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CaMKII at a Central Synapse

α-Calcium/calmodulin protein kinase II and synaptic plasticity at CA3 Schaffer collateral – CA1 synapses in the mammalian hippocampus

by

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Submitted to the Department of Brain and Cognitive Sciences in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

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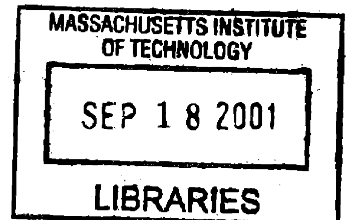

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Abstract

Long term potentiation (LTP) of synaptic transmission at the CA3-CA1 hippocampal synapse is a model synaptic plasticity mechanism that may underlie hippocampal dependent learning and memory. Inhibition of post-synaptic calcium/calmodulin protein kinase II (CaMKII) has been shown to block LTP, and a global knockout of the highly expressed α isoform of CaMKII caused an impairment in LTP and hippocampus dependent learning. We examined the role of CaMKII in CA3-CA1 LTP by selectively deleting α-CaMKII in adult hippocampal CA1 or CA3 pyramidal cells using conditional gene targeting. With this approach, we could investigate the locus of change that underlies LTP expression, as both pre- (CA3) and post- (CA1) synaptic CaMKII dependent mechanisms have been implicated, and further examine how CaMKII dependent plasticity contributes to learning and memory in a background of normal brain development.

CA3-CA1 LTP is reduced in CA1 α-CaMKII knockout mice, suggesting that post-synaptic CaMKII is required for normal LTP. These mice are strikingly reminiscent of the α-CaMKII global knockout mice, demonstrating comparable LTP impairments and abnormal behaviors. In contrast, CA3 α-CaMKII knockout mice have normal LTP at CA3-CA1 synapses, suggesting that CaMKII phosphorylation of pre-synaptic synapsin I is not required for LTP expression. Contextual and cued fear conditioning were also normal in CA3 mutants, demonstrating that one form of hippocampus dependent learning is intact. While several pre-synaptic short term plasticity mechanisms were unaffected in CA3 α-CaMKII knockout mice, repetitive stimulation protocols using short trains of stimuli of increasing frequency revealed enhanced frequency facilitation in mutants compared with controls. This suggests that CaMKII may be acting pre-synaptically as a negative regulator of neurotransmitter release during certain repetitive stimulation conditions, and as a "frequency detector" of calcium spikes, reaching higher levels of activation with increasing frequency of stimulation. Modulation of facilitation could be important to prevent synaptic terminals from depleting their vesicle stores during episodes of repetitive firing, or to maintain synaptic activity in an optimal range for information coding.

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List of Abbreviations

Abbreviation	Definition
ACSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (receptor)
APV	2-amino-5-phosphonovaleric acid (NMDA receptor antagonist)
C/A	Commissural/associational
CaMKII	Calcium/calmodulin dependent protein kinase II
CaMKIV	Calcium/calmodulin-dependent protein kinase IV
CW-2	Forebrain specific Cre recombinase transgenic line
cDNA	Complementary deoxyribonucleic acid
DG	Dentate gyrus
Dox	Doxycycline
EGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol-bis(aminoethylether) N,N,N',N'-tetraacetic acid
EPSC	Excitatory post-synaptic current
EPSP	Excitatory post-synaptic potential
ES	Embryonic stem (cell)
FF	Frequency facilitation
FRT	Flp recognition target
G32-4	CA3 specific Cre recombinase transgenic line
I/O	Input/Output
ISI	Inter-stimulus interval
KII-IN	CaMKII inhibitor, endogenous
KA-1	Kainate-prefering glutamate receptor, subclass I
KO	Knock-out
LFNT	loxP-FRT-Neo-tk
LsL	loxP- *Stop* –loxP cassette
LTD	Long term depression
LTP	Long term potentiation
MAP-2	Microtubule associated protein – 2
mEPSC	Miniature excitatory post-synaptic current
mf	Mossy fibers
mRNA	Messenger ribonucleic acid
n	number of competent pre-synaptic release sites
neo	Neomycin resistance gene
NHS	Normal horse serum
NMDA	N-methyl-D-aspartic acid (receptor)
NR1	NMDA receptor subunit 1
NSF	*N*-ethylmaleimide-sensitive fusion protein
p	Probability of neurotransmitter release
pA	SV40 polyadenylation signal
pAB	Primary antibody
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pKII	α-CaMKII promoter region (8.5kb)
PKA	Protein kinase A
PKC	Protein kinase C
PPF	Paired-pulse facilitation
PTP	Post-tetanic potentiation
PTX	Picrotoxin
rtTA	Reverse tetracycline transactivator

SD	Synaptic depletion
SEM	Standard error of the mean
SNAPs	Soluble NSF attachment proteins
SNARE	Soluble *N*-ethylmaleimide-sensitive attachment factor receptor
synprint	Synaptic protein interaction (site)
tetO	Tet operator promoter element
tk	Thymidine kinase
tTA	Tetracycline transactivator
UTR	Untranslated region
VAMP	Vesicle associated membrane protein (synaptobrevin)

Chapter 1

Introduction

One of the great problems in neuroscience is to understand how a memory is stored in the brain. To form a memory, a representation or "trace" of an experience must be produced and maintained. First, this requires the brain to be "plastic", or able to change its activity in response to experience. Second, these changes in activity must be long lasting for a memory to be efficiently stored. Such changes could include modifications in brain function at the level of molecular interactions, whole-cell function, synaptic connections and/or neuronal network characteristics. Santiago Ramon y Cajal suggested that learning could lead to a reorganization of inter-neuronal connections [1,2], perhaps by changes in synaptic number and strength. This was described using eloquent analogy:

> *...we could say that the cerebral cortex is like a garden planted with innumerable trees – the pyramidal cells – which, thanks to intelligent cultivation, can multiply their branches and sink their roots deeper, producing fruits and flowers of ever greater variety and quality.* [1]

While Hebb [3] also theorized that information was stored by synaptic changes in a network of cells, he additionally proposed a model by which synaptic connections could be strengthened:

> *When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.* [3]

Hebb recognized that a synaptic learning mechanism would by characterized by persistence and specificity, and he further proposed an associative learning demand for coincident pre- and post-synaptic activity. Simply put, a "Hebbian synapse" between two neurons will be strengthened if the two cells involved are active at the same time. A

physiological correlate of this Hebbian model, long term potentiation (LTP), was later discovered in the hippocampal formation of the anesthetized rabbit [4-6]. Following tetanic stimulation of perforant path axonal pathways in the anesthetized rabbit *in vivo*, a long-term enhancement in synaptic strength was observed post-synaptically in dentate gyrus granule cells (Figure 1-1).

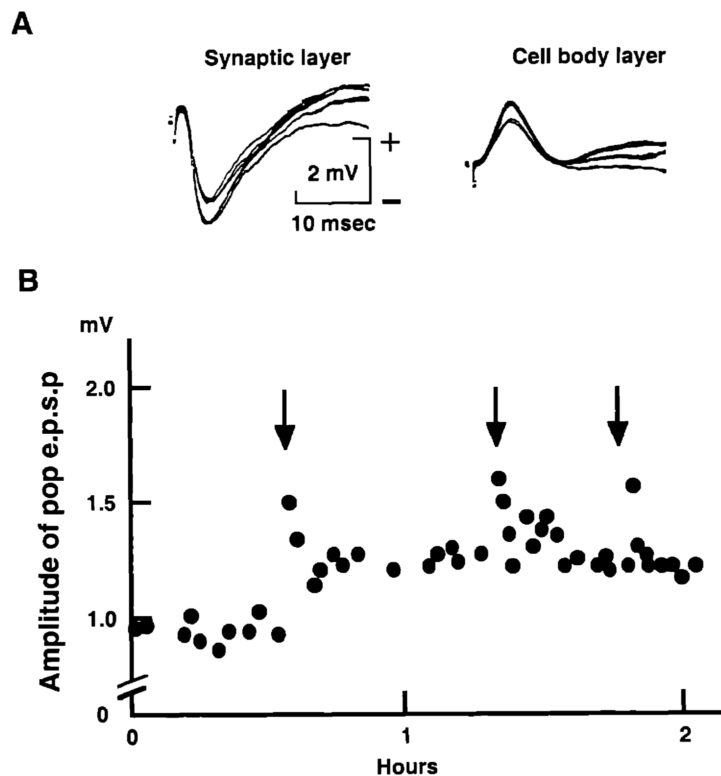


Figure 1-1: Long term potentiation (LTP) at perforant path – dentate granule cell synapses

Perforant path axons from the entorhinal cortex were stimulated and extracellular field recordings of excitatory post-synaptic responses (EPSPs) were recorded in the dentate gyrus of anesthetized rabbits *in vivo*. Stable baseline responses were recorded and LTP was induced by a 100Hz tetanic train of stimuli (4 seconds duration), indicated by the first arrow. Amplitudes of the EPSPs were calculated and plotted over time, normalized to the size of baseline responses. Subsequent tetani did not produce additional potentiation, indicating saturation. Synaptic potentials recorded in both the synaptic layer and in the cell body layer before tetanus and 45min after the first tetanus are superimposed in (A). The time course of potentiation is plotted in (B). [5]

This enhancement lasted for several hours, though later studies showed that LTP could be recorded for up to weeks to months *in vivo* [7].

The rapid induction and long lasting properties of LTP made it an attractive candidate for an information storage and memory formation mechanism. In addition, LTP was shown to have several properties that are reminiscent of Hebbian demands and computational models of synaptic learning [8]. LTP is associative, meaning that temporal coincidence of a weak input and a strong input onto the same post-synaptic cell can result in potentiation of the weak input if the strong input is potentiated [9,10]. This is loosely reminiscent of a type of learning called classical conditioning, which associates a benign conditioned stimulus (CS) with a strong or aversive unconditioned stimulus (US) by presenting the stimuli in close temporal proximity [11,12]. LTP is also cooperative, meaning that multiple inputs can sum to produce a greater stimulus intensity and greater potentiation than each input alone [9]. And finally, LTP has a degree of input-specificity. Only those inputs that are active around the time that LTP is induced will be potentiated [13,14]. However, some evidence has shown that potentiation may be able to spread locally to neighboring synapses [15,16], suggesting that small groups of synapses may be a computational learning unit.

The discovery of LTP in the hippocampal formation was particularly exciting, as this brain region had already been implicated in memory in humans [17-19]. When the hippocampus was removed bilaterally from the patient H.M., who suffered from intractable epilepsy focused in this region, he was left with a profound impairment in the formation of new memories [17]. The hippocampus is a temporal lobe cortical structure that is comprised of the dentate gyrus, the hippocampus proper (regions CA3, CA2 and CA1) and the subiculum. While there are multiple inputs and outputs to these hippocampal subregions, there is a well-characterized tri-synaptic circuit that is thought to be the main information processing pathway of the hippocampus: (1) perforant path axons from the entorhinal cortex synapse on dentate gyrus granule cells, (2) mossy

fiber axons from dentate gyrus granule cells synapse on CA3 pyramidal cells, and (3) Schaffer collateral axons from CA3 pyramidal cells synapse on CA1 pyramidal cells (Figure 1-2).

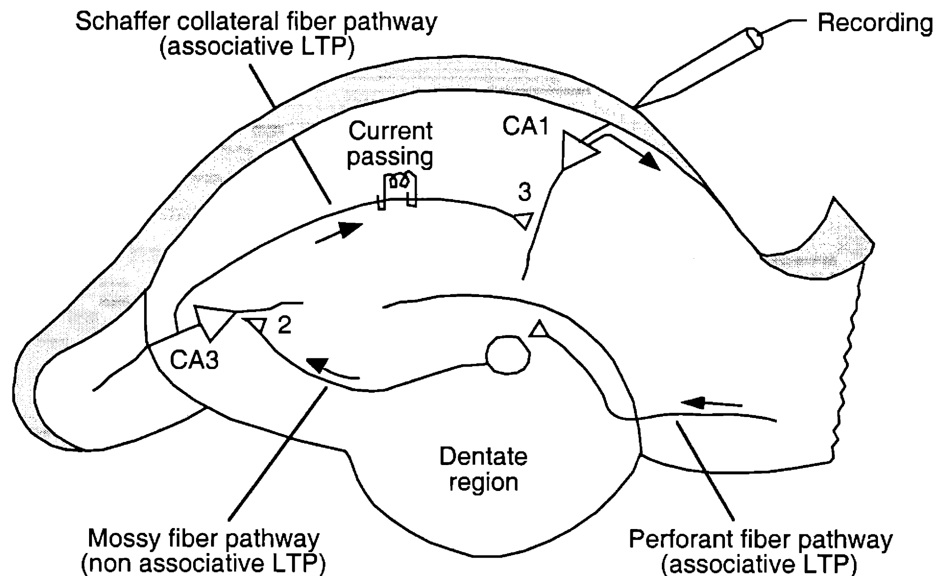


Figure 1-2: Schematic diagram of the trisynaptic circuit in a hippocampal slice

The three major excitatory synaptic pathways in the hippocampus are shown. Arrows indicate the direction of impulse flow through the circuit. While LTP was first described at the perforant path – dentate gyrus granule cell pathway (1), subsequent analysis of LTP mechanisms has focused on the CA3 Schaffer collateral – CA1 pyramidal cell pathway (3). [20]

LTP of synaptic transmission has been observed at all three of these synapses, though these LTP mechanisms are not identical [21]. The hippocampus is particularly amenable to *in vitro* study, as it is possible to make slices of the hippocampus (*e.g.* 250-400μm thick) that retain the trisynaptic circuit, and these slices can be kept alive for several hours in an artificial cerebrospinal fluid solution. The ease and accessibility of this preparation is preferable for the study of hippocampal synapses using whole-cell physiology, imaging, and pharmacological manipulations. However, the hippocampal slice does have disadvantages, including the loss of extra-hippocampal modulatory

projections and variability in slice preparation and experimental techniques among laboratories. Regardless, the hippocampal slice preparation has been invaluable for the study of the potential molecular mechanisms that may underlie LTP, and the greatest effort has focused on characterizing the Schaffer collateral CA3 projections onto CA1 pyramidal neurons in the rat and mouse brain. This CA3-CA1 LTP pathway demonstrates the classic associative properties as predicted by Hebb, and will be the focus of this thesis. Since CA1 is the main output pathway of the hippocampus proper, and has been shown to be critical for memory formation following ischemic lesion in humans [18,22] and genetic lesion in the mouse [23], the study of LTP at the CA3-CA1 synapse could have significant implications for the molecular and cellular basis of learning and memory.

LTP at CA3-CA1 synapses can be induced using several stimulation paradigms, including the traditional high frequency tetanus train (*e.g.* 100Hz) and "theta burst" stimulation (*e.g.* 4 stimuli at 100Hz, repeated several times at 200msec intervals), which is reminiscent of normal physiological hippocampal firing patterns [8,24]. Glutamate is released at these excitatory synapses following such stimuli, where it binds to post-synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and N-methyl-D-aspartic acid (NMDA) receptors co-localized on post-synaptic spines [25-27]. While AMPA receptor activation leads to post-synaptic depolarization by sodium influx through the AMPA channel, the NMDA receptor does not initially contribute to synaptic transmission because it is blocked by magnesium ions at resting membrane potentials [28,29]. The NMDA receptor requires coincident binding of glutamate and post-synaptic membrane depolarization for activation. Once the post-synaptic membrane is sufficiently depolarized, the NMDA channel undergoes a conformational change that removes the magnesium block, allowing calcium and sodium to enter the cell [30]. The ability of the NMDA receptor to detect changes in both pre-synaptic activity (glutamate release) and post-synaptic activity (AMPA receptor driven membrane depolarization)

has led to suggestions that the NMDA receptor is a Hebbian "coincident detector" of associative synaptic changes. Correspondingly, associative LTP at CA3-CA1 synapses is blocked by the NMDA receptor antagonist, APV [31]. High frequency tetanic stimuli used to induce LTP have been shown to produce sufficient post-synaptic depolarization to open NMDA receptor channels. The pairing of low frequency pre-synaptic stimulation with artificial post-synaptic depolarization using whole cell voltage clamp techniques has also been shown to induce LTP, elegantly demonstrating the associative requirements for NMDA channel opening and LTP induction [32-35]

While we know that calcium influx through the NMDA receptor is required for LTP induction, it is unclear what downstream mechanisms lead to LTP expression, or the enhancement of synaptic transmission. Calcium entering through the NMDA receptor can bind to calmodulin and activate calcium/calmodulin dependent protein kinase II (CaMKII), one of the most abundant signaling molecules in the mammalian brain [36]. CaMKII is highly expressed in hippocampal pyramidal cells [37] and has been proposed as a potential "memory molecule" of synaptic activity because of its unusual biochemical properties. CaMKII is a holoenzyme of 12 subunits [38] that rapidly auto-phosphorylates once activated by calcium/calmodulin binding at Thr286 via an intra-holoenzyme, inter-subunit mechanism [39-41] (Figure 1-3). Once autophosphorylated, CaMKII affinity for calmodulin increases dramatically ("calmodulin trapping"), leading to sustained CaMKII activity [42,43]. As calcium levels drop in the cell and calmodulin diffuses off the enzyme, CaMKII activity persists in a calcium-independent autonomous state as long as phosphorylation of Thr286 is maintained [42,44-47]. The ability of CaMKII to become persistently active after a calcium signal is gone led to proposals that CaMKII might serve as a "memory molecule" of activity at synapses and may underlie long term changes in synaptic strength [48-50]. Interestingly, general kinase inhibitors, calmodulin inhibitors and CaMKII inhibitors have all been shown to block CA3-CA1 LTP induction *in*

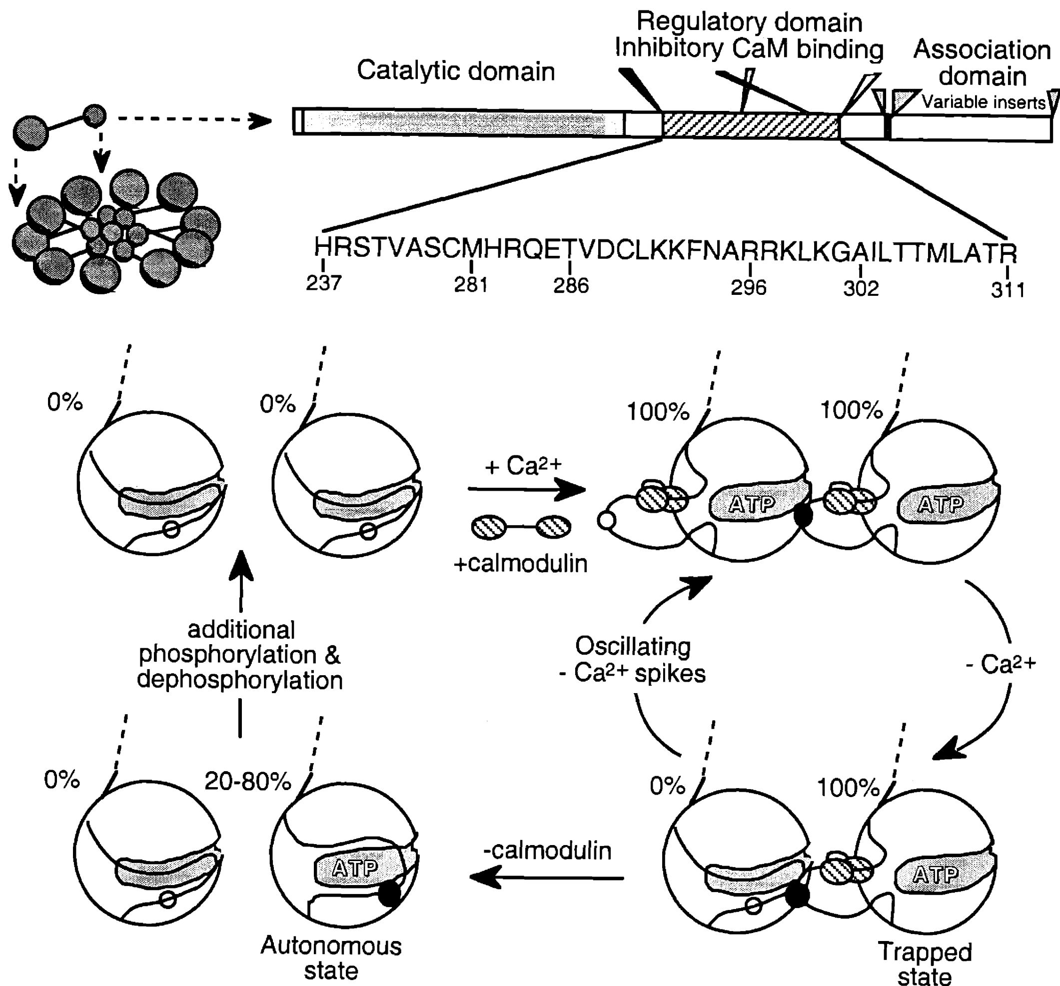


Figure 1-3: Autophosphorylation of subunits within the CaMKII holoenzyme
CaMKII subunits associate in a "hub and spoke" pattern within the CaMKII holoenzyme. Each subunit consists of an N-terminal catalytic domain, a central regulatory domain that inhibits catalytic function until it is disrupted by calmodulin binding, and a C-terminal association domain that facilitates holoenzyme assembly. Calmodulin bound subunits readily autophosphorylate their neighboring subunits at Thr286. Autophosphorylation leads to calmodulin trapping, allowing 100% kinase activity for extended durations. Following calmodulin dissociation, autophosphorylation maintains the kinase in a calcium/calmodulin independent "autonomous" state, at activity levels 20-80% of calmodulin bound levels, until the Thr286 is dephosphorylated by a phosphatase [36].

vitro [51-54]. However, it is not yet clear which CaMKII substrate(s) are involved in LTP expression. CaMKII is highly concentrated in the post-synaptic density and in pre-

synaptic terminals [55-59], and putative CaMKII substrates are present at both sides of the CA3-CA1 synapse whose modification could reasonably enhance synaptic transmission [60].

During the search for the molecular mechanisms that underlie LTP expression, a dizzying number of signaling molecules, receptors, neurotransmitters, structural molecules and miscellaneous proteins have been implicated [61]. This suggests a much more complicated LTP mechanism than was anticipated. Whether these molecules are acting directly in the LTP pathway, as indirect players by modifying other aspects of cell physiology that could affect LTP, or as modulators is often unclear. There has been much debate within the field as to whether CA3-CA1 LTP is expressed as a presynaptic change, such as an increase in the probability of neurotransmitter release, or a post-synaptic change, such as in increase in the responsiveness of the post-synaptic cell, or both [62,63]. A presynaptic change would require a retrograde messenger to be produced post-synaptically following NMDA receptor activation. While recent evidence in support of the modification [64,65] and/or insertion of AMPA receptors into the post-synaptic membrane [66] has turned the tide towards a solely post-synaptic change, previous quantal analysis experiments [67-69] and the discovery of putative retrograde messengers [70,71] have suggested a pre-synaptic contribution. However, many of these studies have shown contradictory results, and are open to multiple interpretations [62,63,71]. For example, the decrease in failure rate and increase in quantal content observed following LTP induction, previously thought to be indicators of pre-synaptic changes, could be explained by the functional recruitment of additional AMPA receptors at previously “silent synapses” (i.e. post-synaptic changes) [72,73]. However, recent evidence further suggests that silent synapses may actually be “whispering synapses” that undergo plastic changes by altering the dynamics of their pre-synaptic fusion pores [74,75]. Clarifying whether pre- or post-synaptic contributions are essential for plastic changes is important to determine which molecules among the numerous ones implicated are likely

to be direct or indirect players in the LTP mechanism. For example, if a post-synaptic mechanism underlies LTP expression, it would suggest that implicated pre-synaptic signaling mechanisms involved in neurotransmitter release play an indirect or modulatory role in LTP. Establishing the locus (or loci) of change that underlies LTP would also clarify where to focus further studies and could give insight into molecules that might be critical for learning and memory in both in mice and humans.

LTP has been identified at many excitatory synapses in the brain and spinal cord, suggesting that synaptic plasticity is a fundamental characteristic of neuronal function [8]. While associative NMDA receptor dependent LTP, as seen at CA3-CA1 synapses, is the most common and widely studied plasticity mechanism, multiple other activity dependent mechanisms have been observed at central synapses that increase or decrease the strength of synaptic transmission [8,76]. This adds a level of complexity to the study of the molecular basis of LTP, as different synapses may utilize different mechanisms, and different induction protocols may mobilize additional signaling pathways. For example, robust stimulation protocols at CA3-CA1 synapses reveal a potentiation that can be parsed into an early phase (E-LTP), which is dependent upon kinase activity and protein phosphorylation, and an additional late phase (L-LTP), which requires transcription and new protein synthesis [77]. It's possible that such de novo protein synthesis provides signaling and structural factors necessary for longer lasting plastic changes. Interestingly, long-term memory has been shown to require the synthesis of new proteins [78]. It seems likely that multiple forms of plasticity work in combination in multiple regions of the brain for efficient information processing and memory formation.

While both early and late phase CA3-CA1 LTP are dependent upon NMDA receptor activation, dentate mossy fiber-CA3 LTP is not [79,80]. Instead, this less well-characterized mechanism appears to involve a pre-synaptic expression locus that is dependent upon PKA activation [81,82], though it is possible that additional potentiation processes involving

post-synaptic mechanisms may occur at this synapse, depending upon stimulation protocols [83-86]. Surprisingly, NMDA receptor independent LTP expressed pre-synaptically has not been observed widely in the brain. Instead, most NMDA independent pre-synaptic plasticity mechanisms involve very short term changes in the strength of synaptic transmission [76]. Repetitive stimulation in the presence of APV, an NMDA receptor antagonist that will block NMDA dependent long-term plasticity mechanisms, reveals an enhancement of post-synaptic responses that rises and decays in three phases: (1) Facilitation occurs with a time constant of less than a second, (2) Augmentation describes any enhancement with a duration of several seconds, and (3) Potentiation elicited by a tetanus (post-tetanic potentiation) of stimuli lasts for minutes. Temporal summation of residual pre-synaptic calcium during repetitive stimulation, leading to an enhancement of calcium dependent neurotransmitter release, is thought to play a role in all three of these mechanisms [76,87].

While enhancing synaptic transmission appears to be a ubiquitous mechanism for activity dependent changes, it is not surprising that the brain has corresponding long term depression mechanisms for decreasing the strength of synapses, perhaps to avoid saturation [88,89]. Information can be more efficiently stored in a network that is able to modulate the weights of inputs in a bi-directional manner, suggesting LTD may also play a role in memory formation. Long term depression (LTD) has been described at multiple synapses, although the mechanism has been most critically examined at CA3-CA1 synapses [90,91]. It is normally induced with a low frequency 1Hz stimulation (900 pulses), which causes a decrease in post-synaptic responses that is long lasting. LTD induction is also dependent upon NMDA receptor opening and calcium influx [92]. Early models suggested that temporal and quantitative differences in calcium influx would determine whether a synapse would be potentiated or depressed [93-95]. While high frequency stimulation, leading to high levels of NMDA receptor dependent calcium influx and kinase activation, causes LTP, low levels of calcium influx following low frequency

stimulation could selectively activate high affinity calcium dependent phosphatase pathways and cause LTD [93]. Subsequent studies using inhibitors of the phosphatases, protein phosphatase I (PP1) and calcineurin, suggest they do play a role in LTD [96]. It is still unclear what role LTD plays *in vivo*, although it is likely that many types of synaptic plasticity are required for efficient information storage of different qualities and durations, and for the selective pruning of synaptic connections during development [97].

Interest in determining the molecular and cellular mechanisms that underlie learning and memory has led to intensive study of synaptic plasticity mechanisms. Studies have focused on hippocampal LTP, often looking for direct or correlative evidence of whether or not LTP is a physiological substrate of hippocampal dependent memory formation. It is still unclear what molecular mechanisms underlie hippocampal LTP expression, and the connection between hippocampal LTP and learning is hotly debated [98-100]. For example, experiments that involve looking *in vivo* for LTP type changes during learning [101,102], or alternatively, examining the effects of tetanizing stimuli on memory formation [103], have been persuasive, but may be difficult to interpret. In vivo recording has its own technical challenges and limitations, such as accurate electrode placement and a limitation to field vs. whole cell recordings, which can mask changes that occur in a small subset of neurons. In addition, it is very difficult to control for confounding variables, such as internal temperature changes, stress, and other associated behaviors that can make data hard to interpret. Alternatively, pharmacological and genetic approaches can address the correlation (or lack of) between synaptic plasticity and memory formation by asking the question "If I disrupt a molecule essential for one process, how does it effect the other?" Doses of the NMDA receptor antagonist AP5, which is known to block the induction of CA1 LTP, have been shown to impair spatial learning in the Morris water maze [104,105] and to have no effect [106] if the animals are pre-trained in a similar pool. While these results, which vary with the precise behavioral assay, complicate the interpretation of AP5 experiments, there is also some concern

that the antagonist may have confounding effects on other critical neurological processes, such as sensorimotor behavior [107,108]. In general, pharmacological approaches are also limited in their ability to optimize and restrict drug delivery to a specific brain region or cell type of interest (for example, the hippocampus vs. the cerebral cortex), and could result in wide-spread cognitive changes that could interfere with learning.

