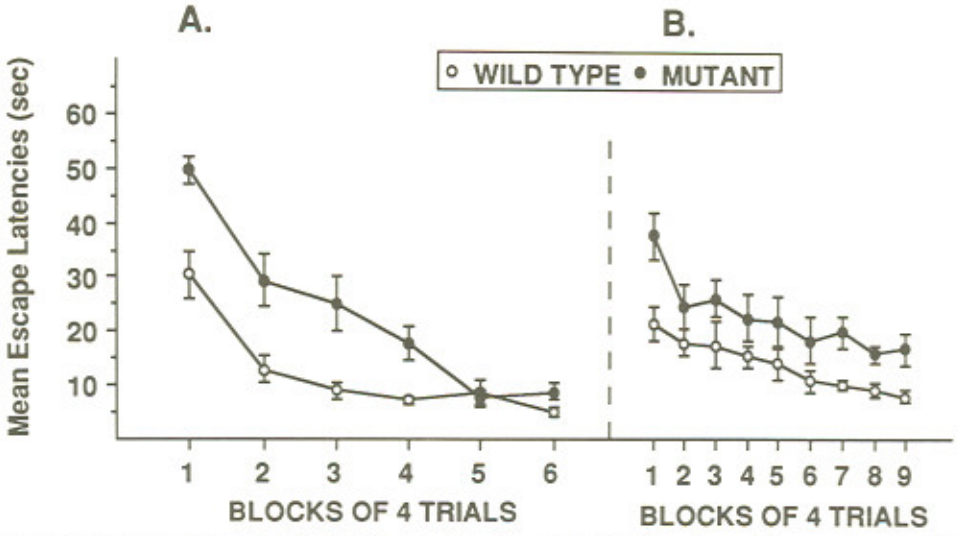

Figure 9. Mean escape latencies for animals in the Morris water task. (*A*) In the first phase of the experiment, wild-type controls ($n = 14$) and α CaMKII mutant mice ($n = 11$) were trained to navigate to a randomly located visible platform. The platform was rendered visible by attaching a small white flag to its top. Each animal was first pretrained to climb onto the platform and given a 15-sec practice swim to ensure that all animals could swim. On each trial, a subject was allowed to search the pool for 60 sec. Once a subject found the platform, it was allowed to remain there for 45 sec. Animals were given 12 trials a day, in blocks of 4 trials, on 2 consecutive days. α CaMKII mutant mice were initially impaired at locating the visible platform but overcame this deficit and learned to locate it just as rapidly as controls. An ANOVA with repeated measures confirms this observation. There was a main effect of Strain ($F[1,23] = 22.778$, $p = .0001$) showing that, overall, wild-type controls were better than the mutant mice. There was also a main effect of Trial Block ($F[5,115] = 43.371$, $p < .00001$) showing that overall, animals' latency to swim to the platform decreased during training. Finally, there was a significant Strain × Trial Block interaction ($F[5,115] = 4.528$, $p = .008$). Post hoc analysis of the interactions (Newman-Keuls, $p < .05$) showed that the wild-type animals had significantly lower escape latencies compared to the mutant mice on trial block numbers 1,2,3, and 4, but were not different on trial blocks 5 and 6. (*B*) All animals were then trained to find a hidden platform located in a fixed location. The top of the platform was 1 cm below the surface of the water. Wild-type and α CaMKII mutant mice were given either 3 or 5 days of training as described above (only 3 days are shown). Wild-type controls had lower escape latencies than the mutants. One wild-type animal floated on all trials, so its data were excluded from the analysis. Thus, 13 wild-type and 11 mutants were used. There was a main effect of Genotype ($F[1,22] = 11.576$, $p = .0024$) showing that, overall, wild-type controls had significantly lower escape latencies than the mutants. The main effect of Trial Block ($F[8,176] = 8.54$, $p < .00001$) was also significant, showing that animals improved during training. The Genotype × Trial Block interaction was not significant. Bars indicate the S.E.M.

tested for 2 consecutive days with three blocks of four trials per day, and the time required to reach the platform was recorded. Figure 9 shows that the α CaMKII mutant mice initially took longer than the wild-type mice to reach the platform, but by the end of training, they were locating it as rapidly as controls. The results show that although α CaMKII mutant mice appear to be initially impaired, they are able to overcome this deficit by training. Therefore, α CaMKII mutant mice (1) are able to learn to associate the flag with the escape platform, (2) are motivated to escape the water, and (3) have the coordinated motor skills needed to swim in water.