An alternative approach is to use genetic methods to examine the correlation between LTP and learning and memory [109]. This traditionally involves producing genetically modified "mutant" mice through global gene knockout or transgenic over-expression of a specific gene of interest. The hippocampal physiology of these mutants can be studied *in vitro* or *in vivo* to clarify what roles the targeted molecule plays in plasticity mechanisms, such as LTP, and behavioral tests can be performed to examine if plasticity deficits correlate with learning performance. This approach is far superior to pharmacological intervention because the molecular targeting is highly specific, and in the case of gene knockout, inhibition is complete. The highly expressed α isoform of CaMKII [110,111] and the tyrosine kinase *fyn* [112] were the first molecules studied via this approach, and analysis of these mutants supported a link between hippocampal plasticity mechanisms and memory formation. Both mice demonstrated deficiencies in hippocampal CA3-CA1 LTP and in hippocampal dependent memory tasks, such as the spatial memory Morris water maze task [105]. Subsequently, a series of interesting mutant mice have been constructed that have generally shown that when CA3-CA1 LTP is specifically deficient, there is an impairment in spatial learning [113,114]. On the contrary, GlurA (GluR1) knockout mice showed deficient LTP, but normal spatial learning [115]. This was particularly interesting because GluR1 phosphorylation [64] and/or insertion of new GluR1 receptors into the post-synaptic membrane [116] may be involved in LTP expression. However, there is some debate as to whether the pre-training methods used in the Morris water maze task may have rendered their behavior NMDA receptor

independent [114]. This suggests that the relationship between NMDA receptor dependent hippocampal CA1 plasticity and learning and memory may still be open for further study. It should be noted that a correlation between LTP and behavior does not imply that LTP is the only synaptic mechanism that contributes to activity dependent changes that underlie learning, or that it is sufficient.

While a genetic approach is powerful for studying the effects of specific molecular changes on behavior and physiology, there are limitations. Global gene knockout removes the gene of interest in all cells at all stages of development. This could lead to abnormal brain development, even lethality, and widespread changes in brain function that could confound the study of plasticity and learning. For example, global α-CaMKII knockout led to deficits in CA3-CA1 hippocampal LTP and LTD, but there were also changes in short term plasticity mechanisms [111,117,118], cell excitability [119], hippocampal anatomy [119], and several general behavioral characteristics, including motor activity levels and anxiety/fear responses [110,120]. While it is possible that these varied phenotypes were due to the immediate absence of normal CaMKII function, it is also possible that they reflect developmental abnormalities and/or "compensatory" mechanisms subsequent to gene knockout. Regardless of their cause, these altered physiological mechanisms and behavioral responses could confound the ability to elicit normal long term synaptic plasticity responses and to study specific learning abilities in these mutant mice. The global knockout of α-CaMKII also did not help to define the exact anatomical regions where normal CaMKII function is required for LTP expression (*e.g.* pre- vs. post-synaptic cell) or which specific brain regions require normal CaMKII function and CaMKII dependent plasticity for learning and memory (*e.g.* specific hippocampal regions vs. cortex).

A new generation of conditional gene knockout has emerged that is able to address some of the limitations of the traditional genetic approach. By adding an element of temporal and spatial control to the onset of genetic deletion, it would be possible to

avoid deficits secondary to gene knockout during early development and to localize the gene deletion to specific cell types or brain regions. This can be done using the Cre/loxP system, a phage P1 derived site specific recombination system that utilizes Cre recombinase to facilitate recombination between two 34bp loxP recognition sequences [121,122]. loxP sequences can be inserted into the mouse genome flanking essential exon(s) of a gene of interest using homologous recombination in embryonic stem (ES) cells. Once a mouse is generated from these recombinant "floxed" ES cells and mated to homozygosity, it is crossed to a second Cre transgenic mouse that expresses Cre in the desired temporal/cell-specific pattern. Ideally, expression of the floxed gene will not be affected by the insertion of the loxP sequences. However, once Cre is expressed, following the spatial and temporal pattern of expression dictated by the transgenic promoter and regulatory sequences proximal to the transgenic insertion site, the DNA located between the loxP sites will be excised. This will result in the conditional knockout of the targeted floxed gene only in cells where Cre is expressed (Figure 1-4).

This conditional gene knockout technology was first used in the brain to selectively delete the NMDAR1 gene (NR1) in post-natal forebrain [123]. A conditional approach was required to study the role of this NMDA subunit in hippocampal plasticity and learning because a global deletion was lethal [124]. The NR1 gene was floxed and crossed to a transgenic mouse expressing Cre recombinase driven by the α–CaMKII promoter [123]. While the α–CaMKII promoter normally drives expression widely in the excitatory neurons of the forebrain [37,125], NR1 was found to be preferentially deleted in post-natal CA1 pyramidal cells when crossed to one Cre transgenic line (T-29). This conditional deletion resulted in a complete loss of CA3-CA1 LTP [23], abnormal firing of CA1 pyramidal neurons in response to the location of the mutant mouse in space ("place field") *in vivo* [126], and impairments in both hippocampus dependent spatial [23] and non-spatial memory tasks [127,128]. This result was particularly compelling, considering the

specificity of the targeted deletion and the known importance of hippocampal CA1 for memory in humans [18,22]. However, it is possible that the NMDAR is involved in other synaptic functions in addition to plasticity, and that the disruption of such mechanisms could have interfered with learning.

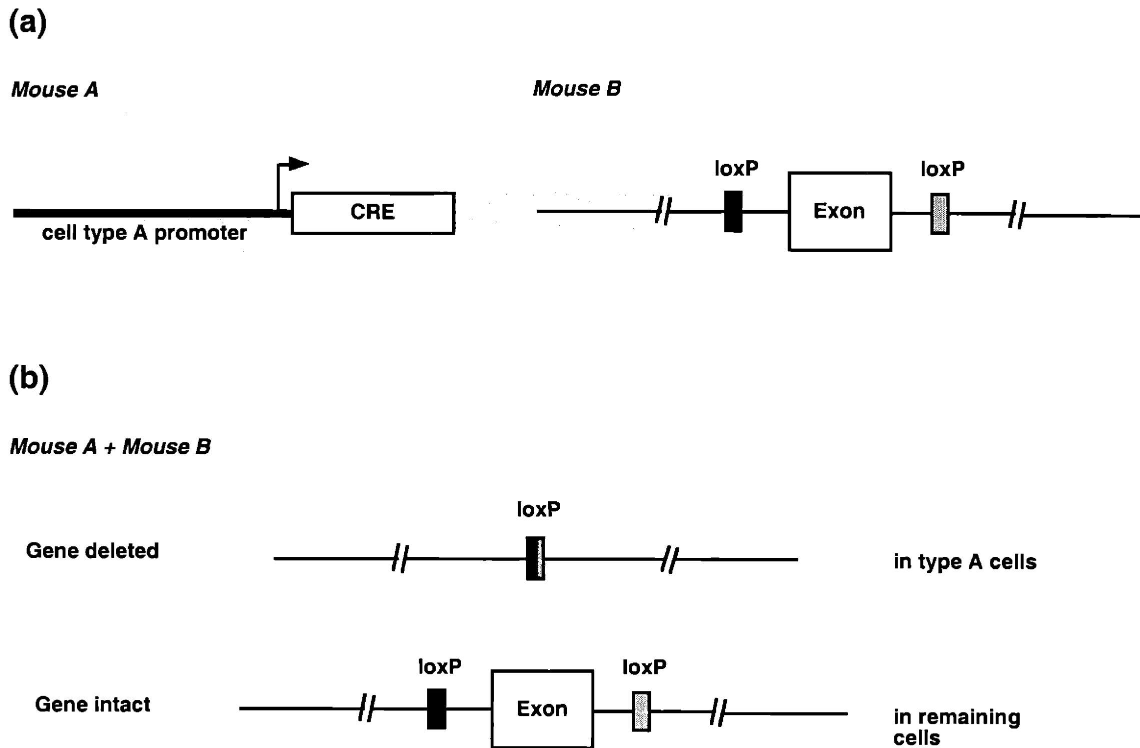


Figure 1-4: Conditional gene deletion using the Cre/loxP system

(a) Two different mouse lines are required for conditional deletion. The first line is a transgenic mouse that expresses Cre in the desired regional/temporal specific pattern. The second line is a recombinant mouse in which loxP sequences have been inserted around critical exons of a gene of interest.
(b) When the two lines are crossed, Cre recombination will drive gene deletion in cells where Cre is expressed.

While the specificity of conditional gene knockout is a major advance in studying neuronal function, the addition of an inducible temporal component is also desirable. This would allow development to continue normally until the exact point of gene knockout is desired. Recently, inducible [129] and "reversible" [130] gene knockout have

been reported in the brain. Most inducible systems take advantage of an inducible promoter to drive the gene of interest (*e.g.* Cre recombinase) or a fusion protein is engineered that will itself respond to an external ligand. The tetracycline transactivator system [131,132] can regulate the expression of a transgene through the administration of the tetracycline analogue, doxycycline (Dox). The simplest variant of this system requires the production of two transgenic mice that are mated to produce double transgenic mice: (1) one expressing the tetracycline transactivator (tTA) from a promoter of choice, and (2) one expressing the gene of interest from the promoter tetO, which consists of multiple repeats of the tet operator linked to a minimal eukaryotic promoter element. tTA is a eukaryotic transcription activator (mutant Tet repressor from *E. coli* fused with VP16 activation domain) that drives expression of genes downstream of a tetO promoter element. In the presence of Dox, this expression is blocked because Dox prevents tTA from binding to the tet operator (Figure 1-5). For an inducible knockout, one option is to cross a third recombinant mouse that has a recombinant floxed gene of interest to the double transgenic. As this is somewhat unwieldy, most inducible studies of gene function in mice involve the regulation of dominant negative transgenes. A constitutively active form of α-CaMKII, made by mutating the normally autophosphorylated threonine to an aspartate (α-CaMKII-Asp286), was overexpressed in this fashion, revealing a shift in the frequency/response curve for the induction of LTP vs. LTD and a deficit in spatial memory [133,134]. This deficit was recoverable when the transgene was suppressed, indicating that the deficits were caused by the acute effects of the transgene and not by permanent developmental effects [135]. Interestingly, the flexibility of temporal control demonstrated that the α-CaMKII-Asp286 transgene not only interfered with memory formation, but that if it was expressed only after a memory was formed, it could block recall of the memory [135].

Conditional gene knockout and transgene expression in the mouse has provided some of the strongest evidence for a role for specific molecules in plasticity and

learning. The two most intensively studied molecules have been the NMDA receptor and CaMKII, which are known to be essential for LTP induction at the CA3-CA1 synapse, the model central synapse for studying plasticity mechanisms and

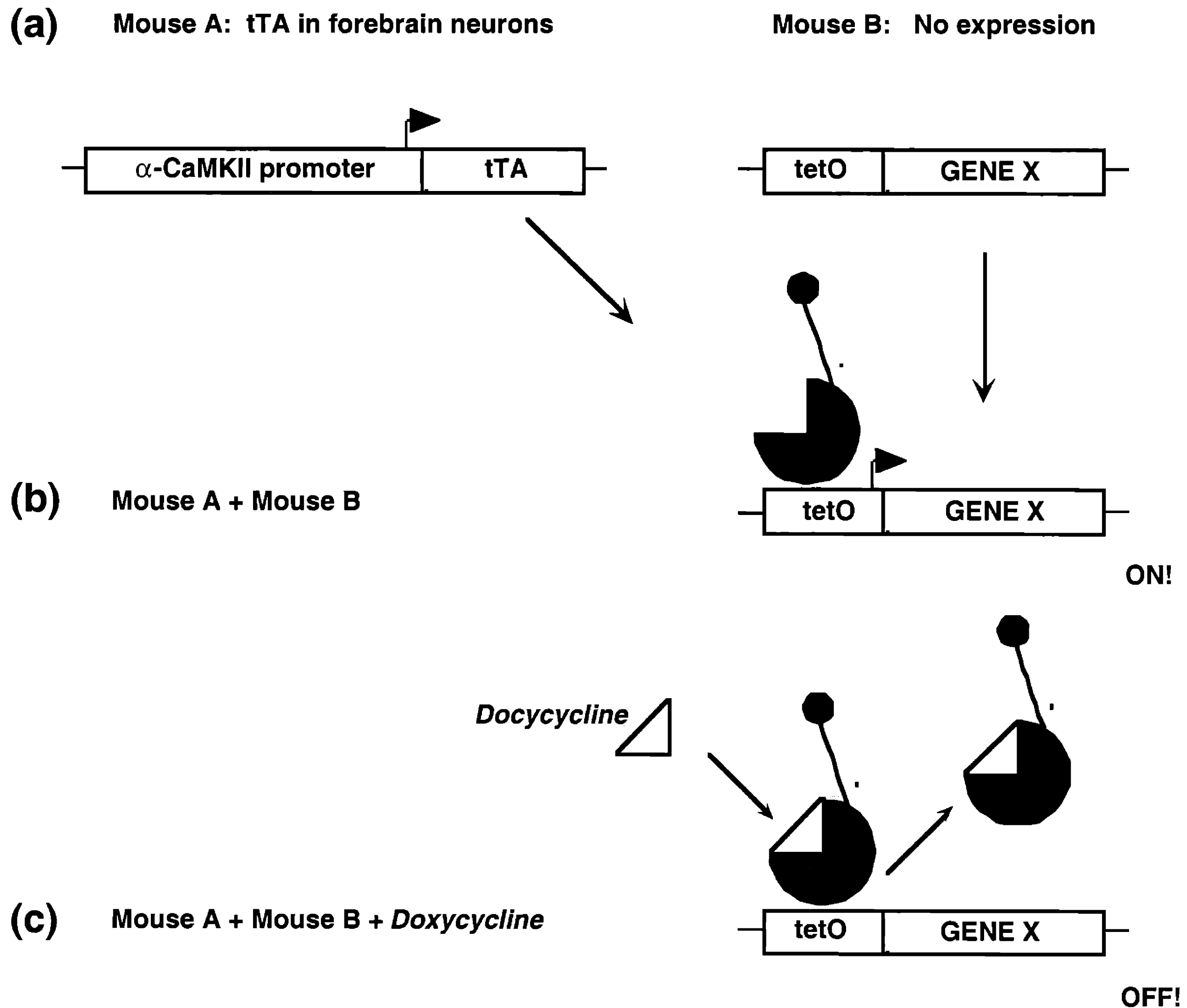


Figure 1-5: Temporal control of transgene expression using the tTA system

(a) Two different lines of mice are required for the inducible expression of a transgene using the tetracycline transactivator system. In the first line of transgenic mice, the tetracycline-responsive transcription factor tTA is driven by a promoter of choice (*e.g.* α-CaMKII promoter). In the second line, the transgene to be regulated (GENE X) is downstream of the tetO promoter.
(b) When these two mice are crossed, GENE X will be expressed only in those cells that express tTA.
(c) The expression of GENE X can be suppressed in the presence of Docycline.

associated memory formation. The CA1 specific knockout of NR1 has given us the strongest argument in favor of a correlation between CA1 synaptic plasticity and spatial learning [23]. Recently, a Cre/loxP system has been successfully developed to delete genes specifically in CA3 pyramidal cells [136]. Utilizing these conditional knockout technologies, we examined the role of CaMKII in CA3-CA1 LTP by selectively knocking out α-CaMKII in the pre- (CA3) or post- (CA1) synaptic cell at this synapse. We were specifically interested in whether a pre-synaptic or post-synaptic deletion of α-CaMKII would affect the ability to induce LTP at CA3-CA1 synapses. This could provide some insight into the locus of change that underlies LTP expression. We were also interested in how deficiencies in CaMKII dependent plasticity at the CA3-CA1 synapse could contribute to learning and memory. Such correlative studies would complement the NR1 conditional knockout, allowing us to study learning and memory in a background of normal NMDA receptor dependent synaptic transmission. It is likely that only through a comprehensive analysis of the relationship between synaptic plasticity mechanisms and learning and memory using very specific genetic techniques in combination with *in vivo* physiology and behavioral analyses will we be able to form strong conclusions about the relationship between LTP and learning.

I began my thesis research with a goal of acquiring a better understanding of the molecular mechanisms that underlie synaptic plasticity in the mammalian brain. Using a genetic approach in the mouse was an innovative method for such studies that offered the additional possibilities of associated behavioral analyses. In Chapter 2, I revisit the α-CaMKII global knockout mouse that established this field and I characterized the LTP deficit at the CA3-CA1 synapse while considering alternatives for genetic rescue experiments to clarify the role of CaMKII in LTP. This work was done in the laboratory of Roberto Malinow at the Cold Spring Harbor Laboratory, where I learned the techniques for extracellular field recording in the mouse hippocampal slice. This

chapter has been published previously [137], and is reproduced here largely as published, except for some minor corrections and the reformatting of citations and figures. Chapter 3 examines a CA1 α-CaMKII conditional knockout mouse that reveals striking similarities with the global knockout, with deficient CA3-CA1 LTP and associated behavioral abnormalities. Chapter 4 examines a CA3 specific α-CaMKII conditional knockout mouse that demonstrates normal CA3-CA1 LTP and behavior, yet reveals a role for CaMKII in the presynaptic short term plasticity mechanism, facilitation. Chapter 5 offers some preliminary data on using a tissue-specific inducible transgenic approach for studying CaMKII function in the brain, using a highly specific endogenous CaMKII inhibitor.

Chapter 2

CA1 long term potentiation is diminished but present in hippocampal slices from α-CaMKII mutant mice

Abstract

Previous work has shown that mice missing the α-isoform of calcium-calmodulin dependent protein kinase II (α-CaMKII) have a deficiency in CA1 hippocampal long term potentiation (LTP). Follow-up studies on subsequent generations of these mutant mice by our laboratories have shown that while a deficiency in CA1 LTP is still present in α-CaMKII mutant mice, it is different both quantitatively and qualitatively from the deficiency first described. Mice of a mixed 129SvOla/SvJ;Balb/c;C57Bl/6 background derived from brother/sister mating of the original α-CaMKII mutant line through multiple generations (>10) were produced using *in vitro* fertilization. While LTP at 60 minutes post-tetanus was clearly deficient in these (-/-) α-CaMKII mice (42.6%, n=33) compared with (+/+) α-CaMKII control animals (81.7%, n=17), α-CaMKII mutant mice did show a significant level of LTP. The amount of LTP observed in α-CaMKII mutants was normally distributed, blocked by APV (2.7%, n=8), and did not correlate with age. This implies that a form of α-CaMKII independent LTP is present in mice that could be dependent upon another kinase, such as the α isoform of CaMKII. A significant difference in input / output curves was also observed between (-/-) α-CaMKII and (+/+) α-CaMKII animals, suggesting that differences in synaptic transmission may be contributing to the LTP deficit in mutant mice. However, tetani of increasing frequency (50, 100, 200Hz) did not reveal a higher threshold for potentiation in (-/-) α-CaMKII mice compared with (+/+) α-CaMKII controls.

Introduction

Calcium-calmodulin dependent protein kinase II (CaMKII) is a serine/threonine protein kinase that is highly expressed in the brain and is thought to be involved in such diverse mechanisms as neurotransmitter synthesis and release, microtubule assembly and disassembly, ion channel modulation and synaptic plasticity [36]. The unique ability of CaMKII to auto-phosphorylate at Thr286, producing a long-lasting calcium independent form of the enzyme [42,44-47], has led to proposals that CaMKII might serve as a "memory molecule" of activity at synapses and may underlie long term changes in synaptic activity [48-50]. Several lines of evidence indicate a role for CaMKII in long term potentiation (LTP) [138], a well-characterized mechanism for increasing synaptic efficacy that has been observed at a number of excitatory synapses in the mammalian central nervous system, and most carefully studied at the CA3-CA1 hippocampal synapse [8,139]. CaMKII is highly expressed in the hippocampus, comprising 2% of total hippocampal protein [140], and is localized in both the pre- and post- sides of the synapse [55,56]. It is thought to be the major protein component of the post-synaptic density (PSD) [57-59,141], where it is strategically located for the modification of proteins that might be involved in the expression and maintenance of LTP. Such proteins include glutamate receptors in the post-synaptic cell membrane and microtubule-associated protein 2 (MAP2), both of which are CaMKII substrates [64,142-145]. CaMKII activation is dependent upon the initial binding of calcium-calmodulin [146], and it is known that calcium influx via the NMDA receptor is necessary for LTP induction [8]. Correspondingly, it is known that LTP induction leads to an increase in CaMKII activity, an increase in auto-phosphorylated CaMKII, and an increase in the phosphorylation of the CaMKII substrates, MAP-2, synapsin I and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors [64,147-149]. CaMKII inhibitors, calmodulin inhibitors, and general

kinase inhibitors have been shown to block LTP induction [Malinow, 1989 #84; [52-54],
while constitutively active CaMKII has been shown to mimic and occlude LTP when
introduced via a viral expression system [150] as well as by direct injection into the post-
synaptic cell [151,152], and to shift the LTP/LTD modification threshold in transgenic animals
[134]. In addition, mice mutant in the α isoform of CaMKII are found to be deficient in
hippocampal LTP, STP and LTD [111,118], deficient in primary visual cortex [153,154] and barrel
cortex plasticity [155], to perform poorly in an assortment of learning [110] and behavioral
paradigms [120], and to exhibit hyperexcitability in multiple limbic structures [119].

We re-examined LTP in the CA1 region of the hippocampus of (-/-) α-CaMKII mice
using field recordings. Preliminary studies indicated that LTP was diminished, but
present, in hippocampal slices from young (-/-) α-CaMKII animals (12-28 days), ages
which had not been examined in previous studies [111,118]. Consequently, we extended
our recordings to older adult animals (up to 108 days) to determine whether a
developmentally dependent variable was affecting the probability of eliciting LTP in
these animals. Basic characteristics of synaptic transmission were also examined to
address whether differences in excitability may be contributing to the LTP deficits in
these CaMKII mutant mice.

Materials and Methods

Mice

The mice used in the following electrophysiology experiments were of a mixed
129SvOla/SvJ;Balb/c;C57Bl/6 genetic background, inbred by brother/sister mating for
over ten generations before the present experiments began. Because natural matings
among heterozygous and homozygous α-CaMKII mutant animals were often found to be
unproductive, animals were produced using *in vitro* fertilization. Hippocampal slices
from mutant and wild-type animals were interleaved during experiments, using wild-type

littermates and/or wild-type animals matched for background and age as controls. No significant differences were seen between those animals tested blindly and those tested with knowledge of phenotype, so data from these experiments were pooled. The genotypes of animals used were determined before, and confirmed after, the experiments using a PCR assay.

Slice preparation and recording

Transverse hippocampal slices (400μm) were prepared from 12-108 day old mice using the tissue chopper method (Malinow and Tsien 1990). CA3 and CA1 regions were surgically separated to prevent epileptiform activity. The slices were submerged and superfused with a bath solution containing 119mM NaCl, 2.5mM KCl, 2.5mM $CaCl_2$, 26.2mM $NaHCO_3$, 1.3mM $MgCl_2$, 1mM NaH_2PO_4, 11mM glucose and equilibrated with 95% O_2 and 5% CO_2. Picrotoxin (100μM) was added to the bath to block $GABA_A$ inhibitory postsynaptic currents. The temperature of the recording chamber was maintained at 30°±.5°. Slices were allowed to recover at least one hour before recording was initiated.

Using visual guidance, a glass stimulating electrode filled with 2M NaCl was placed in Schaffer collateral afferents to elicit field excitatory postsynaptic potentials (f-EPSPs) using a brief (<200μsec) current pulse. An extracellular glass recording electrode, filled with the above bath solution, was placed in the CA1 stratum radiatum about 150-200μm from the stimulating electrode. Visual guidance allowed specific and consistent placement of electrodes and rapid superficial assessment of the health of the slice. Stimulus intensity was adjusted to evoke baseline pre-tetanic responses of similar sizes for all of the slices (.2-.3mV). Baseline stimulation frequency was .033Hz and LTP was typically induced using a 100Hz tetanus (5 trains, 20 pulses per train, 10 seconds between trains), unless otherwise stated. To block NMDA receptors in some experiments, 100μM DL-APV was added to the bath solution, as appropriate.

Individual field EPSP peak amplitudes were determined by averaging the response size over a fixed 1-5ms window encompassing the period of negative slope just preceding the peak amplitude of the signal, and subtracting a baseline estimate. For each experiment, the same windows were used on recorded sweeps in all experimental conditions. At the beginning of each experiment, the stimulating intensity (input) was varied and the amplitude of the field EPSP response was measured (output). The intensity of input stimulation began at a level where no responses were observed and increased until responses were maximal. These paired stimulation/response values were used to plot input/output curves of representative experiments (Figure 5).

For LTP experiments using the above tetanus paradigm, the magnitude of LTP was measured by comparing the average of responses over a 5 minute interval 60 minutes after the tetanus with the average of responses over a 5 minute interval preceding the tetanus. Consequently, the magnitude of LTP was expressed as a relative change (%) in the response amplitude.

Results

We began by examining LTP in the CA1 region of hippocampal slices from young (-/-) α-CaMKII mice (12-28 days) from our mixed background inbred line. Synaptic transmission was monitored for stable baseline periods of up to 30 minutes before LTP was induced using a tetanus protocol (100Hz, 5 trains, 20 pulses per train, 10 seconds between trains). Surprisingly, LTP was observed in mutant slices, although it appeared to be smaller in magnitude than that observed in wild-type control experiments (Figure 2-1). This was in contrast to previously published analysis of the original (-/-) α-CaMKII line, in which 14 out of 16 mutant slices showed no LTP (-1% ± 3 at 30 minutes post-tetanus), while the remaining 2 mutant slices showed significant LTP of a magnitude comparable to that observed in (+/+) α-CaMKII animals (68% ± 32 at 30 minutes post-

tetanus), under given experimental conditions [111]. Subsequent studies also concluded that α-CaMKII mutants show significantly diminished LTP [118], suggesting that it may be completely absent in these mice.

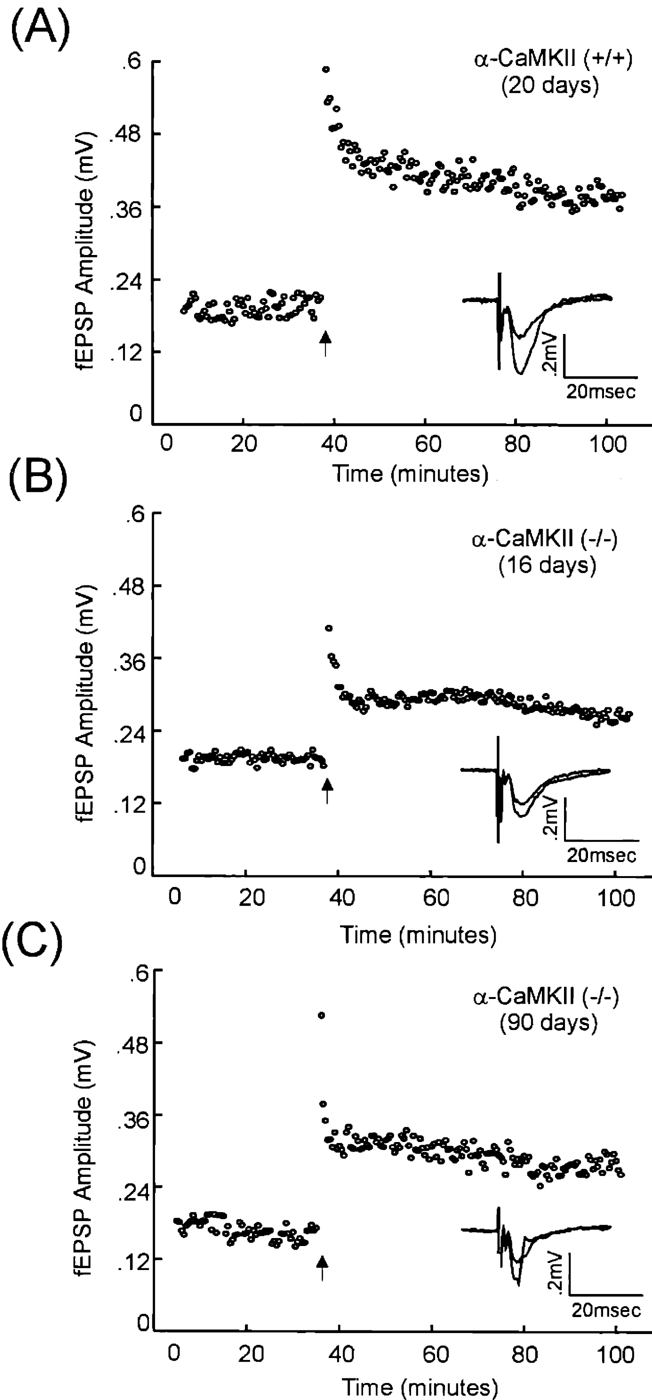
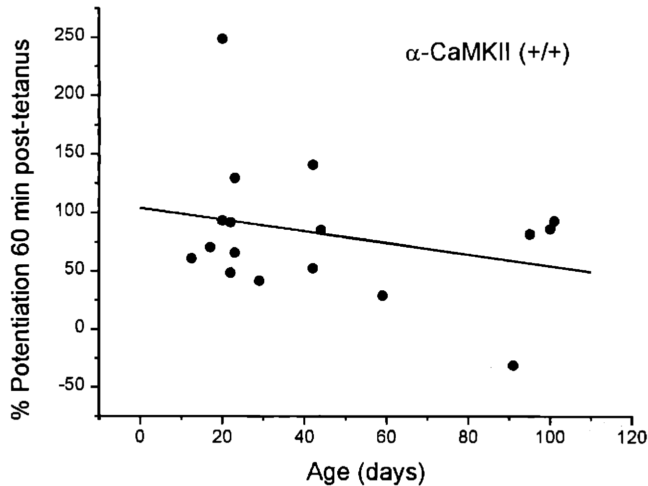


Figure 2-1: Tetanus-induced LTP of field EPSPs in the CA1 region of the hippocampus is present, but diminished, in (-/-) α–CaMKII mutant mice.

Field EPSP amplitude (mV) is plotted versus time (minutes), with the arrow indicating the time of tetanus delivery (see Materials and Methods). Average field EPSP responses before (10 consecutive trials, 5 minutes prior) and after tetanus (10 consecutive trials 60 minutes after) are shown, overlapping, in the inset. Three representative experiments are shown, demonstrating CA1 LTP in slices from a wild-type mouse (A), a young (16 days) mutant mouse (B), and an adult (90 days) mutant mouse (C).

As the original analysis of (-/-) α-CaMKII mutant mice had been performed on animals primarily 1.5 to 3 months of age (Silva et al. 1992a), we considered that a developmentally dependent variable could be affecting the probability of eliciting LTP in young (-/-) α-CaMKII animals. To address this, we examined LTP in older animals (28 - 108 days) and found that LTP was also diminished, but present, in older (-/-) α-CaMKII mice compared to (+/+) α-CaMKII control animals (Figure 1A,C), and there was no correlation between age and the amount of potentiation post-tetanus in either (-/-) α-CaMKII or (+/+) α-CaMKII mice (Figure 2-2).

(A)



(B)

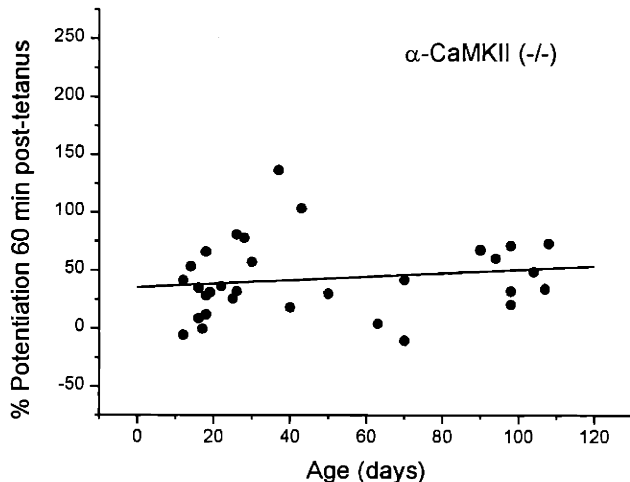


Figure 2-2: No correlation between age and % potentiation post-tetanus was observed in (+/+) α–CaMKII or (-/-) α–CaMKII mice.

Potentiation of field EPSP amplitude at 60 minutes post-tetanus is plotted versus animal age (days). Synaptic potentiation is not correlated with age in either wild-type (A: n = 17, R = 0.28, P = 0.29) or mutant (B: n = 33, R = 0.27, P = 0.36) slices.

Cumulative data across all age groups showed that while LTP at 60 minutes post-tetanus was clearly deficient in (-/-) α-CaMKII mice (42.6% ± 5.5, n = 33) compared with (+/+) α-CaMKII control animals (81.7% ± 14.1, n = 17), α-CaMKII mutant mice did show a significant level of LTP (Figure 2-3).

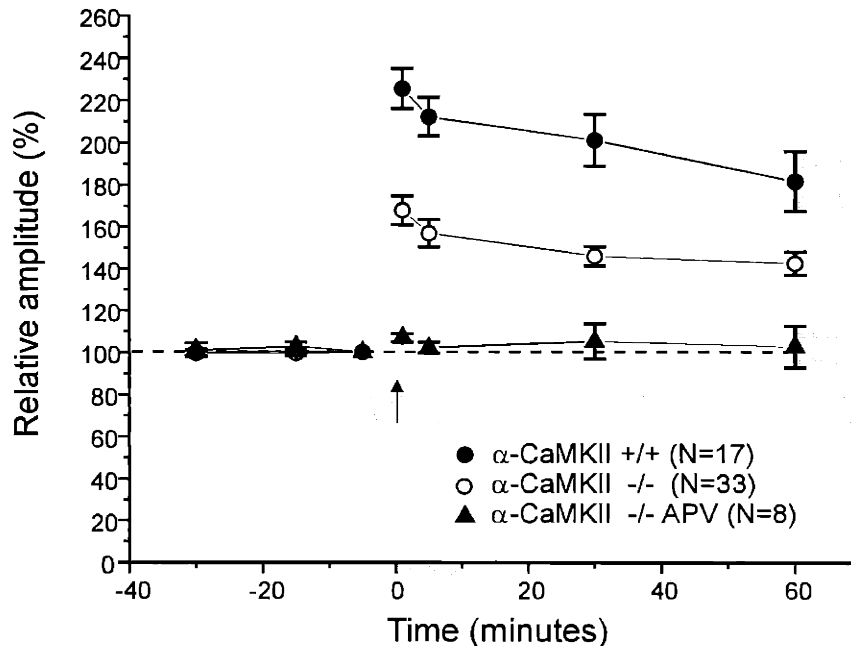


Figure 2-3: α–CaMKII independent CA1 LTP is about 50% of that observed in (+/+) α–CaMKII mice and is blocked by APV.

Cumulative data from tetanus induced CA1 LTP of field EPSP responses in wild-type (closed circles, n = 17) and mutant (open circles, n = 33) slices, and in mutant slices in the presence of 100μM DL-APV (closed triangles, n = 8). The relative mean field EPSP amplitudes ± SEM (mV) are plotted versus time (minutes), with the arrow indicating the time of tetanus delivery. The mean values are indicated for time points 30 min, 15 min and 5 min before tetanus, and 1 min, 5 min, 30 min and 60 min after tetanus and are calculated from field EPSP amplitude values over a 5 minute period, beginning at the time point indicated.

This α-CaMKII independent LTP was blocked when DL-APV (100μM) was included in the bath (2.7% ± 10, n = 8) (Figure 2-3), indicating that the potentiation is NMDA receptor dependent. The amount of potentiation observed in both (-/-) α-CaMKII and (+/+) α-CaMKII slices followed a normal distribution around a mean value (Figure 2-4).

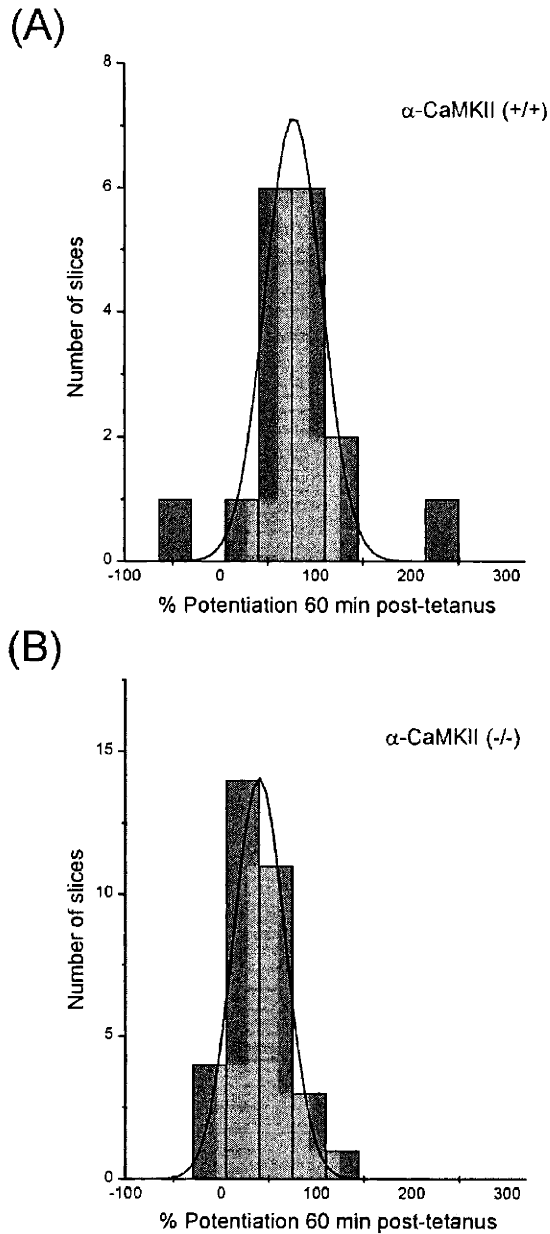


Figure 2-4: Potentiation in (-/-) α–CaMKII and (+/+) α–CaMKII slices 60 minutes post-tetanus follows a normal distribution.

Histograms depicting the distribution of slices with the indicated amount of potentiation 60 minutes post-tetanus in mutant (A) and wild-type (B) slices.

The mean value for mutant slices is about 50% of that observed in wild-type animals, with a comparable standard deviation/mean for both groups of animals (s.d./mean: (+/+) = .71; (-/-) = .75).

It is possible that the LTP deficit observed in (-/-) α-CaMKII mutant mice might reflect differences in the basic properties of synaptic transmission between mutant and wild-type mice, which could consequently confound attempts to produce LTP efficiently in mutant animals. To examine this, we plotted input /output curves for data obtained from (-/-) α-CaMKII and (+/+) α-CaMKII animals (Figure 2-5a). Synaptic input / output curves from age matched mutant and wild-type mice (6-10 weeks old) are significantly different, with the mean slope, calculated by linear regression, of wild-type slices (n = 7; 34 ± 7.2μV/V) being over twice that observed in mutant slices (n = 7; 16 ± 2.7μV/V).

If differences in some aspect of depolarization and/or cell excitability are present in the (-/-) α-CaMKII mutant mice, it might be possible to "overcome" these deficits when trying to induce LTP by using stimuli of increasing strength. To address this, stable baseline field EPSP responses were recorded for 30 minutes in (-/-) α-CaMKII (n = 5) and (+/+) α-CaMKII slices (n = 4), followed by successive tetani of 50Hz, 100Hz and 200Hz (each 5 trains, 10-20 pulses per train, 10 seconds between trains), with each tetanus separated by 30 minutes of recording (Figure2-5b). The amount of potentiation at 30 minutes following each tetanus was normalized to the maximum level of potentiation obtained following the 200Hz tetanus. The resulting "frequency - potentiation" curves are shown, and they do not reveal a higher threshold for potentiation in (-/-) α-CaMKII mice compared to (+/+) α-CaMKII mice. While mutant mice show decreased responses following each tetanus when compared with wild-type, these responses appear to be saturated with tetani of similar strengths in both mutant and wild-type slices.

Discussion

We have examined LTP in the CA1 region of hippocampal slices from mice lacking the α isoform of CaMKII and find that LTP is deficient in these mutant mice. However,

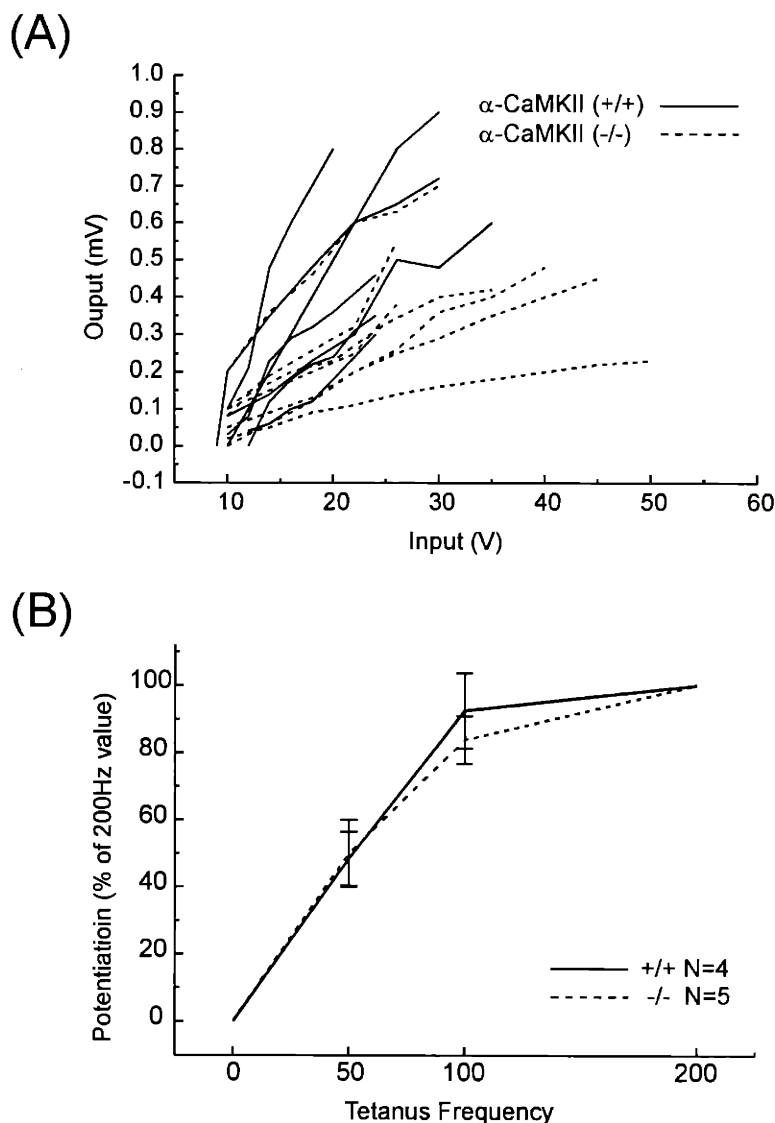


Figure 2-5: Synaptic input-output curves are steeper in (+/+) α–CaMKII slices than in (-/-) α–CaMKII slices.

(A) Elicited fEPSPs (mV) as a function of stimulation strength (V) are shown for a sampling of wild-type (broken lines, n = 7) and mutant (solid lines, n = 7) slices. For each curve, a slope was calculated by linear regression. The mean slope of the input-output curves is greater in wild-type slices (34 ± 7.2μV/V) than in mutant slices (16 ± 2.7μV/V). T-test, $p < 0.05$.

(B) Successive tetani of increasing frequency do not reveal a higher threshold for potentiation in (-/-) α–CaMKII mice compared to (+/+) α–CaMKII mice. Following 30 minutes of stable recording of baseline field EPSPs, three successive tetani of 50, 100 and 200 Hz (each 5 trains, 10-20 pulses per train, 10 seconds between trains) were delivered to the slice, each separated by 30 minutes of recording. The amount of potentiation at 30 minutes following each tetanus was normalized to the potentiation following the 200Hz tetanus and was plotted relative to the tetanus frequency for both wild-type (solid line, n = 4) and mutant (broken line, n = 5) slices.

the deficiency is different from that first described [111]. We find that there is a significant amount of potentiation in (-/-) α-CaMKII mutant mice, although it is reduced to about 50% of that observed in wild-type animals. This α-CaMKII independent potentiation is blocked by APV, suggesting that it is traditional NMDA receptor dependent LTP, and is consistent in quantity and distribution (normal) across all ages examined. α-CaMKII is therefore not absolutely required for CA1 LTP. This result is not in opposition to the idea that the CaMKII holoenzyme does play a critical role in the induction and maintenance of LTP, or that perhaps the α-CaMKII isoform, specifically, is the dominant contributing molecule in wild-type mice. The residual LTP observed in α-CaMKII mutant mice does suggest that some other molecule(s), such as other isoforms of CaMKII or other implicated kinases, are able to complement the CaMKII deficiency. In addition, it is possible that in the wild-type animal, it is the α-CaMKII isoform alone that contributes, due to its high expression levels and optimal localization, and that only in its absence are the potential contributing functions of other molecules revealed.

The α isoform of CaMKII is the most abundant subunit expressed in the adult mammalian hippocampus, where it is found at a concentration three times higher than the β subunit, the next most highly expressed brain isoform [37,141,156]. Previous results have shown that total CaMKII activity in forebrain from (-/-) α–CaMKII mutant mice is decreased to 45% ± 4, when measured by an in vitro assay, indicating that substantial CaMKII activity still remains in these mutant mice [111]. Recent studies have also shown that the β isoform is able to target to synapses in the absence of the α isoform, as it is observed to be present at normal levels in the post-synaptic densities of α–CaMKII mutant mice (M. Kennedy, manuscript in preparation). These levels are low compared with the wild-type levels of the α–isoform, as previous studies have also indicated that mRNA for the α but not the β isoform is localized in dendrites [37] and that the 3' untranslated region of the α subunit mRNA is critical for dendritic targeting [125].

Consequently, the α isoform may be positioned appropriately to receive an incoming calcium transient in α–CaMKII mutant animals, though its relative lower abundance and perhaps lack of efficient mRNA targeting would still lead to a less efficient subsequent signaling response, and decreased LTP.

The α isoform of CaMKII is expressed post-natally in the hippocampus, initially at very low levels, increasing five-fold to high adult levels by three weeks of age [37,157]. It contrast, the β isoform is expressed at moderate levels at birth and increases just slightly during development. During the first week of post-natal development, tetanus-induced LTP is diminished but present [67,158-161], and increases in a fashion that parallels the expression of α–CaMKII. Interestingly, in one study the amount of tetanus induced LTP observed in the youngest animals tested (post-natal day 4-9) was about 50% of that observed in older animals (post-natal day 10-14) [161], comparable to the difference we observe between α–CaMKII mutant and wild-type mice (Figure 2). Since LTP, though reduced, is still attainable in young wild type animals when the α isoform is known to be poorly expressed, it suggests that the α–isoform of CaMKII (or other mediators) can partially complement the absence of α–CaMKII.

While tetanus induced LTP is reduced in very young rats, LTP induced by low frequency stimulation paired with depolarization of the post-synaptic cell during whole cell recording produces a robust potentiation similar to that observed in older animals [161-163]. This result, combined with the observation that the majority of CA1 transmission in young animals acts through NMDA receptors, making them effectively "silent" at hyperpolarized potentials, led to the hypothesis that an insufficient depolarization during LTP induction is responsible for the diminished LTP observed in immature animals. To address whether our deficiency in LTP is attributable to similar problems with induction or with subsequent expression mechanisms, we examined other parameters of synaptic transmission. Basal transmission, as indicated by the slope of input-output curves, is diminished in our mutant animals, compared to wild-type animals. This also parallels

what was observed in young wild-type rats, which show reduced input-output slopes when compared with older animals [161]. These similarities suggest that ineffective depolarization could be contributing to LTP induction deficits in α–CaMKII mutant mice. It will be interesting to examine in future studies if α–CaMKII mutant mice have increased numbers of silent synapses. While a high proportion of pure NMDA silent synapses could be a barrier to eliciting LTP, it could also be a reflection of deficient LTP during the development of α–CaMKII mutant mice, as it is thought that an LTP-like process is necessary to convert pure NMDA-receptor synapses to synapses with mixed AMPA and NMDA receptor function [72,73,163,164].

If α–CaMKII mutant mice have a deficit in LTP induction mechanisms, then one may be able to overcome this deficit by delivering stronger tetani. Our results, however, indicate that increasing tetanic strength or number does not overcome the mutant phenotype. Indeed, mutant mice appear to have a similar threshold for LTP induction and their LTP saturates with similar tetanic strengths as wild-type mice. While this implicates deficient expression mechanisms in α–CaMKII mutant mice, it is possible that tetanus delivered stimulation does not produce effective maximal depolarization. Whole cell experiments using a pairing protocol for LTP induction would be useful to determine if such a limit to effective post-synaptic membrane depolarization exists in these animals.

The completely lack of LTP and/or bimodal distribution of LTP values observed in previous studies [111,118] is in contrast to that observed in our present studies, in which LTP was typically present in a normal distribution across mutant slices, but reduced compared to wild-type values. The bimodal distribution suggests that a variable at the level of the entire slice or the entire animal may be working to affect the probability of eliciting LTP in these experiments. A normal distribution of LTP would be expected if there is a large number of synapses acting independently, some producing LTP and some not, with the absence of α–CaMKII decreasing the likelihood or amount of LTP

that can be obtained at any synapse.

While the uncontrolled variables that underlie hippocampal slice preparation and recording conditions cannot be ruled out, the differences between present and past analyses of α–CaMKII mutant mice are likely to result from known differences in the genetic background of the mice used [165-169]. The earlier study was carried out using F2 and F3 mice produced between the 129Sv and Balb/c strains, while the present study utilized progeny generated by repeated sister/brother mating of litters that included contributions from the C57Bl/6 strain. It is quite possible that each of these mouse strains contains a unique assortment of alleles at multiple loci, and that these alleles could play roles directly in the LTP mechanism, peripherally as modifiers of LTP, or indirectly by changing other aspects of neurologic function that could affect plasticity. In fact, it has recently been shown that the amount of hippocampal LTP produced following theta burst stimulation varies among different inbred strains of wild-type mice [170]. For example, a129Sv inbred line has been shown to exhibit reduced levels of hippocampal LTP following theta-burst stimulation [170]. Since a 129Sv embryonic stem (ES) cell line was used to create the original α–CaMKII mutant mice, it is possible that 129Sv genes that account for this reduction in LTP could be closely linked to the mutated α–CaMKII locus, and therefore more readily retained in early generations of animals. For this reason, as well as the numerous behavioral deficits that are known to be associated with the 129Sv background [165], ES cells from the C57Bl/6J line are being used in our laboratory for creating future mutant mice.

It is possible that in the mice employed in the earlier LTP study, the combinations of the alleles of the relevant genes fell into two classes: one in which α–CaMKII is essential for LTP and another in which α–CaMKII is entirely dispensable, explaining the bi-modal distribution of LTP values. As the C57Bl/6 background was introduced and brother-sister mating was repeated, a combination of alleles that permits partial dependency on α–CaMKII may have been established in the pedigree. Recently, a

bimodal distribution of other phenotypes was also observed among the progeny of early crosses of the original α–CaMKII mutant line that was repeatedly crossed into C57Bl/6 [154]. Visual cortical plasticity experiments revealed a severe deficit following monocular occlusion in only about half of the α–CaMKII mutants of this line, with a similar fraction of animals showing a deficit in spatial learning in the Morris water maze following a period of intensive training. Mapping the genetic loci responsible for phenotypic differences both within, and between, these strains could be a valuable tool for identifying other genes that influence hippocampal LTP, and other α–CaMKII associated phenotypes.

In conclusion, we find that NMDA receptor dependent CA1 LTP is present, but reduced in mice lacking the α–subunit of CaMKII in the present background examined. Therefore, α–CaMKII is not absolutely required for LTP. However, this does not preclude an absolute requirement for CaMKII in LTP, as other isoforms of CaMKII could be complementing the deficiency in the α isoform. While we cannot conclude whether the deficit is in the induction or expression of LTP, future whole cell recording experiments looking at silent synapses and post-synaptic depolarization in α–CaMKII mutant mice could be enlightening.

Acknowledgments

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Chapter 3

Synaptic plasticity at CA3-CA1 hippocampal synapses in the CA1 specific α-CaMKII conditional knockout mouse

Introduction

CaMKII is activated following calcium entry through the NMDA receptor and this post-synaptic activation has been shown to be important for the induction of LTP at CA3-CA1 hippocampal synapses [60,171]. Delivery of a CaMKII inhibitory peptide specifically to post-synaptic CA1 has been shown to block LTP induction [51,52], while delivery of activated CaMKII to CA1 either through a patch-pipette [151,152] or by using a viral delivery system [150] has been shown to potentiate synapses. NMDA receptor activation has also been shown to promote translocation of CaMKII to the post-synaptic density (PSD) [172,173], where it is transiently "trapped" and strategically located for modifying post-synaptic substrates which could change the strength of synaptic transmission. However, CaMKII is also present at pre-synaptic terminals, including CA3 terminals, where it phosphorylates synapsin I following LTP induction [148]. Such pre-synaptic modification following LTP induction would require a retrograde signal, the evidence for which remains controversial, although a pre-synaptic requirement for kinase activity has been shown to critical for LTP maintenance [51].

It is still unclear whether the increase in synaptic strength that underlies LTP is primarily due to pre- or post-synaptic change. A purely post-synaptic locus for LTP expression is attractive for its simplicity. For example, a modification of AMPA receptor number and/or conductance could reasonably underlie an increase in synaptic transmission. CaMKII has been shown to phosphorylate GluR1, increasing the ion channel conductance, and GluRI phosphorylation has been observed following LTP

induction in CA1 [64,65,174]. Recent studies have also uncovered a mechanism for calcium evoked dendritic exocytosis (CEDE) in dendrites [175] which may contribute to the delivery of additional AMPA receptors to spines following a tetanic stimulation [116]. CEDE appears to be CaMKII dependent and is blocked by an inhibitor of microtubule polymerization [175,176]. While the exact function of MAP2 (microtubule associated protein 2) is unknown, it may be involved in organelle transport and microtubule stabilization in dendrites [177], and it is also a CaMKII substrate phosphorylated following CA1 LTP induction in slices [148]. Recent imaging studies are providing additional evidence for cytoskeletal remodeling of dendritic spines with synaptic plasticity, with CaMKII possibly adding new synapses following LTP induction [178-180].

While an abundance of evidence implicates post-synaptic CaMKII in LTP induction and possible expression mechanisms, global knock-out [110] and knock-in [50] studies of the α-CaMKII gene have shown that normal CaMKII activity is also required for learning associated behaviors. Initial experiments using CaMKII inhibitors in *Drosophila* [181] and in the rat [182] had suggested a role for CaMKII in learning and memory formation. Genetic approaches in the mouse extended these findings by showing that α-CaMKII activity and Thr286 autophosphorylation of α-CaMKII were specifically required for normal CA3-CA1 LTP production and for hippocampal dependent spatial learning [50,110]. These data support the possibility that Hebbian mechanisms of synaptic plasticity, such as LTP and LTD, could be critical for memory formation [98,100]. Conditional knock-out of NMDAR, a molecule essential for the induction of LTP in CA1, gave the strongest argument in favor of a correlation between CA1 synaptic plasticity and learning and memory, free of many of the unwanted variables that must be addressed using the traditional global knock-out approach [23]. However, it is possible that the NMDAR is involved in other synaptic functions in addition to plasticity, and perhaps the disruption of these mechanisms could have interfered with behavior in these mice. While the NMDAR does not appear to play a large part in normal synaptic transmission at CA1

synapses (Coan & Collingridge, 1985), it does contribute some current, particularly at depolarized potentials

As we believe that a genetic approach is a powerful method for examining the molecules underlying synaptic plasticity mechanisms and learning and memory, we have chosen to re-examine the role of CaMKII in CA1 hippocampal plasticity and associated behaviors. Regionally restricted conditional knock-out of α-CaMKII, similar to the CA1 regionally restricted knock-out of NMDAR1 [23,123], will allow us to study the role of CaMKII in a specific region of the hippocampus while ideally avoiding developmental deficits and confounding problems due to global deletion. It will complement the results of our NMDAR1 studies, as it will preserve normal synaptic transmission at depolarized potentials that may be dependent upon NMDAR1. While CaMKII has multiple putative substrates, it is not completely clear which ones are involved in synaptic plasticity at CA1 *in vivo.* Conditional deletion of α-CaMKII in either the pre- (CA3) or post-synaptic (CA1) side of the CA3-CA1 synapse using the Cre/loxP system will give us some insight into the mechanisms that underlie expression. While a tissue specific knock-out of CaMKII in CA1 will allow us to confirm a post-synaptic involvement of CaMKII in LTP (Chapter 3), a pre-synaptic knock-out of α-CaMKII will address unknown contributions of this kinase at pre-synaptic terminals to plasticity mechanisms at CA3-CA1 synapses (Chapter 4).