The exact nature of the initial impairment is not clear but is likely due to the fact that α CaMKII mutant mice initially responded differently from the wild-type animals when placed on the platform. Unlike wild-type mice, the α CaMKII mutant mice immediately jumped into the water. This "jumping response" occurred a number of times before the first trial, and thus by the time their jumping response habituated, they appeared fatigued. Therefore, they may have taken longer on the first day of "visible-platform" training simply because they were tired. On the second day, however, they did not show the jumping response and, hence, were not fatigued before their trials were given.

Phase 2: Training on the hidden-platform task. Seven to twelve days after the visible-platform task, animals were trained on the Morris hidden-platform task. Animals received the standard 3 days of training used previously for inbred strains of mice (Upchurch and Wehner 1988). As can be seen in Figure 9, wild-type controls quickly learned to locate the hidden platform, and by the end of the third day of training, they were navigating directly to it in less than 10 seconds. The α CaMKII mutant mice took longer than the wild-type mice to locate the hidden platform, but their performance improved during training. However, after performance reached plateau levels, the mutant mice still took approximately 20 seconds to locate the platform, approximately twice as long as the wild-type mice. To ensure that mice were at asymptotic levels of performance, some animals from each group ($n = 5$) were given an additional 2 days of training. Although wild-type animals were significantly better than the mutant mice at locating the platform during extended training, neither group improved significantly com-

pared to day 3 of training. In fact, in all measures of performance during each phase of this study, extended training did not significantly alter the performance of wild-type or α CaMKII mutant mice.

Phase 3: Probe trial. To evaluate whether the mice indeed located the hidden platform by learning the multiple spatial relationships between distal cues and the hidden platform, we subjected them to a probe trial. In this trial, the platform was removed and mice were allowed to search the pool for 1 minute. If an animal located the platform during training by using distal cues, it should selectively search the place where the platform was located during training more than other places in the pool. Animals were given their probe trial immediately following the last training trial (i.e., following 3 or 5 days of training). The results are shown in Figure 10.

The wild-type mice spent a larger percentage of their time in the quadrant where the platform had been during training (training quadrant) than in the other three quadrants. They also crossed the exact spot where the platform had been located during training (training site) more often than equivalent locations in other quadrants. In contrast, α CaMKII mutant mice did not spend more time searching in the training quadrant than in the other quadrants; they spent an equal amount of time in all four quadrants. The mutant mice did not cross the training site more often than equivalent locations in the adjacent quadrants. They did cross the equivalent site in the opposite quadrant less frequently than the training site. This difference, however, may be an artifact of the testing protocol in which the animals always started the probe trial from positions in the opposite quadrant. α CaMKII mutant mice may have learned to swim directly away from the start location before implementing any search strategy. Since they were always started from places opposite to the training site, their "starting strategy" reduced the likelihood of crossing the opposite quadrant site early