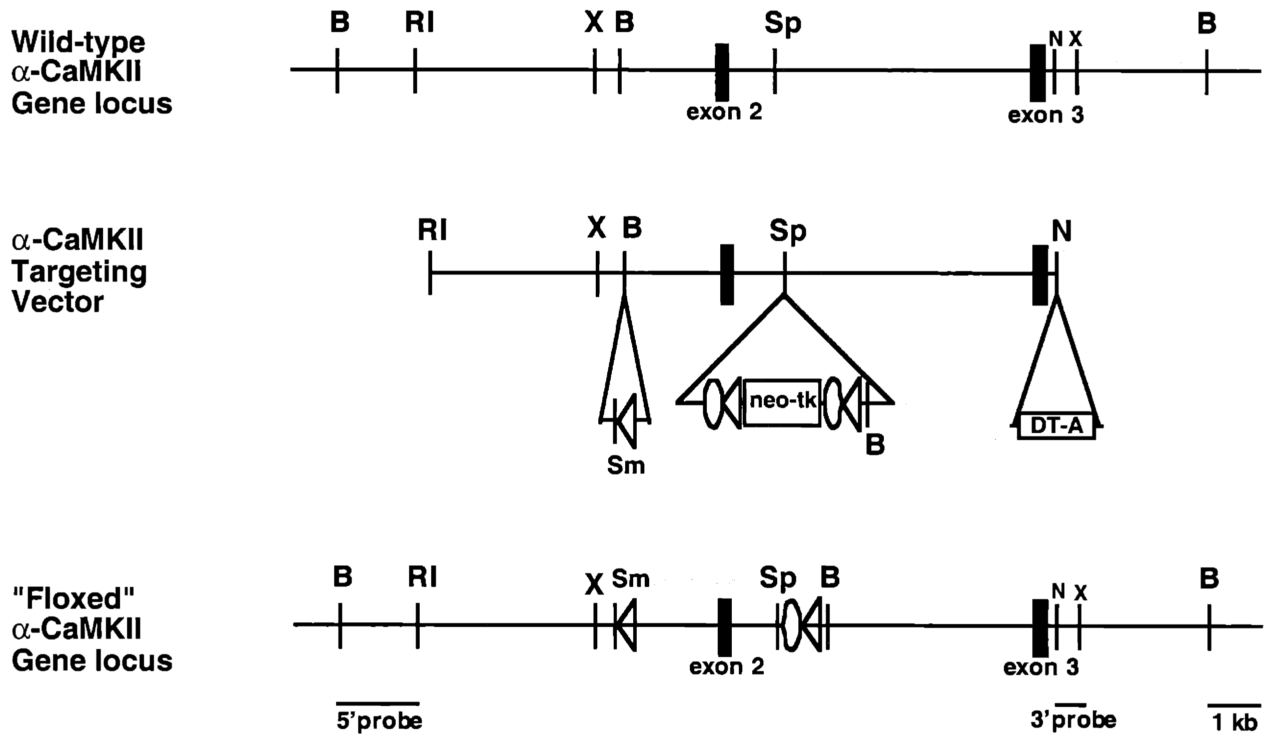
Results

Production of CA1 specific α-CaMKII knockout mice

To generate conditional α-CaMKII mutant mice, we first produced a recombinant "floxed" (*i.e.* "flanked by loxP") α-CaMKII mouse (*f*α-CaMKII) in which Cre recombinase recognition sequences (loxPs) [121] were inserted into the introns flanking exon 2 of the α-CaMKII gene. Cre mediated excision of this essential exon will delete the putative ATP

binding site from the molecule [183-185] and cause a frame shift that will result in a truncated nonfunctional peptide. We prepared a targeting vector for homologous recombination into the desired target region of the α-CaMKII gene that consisted of regions of homology flanking exon 2, strategically placed positive (neomycin resistance) and negative (thymidine kinase, diptheria toxin) selectable markers, and appropriate recombinase target sites (Figure 3-1). These recombinase target sites included a pair of FLP recombinase recognition targets (Frts) [186] flanking the neo-tk genes, with an additional loxP recombination site at the 3' end [187]. This "FLNT" cassette was cloned into a SpeI site in the intron 3' to α-CaMKII exon 2 in an α-CaMKII genomic clone (C57Bl/6). An appropriately directed second loxP sequence was then placed in a BamHI site in the intron 5' to α-CaMKII exon 2. The linearized targeting vector was transfected via electroporation into embryonic stem (ES) cells of C57Bl/6 background [188], and positive transfectants were selected with G418, a derivative of neomycin. Surviving ES cell clones were screened by Southern hybridization to identify clones in which the vector DNA replaced the endogenous α-CaMKII gene via homologous recombination. We transfected 6 homologously recombined ES clones with a Flpe recombinase expression vector [189] and grew the cells in the presence of gancyclovir. Surviving ES cell clones were screened by Southern hybridization to identify clones in which the neo-tk cassette had been excised, leaving a loxP sequence in the intron 3' to exon 2. Removal of the neo-tk cassette is important because the presence of the tk gene interferes with germline transmission of the homologous integrant (H. Prosser, personal communication). We injected 8 independently derived ES cell clones into blastocysts (Balb/c background), produced 37 chimeric progeny in total from 6 of the 8 ES cell clones, and identified 5 chimeras (from 3 ES cell clones) that transmitted the floxed α-CaMKII allele (*f*α-CaMKII) to their progeny when crossed with C57Bl/6 breeders.

A



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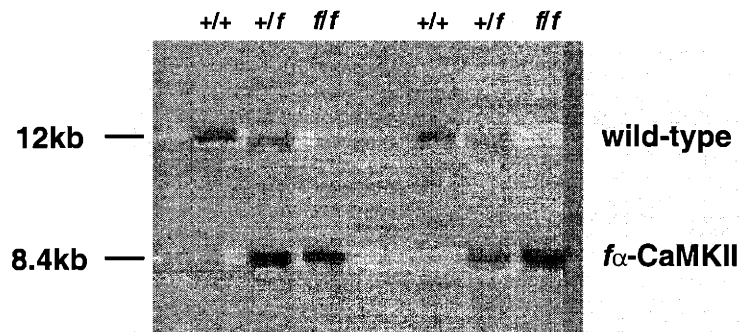


Figure 3-1: Generation of ***f*****α-CaMKII recombinant mice**

(a) *Top*: Region of the wild-type α-CaMKII locus containing exon 2. *Middle*: α-CaMKII targeting vector in which a loxP site was inserted into intron 1 (BamHI site) and the LFNT cassette was inserted into intron 2 (SpeI site). The DT-A marker [190] was inserted at the 3' end to select against non-homologous recombinants. Homologous recombination of this construct into the genomic locus introduces a BamHI site on the 3' side that can be detected with both the 3' and 5' probes. Transfection of homologous recombinant ES cells with FLP recombinase removes the neo-tk genes. *Bottom*: *f*α-CaMKII locus.

Cre/loxP mediated recombination at the *f*α-CaMKII locus will excise 4kb of sequence that includes exon 2 of α-CaMKII. Open triangle, loxP. Open oval, FRT. neo = neomycin resistance gene. tk = thymidine kinase gene. DT-A = diptheria toxin gene. Restriction enzymes: B = BamHI; R1 = EcoRI; Sp = SpeI; N = NcoI, X = XhoI, Sm = SmaI. Not all sites are shown.
(b) Representative southern blot analysis of genotype. Tail DNA was digested with BamHI and screened with a 3' probe external to the recombined locus. Homologous recombination introduced an additional BamHI site in the floxed allele whose presence will be detected with this probe. The wild-type allele is 12kb and the floxed allele is 8.4kb. +/+, wild type; +/*f*, heterozygous; *f*/*f*, homozygous *f*α-CaMKII.

To generate a CA1 specific knockout of the α-CaMKII gene, heterozygous floxed progeny (*f*α-CaMKII/+) from two different lines were crossed to the CW-2 transgenic line, which expresses Cre recombinase under the control of the α-CaMKII promoter, in a pure C57Bl/6 genetic background. When crossed to a floxed lacZ reporter line [191], CW2 mice showed a surprising pattern of recombination that was specific to CA1 hippocampal pyramidal cells (Figure 3-2). This pattern is reminiscent of the CA-1 restricted pattern of our previously published α-CaMKII promoter-Cre transgenic mouse, of mixed genetic background (T29-1) [123]. We crossed fα-CaMKII mice to the CW-2 Cre line rather than our previously published T29-1 line to maintain our conditional mutant mouse in a pure genetic background. C57Bl/6 mice are known to perform well in several learning paradigms [192] and to exhibit robust CA1 hippocampal LTP [193], and therefore C57Bl/6 is an optimal genetic background choice for studying mutations that affect hippocampal plasticity and associated behaviors. (*f*α-CaMKII/+, +/+) x (*f*α-CaMKII /+, Cre /+) matings were established to produce (*f*α-CaMKII/fα-CaMKII, Cre /+) mutant mice (CA1 α-CaMKII KO) for analysis. All expected genotypes were observed in the progeny of such matings, in the expected Mendelian proportions.

Expression studies of CA1 α-CaMKII KO mice

We used in situ hybridization to determine the spatiotemporal pattern of α–CaMKII gene deletion in CA1 α–CaMKII KO mice. In situ hybridization was first performed on brain sections using a ^{33}P labeled cRNA probe derived from the 3' untranslated region

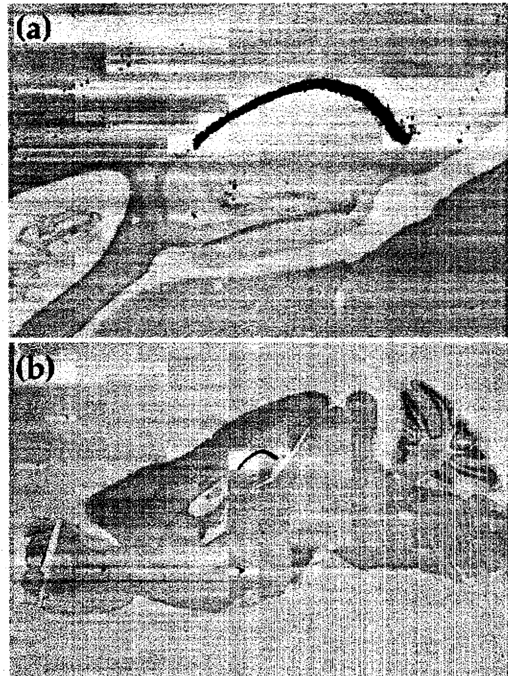


Figure 3-2: α-CaMKII promoter – Cre recombinase transgenic line, CW-2

CW-2 was crossed to a chicken β-actin promoter driven – loxP *Stop* loxP – lacZ reporter line [191]. The transcription and translation of lacZ is prevented by the upstream *Stop* sequence, which includes a stop codon and a polyadenylation signal. In the presence of Cre recombinase, the Stop sequence is excised. Since the chicken β-actin promoter is a ubiquitous promoter, the pattern of lacZ expression and subsequent X-gal staining reflects the pattern of Cre recombination in double transgenic mice. Cre/loxP recombination in CW-2 mice is largely restricted to the hippocampal CA1 region in 1.5 month old mice, when crossed to this reporter line. Sagital brain section (b) and high magnification of the hippocampus (a) are shown. This image was produced and generously provided by Chanel Lovett.

of the α-CaMKII cDNA. This probe detected RNA in adult CA1 α-CaMKII KO mice in a normal α-CaMKII expression pattern, suggesting that Cre mediated recombination of exon 2 did not destabilize the α-CaMKII mRNA, and that splicing occurred the missing essential exon (data not shown). *In situ* hybridization was repeated with a 46bp cRNA probe specific for the floxed exon 2. This probe detected the deletion of the *f*α-CaMKII gene in multiple areas of the forebrain in adult mice, including hippocampal CA1, neocortex and the amygdala in CA1 α-CaMKII KO mice (Figure 3-3; Figure 3-7).

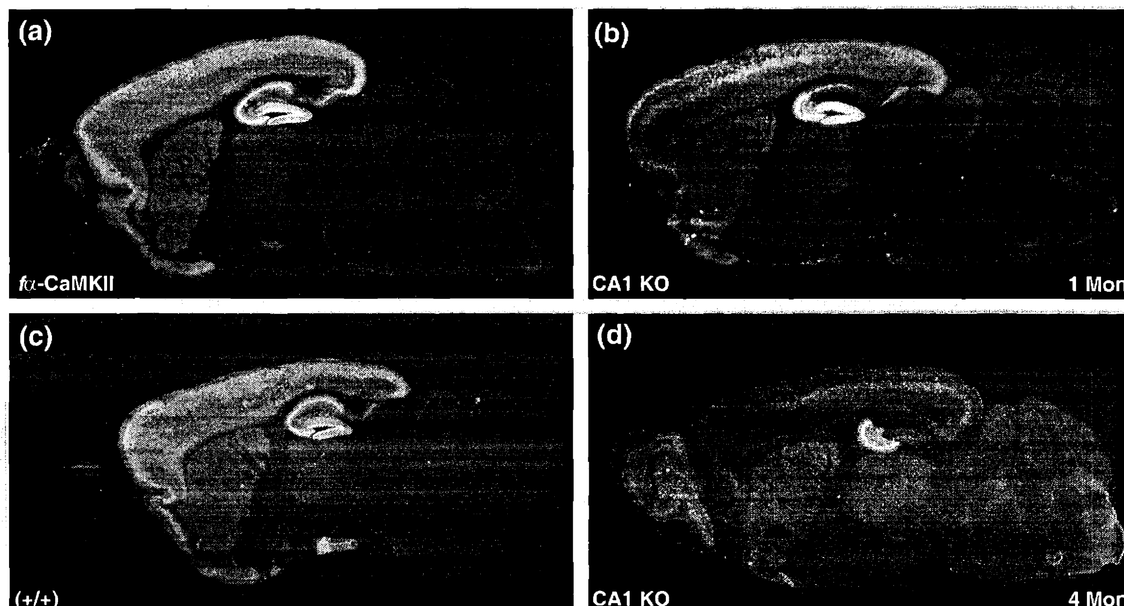


Figure 3-3: *In situ* hybridization revealed widespread deletion of *f*α-CaMKII exon 2 in the forebrain of CA1 α-CaMKII KO mice

Dark field images of sagital brain sections from floxed (a), wild-type (c) and CA1 α-CaMKII KO mice (b), (d) following *in situ* hybridization with an exon 2 specific probe. Silver grains representing mRNA signals are in white. Deletion of the *f*α-CaMKII gene was visible in the cortex and hippocampal CA1 at 1 month of age in CA1 α-CaMKII KO mice, becoming more complete and spreading to the dentate by 4 months of age. No difference in α-CaMKII mRNA expression was detected between wild-type (+/+) and floxed (*f*α-CaMKII) mice.

The deletion started in a small percentage of cells in a restricted pattern that became more widespread with increasing mouse age (Figure 3-3, Figure 3-4). The pattern of deletion represented a subset of areas where the α-CaMKII promoter, which was used to make the CW-2 Cre transgenic mice, is normally expressed [37]. Surprisingly, the deletion was much more broad than the CA1 pyramidal cell-specific pattern we observed after crossing CW-2 mice to a chicken β-actin promoter driven Lox-stop-Lox-lacZ reporter line [191]. We suspect that the susceptibility of a target locus to Cre recombination is variable, which would explain the spatiotemporal differences in deletion patterns observed when either the *f*α-CaMKII mouse, the lacZ reporter line [191] or a calcineurin floxed *f*CNB1 mouse [194] were crossed with CW-2. *In situ* hybridization

studies of floxed ($f\alpha$-CaMKII/$f\alpha$-CaMKII, +/+) mice demonstrated that the insertion of loxP sequences alone did not affect normal expression from the α-CaMKII locus (Figure 3-3), and consequently these animals were used as controls ($f\alpha$-CaMKII) in subsequent experiments.

Deletion of the $f\alpha$-CaMKII gene was first detected in pyramidal cells in the CA1 region of the hippocampus in 4 week old mice (Figure 3-4).

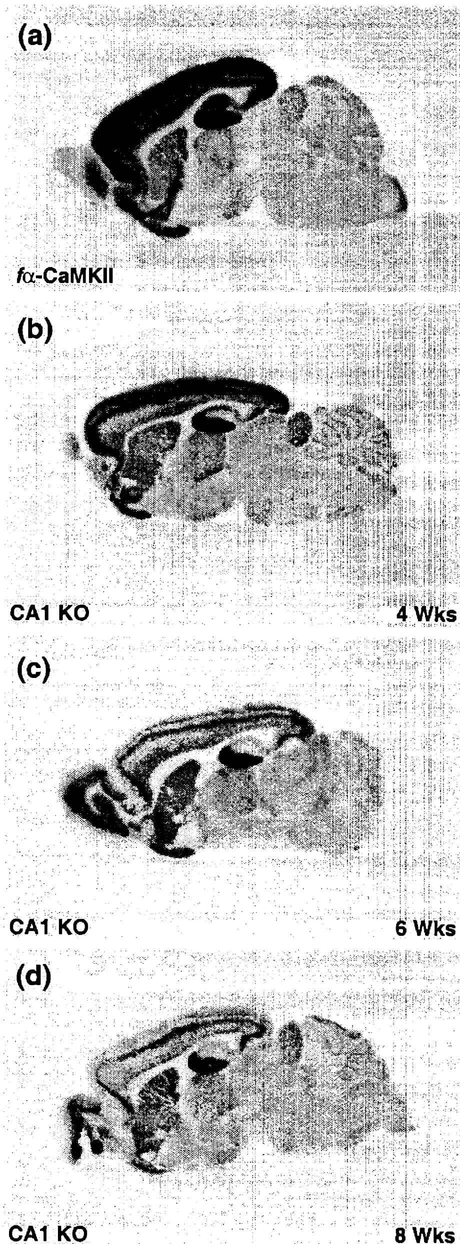


Figure 3-4: $f\alpha$-CaMKII deletion in CA1 is complete by 8 weeks of age in CA1 α-CaMKII KO mice

Scanned images of sagital brain sections from floxed (a) and CA1 α-CaMKII KO mice (b) (c) (d). CA1 α-CaMKII KO mice at 4 wks (b), 6 wks (c), and 8 wks (d) of age were examined in this experiment. Positive mRNA signals are in black. A dramatic reduction in mRNA signal was evident in hippocampal CA1 at 1 month of age, with nearly a complete loss of signal in CA1 cell bodies (stratum pyramidale) and dendrites (stratum radiatum, stratum oriens) by 2 months of age.

By 8 weeks, the deletion in CA1 was nearly complete. *f*α-CaMKII mRNA was absent from the CA1 cell body layer (stratum pyramidale) as well as the dendritic layers (stratum radiatum, stratum oriens) which normally contain large amounts of α-CaMKII mRNA. At this age, recombination was also observed in the dentate gyrus in a relatively small proportion of granule cells. However, recombination in the dentate accumulated over time, leading to near complete deletion of *f*α-CaMKII in this region in animals older than 5 months (Figure 3-5).

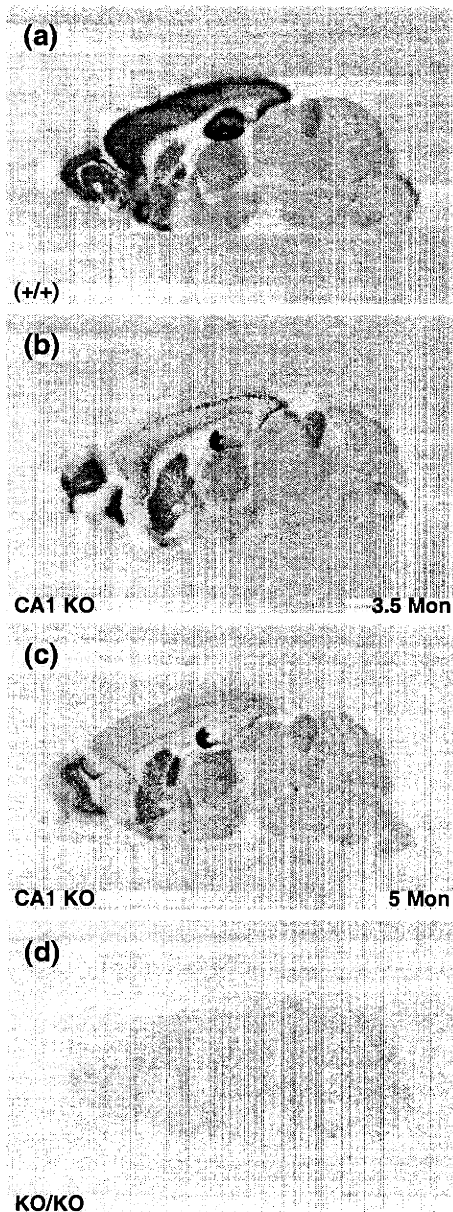


Figure 3-5: *f*α-CaMKII deletion spreads to the dentate gyrus, but expression in CA3 is unaffected in CA1 α-CaMKII KO mice

Scanned images of sagital brain sections from wild-type (a), CA1 α-CaMKII KO mice (b) (c), and a global α-CaMKII KO (d) were examined in this experiment. With increasing age, deletion spread dramatically in the dentate gyrus of CA1 α-CaMKII KO mice. No deletion was observed in the CA3 region at all ages examined.

The *f*α-CaMKII gene was also dramatically deleted in the subiculum, following a developmental time course reminiscent of the CA1 deletion. However, absolutely no significant deletion of *f*α-CaMKII was observed in the pyramidal cells of the CA3 region of the hippocampus. Consequently, we have produced a specific knockout of *f*α-CaMKII in only the post-synaptic cell at CA3 Schaffer collateral – CA1 synapses, and we will continue to refer to these mice as CA1 α-CaMKII KO.

As mentioned, *f*α-CaMKII gene deletion was not restricted to the hippocampal formation. Recombination was first observed in the deep layers of frontal cortex in 3 week old mutant mice (Figure 3-6).

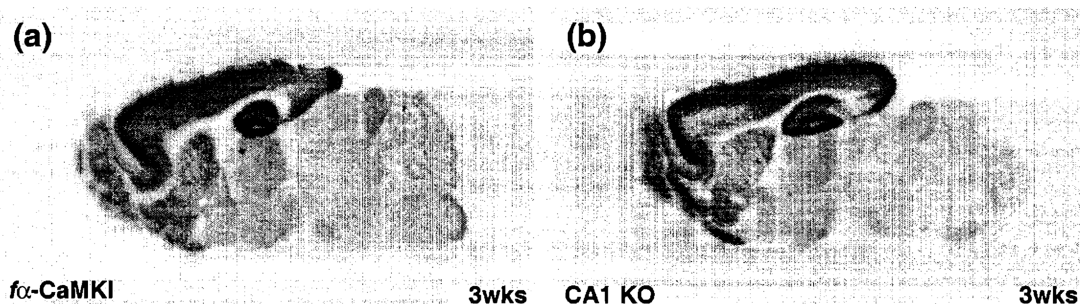


Figure 3-6: *f*α-CaMKII deletion begins in the inner layers of the cortex

Scanned images of sagital sections from 3 week old floxed (a) and CA1 α-CaMKII KO mice (b). Deletion is first observed in the inner cortical layers, and is most pronounced in frontal cortex. The hippocampus proper is not affected at this age, although some deletion is present in the subiculum.

Deletion was widespread in the cortext with increasing age, progressing to near completion in layers 2/3 and layers 5/6 by 4 months of age, with the deletion accumulating more slowly in layer 4 and the deepest cells of layer 6 (Figures 3-4, 3-5, 3-7). Additional recombination was observed in lateral and basolateral nuclei of the amygdala and in the piriform cortex, which followed a comparable time course of deletion (Figure 3-7).

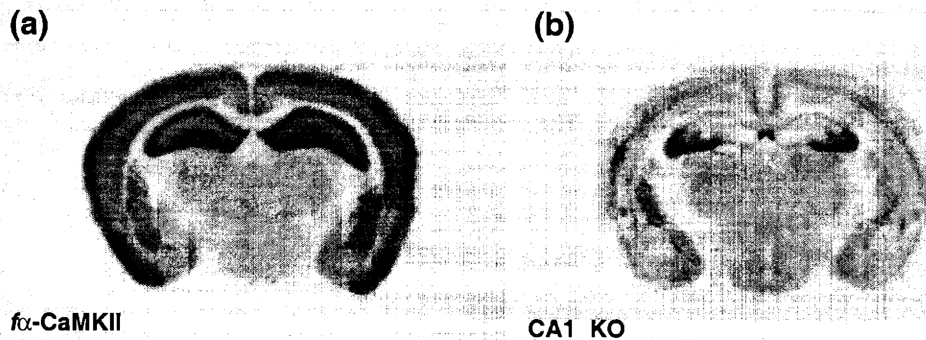


Figure 3-7: fα-CaMKII deletion is widespread in the cortex and the amygdala in CA1 α-CaMKII KO mice

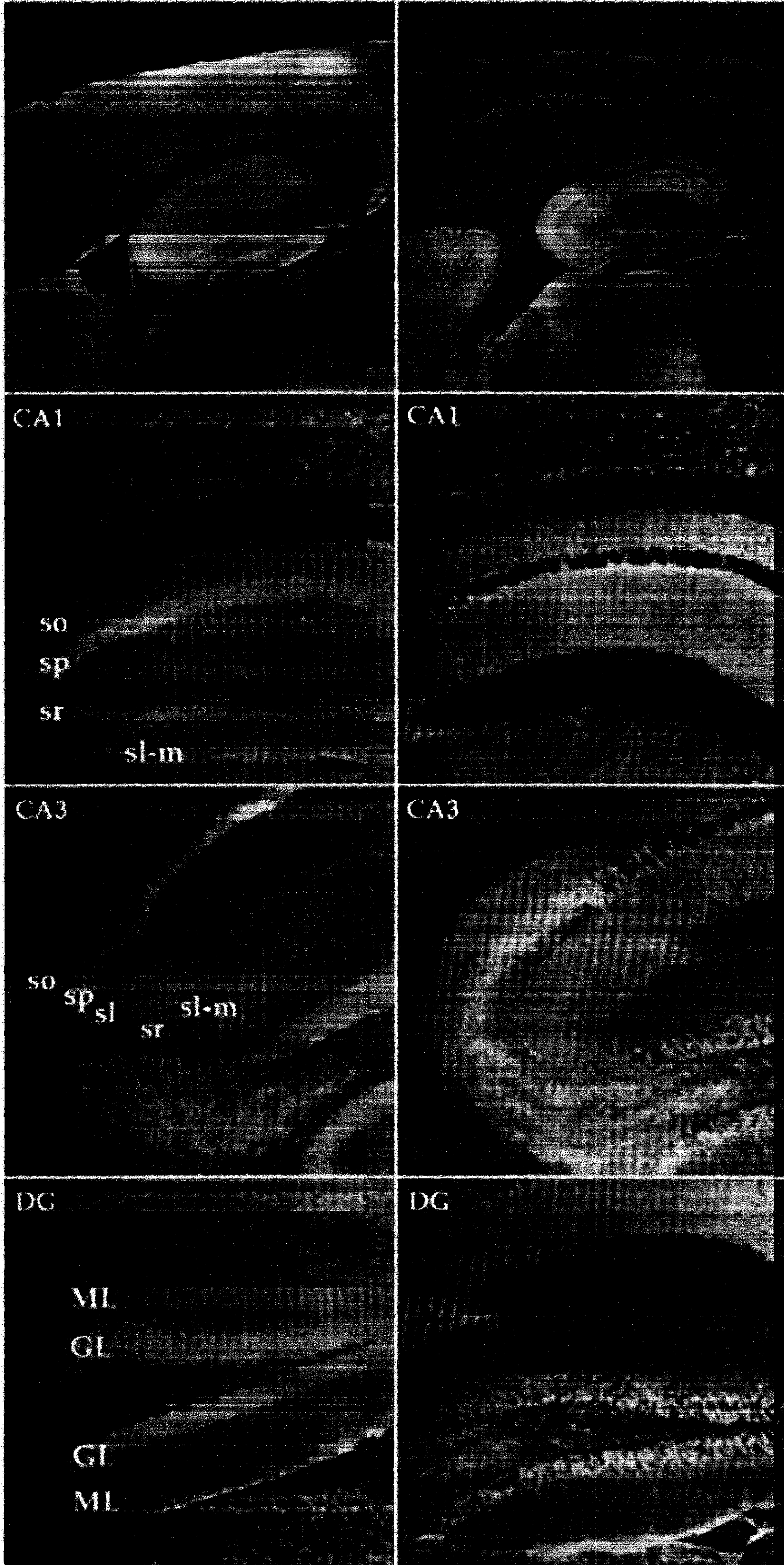
Scanned images of coronal sections from 4 month old floxed (a) and CA1 α-CaMKII KO mice (b). Deletion is widespread in cortex, with some preservation of layer 4, and in the lateral amygdala nuclei of CA1 α-CaMKII KO mice.