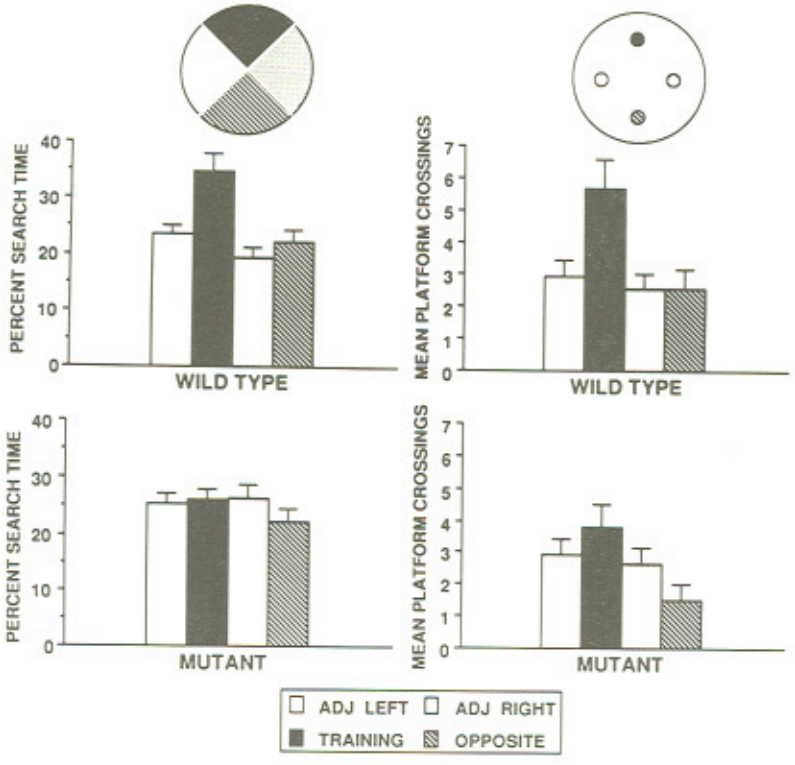

Figure 10. Data from animals given a probe trial after 3 or 5 days of training. Since there were no differences in performance for wild-type or α CaMKII mutant mice, the data were analyzed together. These data show that wild-type animals selectively searched the place where the platform had been located during training, whereas, in general, the α CaMKII mutants' search was not selective. The upper panel represents data from the wild-type animals. An ANOVA showed that wild-type mice spent significantly more time in the quadrant where the platform had been located during training than in the other three quadrants ($F[3,36] = 7.29$, $p = .006$; Newman-Keuls post hoc analysis: Training > Opposite, Adjacent Left, Adjacent Right, $p < .01$). Similarly, an ANOVA showed that wild-type mice crossed the exact site where the platform had been located significantly more often than equivalent locations in the other three quadrants ($F[3,36] = 13.673$, $p < .0001$; Newman-Keuls analysis: Training > Opposite, Adjacent Left, Adjacent Right, $p < .01$). The lower panel shows the data for the α CaMKII mutant mice. In contrast to wild-type animals, α CaMKII mutant mice did not selectively search any quadrant of the pool (ANOVA $F[3,30] = .689$, $p > .05$). The α CaMKII mutant mice also failed to cross the exact place where the platform had been located compared to both adjacent sites. However, they did cross the opposite site less often than the training site. ANOVA $F[3,30] = 3.85$, $p < .05$; Newman-Keuls analysis: Training = Adjacent Left and Adjacent Right, but Training > Opposite, $p < .05$). Above the panels on the right, we show a schematic drawing of the pool with the four virtual quadrants used in the analysis, and on the left we show the four platform sites. Bars indicate the S.E.M.

in the probe. In future studies, the probe trial start location should be randomly chosen.

These data indicate that the wild-type mice learned the spatial relationships between distal cues and the hidden platform (spatial strategy). In addition, these results suggest that the mutant mice improved their performance by developing an alternate strategy that does not depend on specific cues surrounding the pool. For example, mutant mice may have learned the distance between the wall of the pool and platform. Since the pool is circular and the wall is uniform, this strategy would not allow the mutant mice to distinguish among the four quadrants of the pool.

Phase 4: Random-platform task. To confirm that mutant mice are impaired at spatial learning, they were subjected to a "random platform" task. In this task, mice were given trials with the platform in its original location intermixed with trials where the platform was moved to any one of the seven other locations as shown in Figure 11. If the mutant mice were impaired in spatial learning, they should be able to find the platform in the new locations as readily as when it was in the original training site.

Figure 11 also shows that the wild-type mice took significantly less time to locate the platform when it was in its original location (training trials) compared to new locations (random trials). In contrast, the α CaMKII mice took just as long to locate the platform when it was in new locations as when it was in its training site. These results support the hypothesis that the mutant mice developed a "nonspatial" strategy to find the platform. Another observation which confirms the validity of our hypothesis is that on the random trials, wild-type mice crossed the training site significantly more often than the α CaMKII mutant mice (Fig. 12).

In agreement with the data shown in Figure 9B, mutant mice took approximately twice as long as the wild-type mice to locate the platform when it was in its training site. This is most probably a reflection of the strategy employed by the mutant mice, which does not allow as precise a localization of the platform as a spatial strategy does.

Phase 5: "+" (Plus) maze task. The results described thus far suggest that the mutant mice are impaired in learning the spatial relationships between distal cues and the escape platform (i.e., true impaired spatial learning). However, an alternative explanation of the results is that they are impaired in another process(es), such as in the ability to see and attend to distal cues or to make an association between the distal environment and the escape platform. To exclude these latter possibilities, we tested the mice in a water-filled "+" maze (L. Baskall et al., in prep.).

The "+" maze is a four-armed clear Plexiglas maze filled with opaque water. An escape platform is placed in one arm of the maze with its top 1 cm below the surface of the water and is kept there throughout all trials. The + maze was placed directly on top of the Morris pool to ensure that the same distal cues were

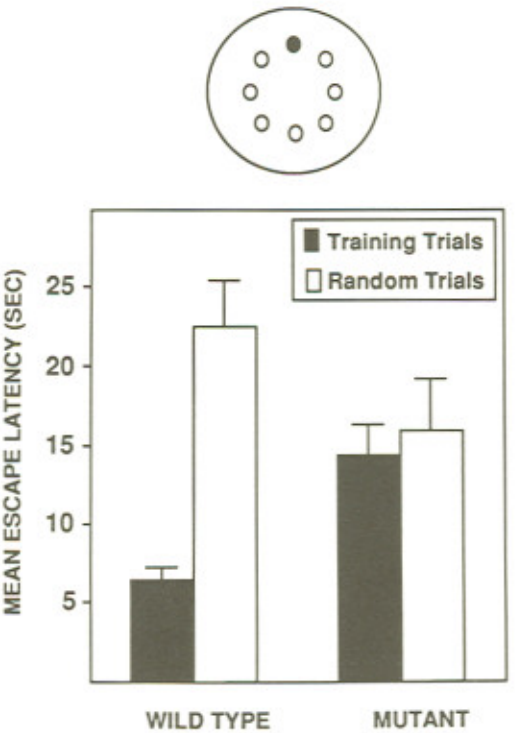

Figure 11. Latency analysis of the "random-platform" task. Two days after the animals were given 3 or 5 days of training and subsequent probe trials, they were given a block of four trials with the platform in its original training location. On the next block of four trials, the platform was in the original location for the first two trials but was in two new ("random") locations on the next two trials. On the final block of four trials, the platform was placed in the original spot on the first trial and then in different random locations on the next three trials. At the top of the panel, we show a schematic drawing of the locations of the platform in the training trials (●) and in the random trials (○). In each random trial, only one of the seven locations was used. Represented in the figure is the mean latency to find the platform on the seven trials when the platform was in its original location (black bars) compared to the five trials when it was in random locations (white bars). The wild-type controls clearly took less time to find the platform when it was in its original location compared to when it was in random locations (correlated T-test; $t[12] = -6.504$, $p < .001$). The α CaMKII mutant mice, however, were just as proficient at locating the platform in random locations as when it was in its original location (correlated T-test $t[10] = -.336$, $p > .05$). One α CaMKII mutant mouse appeared to have had a seizure, and it failed to swim normally on the day of random platform training. Thus, its data were not analyzed. Bars indicate the S.E.M.

utilized. On each trial, a mouse was placed in one of the three arms that did not contain the platform and allowed to swim toward the intersection. At this point, the mouse must choose one of the three remaining arms to enter. If the mouse chose the correct arm containing the platform, it was allowed to climb onto the platform and then was removed from the maze and scored as a correct choice. When the animal swam into another arm of the maze that did not contain the platform, it was trapped in that arm and the trial was scored as a mistake. The arm used to start the animal on each trial was chosen pseudorandomly with the restriction that

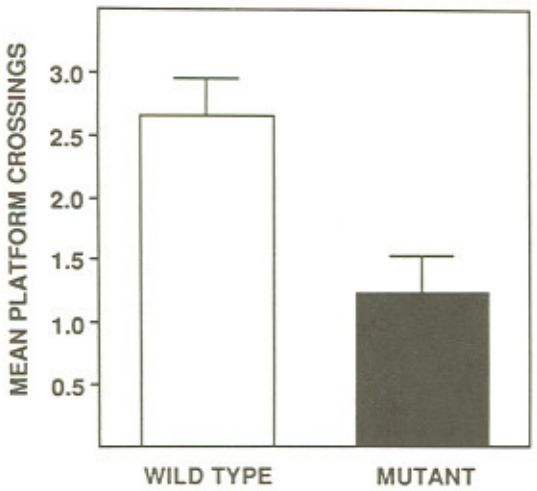

Figure 12. Platform-crossing analysis in the random-platform task. Represented in this figure are the mean number of times mice crossed the original training site on the five trials with the platform in random locations. Wild-type controls crossed the training site significantly more often than α CaMKII mutant mice on these five trials ($t[21] = 3.318$, $p < .01$). Bars indicate the S.E.M.