No significant deletion of the fα-CaMKII gene was observed in the striatum, thalamus, cerebellum, olfactory bulb or other subcortical regions.

We performed immunohistochemistry using an anti-mouse α-CaMKII monoclonal antibody on formaldehyde fixed 50μm vibratome brain sections and found staining patterns consistent with the *in situ* hybridization studies (Figure 3-8; see subsequent page for figure).

Figure 3-8: Immunohistochemical staining with anti-α-CaMKII antibody shows α-CaMKII protein is absent in cells where fα-CaMKII deletion has occurred in CA1 α-CaMKII KO mice

Confocal images from 3 month old floxed (left column) and CA1 α-CaMKII KO (right column) mice following immunohistochemical staining with anti-α-CaMKII antibody. Lower magnification sagital image on top, followed in descending order by high magnification images of hippocampal CA1, CA3 and dentate gyrus (DG). Fluorescent green labeling indicates α-CaMKII protein. In the CA1 region, protein is absent from the vast majority of pyramidal cells. No protein is absent from CA3 pyramidal cells. Scattered dentate granule cells are not stained; correspondingly, there is decreased staining in mossy fiber axons from these cells that project to the stratum lucidum of CA3. sp, stratum pyramidale; sr, stratum radiatum; so, stratum oriens; sl, stratum lucidum; sl-m., stratum lacunosum-moleculare; GL, granule layer; ML, molecular layer.


fα-CaMKII
CA1 α-CaMKII KO
CA1
CA1
so
sp
sr
sl-m
CA3
CA3
so
sp
sl
sr
sl-m
DG
DG
ML
GL
GL
ML

In 3 month old CA1 α-CaMKII KO mice, α-CaMKII protein was visible in only a few scattered CA1 pyramidal cells. α-CaMKII protein is normally found in the cell bodies, dendrites and axons of hippocampal pyramidal cells. Consequently, we saw a dramatic reduction in staining in the CA1 cell body layer (stratum pyramidale), and a moderate reduction in dendritic layers (stratum radiatum and stratum oriens). Residual staining in these dendritic layers was from CA3 Schaffer collateral axons projecting to CA1 pyramidal cells. No CA3 pyramidal cells showed a loss of α-CaMKII protein. A small reduction in staining was detectable in the stratum lucidum of CA3 due to the increasing deletion of the *f*α-CaMKII gene in dentate granule cells, which project their mossy fiber axons to this region. Interestingly, α-CaMKII protein was absent from perforant path axons, indicating that the *f*α-CaMKII gene was deleted in entorhinal cortex. Consistent with this, staining is dramatically reduced in the strata lacunosum and moleculare of CA1, with residual staining in corresponding layers of CA3 and dentate gyrus reflecting dendritic proteins from pyramidal cells and granule cells, respectively. α-CaMKII staining was also substantially reduced in cortex, although much less reduced in layer 4. Neither immunohistochemical nor *in situ* hybridization studies revealed any gross anatomical abnormalities in CA1 α-CaMKII KO.

Because the CW2 Cre transgene contains an additional 'copy' of the α-CaMKII promoter, we were concerned that it may compete with the endogenous α-CaMKII gene for limited transcription factors/regulatory proteins. This could cause a decrease in endogenous α-CaMKII in all cells that express the transgene. To address this issue, we performed *in situ* hybridization on fixed brain sections from (+/+, Cre/+) CW2 mice using an α-CaMKII specific cRNA probe (Figure 3-9). Endogenous α-CaMKII expression was not affected in CW2 mice.

While the α isoform is by far the most abundant neuronal CaMKII isoform, the highly homologous β isoform of CaMKII is also expressed in the brain, with moderate levels of expression in the hippocampus, cortex, striatum, and cerebellum (Burgin *et al.,* 1990).

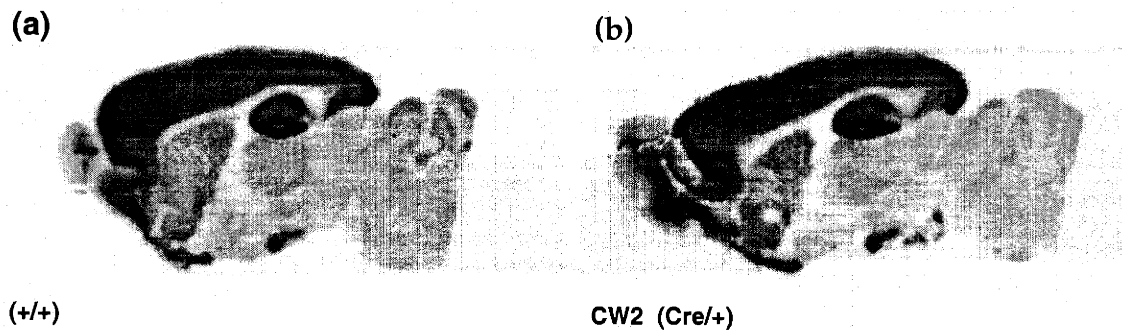


Figure 3-9: α-CaMKII expression is not affected by the presence of the CW2 Cre transgene

Scanned images of sagital sections from wild-type (a) and CW2 (Cre/+) (b) mice following *in situ* hybridization with an α-CaMKII exon 2 probe.

There are differences in the cell/tissue distribution of the α and β isoforms, and some differences in their calmodulin affinity and intracellular targeting, yet it is unclear if they have unique substrates (Braun and Schulman, 1995). It is possible that up-regulation of β-CaMKII could occur to compensate the absence of the α-isoform in α-CaMKII KO mice. To check if expression of the β-CaMKII isoform was up-regulated following recombination in CA1 α-CaMKII KO mice, we performed *in situ* hybridization on fixed brain sections from mutants and wild-type mice using a probe specific for exon 2 of β-CaMKII (Figure 3-10). No change in β-CaMKII expression was detected in CA1 α-CaMKII KO mice. To confirm that β-CaMKII protein is not upregulated in CA1 α-CaMKII KO mice, we performed immunohistochemistry using an anti-mouse β–CaMKII monoclonal antibody and saw no difference in expression levels and protein localization compared with controls. Antibodies specific for the α-CaMKII substrates synapsin I and Glur1 were also used to look for changes in substrate distribution following α-CaMKII KO, and no differences were observed (data not shown).

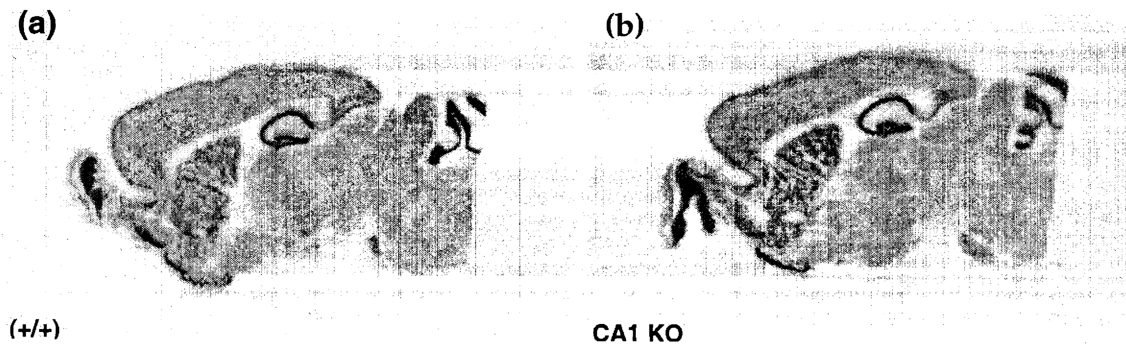


Figure 3-10: β-CaMKII expression is not affected by α-CaMKII deletion in CA1 α-CaMKII KO mice

Scanned images of sagital sections from wild-type (a) and CA1 α-CaMKII KO (b) mice following *in situ* hybridization with a probe specific for exon 2 of the β-CaMKII gene. β-CaMKII mRNA is highly localized to cell bodies, while α-CaMKII mRNA is also present in dendritic processes. Consequently, the hybridization signal for β-CaMKII is more punctate while the signal for α-CaMKII mRNA is more diffuse.

General behaviors of CA1 α-CaMKII KO mice

CA1 α-CaMKII KO mice are viable and their mean survival is similar to littermate control mice; the oldest CA1 α–CaMKII KO mouse in our colony is almost one year old. A small number of motor seizures were observed in the CA1 α-CaMKII KO colony (6 mice out of 430). While this may not be statistically significant, it is worth noting since the global α-CaMKII knockout mouse was susceptible to seizure [119]. We were unable to evoke motor seizures using auditory [195] or vestibular [196] stimulation.

CA1 α-CaMKII KO mice mate and care for their litters, although female mutants may have decreased fertility with age. We did observe germline deletion of the floxed allele in the pups of (*f*α-CaMKII /+, *Cre* /+) males or (*f*α-CaMKII/*f*α-CaMKII, *Cre*/+) males. To clarify, recombination appears to be occurring in the sperm of *Cre*/+ males, such that their progeny are at risk for inheriting a fully recombined allele and essentially, inheriting a "straight" heterozygous knock-out genotype. The frequency of germline deletion increased with the age of the male, to near 90-100% in (*f*α-CaMKII/*f*α-CaMKII, *Cre*/+) males. It is known that α-CaMKII is expressed during spermatogenesis [197].

Correspondingly, the CW2 Cre gene may be expressed in sperm, since it is driven by the α-CaMKII promoter. The endogenous α-CaMKII locus would also be decondensed during spermatogenesis because it is transcriptionally active, and likely more susceptible to Cre recombination. Because of the high frequency of germline deletion in (*f*α-CaMKII/*f*α-CaMKII, *Cre*/+) males, we tried to use (*f*α-CaMKII/*f*α-CaMKII, *Cre*/+) females, rather than males, as much as possible for breeding purposes. We did not perform electrophysiological or behavioral analyses on female mice to avoid the complicating effects of the estrus cycle on plasticity [198,199] and behavior [200].

CA1 α-CaMKII KO mice behaved unusually both in their home cage and during routine handling. They were very active in the home cage and were extremely "jumpy" and easily startled during routine handling. At times, they were difficult to catch, reflexively recoiling from human touch and occasionally jumping out of the home cage. Hippocampal lesions have been shown to cause hyperactivity in rats [201]. Activity levels of CA1 α-CaMKII KO male mice were monitored using an automated open field system for a 30 minute trial (mid-light cycle). In the Digiscan open field system, photocell beam breaks are used to monitor horizontal and vertical movements, and total distance traveled can be assessed. As expected, we saw a dramatic difference in the total activity of CA1 α-CaMKII KO mice during this period, with mutants covering nearly 100% more distance than controls ($p > 0.001$, t-test) (Figure 3-11a). This is comparable to the hyperactivity observed in the global α-CaMKII mice [110]. CA1 α-CaMKII KO hyperactivity was not associated with a difference in weight when compared with controls ($p > 0.05$ at 2, 3, and 4 months of age, t-tests). It seems likely that the mutant mice eat more food to sustain their increased activity level, although exact food intake was not quantitatively monitored. Interestingly, CA1 α-CaMKII KO mice also spent about 50% less time in the center of the open field compartment compared with controls ($p < 0.0002$, t-test) (Figure 3-11b). Mice tend to avoid the center of an exposed, open field environment, possibly as a protective measure to avoid being spotted by a

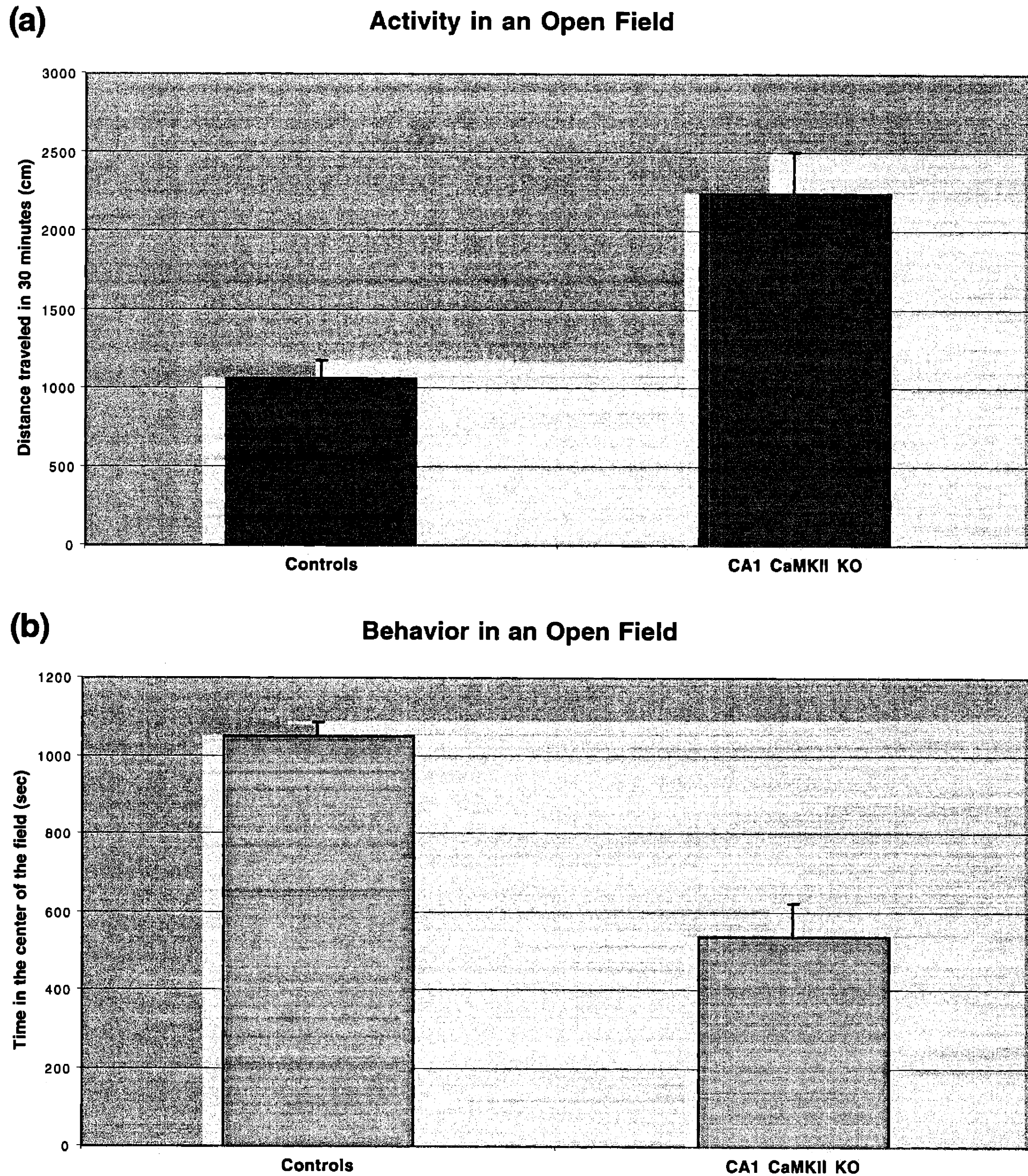


Figure 3-11: CA1 α-CaMKII KO mice are hyperactive in an open field environment

(a) Naïve CA1 α-CaMKII KO (n = 12) and littermate control (n = 10) male mice were exposed to an open field environment and their total activity was monitored for 30 min. CA1 KO mice were hyperactive (2241 ± 116cm) compared with controls (1065 ± 261cm; $p < 0.001$). Error bars represent SEM.
(b) CA1 α-CaMKII KO (n = 7) mice also spend significantly less time in the center of the open field compartment (536.4 ± 87.6 sec) compared with control littermates (n = 7) (1050.5 ± 36.6 sec; $p < 0.0002$). Error bars represent SEM.

predator [202]. Increased thigmotaxis (movement at the perimeter) could indicate a higher level of anxiety in CA1 α-CaMKII KO mice, although other factors contributing to their hyperactivity could be involved.

Synaptic transmission and plasticity at CA3-CA1 synapses

To examine the role of post-synaptic CaMKII in CA3-CA1 synaptic transmission and plasticity, we performed extracellular field recordings at CA3 Schaffer collateral – CA1 synapses in acute hippocampal slices from CA1 α-CaMKII KO mice. We induced LTP using 100Hz tetanic stimulation of Schaffer collateral axons (Figure 3-12).

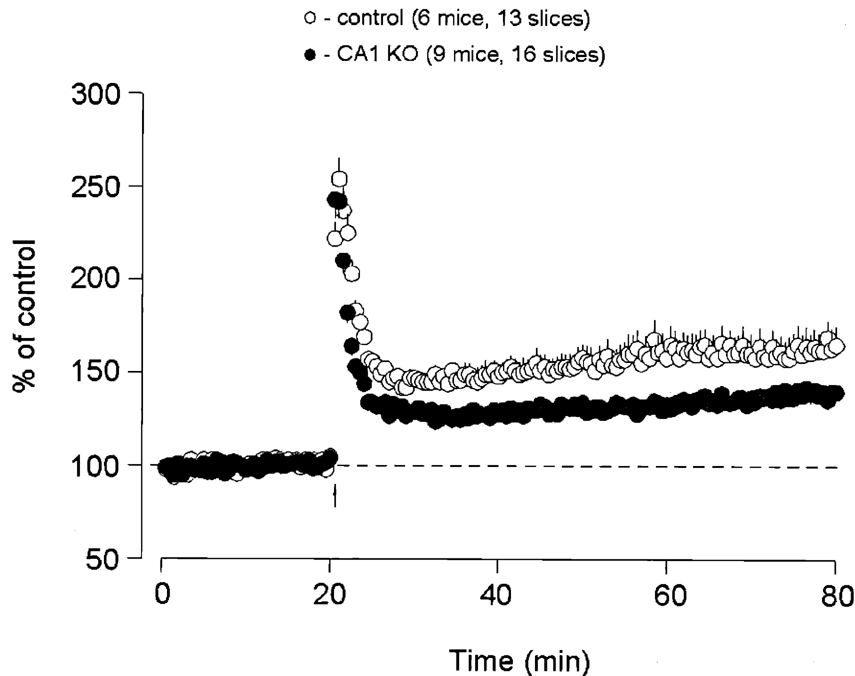


Figure 3-12: Tetanus induced LTP at CA3-CA1 synapses is deficient in CA1 α-CaMKII KO

Extracellular field recordings were used to sample EPSPs every 30 sec and a stable baseline was recorded for 20 min. LTP was induced by two stimulus trains (100Hz for 1 second) spaced 20 sec apart. Amplitudes of EPSPs were normalized to baseline values and are plotted versus time (min). CA1 α-CaMKII KO mice show reduced potentiation at 60 min post-tetanus (139.6 ± 4.6%; n = 16) compared with controls (163.4 ± 10.1%; n = 13; $p < 0.04$, t-test). All potentiation values are shown relative to a 100% baseline value. Mean values are shown and error bars are ± SEM. Arrow indicates time of tetanus.

LTP was clearly reduced in CA1 α-CaMKII KO mice (139.6 ± 4.6%) compared with littermate control mice (163.4 ± 10.1%; $p < 0.04$, t-test). This LTP deficiency is reminiscent of earlier studies of the global α-CaMKII KO, as described in Chapter 2 [137]. LTP was generally not absent in hippocampal slices from CA1 α-CaMKII KO mice, but was present in reduced levels, clustered around a shifted mean value (Figure 3-13).

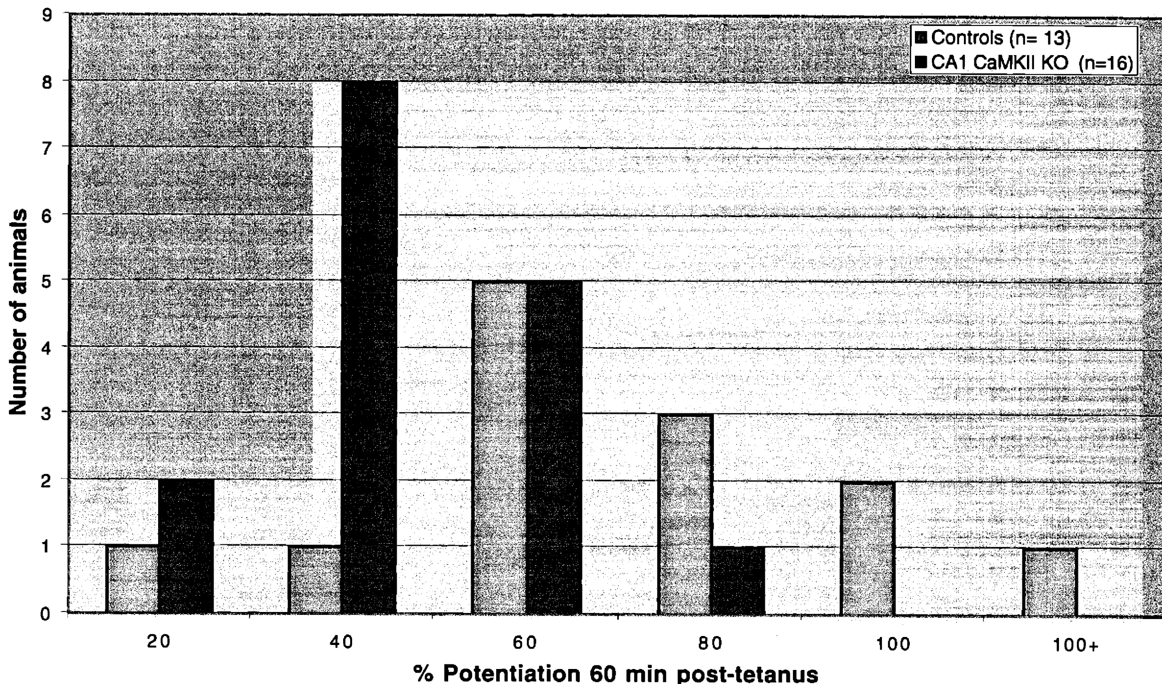


Figure 3-13: Potentiation in CA1 α-CaMKII KO and control mice follows a normal distribution

Histograms depicting the distribution of slices with the indicated amount of potentiation (bins of 20%) 60 min post-tetanus in mutant and control slices. % Potentiation indicates the % change over baseline vales.

It is possible that differences in baseline synaptic transmission parameters could be contributing to the LTP deficit in CA1 α-CaMKII KO mice and/or could reflect changes in parallel pathways. One basic method for assessing synaptic transmission is to examine the strength of post-synaptic responses (output) following comparable strengths of pre-

synaptic stimulation (input). A decrease in the slope of input/output curves was observed in global α-CaMKII KO mice [137]. We plotted input/output curves for CA1 α-CaMKII KO and control mice after stimulating over a range of stimulus intensities (Figure 3-14).

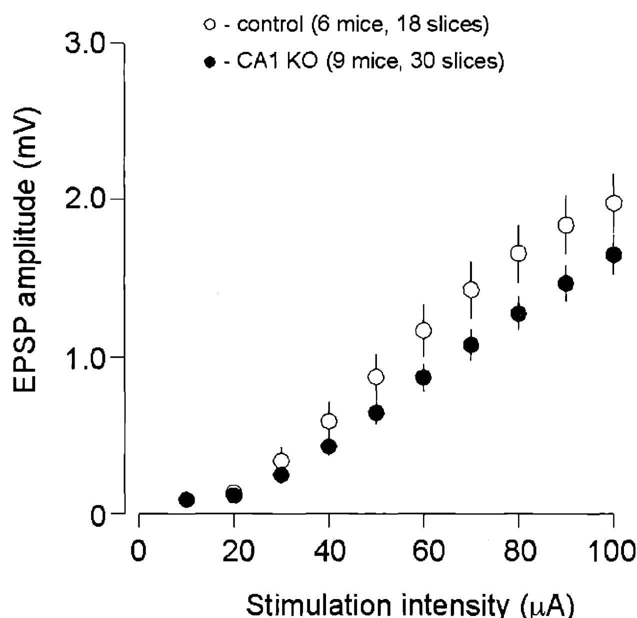


Figure 3-14: Input/output curves in CA1 α-CaMKII KO mice are reduced compared with control mice

The strength of presynaptic stimulation (input) was varied from 10-100μA in 10μA steps and excitatory post-synaptic potentials (EPSPs) were measured extracellularly (output). CA1 α-CaMKII KO (n = 24) show reduced input/output curves for stimuli greater than 40μA compared with controls (n = 12; $p < 0.004$, $F_{(1,46)}$ two-way ANOVA). Mean values for each genotype are shown and error bars are ± SEM.

Input/output curves from CA1 α-CaMKII KO mice (n = 22) were significantly reduced compared with control littermates (n = 12; $p < 0.004$, $F_{(1,46)}$ two-way ANOVA).

Post-tetanic potentiation (PTP) is a short term form of potentiation independent of post-synaptic NMDA receptor function [76]. This pre-synaptic potentiation is thought to be dependent upon mobilizing vesicles from the reserve pool, and requires high frequency stimulus for sufficient calcium loading of pre-synaptic terminals. We would not expect

that a specific deletion of α-CaMKII in the CA1 post-synaptic cell would affect pre-synaptic plasticity at the CA3-CA1 synapse. To confirm this, we stimulated CA3-CA1 synapses at 100Hz for 1 second in the presence of D-APV and saw no difference in the amplitude or time course of PTP in CA1 α-CaMKII KO mice (158 ± 6.8%) compared with controls (150 ± 4.8%; p = 0.41) (Figure 3-15).

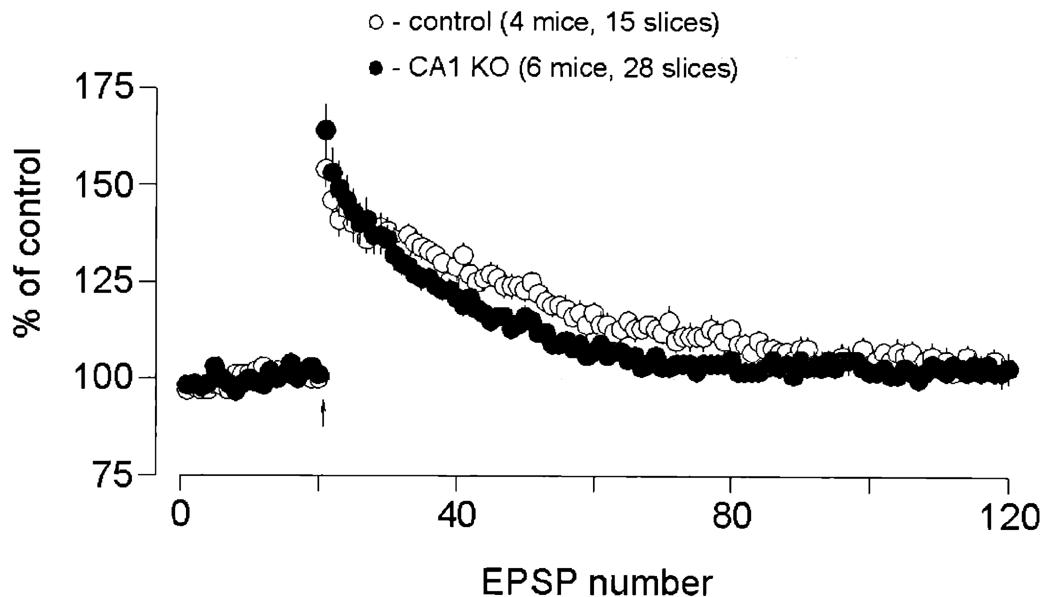


Figure 3-15: PTP is normal in CA1 α-CaMKII KO mice

PTP was induced by a 1 sec 100Hz stimulus train and 100 EPSPs were collected at 0.33Hz. Amplitudes of EPSPs were plotted over time, normalized to the size of baseline responses. Maximum value of PTP was normal in CA1 α-CaMKII KO mice (158 ± 6.8%; n = 28 slices) compared with controls (150 ± 4.8%; n = 15 slices; p = 0.41, t-test). Mean values are shown and error bars are ± SEM. Arrow indicates time of stimulus train.

PTP was enhanced [117] in studies of the global α-CaMKII KO mouse, suggesting that pre-synaptic CaMKII may play a role in pre-synaptic plasticity mechanisms. Normal PTP in CA1 α-CaMKII KO mice is indicative of the specificity of the post-synaptic CaMKII mutation.