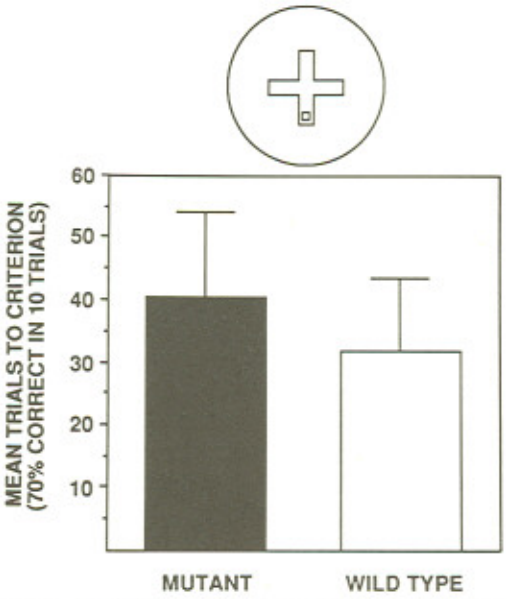

Figure 13. Analysis of performance on the "+" maze. The figure shows the mean number of trials to reach the learning criterion of 70% correct in 10 trials in a water-filled "+" maze. On each trial, an animal was placed facing the center of the maze in one of the three arms that did not contain the platform. An animal was allowed to swim into any of the arms, but if it chose an incorrect arm, it was trapped for 20 sec. If it chose the correct arm, it was allowed to remain on the platform for 10 sec. Each animal was given 15 trials on day 1, and 25 trials on each subsequent day. Each animal was given one trial at a time with an intertrial time of 1–3 min. These animals had been given extended training in the Morris task. The data indicate that both wild-type and α CaMKII mutant mice were able to learn the location of a hidden escape platform in the + maze. The difference between the two groups' performance was not significant ($t[8] = .469$, $p > .05$). A schematic representation of the + maze placed on top of the Morris pool and the location of the hidden platform are shown at the top of the panel. Bars indicate the S.E.M.

each arm which did not contain the platform was used in a block of three trials. Since on each trial there are three possible arms into which an animal can swim, random choices should result in the correct choice 33% of the time. Likewise, other predictable strategies such as always swimming left, right, or straight would also produce correct choices about 33% of the time, because the start position is rotated such that in each ten trials, each of the three start arms are used at least three times. The criterion used to assess whether mice had learned to locate the platform using a distal cue was 70% correct choices in ten trials.

The + maze task has several features in common with the hidden-platform version of the Morris task. Both tasks require subjects to navigate through water and locate a hidden platform by using the extramaze environment. Thus, in both tasks, animals must be able to see and attend to distal cues. The difference between the tasks is that the hidden-platform version of the Morris task requires an animal to learn multiple spatial relationships between distal cues and the escape platform. In the + maze, however, an animal always takes the same path to the platform, thus, it always has the same distal environment to swim toward for escape. Therefore, the maze can be solved by learning the single relationship between a particular distal environment and the escape platform.

Figure 13 shows that both the wild-type mice and the α CaMKII mutant mice learned to solve the task and that there was no significant difference in the number of trials to reach criterion between the two genotypes ($t[8] = 0.469$, $p < 0.05$). These results demonstrate that the impairment of the α CaMKII mutant mice in the Morris hidden-platform task was not due to an inability to see distal cues, attend to the extramaze environment, or learn a simple association between escape and distal environment.

DISCUSSION

In this study, we have constructed mutant mice that are deficient in α CaMKII by the gene targeting technique. The mutant mice exhibit no obvious neuroanatomical defects and retain intact postsynaptic mechanisms, including NMDAR function, but are strikingly deficient in their ability to produce LTP in the CA1 region of the hippocampus. Thus, these mice provided a highly suitable animal model for studying the relationship between LTP and learning processes. Indeed, we have shown that the mutant mice have remarkably specific learning impairments, which leads to the conclusion that α CaMKII has a prominent role in spatial learning, but that it is not essential for some types of nonspatial learning. Our results considerably strengthen the contention that the synaptic changes exhibited in LTP are the basis for spatial memory.