Fear conditioning in CA1 α-CaMKII KO mice

As we are interested in the relationship between hippocampal synaptic plasticity mechanisms and memory formation, we examined CA1 α-CaMKII KO mice using a Pavlovian fear conditioning paradigm that is sensitive to hippocampal lesions [203-205]. For this task, a mouse is placed into a novel context (A) and after a brief exploration period, a conditioned stimulus (CS = tone) is paired with an aversive unconditional stimulus (US = foot shock). This single training event leads to a long lasting memory and an associated fear response when the mouse is re-exposed to either the tone (cue) or the conditioning context (A) (Figure 3-16).

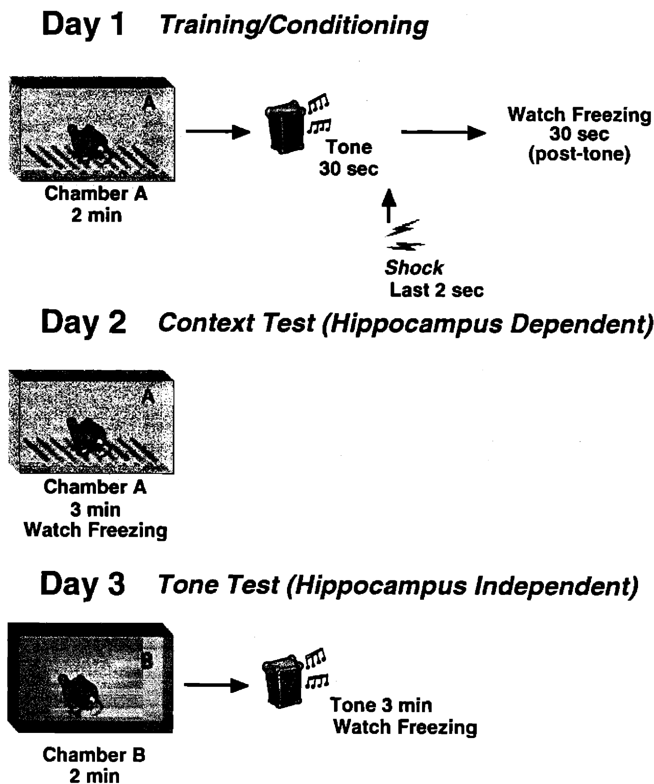


Figure 3-16: Contextual and cued fear conditioning

Naïve mice are placed in a novel context A and are exposed to a tone paired with a foot-shock (training). Mice are returned to their home cage after a brief observation period. To test hippocampus dependent memory of the associated context, mice are re-exposed to context A and freezing behavior is assessed. To test hippocampus independent memory of the associated tonal cue, mice are exposed to a different context B, the tone is re-played, and freezing behavior is assessed.

Fear is quantitated by measuring the proportion of time the mouse spends "frozen" following the appropriate conditioned stimulus. Freezing, defined as no movement except for respiration, is an adaptive defense reaction and is thought to be an expression of fear [206,207]. Cued and contextual conditioning, as well as the freezing response itself, are dependent upon intact amygdala function [203,204]. However, context

dependent conditioning additionally requires intact hippocampal function [203-205].

We trained CA1 α-CaMKII KO and control male mice by placing them into a novel context (A) and pairing a single tone cue with a footshock (2 sec, 0.27mA). Mice were returned to the novel context 1 day and 7 days after training (context tests) and freezing was monitored (Figure 3-17).

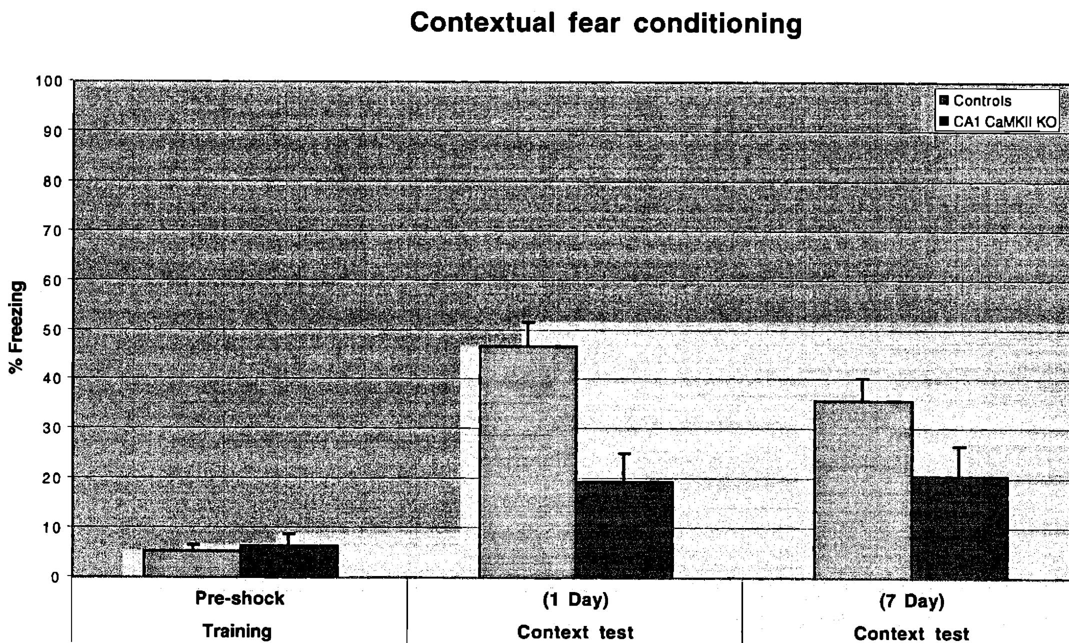


Figure 3-17: Contextual fear conditioning is abnormal in CA1 α-CaMKII KO mice

CA1 α-CaMKII KO mice (n = 25) and littermate controls (n = 17) were trained using a single tone/shock pairing (2 sec, 0.27mA) in a novel context. Pre-shock background freezing levels are shown. 1 day after training, CA1 α-CaMKII KO mice showed reduced freezing to context (19 ± 5.9%) compared with controls (47 ± 4.7%; p = 0.0017). 7 days after training, freezing was still reduced in mutants (20.6 ± 6%; n = 21) compared with controls (36 ± 5.5%; n = 16), but this difference just exceeded statistical significance (p < 0.06, t-test). % freezing mean values are shown ± SEM.

CA1 α-CaMKII KO mice froze significantly less than control mice during a 3 minute context test 1 day after training. While control mice remembered the context as a potentially unpleasant place and froze for 47 ± 4.7% of the 3 minute testing period, CA1

mutant mice froze for only 19 ± 5.9% of the time ($p = 0.0017$, t-test). When tested for memory of the conditioned context 7 days after training, CA1 α-CaMKII KO mice froze less (20.6 ± 6%) than controls (36 ± 5.5%), but this difference was just above statistical significance ($p < 0.06$, t-test). These data suggest that CA1 α-CaMKII KO mice have a deficiency in hippocampal-dependent memory.

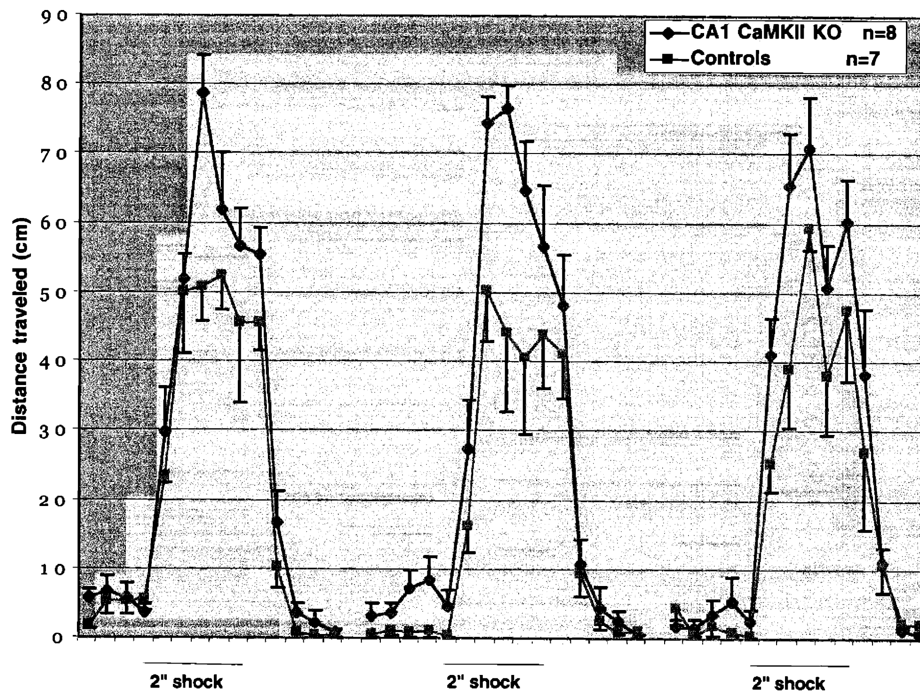


Figure 3-18: CA1 α-CaMKII KO mice are hypersensitive to footshock

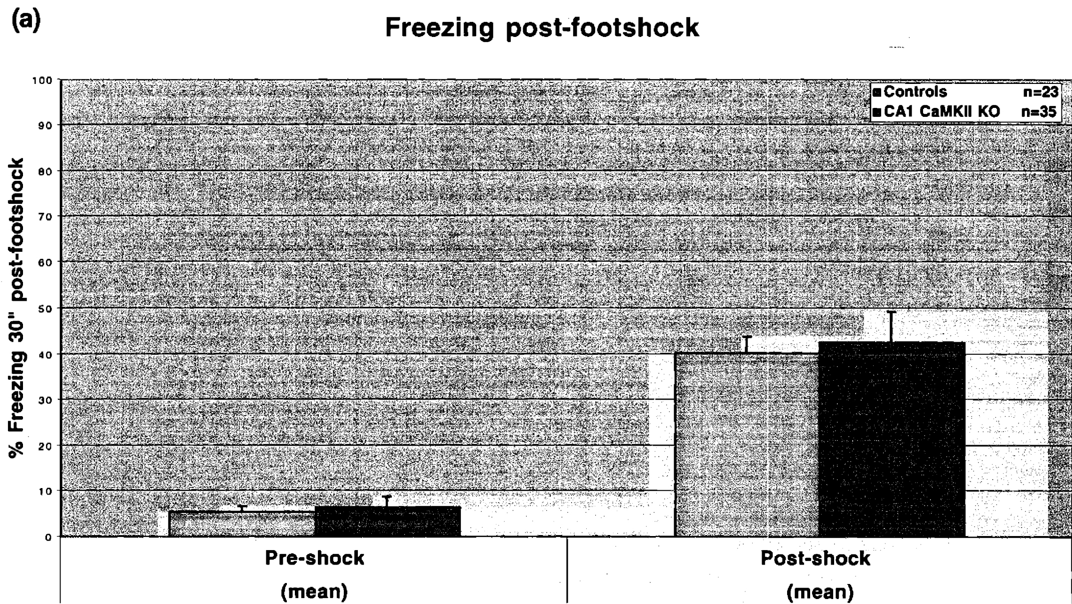
Footshocks (bars) of 0.27mA were presented 3 times, each for 2 seconds. The distance traveled during the 2 seconds before, during and after the footshock was calculated. The distance traveled by CA1 α-CaMKII KO mice (n = 8) was significantly increased compared with control mice (n = 7; $p < 0.0001$, t-test). Mean values are shown ± SEM.

CA1 α-CaMKII KO mice have decreased CaMKII activity in multiple areas of the brain including the amygdala, which is known to be essential for normal fear responses and conditioning [203,204]. To test the specificity of the hippocampal learning deficit and to examine if amygdala deficits might be contributing to the abnormal conditioning we observed in CA1 α-CaMKII KO mice, we examined several other behaviors that are

essential for context conditioning, including (a) shock sensitivity, (b) freezing response to shock, and (c) tone dependent fear conditioning. Since decreased memory for a conditioned context-shock association could reflect decreased sensitivity to footshock, we measured the distance traveled during and immediate after footshocks were applied as an indicator of shock sensitivity (Figure 3-18). CA1 α-CaMKII KO mice responded dramatically to footshock, traveling a significantly greater distance both during and immediately after footshocks were given ($p < 0.0001$, t-test). While this hypersensitivity to shock is abnormal, it does indicate that CA1 α-CaMKII KO mice sense footshock and respond significantly to it.

We observed the behavior of CA1 α-CaMKII KO mice immediately after a footshock to determine if a normal freezing response to a fearful situation was intact. Mice were trained using 1 tone-shock pairing in a novel context (Figure 3-16) and both mutant and control mice froze at comparable levels (mean) during the 30 seconds immediately following the shock (Figure 3-19a). This suggested that CA1 α-CaMKII KO mice could freeze in response to fearful stimuli, despite the absence of α-CaMKII in the amygdala. Global α-CaMKII knockout mice did not freeze comparable to controls immediately following footshocks [120]. However, closer examination revealed a difference in the total freezing distribution between CA1 α-CaMKII KO mice and controls. While both types of mice were frozen for about 40% (mean) of the time during the 30 sec following the shock, control mice showed a unimodal distribution around this mean value, while mutant mice freezing levels fell into a bimodal distribution (Figure 3-19b). All controls tended to freeze at moderate amounts (median = 44.4%, n = 23), but mutants either froze very little or froze a dramatic 90-100% of the time (median = 22.2%, n = 35; or two modes: 16.1%, n = 24; 99.9%, n = 11). The control distribution did not overlap at all with the high mode of the mutant distribution. This distribution could reflect two alternative strategies chosen by fearful mutants – to freeze completely or to flee – or could reflect other widespread deficits in CA1 α-CaMKII KO mice that confound a

(a)



(b)

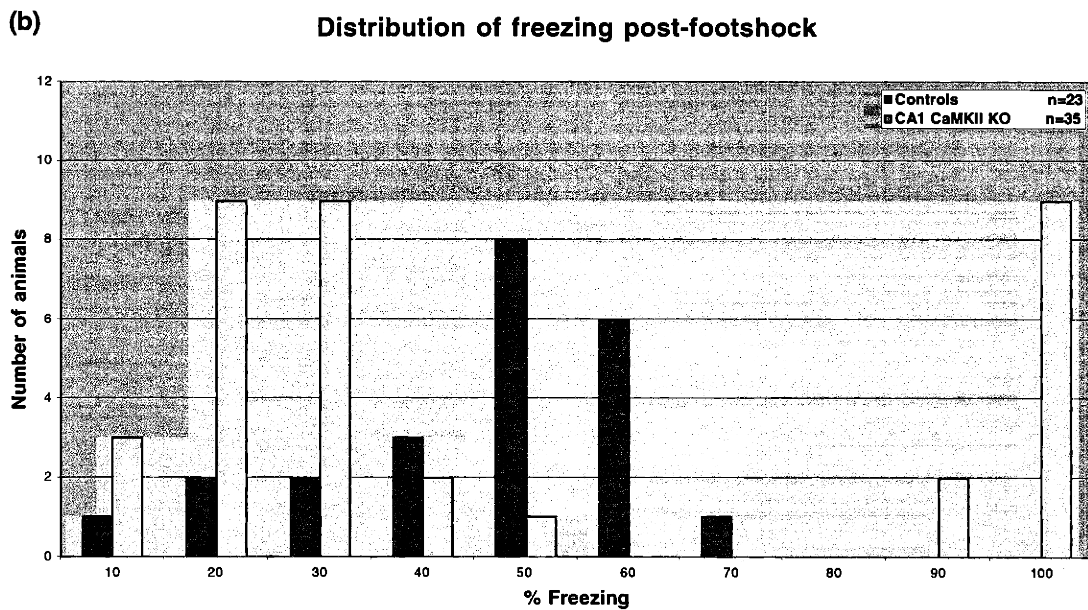


Figure 3-19: CA1 α-CaMKII KO mice have an abnormal freezing response following a fear provoking footshock

(a) CA1 α-CaMKII KO mice (n = 35) and controls (n = 23) were placed in a novel chamber and allowed to explore for 2 min before a 30 sec white noise tone was delivered. A 2 sec footshock (0.27mA) was delivered during the last 2 sec of the tone, and freezing activity was monitored for 30 additional seconds. CA1 α-CaMKII KO and controls did not show a significant difference in mean % freezing post-shock (p = 0.779, t-test). Mean values are shown ± SEM.

(b) Mice were binned according to % freezing (10% interval bins) and their distributions were plotted. During the 30 sec post-shock period, CA1 KO mice (n = 35, median = 22.2%) showed a much wider distribution than littermate controls (n = 23, median = 44.4%). CA1 KO mice are most accurately described by a bimodal distribution of mice falling into a group of low freezers (median 16%, n = 24) and a group of super freezers (median 99.9%, n = 11).

normal freezing response in a bimodal pattern. It should be noted that CA1 α-CaMKII KO mice also showed dramatically less median freezing behavior (5.55% median) during the 24 hour context test compared with control mice (39% median).

Finally, we examined CA1 α-CaMKII KO mice in the cued fear conditioning task. Since cued conditioning is not dependent upon the hippocampus [203,204], it should remain intact if the contextual conditioning deficit is specific. After training, mice were placed into a new unfamiliar context (B) that should not provoke an associated fear response, and were allowed to explore for 2 minutes. Then the conditioned tone was played for 3 minutes and freezing behavior was assessed. While mutants did appear to freeze (40 ± 5.6%) in response to the conditioned tone on the 2 day tone-test, this freezing was not significantly more than pre-tone levels (27.9 ± 6.3%; $p > 0.15$, t-test) (Figure 3-20). A moderate level of background freezing was observed before the tone in both control (18.1 ± 3.1%) and mutant (27.9 ± 6.3%) mice, but control mice showed significantly greater freezing when exposed to the conditioned tone ($p < 0.0000003$, t-test). Some mutant mice appeared to have particularly poor context discrimination and consequently, particularly high levels of freezing were observed during the pre-tone period in context B. These data suggest that CA1 α-CaMKII KO mice may have difficulty with both hippocampal and non-hippocampal dependent versions of the fear conditioning task, as well as with the basic freezing response. This could reflect a CaMKII deficit in the amygdala [182,203,204]. While this is interesting, it makes it difficult to assess hippocampus-specific memory function in CA1 α-CaMKII KO mice using this task. In retrospect, this is not surprising, since the α-CaMKII deletion is not restricted to the hippocampus, and other behavioral abnormalities, such as hyperactivity and seizure

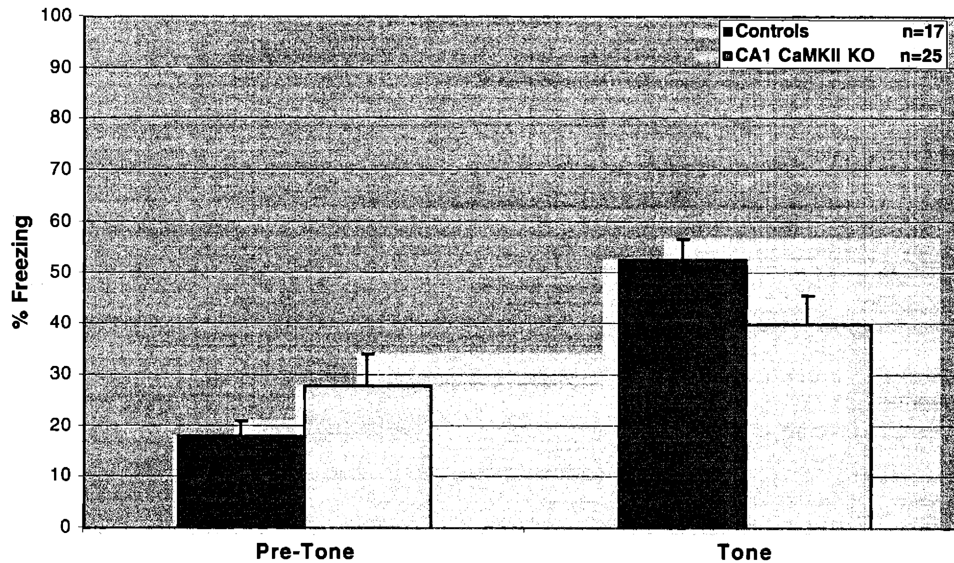


Figure 3-20: Cued dependent fear conditioning is abnormal in CA1 α-CaMKII KO mice

CA1 α-CaMKII KO mice (n = 25) and littermate controls (n = 17) were trained using a single tone/shock pairing (2 sec, 0.27mA) in a novel context. 2 days after training, mice were introduced to a new context, were allowed to explore for 2 min, and the conditioning tone was played for 3 additional min. CA1 α-CaMKII KO mice did not show significantly more freezing in response to the tone ($p > 0.15$, t-test). % freezing mean values are shown ± SEM.

susceptibility could also be confounding. However, there is some indication that a memory impairment exists (Figure 3-17), so it would be interesting to explore the behavior of CA1 α-CaMKII KO mice in other learning tasks that are less effected by hyperactivity or abnormal amygdala dependent emotional responses.

Discussion

We have examined the role of CaMKII in the brain using Cre/loxP conditional gene knockout of the dominant α-CaMKII isoform. By restricting gene knockout to CA1 pyramidal cells at the CA3-CA1 hippocampal synapse, we could address the role of

post-synaptic CaMKII in synaptic plasticity mechanisms and associated behaviors. LTP induced by tetanic stimulation was deficient at the CA3-CA1 synapse in CA1 α-CaMKII KO mice, confirming that post-synaptic CaMKII plays a role in LTP. This partial LTP deficiency was strikingly reminiscent of the reduction in LTP observed in the global α-CaMKII knockout mouse [137], re-examined in Chapter 2. Similarly, we observed a reduction in input/output curves in CA1 α-CaMKII KO mice, suggesting post-synaptic cell responsivity might be altered in α-CaMKII deficient mice. While this could impair induction mechanisms by reducing post-synaptic depolarization in response to transmitter, it could also reflect a deficiency in putative plasticity expression mechanisms, such as in the delivery of AMPA receptors to potentiated synapses [66] or GluR1 phosphorylation [65]. Normal pre-synaptic plasticity mechanisms (PTP) in CA1 α-CaMKII KO mice confirm the specificity of the mutation on post-synaptic aspects of long term plasticity pathways. It is still possible that pre-synaptic CaMKII contributes to LTP expression mechanisms, and that other CaMKII isoforms may be functioning post-synaptically to maintain the residual levels of long term potentiation observed in CA1 α-CaMKII KO mice.

Using the Cre transgenic mouse CW-2, we had anticipated that we would obtain gene knockout only in the hippocampal CA1 region of the brain when crossed to *f*α-CaMKII mice. Previous studies of CW-2 crossed to a lacZ reporter line (Figure 3-2) and of similar transgenic lines crossed to *f*NR1 mice [123], suggested that such serendipitous specific gene knockout could be obtained, at least during younger adult ages (A. Kato, unpublished observations). However, gene deletion was widespread throughout the cortex, amygdala, dentate gyrus and subiculum, although CA3 was spared, in *f*α-CaMKII mice crossed to CW-2 transgenics (CA1 α-CaMKII KO). As Cre-mediated gene targeting is subject to significant positional effects [187], it is possible the lacZ reporter line floxed locus [191] was less susceptible to recombination, giving a much more limited pattern of Cre recombination. We have some suspicion that the distance between loxP

sites may also contribute to the frequency of recombination, as the relatively restricted fNR1 knockout allele had over 12kb separation between loxP sites [23,123], compared with the 3.3kb separation in the fα-CaMKII line. It is still unclear why recombination occurs preferentially in certain brain subregions compared with others (*e.g.* CA1 vs. CA3), although there is an indication that variable levels of Cre activity could contribute [123].

While widespread gene deletion in other cortical regions complicates the correlative interpretation of behavior changes in CA1 α-CaMKII KO mice, conditional knockout still offers vast improvements compared to global knockout approaches. α-CaMKII is normally expressed postnatally at low levels on day 4, but increases dramatically (10 fold) by day 16 to near adult levels [37]. This period of early development is critical for the formation of synaptic connections, and there is some evidence that CaMKII is important for neurite outgrowth and growth cone motility [208-210] and for stabilizing and regulating the growth of dendritic arbors [211,212]. Cre/loxP deletion did not occur until after 3 weeks in CA1 α-CaMKII KO mice, with dramatic knockout only visible in the hippocampus by 4 weeks of age. This likely avoided most if not all of the developmental effects of disrupting CaMKII levels during early stages of neuronal development and synaptogenesis. There was also significant spatial restriction of gene knockout, with dramatic knockout in hippocampal and cortical regions, and negligible deletion in thalamus, striatum, cerebellum and midbrain nuclei. Clearly the temporal and spatial restriction of Cre recombination will avoid some of the confounding effects of abnormal development, early initiated compensatory mechanisms, and ubiquitous gene knockout. While deletion was not restricted solely to CA1 in the "CA1" α-CaMKII KO, it was still possible to study the specific effects of post-synaptic deletion of α-CaMKII on CA3-CA1 physiology since CA3 α-CaMKII expression was intact. This was a significant advance over previous global deletion studies, and particularly useful, as the LTP field has invested a tremendous effort in understanding the LTP mechanism specifically at the model CA3-CA1 synapse.

Original analyses of global α-CaMKII KO mice showed dramatically decreased or absent CA3-CA1 LTP in the majority of slices/cells [111,118], with some potentiation in a small subset of experiments. This bimodal distribution suggested that basic LTP mechanisms were functioning in global α-CaMKII mutants, but that α-CaMKII was critical for regulating LTP production. Of course, it was possible that compensatory mechanisms involving the β-CaMKII isoform or other kinases, could substitute in the instances where mutant mice demonstrated LTP. Later experiments of the global knockout in an inbred mixed genetic background showed that LTP was generally present in mutant slices, but in a significantly reduced amount [137]. This confirmed that α-CaMKII alone was not essential for LTP production, and that other modifying genes likely clustered in this new genetic background to partially compensate for the absence of α-CaMKII. Interestingly, our conditional deletion of α-CaMKII in post-synaptic CA1 showed a similar downward shift in the magnitude of LTP. This phenotype may directly reflect the deficiency in post-synaptic CaMKII function, since neuronal development should be intact and early compensatory mechanisms less significant in the conditional knockout mice. CA1 α-CaMKII KO mice were produced in a pure C57Bl/6 genetic background, which is optimal for LTP analyses [193] and learning and memory studies [165,192], and will become a new standard to avoid the complication of variable genetic backgrounds. It is possible that the inbred mixed genetic background global knockout examined in Chapter 2 shared more of these C57Bl/6 alleles, as several C57Bl/6 backcrosses were performed before brother/sister mating was established in this line.

Our studies confirm that α-CaMKII plays a role in the regulation of LTP in the post-synaptic cell. Residual LTP present in knockout mice may reflect contributions from the β isoform. It would be interesting to examine CA3-CA1 LTP in α-CaMKII and β-CaMKII double conditional knockout mice to see if post-synaptic CaMKII is absolutely required for LTP *in vivo.* Contrary to early models of CaMKII as a putative long lasting "memory molecule" [138], recent studies suggest that post-synaptic CaMKII may be involved in the

early stages of LTP production (induction/expression) but not in the maintenance of LTP [213]. However this question is open for further study, as is the role of CaMKII in LTP expression mechanisms in the pre-synaptic terminal (see Chapter 4). It is possible that LTP expression may involve activation of pre-synaptic CaMKII mechanisms either in parallel to post-synaptic CaMKII pathways, or directly downstream of post-synaptic CaMKII. For example, CaMKII is known to phosphorylate nitric oxide synthase (NOS), leading to the production of nitric oxide (NO), a putative, though controversial, retrograde messenger [70].