Our observations also extend earlier conclusions based on pharmacological experiments that CaMKII holoenzyme is involved in LTP (Malinow et al. 1989); we have now shown that the α form of this enzyme is involved in LTP. Because LTP was greatly diminished

but apparently normal when present in the mutant mice, we favor the notion that α CaMKII is involved in a regulatory pathway for the processes responsible for LTP. In this view, the magnitude of potentiation would be altered in the mutants, but any LTP that appeared would have usual characteristics. We cannot rule out, of course, that α CaMKII is directly in the pathway for the production of LTP and that some other kinase (β CaMKII, for example) is substituting in the instances where mutant mice exhibited LTP.

A deficit in LTP could be caused by a decreased number of connections between CA3 and CA1 neurons in the mutant mice. Although we have no anatomical evidence relating to number of connections, our electrophysiological results make this possibility highly unlikely: (1) Comparable stimulus intensities in normal and mutant mice produced comparable-sized responses; (2) the total depolarization for normal and mutant preparations was similar because the response sizes were the same for both; and (3) for the whole-cell experiments, we did not rely on synaptic depolarization for unblocking NMDA receptors at activated synapses; rather the cells, which were voltage-clamped, were held at membrane potentials of −30 or −40 mV during the tetanus, voltages that produce LTP even for exceedingly small inputs.

The precise role of α CaMKII in LTP induction remains unknown. We hope that our α CaMKII mutant mice will be useful in the identification of this role. To date we have tested whether α CaMKII modulates the activities of protein kinase C, a kinase that has also been implicated in LTP induction (Malinow et al. 1989). We found that normal levels of activity of this kinase are present in homogenates from forebrain of mutant mice, suggesting that the targets of α CaMKII are elsewhere. Synapsin I was shown to be one target for this kinase (McGuiness et al. 1985), and our observation of diminished paired pulse facilitation raises the possibility that the effect might be a presynaptic one. We also tested the hypothesis that α CaMKII has the unique capacity to target the CaMKII holoenzyme to the membrane (Erondu and Kennedy 1985), where it can respond to localized Ca^{++} rises (Connor and Muller 1991; Guthrie et al. 1991). We found that the proportion of the CaMKII holoenzyme activity associated with the membrane is unaltered in the forebrain of the mutant mice, which does not support the hypothesis (A.J. Silva, unpubl.).

The most remarkable feature of the α CaMKII mutant mice is the apparent lack of widespread abnormalities. However, this does not mean that abnormalities are restricted to those described here. At the microscopic level, a high-resolution analysis may very well reveal fine anatomical differences between the mutant mice and the wild-type control. At the electrophysiological level, we have thus far analyzed only the hippocampal CA1 field cells. In light of the known distribution of α CaMKII, cells in the other fields of the hippocampus, as well as in amygdala and neocortex, should be examined for possible deficit. At the behavioral level, we suspect that the modulation of the acoustic startle response (Davis 1992) is impaired in these mice, since they seem to have an abnormally enhanced acoustic startle response. Hence, it is interesting that the amygdala is another prominent site of α CaMKII expression (Erondu and Kennedy 1985; Burgin et al. 1990) and that LTP in this part of the brain might be involved in the modulation of the acoustic startle response (Davis 1992). On the other hand, our behavioral work did show that α CaMKII is not essential for all learning. For instance, although it has previously been shown that α CaMKII is expressed in the basal ganglia (Erondu and Kennedy 1985), and that this structure is essential for learning the visible-platform version of the Morris task (Wishaw and Kolb 1984), our study indicated that the α CaMKII mutant mice can learn this task.

Finally, we have demonstrated that α CaMKII mutant mice are a very useful model for studying the relationship between a particular gene product (α CaMKII), LTP, and behavior. We view our results as a promising start in the application of the gene targeting technique for the study of mammalian learning and memory. We expect that other similarly constructed mice with mutations in judiciously chosen genes will be useful tools for studying mammalian behavior. In this regard, perhaps even more useful would be the mice with subtle rather than null mutations or mice with mutations directed to specific regions of the brain. Construction of these mutant mice may be feasible (Hasty et al. 1991; O'Gorman et al. 1991).

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