One of the advantages of using mouse genetics to dissect biochemical pathways is that the analysis of genetically modified animals can be performed at several levels of complexity. For example, *in vitro* slice physiology can be used to study plasticity mechanisms directly, and whole animal behavioral studies can be performed to examine associated changes in learning and memory. CA1 α-CaMKII KO mice demonstrated several abnormal behaviors, including some thought to be hippocampal dependent. However, the widespread knockout of α-CaMKII in cortex, amygdala, and dentate gyrus, in addition to CA1, makes it difficult to correlate abnormal learning behavior with deficient hippocampal physiology. Similar to the global α-CaMKII knockout mouse [110], CA1 α-CaMKII KO mice are hyperactive both in their home cage and in an open field environment. Hippocampal lesions can cause hyperactivity [201]. While striatal function is also critical for the control of locomotor activity [214], α-CaMKII is not deleted in the striatum in CA1 α-CaMKII KO mice, although cortical-striatal inputs could be affected. Hyperactivity could be a confounding variable for mutant mice in the performance of behavioral tasks that rely upon control of movement. The hippocampus is also prone to seizure [215], and an increased seizure frequency was observed in both CA1 α-CaMKII KO and global knockout mice [119]. While hippocampal developmental malformation is less likely to be a cause of seizure activity in CA1 α-CaMKII KO mice due to the delayed onset of deletion, inhibition of α-CaMKII has recently been

associated with cellular hyperexcitability in cultured hippocampal neurons [216]. Since some forms of seizure activity have been shown to interfere with hippocampal LTP [217] and learning [218], this must be considered when examining mice susceptible to seizure. However, this does not preclude the study of plasticity and behavior in such mice. Identifying consistent, specific physiological and behavioral phenotypes in a background of increased seizure susceptibility is still possible. CA1 α-CaMKII KO mice show reduced levels of hippocampal LTP and decreased input/output curves, but normal PTP. This argues against broad, non-specific changes in cell excitability. Global knockouts of the pre-synaptic molecules synapsin I [196,219,220] and synapsin II [196] also show an increase in seizure propensity. However, hippocampal LTP was unaffected in these mice, which had abnormal short term plasticity. Paired pulse facilitation was enhanced and post tetanic potentiation was decreased in synapsin I and synapsin II, respectively [196]. Furthermore, synapsin II mutant mice showed a severe memory deficit in contextual fear conditioning (1 tone/shock pairing) while synapsin I mice performed normally in this hippocampal dependent task [221]. As alterations in calcium homeostasis have been implicated in epilepsy, it is interesting to note the highly expressed calcium dependent phosphatase calcineurin is also associated with seizures in a conditional knockout mouse [194]. However this "CA1 calcineurin KO" mouse had very specific deficits in CA3-CA1 plasticity and hippocampus dependent learning.

CA1 α-CaMKII KO mice were examined using the contextual and cued fear conditioning paradigms to see if α-CaMKII deletion, associated with CA3-CA1 LTP disruption, had specific effects on learning and memory formation. While hippocampal dependent contextual fear conditioning was dramatically impaired in CA1 α-CaMKII mutant mice, some confounding variables complicate interpretation. First, the hyperactivity and hypersensitivity to shock exhibited by these mice could have prevented them from exhibiting the typical freeze - fear response, and/or may reflect alternative strategies chosen by some mice to confront the fearful situation. For

example, while some mice exhibited an extreme freeze response after footshock, others froze little if at all, perhaps because they chose an alternative strategy and were trying to escape the fearful situation. Second, the CA1 α-CaMKII KO mice are not very regionally restricted in their gene knockout. While α CaMKII is absent only in CA1 at the CA3-CA1 synapse, it is also dramatically knocked out in the dentate gyrus (DG) of the hippocampus, multiple layers of the cortex, and multiple nuclei of the amygdala. The absence of CaMKII in these complementary regions could have contributed to the abnormal freezing behavior and deficient cued conditioning performance of this task [203,204]. Fear conditioning induces a lasting synaptic potentiation in the lateral nuclei of the amygdala, demonstrating a correlation of LTP with learning in this brain region [102,222]. Global α-CaMKII KO mice [120] and an α-CaMKII-Asp286 transgenic line with high expression in the amygdala [135] both showed impairments in contextual and cued conditioning. To analyze the specific affects of CA1 α-CaMKII KO on hippocampal dependent learning and memory, we should explore additional tasks that are less complicated by hyperactivity and abnormal fear related behaviors. However, to study the correlated relationship between CA3-CA1 plasticity and hippocampus dependent learning, additional Cre expressing transgenic mice should be made to enable the production of truly CA1 specific, or at least hippocampus specific, conditional CaMKII knockout mouse. It would also be interesting to explore the role of CaMKII in LTP in the amygdala, perhaps by conditional gene knockout, as an association between synaptic plasticity and conditioned learning in this region may prove to be enlightening.

Materials and Methods

Generation of conditional knock-out mice

A mouse C57Bl/6 BAC library (Genome systems) was screened using an EcoRI-Xma1 440bp fragment from the 5' end of the α-CaMKII cDNA [184]. A BAC was isolated that

contained the 5' end of the α-CaMKII genomic locus and it was mapped by restriction analysis to locate the position of the exon containing the ATP binding site, exon 2. Because of the small size of the exons (60-100bp, on average) and their wide spacing within the α-CaMKII locus, oligonucleotide probes specific for exon 2 and flanking exons were used for fine mapping studies. Two subclones were isolated that encompassed the region flanking exon 2: a 5' 6kb BamHI fragment and a 3' 9kb XhoI fragment that contained exon 2 and exon 3. These two subclones were combined to make the targeting construct and sequences were removed from the 5' and 3' ends to be used as external probes for screening: a 1.3kb BamHI-EcoRI fragment was removed for a 5' probe and a .4kb NcoI-XhoI fragment was removed for a 3' probe. Sequencing was performed in the regions flanking exon 2 to enable careful placement of loxP sites/FLNT cassette, avoiding nearby exons and critical splicing sequences. A loxP site was inserted into a BamHI site in intron 1 about 2.2kb upstream of exon 2, removing that site and creating a new SmaI site. The FLNT cassette was inserted into a SpeI site in intron 2 approximately 1kb downstream of exon 2, creating several new sites, including a BamHI site. The FLNT cassette consisted of a central pgk-neo gene and a hsv-tk gene, a pair of flanking loxP sites, and a pair of flanking FRT sites, as shown in Figure 3-2. The DT-A gene was inserted at the 3' end of the construct to select against nonhomologous recombinants. The targeting vector contained 5kb of homologous DNA upstream of the first loxP site (left arm) and 5kb of homologous DNA downstream of the FLNT cassette (right arm). 3.3kb separated the 3' loxP site and the FLNT cassette.

50μg of the targeting vector was linearized with SalI and was transfected into C57Bl/6 ES cells by electroporation (Biorad Gene Pulser set at 800V and 3μF). G418 selection was applied 24 hours after transfection, and G418 resistant colonies were isolated on days 5-8 of selection. 300 resistant colonies were screened for homologous recombination by Southern hybridization by digesting with BamHI and checking for loss of a BamHI site with the 5' probe and for the presence of a new BamHI site with the 3'

probe (Figure 3-1). 16% of clones were homologously recombinant. 6 of these successful recombinants were expanded and transfected again by electroporation with 50μg Flpe [189] to excise the neo-tk cassette. Gancyclovir selection was applied 24 hours after transfection, and gancyclovir resistant colonies were isolated on days 5-10 of selection. 48 resistant colonies were screened for removal of the neo-tk, and 52% showed successful Flpe mediated deletion. In these colonies, only a single loxP site and a single FRT site remained in intron 2, and a single loxP was still present in intron 1 (*f*α-CaMKII). 8 different ES cell clones were chosen for injection into blastocysts (Balb/c background). Blastocysts were transferred into psuedopregnant mothers and pups were examined for successful incorporation of the modified ES cells, indicated by black coat color/black eye color in a background of normal Balb/c white coat color/red eye color. We produced 37 such "chimeric" progeny in total from 6 of the 8 ES cell clones, and identified 5 chimeras (from 3 ES cell clones) that transmitted the floxed α-CaMKII allele (*f*α-CaMKII) to their progeny when crossed with C57Bl/6 breeders. Two of these lines were expanded and crossed with CW-2 Cre recombinase transgenic mice to produce conditional knockout mice, and one of these lines (line CF) was used for all studies described in this chapter.

Mice

Mating pairs of (*f*α-CaMKII/+, +/+) x (*f*α-CaMKII/+, *Cre* /+) mice were set up to produce (*f*α-CaMKII/ *f*α-CaMKII, *Cre*/+) mice (CA1 α-CaMKII KO) and three types of littermate controls: wild-type (+/+, +/+), CW2 (+/+, *Cre*/+), and homozygous *f*α-CaMKII (*f*α-CaMKII/*f*α-CaMKII). Mating pairs of (*f*α-CaMKII/*f*α-CaMKII, *Cre*/+) x (*f*α-CaMKII/*f*α-CaMKII, +/+) were also used to facilitate the expansion process. When available, female (*f*α-CaMKII/*f*α-CaMKII, *Cre*/+) mice were used in these mating pairs. Initial genotyping was done by Southern hybridization of tail DNA. Subsequently, PCR primers were designed that could discriminate amongst the wild-type, floxed, and deleted alleles to

aid in rapid typing and in identifying germline deleted pups. Genotypes were confirmed both before and after experimentation. Electrophysiology and behavior experiments were performed on male mice between the ages of 3-6 months. *f*α-CaMKII littermates were used as controls and all experiments were performed blind to genotype. All animal handling was performed in compliance with NIH and MIT guidelines.

In situ hybridization

Freshly dissected mouse brains were rapidly frozen on dry ice and were cut into 14μm sagital and coronal sections in a cryostat and mounted onto pre-coated glass slides. The sections were postfixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 minutes before treatment with 10μg/ml proteinase K at 37° for 30 min . Following a second fixation for 15 min in 4% PFA, sections were serially treated with 0.2M HCl for 10 min and 0.1M TEA/0.25% acetic anhydride for 10 min, with PBS washes in between. Following dehydration in ethanol, treated slides were hybridized at 55°C for 18 hr in hybridization buffer (50% formamide, 10mM Tris-HCl, pH 8.0, 200μg/ml tRNA, 1xDenhardt's solution, 10% dextran sulfate, 600mM NaCl, 0.25% SDS, 1mM EDTA) with $5x10^5$ cpm of ^{33}P-labeled cRNA probe (Riboprobe system kit, Promega). Probes used included a 550bp EcoRI fragment from the 3' untranslated region of α-CaMKII, and a 46bp oligonucleotide specific for the deleted exon 2 of α-CaMKII. Hybridized sections were washed at 55°C in a series of SSC buffers of increasing stringency and were treated with RNase A at 37°C for 30 min. After dehydration, slides were exposed to Biomax MR film (Kodak) for 1-3 days, and then were dipped in Kodak NTB3 nuclear emulsion and exposed for 2 weeks. X-ray film was scanned using an Epson Expression 836XL scanner, with mRNA signal indicated by dark grains. Alternatively, emulsion dipped slides were developed with D19 (4 minutes, 16°) and fixed (8 minutes, 16°). Dark field images were captured and digitized using a

Spot CCD camera and software system (SPOT Diagnostic Instruments, Inc.), with mRNA signal shown by white grains.

Immunohistochemistry

Mice were perfused transcardially with 4% PFA in PBS for 30 min. Brains were removed and post-fixed in 4% PFA overnight at 4°C. 50μm thick sagital vibratome sections were prepared in PBS, and were permeabilized with 50% ethanol/PBS for 30 min, and then incubated in 1% hydrogen peroxide/PBS to inactivate endogenous peroxidases. After blocking in 10% normal horse serum (NHS) / 0.5% blocking reagent (TSA kit, NEN) for 30 min, sections were incubated with primary antibodies overnight at 4° (anti-α-CaMKII pAB, 1:500, Boehringer/Chemicon; anti-β-CaMKII pAB, 1:100, Gibco; anti-GluR1 pAB, 2μg/ml, Chemicon) in 3% NHS/0.5% blocking reagent. The sections were then incubated with a biotinylated horse anti-mouse IgG (1:200, Vector) at room temperature for 2 hours, and then with streptavidin-biotin/horseradish peroxidase complex (Vector) for 30 min. Immunoreactivity was amplified by 5 min treatment with fluorescein isothiocyanate (FITC)-tyramide (NEN) at room temperature. Fluorescent images were captured using a confocal laser scanning microscope (BioRad).

Extracellular recordings

Hippocampal slices (250μm) from 3-4 month old control and mutant mice were prepared using a vibratome. Slices were submerged and continuously superfused with artificial cerebrospinal fluid (ACSF) containing (in mM) 119 NaCl, 2.5 KCl, 1.0 $MgSO_4$, 2.5 $CaCl_2$, 1.25 NaH_2PO_4, 26.2 $NaHCO_3$ (pH 7.4), 10 D-glucose equilibrated with 95% O_2 and 5% CO_2 at 20-23°C. Slices were allowed to recover for at least one hour before recording was initiated. In all experiments, 50μM picrotoxin was added to the bath solution to block $GABA_A$ inhibitory postsynaptic currents. An NMDA receptor antagonist (D-APV, 50μM) was present in the bath solution in the experiments measuring PTP to block post-

synaptic plasticity mechanisms typically activated by high frequency tetani. For recordings, slices were placed in a submersion chamber kept at room temperature and perfused with oxygenated ACSF at a rate of 2 ml/min. To elicit synaptic responses, Schaffer collateral/commissural fibers were stimulated with bipolar tungsten electrodes using brief current pulses. Field potentials from the CA1 stratum radiatum region were recorded with an extracellular recording electrode filled with ACSF. EPSP amplitudes were measured as the difference between the mean potential during a prestimulus baseline and the mean potential over a 2ms window at the peak of the response. I/O curves were determined by stimulating with intensities of 0-100μA, using 10μA current steps. 3 pulses were given at each stimulus intensity; mean values were calculated and were averaged across genotype. After collecting a short baseline (20 pulses, 0.33Hz), PTP was induced using a 1 sec stimulation train of 100Hz. 100 more pulses (0.33Hz) were collected following the tetanus. The two peak responses post-tetanus were averaged and mean values were calculated across genotype. For tetanus induced LTP experiments, a stable baseline was collected (20 min, 0.033Hz) and LTP was induced using two 1 second trains of 100 Hz stimulation delivered 20 sec apart. The magnitude of LTP was measured by comparing the average of responses over a 5 minute interval 55-60 min post tetanus with the average of responses over a 5 min interval preceding the tetanus. All experiments were performed on male littermates 3-4 months old, and data was collected and analyzed blind to genotype.

Analysis of locomotor behavior

We monitored spontaneous locomotor activity of mice in an open field environment using an automated Digiscan apparatus (Accuscan Instruments). In this system, photocell beam breaks are used to monitor horizontal and vertical movements and total distance traveled can be assessed. A 42 cm^2 plexiglass monitoring chamber was divided into 4 quadrants and two animals were tested per monitor, in diagonal

quadrants. Mice were allowed to acclimated to the room for at least one hour before being tested in the monitor. During monitoring, the bottom of the chamber was covered with a single piece of 3mm chromatography paper (Whatman) to facilitate cleaning. The chamber was thoroughly cleaned with quatricide PV between experiments. A sleep machine (Radio Shack) was used during experiments to generate a low level of background noise. Mice were monitored for a 30 minute trial during mid-light cycle and total distance traveled and time in the center of the quadrant were assessed. All mice analyzed were male littermates 4.5-6 months old. Experiments were performed blind to genotype.

Fear conditioning

We performed fear conditioning using the 1 tone-shock pairing training protocol and tested whether the mice remembered the association of the shock with the context and with the tone (cue). Each mouse was placed in a shocking chamber (Chamber A, metal grid floor, clydox odor) (Coulbourn Instruments), and after 2 minutes, a 30 sec white noise tone (80dB) was delivered. A 2 sec footshock (0.27mA) was delivered during the last 2 sec of the tone, and the mouse was observed for 30 additional seconds before it was returned to the home cage. On day 2, the mice were returned to Chamber A for 3 minutes and were assessed for freezing in response to the context. On day 3, the mice were placed into a different plexiglass chamber (Chamber B, smooth flat floor, alcohol odor) and after 2 min, the tone was delivered for 3 min, and freezing was assessed in response to the tonal cue. Some groups of animals performed an additional context test one week after training. Freezing was scored every 3 seconds and was defined as no movement other than respiration. Shock sensitivity was assessed by comparing the mean distance covered by mutant and control mice during and immediately after each of a series of 3 footshocks. Mice were prehandled for 2 min per day for one week prior

to training. All mice analyzed were male littermates 4.5-6 months old. Experiments were performed blind to genotype.

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Chapter 4

Synaptic plasticity at CA3-CA1 hippocampal synapses in the CA3 specific α-CaMKII conditional knockout mouse

Introduction

Multiple studies have demonstrated that CaMKII in post-synaptic CA1 is necessary and sufficient for CA3-CA1 LTP induction [51,52,54,150,152], yet it is still unclear whether there is such a requirement for CaMKII in pre-synaptic CA3. CaMKII is highly expressed in CA3 pyramidal cells and is present in pre-synaptic terminals [55,56], where it can be found associated with synaptic vesicles [223]. There are multiple putative CaMKII substrates present in synaptic terminals of CA3-CA1 synapse whose modification could reasonably transduce the expression of LTP [60,224]. However, very little is known about the role of CaMKII in synaptic transmission or synaptic plasticity at presynaptic sites in vivo. A conditional knockout of α-CaMKII in CA1 confirmed a post-synaptic role for CaMKII in LTP (see Chapter 3), and a knockout of α-CaMKII in CA3 could provide evidence for or against a pre-synaptic role in CA3-CA1 LTP expression.

While an essential role for post-synaptic CaMKII lends support to post-synaptic LTP expression mechanisms, it does not exclude the possibility of pre-synaptic changes. Pre-synaptic synaptic transmission could be enhanced by a decrease in the failure rate [63,68] or by an increase in the amount or temporal dynamics of neurotransmitter release [74,75]. Such pre-synaptic changes would require a retrograde messenger to be produced post-synaptically following NMDA receptor activation. Multiple retrograde messengers have been investigated, including NO, CO, arachadonic acid, and platelet activating factor [71], yet it has been difficult to conclude that any of these molecules are absolutely required for the generation of LTP. Although a mechanism for pre-synaptic activation of

CaMKII by such putative retrograde messengers is as yet unclear, studies have shown that pre-synaptic kinases may be important for LTP maintenance [51].

Paired pulse facilitation (PPF) [111] and post-tetanic potentiation (PTP) [117], short term plasticity mechanisms that are thought to be transduced pre-synaptically, are abnormal in global α-CaMKII knockout mice, which suggests a contribution of CaMKII to pre-synaptic function. CaMKII has several putative substrates that are involved in pre-synaptic neurotransmitter release. These include N-type voltage dependent calcium channels [225], the putative calcium sensor synaptotagmin [226], the SNARE proteins SNAP-25, syntaxin 3 and synaptobrevin/VAMP [227-229], α-SNAP and NSF [228], synaptophysin [230], Rabphilin-3A [231], and synapsin I [232]. It is not known whether the phosphorylation of these substrates, which include molecules in all stages of the vesicular release pathway, is critical for normal synaptic transmission or for the modulation of synaptic transmission during plastic changes, or both. Most of the studies involving CaMKII phosphorylation of pre-synaptic substrates have been done *in vitro,* and little is known about the significance of these phosphorylation reactions *in vivo*, except for synapsin I. Synapsin I crosslinks synaptic vesicles to the cytoskeleton, maintaining a reserve pool of vesicles away from the synaptic membrane and regulating their mobilization to active zones in a phosphorylation dependent manner [233]. Phosphorylation of synapsin I by CaMKII reduces its affinity for actin [234-236], and for synaptic vesicles [237], perhaps increasing the availability of vesicles for recruitment to the active zone for release. Studies in the squid giant axon have shown that injecting dephosphorylated synapsin I inhibits neurotransmitter release, while CaMKII phosphorylated synapsin I has no effect [238,239]. An increase in CaMKII dependent phosphorylation of synapsin I has been observed with LTP induction [148,240] and this phosphorylation was blocked by APV and by KN-62, an inhibitor of CaMKII [241]. However, traditional knockout studies in mice have shown that synapsin I and II are not essential for LTP [219,242], although they are important for regulating neurotransmitter release during short term plastic changes [243]. It is

possible that other molecules, such as newly discovered synapsin isoforms [244], could have redundant functions that complement the synapsin I and II knockouts. Regardless, the synapsin I data demonstrate that CaMKII is activated pre-synaptically following LTP induction where it can phosphorylate multiple substrates that could be involved in enhancing neurotransmitter release [36,224]. A conditional knockout of α-CaMKII in CA3 alone may determine whether CaMKII is only essential for post-synaptic induction of CA1 LTP, or if pre-synaptic CA3 CaMKII is also required for normal synaptic plasticity at the CA3-CA1 synapse.

Results

Production of CA3 specific α-CaMKII knockout mice

To generate a CA3 specific knockout of the α-CaMKII gene, heterozygoud floxed α-CaMKII mice (*f*α-CaMKII/+) were produced (see Chapter 3) and were crossed to the G32-4 transgenic line, which expresses Cre recombinase under the control of the KA-1 promoter in a pure C57Bl/6 genetic background.

Figure 4-1: KA-1 promoter – Cre recombinase transgenic line, G32-4

G32-4 was crossed to a loxP – *Stop* - loxP – lacZ reporter line, and subsequent X-gal staining reflects the pattern of Cre recombination in double transgenic mice. Cre/loxP recombination is largely restricted to the hippocampal CA3 region in 2 month old mice, when crossed to this reporter line. Lower levels of staining are present in the dentate gyrus and the facial nerve nuclei (data not shown) [135]. This image was generously provided by K. Nakazawa.

When crossed to the floxed lacZ reporter line Rosa26 [245], G32-4 mice showed a pattern of recombination that was primarily restricted

to the CA3 region of the hippocampus, with lower amounts of recombination in the dentate gyrus (Figure 4-1) [136]. (*f*α-CaMKII/+, +/+) x (*f*α-CaMKII /+, *Cre* /+) matings were established to produce (*f*α-CaMKII/*f*α-CaMKII, *Cre* /+) mutant mice (CA3 α-CaMKII KO) for analysis. All expected genotypes were observed in the progeny of such matings, in the expected Mendelian proportions.

Expression studies of CA3 α-CaMKII KO mice

We used *in situ* hybridization to determine the spatiotemporal pattern of α–CaMKII gene deletion in CA3 α-CaMKII KO mice. A 46bp cRNA probe specific for the floxed exon 2 was hybridized to parasagital fixed brain sections. This probe detected the deletion of *f*α-CaMKII exon 2 in the CA3 region of the hippocampus in CA3 α-CaMKII KO mice (Figure 4-2).

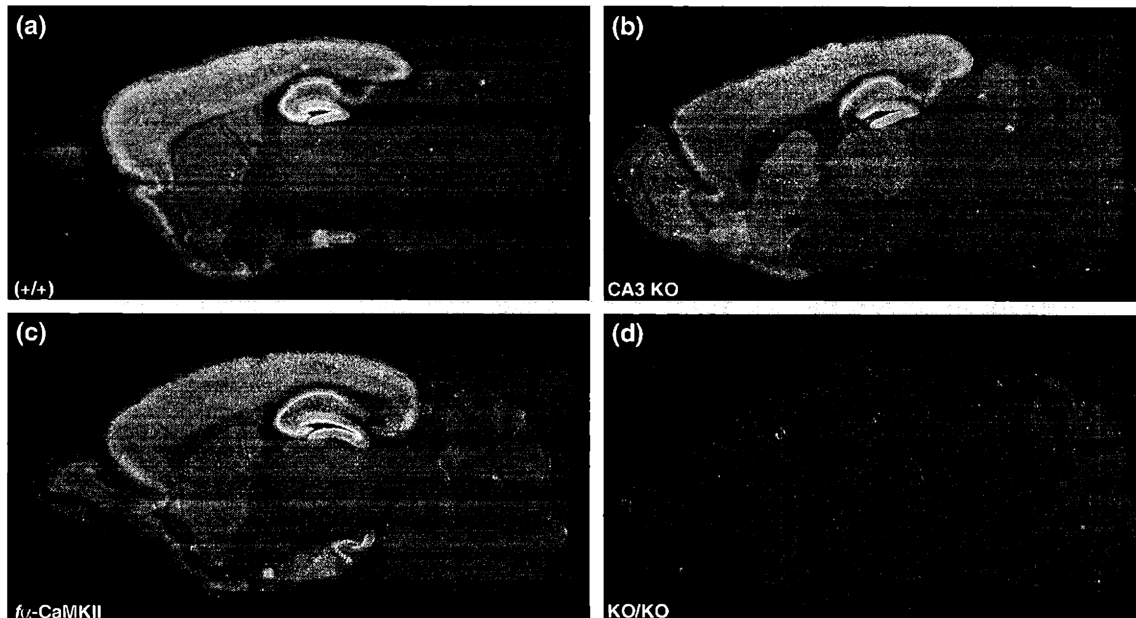


Figure 4-2: *In situ* hybridization revealed specific deletion of *f*α-CaMKII exon 2 in hippocampal CA3 of CA3 α-CaMKII KO mice

Dark field images of sagital brain sections from 4 month old wild-type (a), floxed (c), CA3 α-CaMKII KO (b), and global α-CaMKII knock-out mice (d) following *in situ* hybridization

with an exon 2 specific probe. Silver grains representing mRNA signals are in white. Deletion of the *f*α-CaMKII gene was specific to hippocampal CA3.

Recombination was first detected in CA3 pyramidal cells at 1 month (Figure 4-3).

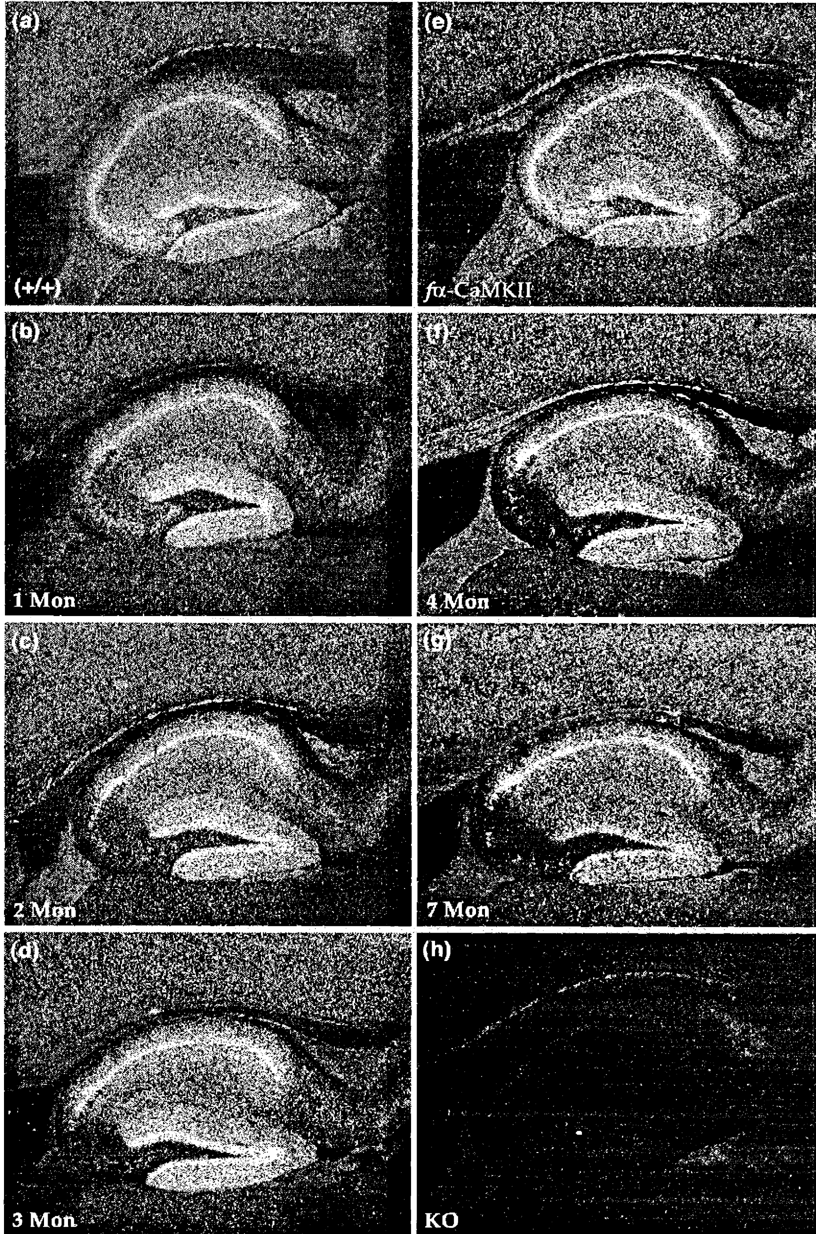


Figure 4-3: *f*α-CaMKII deletion in CA3 is complete in 3 month old CA3 α-CaMKII KO mice

Dark field images of the hippocampus from wild-type (a), floxed (e), global α-CaMKII knock-out (h) and CA3 α-CaMKII KO mice (b, c, d, f, g). Deletion of *f*α-CaMKII exon 2 is first visible at 1 month and increases to near completion by 3 months of age in CA3 α-CaMKII KO mice. While a small amount of deletion appears in the dentate of older animals, CA1 expression is unaffected at all ages observed. No expression is visible in an image from a global α-CaMKII mutant mouse (KO) in panel (h).

Deletion in CA3 increased with age, reaching a plateau of 90-95% gene knockout in 3 month old CA3 α-CaMKII KI mice. A very small amount of recombination (<5%) was detected in dentate granule cells in 4 month old animals. However, absolutely no

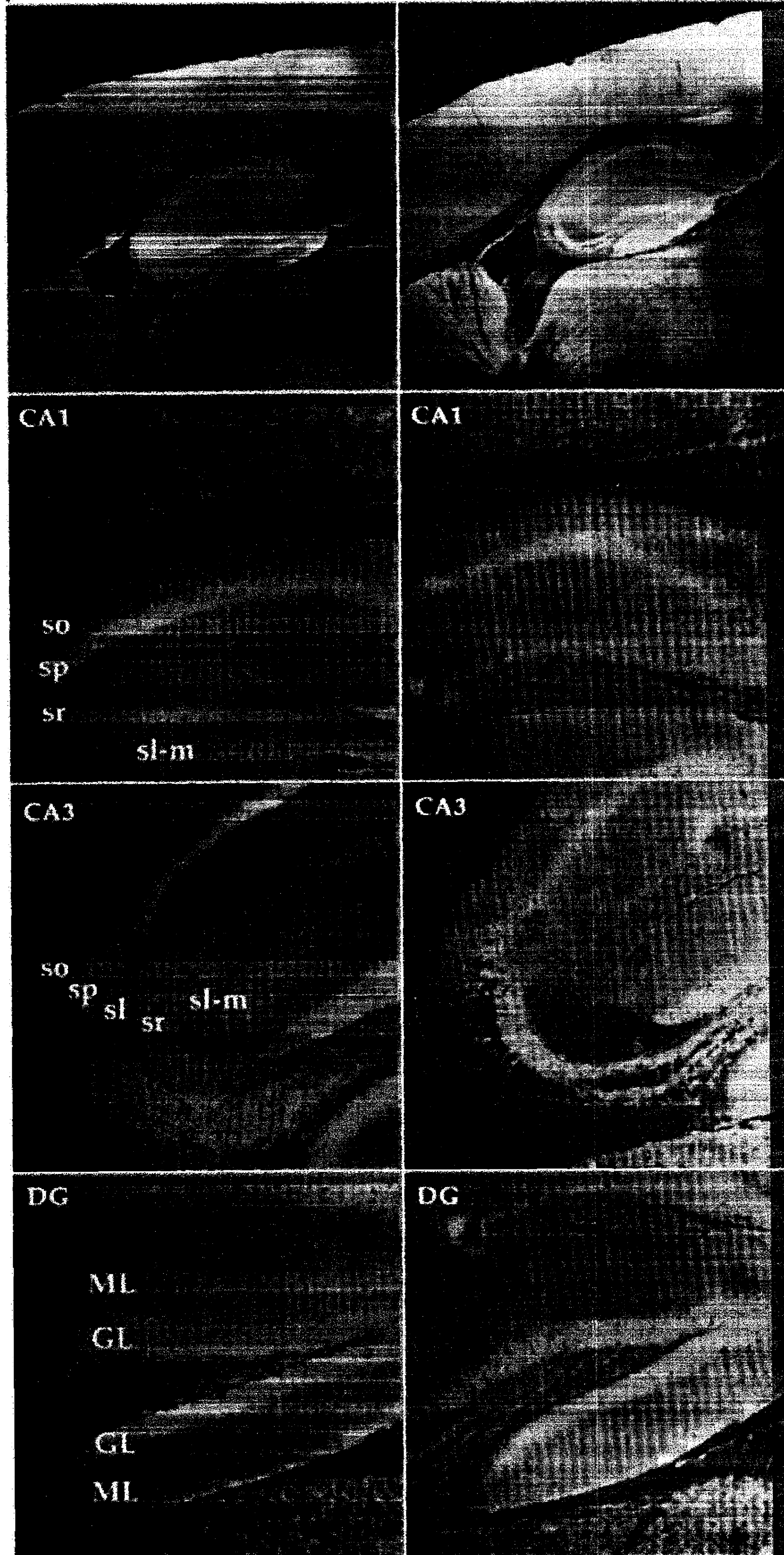
significant deletion of *f*α-CaMKII was observed in the pyramidal cells of the CA1 region of the hippocampus. Consequently, we have produced a specific knockout of *f*α-CaMKII only in pre-synaptic cells at CA3 Schaffer collateral – CA1 synapses, and we will continue to refer to these mice as CA3 α-CaMKII KO. *In situ* hybridization studies of floxed (*f*α-CaMKII/*f*α-CaMKII, +/+) mice again demonstrated that the insertion of loxP sequences alone did not affect normal expression from the α-CaMKII locus (Figure 4-2c), and consequently these animals were used as controls (*f*α-CaMKII) in subsequent experiments.

We performed immunohistochemistry using an anti-mouse α-CaMKII monoclonal antibody on formaldehyde fixed 50μm vibratome brain sections and found staining patterns consistent with the *in situ* hybridization studies (Figure 4-4; see subsequent page for figure).

Figure 4-4: Immunohistochemical staining with anti-α-CaMKII antibody shows α-CaMKII protein is absent in cells where *f*α-CaMKII deletion has occurred in CA3 α-CaMKII KO mice

Confocal images from 3 month old floxed (left column) and 4 month old CA1 α-CaMKII KO (right column) mice following immunohistochemical staining with anti-α-CaMKII antibody. Lower magnification sagital image on top, followed in descending order by high magnification images of hippocampal CA1, CA3 and dentate gyrus (DG). Fluorescent green labeling indicates α-CaMKII protein. In the CA3 region, protein is absent from the vast majority of pyramidal cells and CA3 dendritic regions. Intense staining adjacent to the CA3 stratum pyramidale is from mossy fiber axons projecting from the dentate. No protein is absent from CA1 pyramidal cells. A small percentage of dentate granule cells are not stained. sp, stratum pyramidale; sr, stratum radiatum; so, stratum oriens; sl, stratum lucidum; sl-m., stratum lacunosum-moleculare; GL, granule layer; ML, molecular layer.

α-CaMKII protein was visible in only 5-10% of CA3 pyramidal cells in 4 month old CA3 α-CaMKII KO mice. Consequently, we saw a dramatic reduction in staining in the CA3 cell body layer (stratum pyramidale) and in dendritic layers (stratum radiatum and


fα-CaMKII
CA3 α-CaMKII KO
CA1
CA1
so
sp
sr
sl-m
CA3
CA3
so
sp
sl
sr
sl-m
DG
DG
ML
GL
GL
ML

stratum oriens). CA3 pyramidal axons also project into the stratum oriens and stratum radiatum to form associational connections with CA3 dendrites, contributing further to the reduced staining in these layers. The CA3 Schaffer collateral axons travel to the CA1 stratum radiatum and oriens and form synapses on CA1 dendrites, leading to a decrease in staining in a high background of CA1 dendritic α-CaMKII protein in these areas. No CA1 pyramidal cells showed a loss of α-CaMKII protein. A small reduction in staining was detectable in scattered dentate granule cells (<5%), reflecting a lower rate of Cre recombination in cells of this region. Consequently, mossy fiber projections from dentate granule cells to the stratum lucidum of CA3 were still highly stained. Neither immunohistochemical nor *in situ* hybridization studies revealed any gross anatomical abnormalities in CA3 α-CaMKII KO mice.

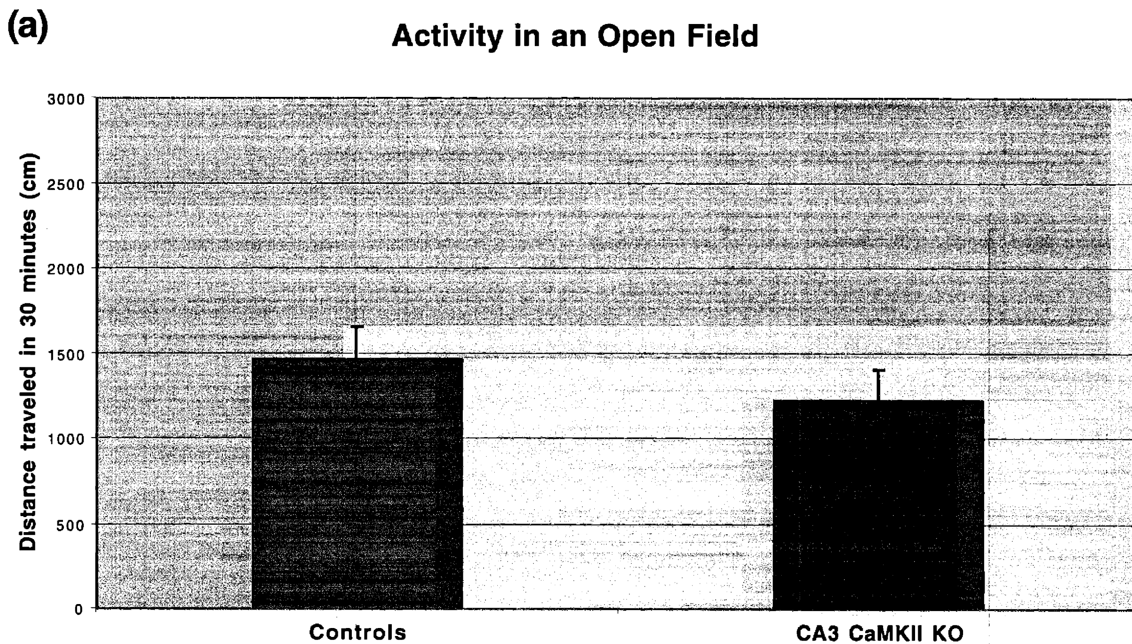
General behaviors of CA3 α-CaMKII KO mice

CA3 α-CaMKII KO mice are viable and they appear grossly normal in appearance and behavior in the home cage. Their mean survival is similar to wild-types; the oldest CA3 α-CaMKII KO mouse in our colony is over 10 months old. In contrast with the CA1 α-CaMKII KO, no motor seizures were observed in CA3 α-CaMKII KO mice. Mutants are fertile and mate with normal frequency. While we expected that Cre recombination would only occur in the brain, based on previous crosses of the KA-1 Cre mice with the NMDA receptor NR1 floxed mouse [136], we did observe some germline deletion of the floxed allele in (*f*α-CaMKII /+, *Cre* /+) females and (*f*α-CaMKII/*f*α-CaMKII, *Cre*/+) females. With increasing mouse age, the frequency of germline deletion increased to 90-100% of gametes in (*f*α-CaMKII/*f*α-CaMKII, *Cre*/+) females. We expect that there is some "leaky" CRE expression in the oocytes of *Cre*/+ females that is causing the recombination with increasing probability over time. While it is unknown if the KA-1 promoter is expressed in oocytes, it seems most likely that this leaky CRE expression is due to positional effects at the transgene insertion site. This germline recombination

may also be dependent upon the floxed allele, as it was not observed in the NMDAR1 floxed mouse (unpublished observations). Since CaMKII is expressed in oocytes following fertilization, and the NMDAR1 gene likely is not, it is possible that the relaxed chromatin structure of the active CaMKII gene would leave it more susceptible to Cre mediated recombination. Because of the high frequency of germline deletion in (*fα*-CaMKII/*fα*-CaMKII, *Cre*/+) females, we tried to use (*fα*-CaMKII/*fα*-CaMKII, *Cre*/+) males, rather than females, as much as possible for breeding purposes. This was quite inconvenient, as only (*fα*-CaMKII/*fα*-CaMKII, *Cre*/+) males were used for electrophysiology and behavioral studies.

While no gross home cage differences were observed between CA3 α-CaMKII KO and control mice, we monitored spontaneous locomotor activity of these mice in an automated open field environment. We observed mice during mid-light cycle for a 30 minute trial and saw no significant difference in the total distance traveled between CA3 α-CaMKII KO and control mice ($p = 0.186$, t-test) (Figure 4-5).

(a)



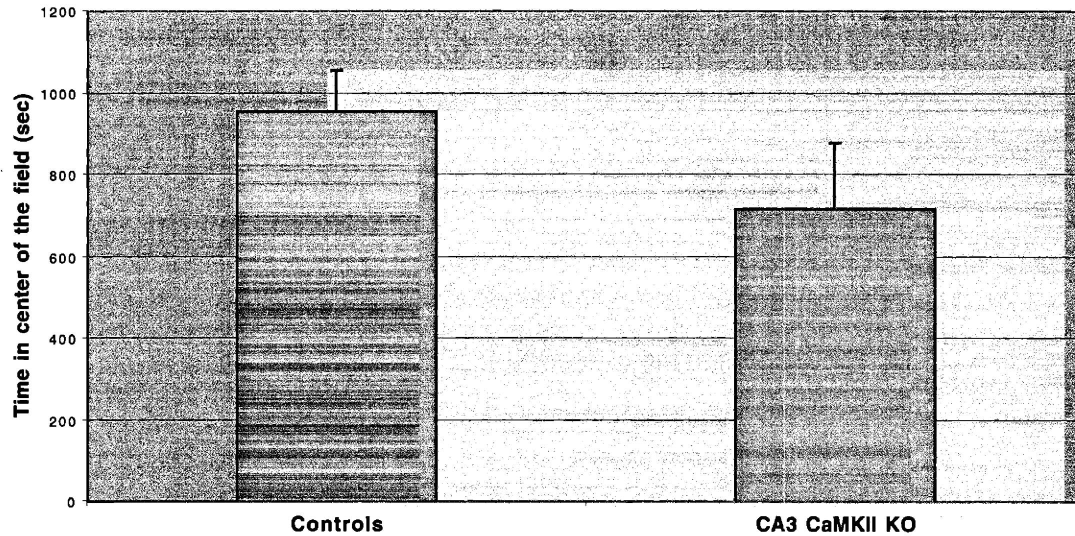


Figure 4-5: CA3 α-CaMKII KO mice show normal activity in an open field environment

(a) Naïve CA3 α-CaMKII KO (n = 11) and littermate control (n = 9) male mice were exposed to an open field environment and their total activity was monitored for 30minutes. CA3 KO mice were active at levels (1229 ± 178cm) comparable with controls (1466 ± 190cm; p = 0.186, t-test). Error bars represent ± SEM.
(b) CA3 α-CaMKII KO (n = 7) mice a similar amount of time in the center of the open field compartment (717.83 ± 161.8) compared with control littermates (n = 7) (954.87 ± 102.46 sec; p = .24, t-test). Error bars represent ± SEM.

In addition, no increased thigmotaxis was observed in CA3 α-CaMKII KO mice (Figure 4-5b).

Synaptic transmission and plasticity at CA3-CA1 synapses

To examine the role of pre-synaptic CaMKII in CA3-CA1 synaptic transmission and plasticity, we performed recordings at CA3 Schaffer collateral – CA1 synapses in acute hippocampal slices from CA3 α-CaMKII KO mice. Since CaMKII can phosphorylate several molecules that are involved in neurotransmitter release *in vitro* [224], we first examined baseline synaptic transmission in CA3 α-CaMKII KO mice. Presynaptic fibers were stimulated and EPSPs were evoked over a range of stimulus intensities.

Input/output curves were plotted for mutant and control mice to compare the size of post-synaptic responses following comparable levels of pre-synaptic activity (Figure 4-6).

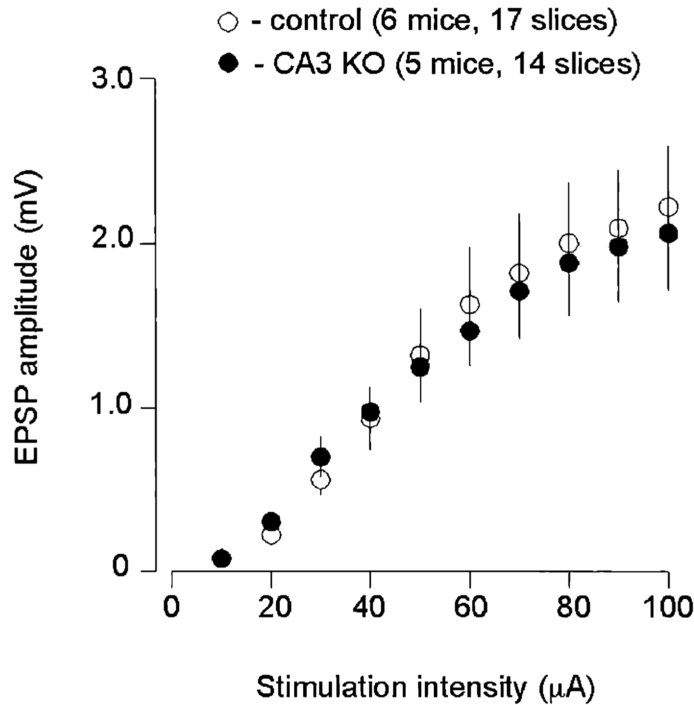


Figure 4-6: Input/output curves are normal in CA3 α-CaMKII KO mice

The strength of presynaptic stimulation (input) was varied from 10-100μA in 10μA steps and excitatory post-synaptic potentials (EPSPs) were measured extracellularly (output). CA3 α-CaMKII KO (n = 14) show normal input/output curves compared with controls (n = 17; p = 0.89, $F_{(1,29)}$ two-way ANOVA). Mean values for each genotype are shown and error bars are ± SEM.

Normal synaptic responses of comparable amplitude per stimulus strength were obtained in both mutant and control slices (p = 0.89, $F_{(1,29)}$ two-way ANOVA). This suggested that the basic release machinery for evoked neurotransmitter release is intact in CA3 α-CaMKII KO mice. Since synaptic transmission is a probabilistic process [246], the size of post-synaptic responses is dependent upon the number of competent transmitter release sites (n) and the probability of neurotransmitter release at those sites

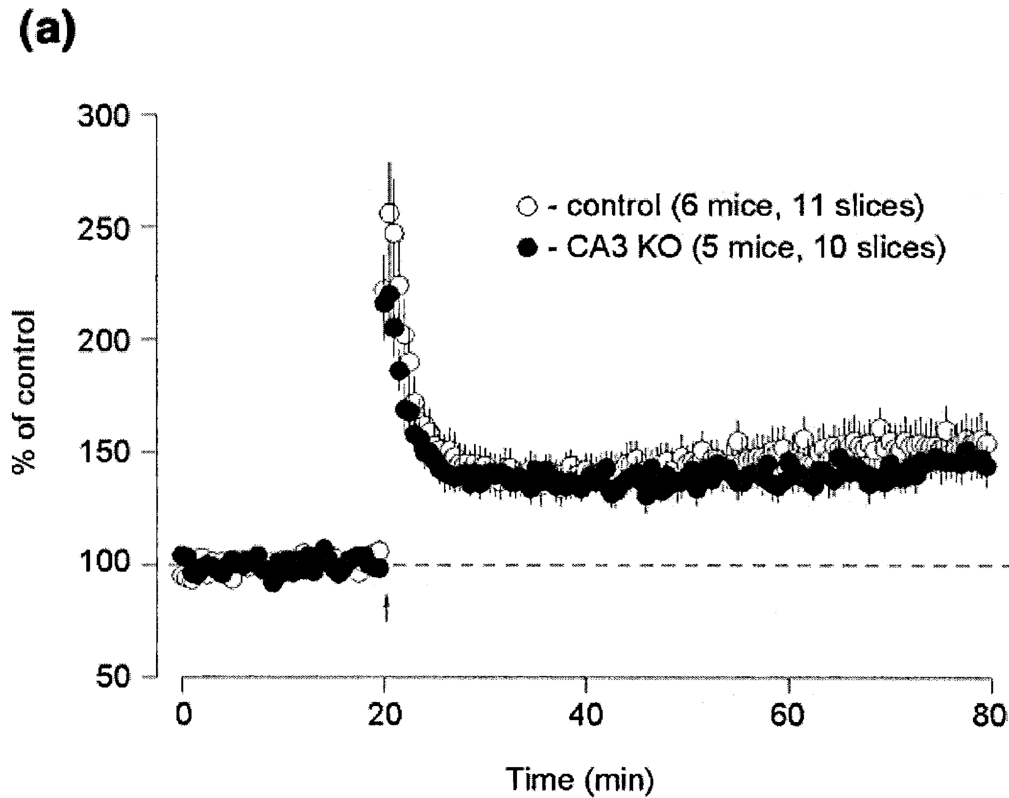
(p), in addition to post-synaptic sensitivity to transmitter. As comparable synaptic responses were observed in mutant and control mice, it is unlikely that either p or n is grossly abnormal under baseline conditions in CA3 α-CaMKII KO.

Using whole-cell recording techniques, spontaneous neurotransmitter release was examined by comparing the frequency of spontaneous miniature EPSCs (mEPSCs) in CA3 α-CaMKII KO and control mice. Since minis reflect spontaneous release events from pre-synaptic terminals that are at least partially calcium-independent, it is likely that a difference in the frequency of such release would reflect differences in the number of docked vesicles available (n). The mean frequency of mEPSCs recorded in the presence of tetrodotoxin (1μM) to block action potentials was not significantly different between controls (0.61 ± 0.13Hz; n = 6 cells) and CA3 α-CaMKII KO mice (0.705 ± 0.18Hz; n = 4 cells; p = 0.67, t-test). These data further suggest that neurotransmitter filled vesicles are present at active zone release sites and are competent for both spontaneous and evoked release in CA3 α-CaMKII KO mice.

As LTP induction is associated with an increase in CaMKII phosphorylation of the pre-synaptic substrate synapsin I [148,240], we examined LTP at CA3-CA1 synapses in CA3 α-CaMKII KO mice. LTP was normal in time course and magnitude in CA3 α-CaMKII KO mice following both tetanus (p = 0.61, t-test) and pairing (p = 0.88, t-test) induction protocols (Figure 4-7). This suggests that LTP expression is not dependent upon pre-synaptic α-CaMKII activity at the CA3-CA1 synapse.

Since long term synaptic plasticity mechanisms appeared to be unaffected in CA3 α-CaMKII KO mice, we examined short term plasticity mechanisms that are known to include pre-synaptic components. Tetanic stimulation in the presence of D-APV at hippocampal synapses leads to a pre-synaptic potentiation of responses that lasts for tens of seconds [87,247]. This post-tetanic potentiation (PTP) is thought to be dependent upon mobilizing vesicles from the reserve pool, and some molecular models propose a

(a)



(b)

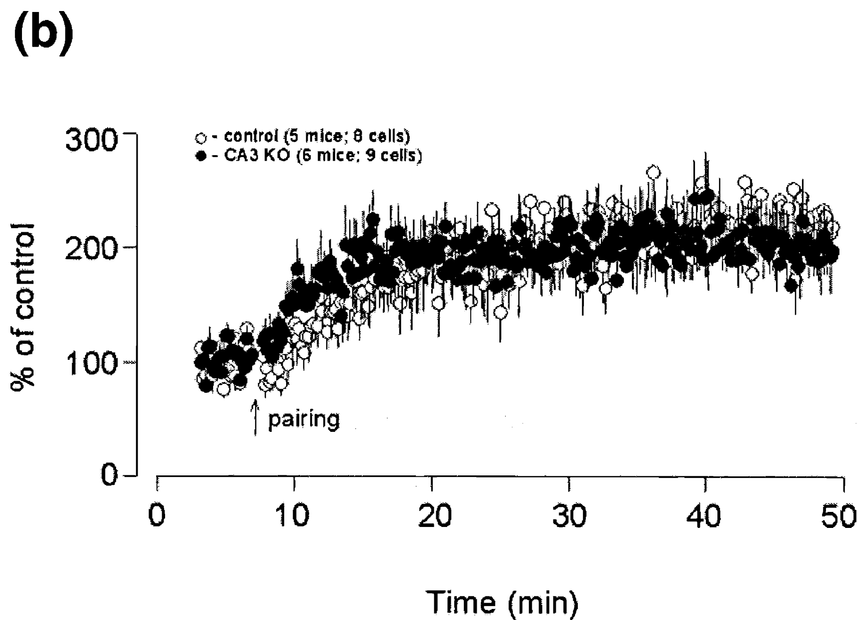


Figure 4-7: Long term potentiation is normal in CA3 α-CaMKII KO mice

(a) LTP was induced by two stimulus trains (100Hz for 1 second) spaced 20 sec apart. Amplitudes of ESPSs were normalized to baseline values and averaged across genotype and are plotted versus time (min). CA3 α-CaMKII KO mice (n = 10 slices) show normal potentiation at 60 min post-tetanus (147 ± 9%) compared with controls (154 ± 11%; p = 0.61, t-test) (n = 11 slices). Mean values are shown and error bars are ± SEM. Arrow

indicates time of tetanus.
(b) Whole cell recordings (-70mV) were used to sample EPSCs every 10 seconds and a stable baseline was recorded for 5'. LTP was induced by a voltage pairing protocol (+30mV, 80 pulses, 2Hz). Amplitudes of EPSCs were normalize to baseline values and averaged across genotype. CA3 α-CaMKII KO mice (n = 9) show normal potentiation at 35 min post-pairing (216 ± 31%) compared with controls (206 ± 33%; p = 0.88, t-test) (n = 8). Mean values are shown and error bars are ± SEM. Arrow indicates LTP pairing induction time point.

role for synapsin I – CaMKII [76,232]. We examined PTP at CA3-CA1 synapses and found it was unaffected in CA3 α-CaMKII KO mice (Figure 4-8).

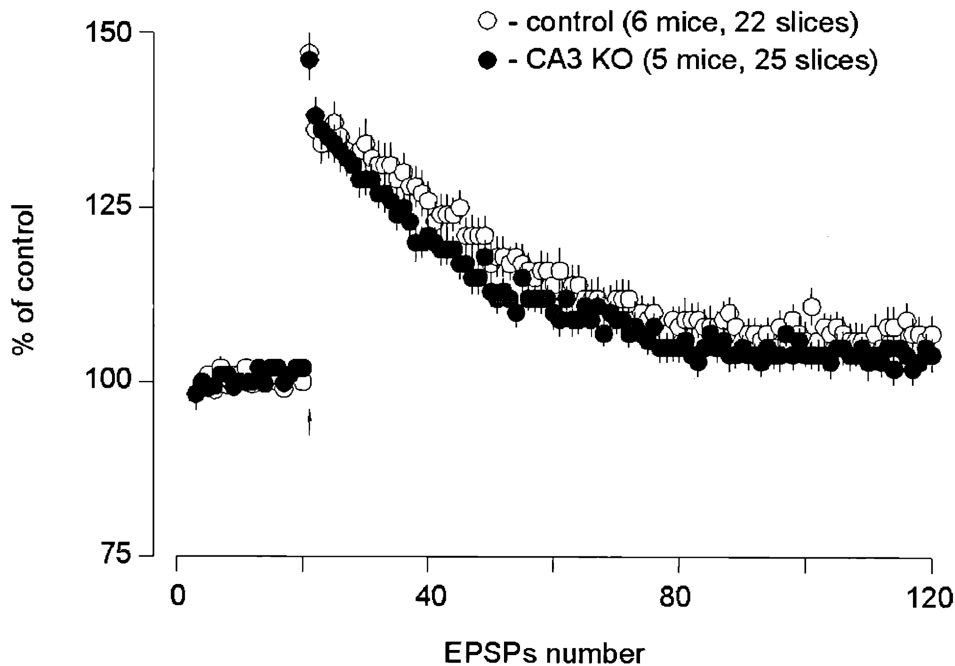


Figure 4-8: Post-tetanic potentiation is unaffected in CA3 α-CaMKII KO mice

PTP was induced by a 1 sec 100Hz stimulus train and 100 EPSPs were collected at .33Hz. Amplitudes of EPSPs are plotted over time, normalized to the size of baseline responses. PTP was normal in CA3 α-CaMKII KO mice (142 ± 2.7%; n = 25 slices) compared with controls (141 ± 3%; p = 0.83, t-test; n = 22 slices). Mean values are shown and error bars are ± SEM. Arrow indicates time of stimulus train.

100Hz tetanus in the presence of D-APV (50μM) revealed similar time courses and PTP peak values in mutants (142 ± 2.7%) compared with control mice (141 ± 3%; p = 0.83, t-test). Surprisingly, PTP was reported to be enhanced in one study of the α-CaMKII

global knockout mouse [117], although our studies indicate pre-synaptic α-CaMKII should not be required for this pre-synaptically transduced plasticity mechanism.

Facilitation refers to the short term regulation of neurotransmitter release that occurs with a rapid time course (time constant < 1 second) following repetitive stimulation [87,247]. Paired pulse facilitation (PPF) is an increase in a second EPSP when it is elicited soon after the first [247-249]. This facilitation declines with increasing inter-pulse interval [250], and is thought to be due to residual pre-synaptic calcium from the first elicited response adding with calcium influx from the second. The result is a greater release of transmitter (higher p) in response to the second stimulus. PPF was also found to be reduced in field recording experiments in α-CaMKII global knockout mice [111,117] further implicating CaMKII in the regulation of neurotransmitter release. We elicited PPF in CA3 α-CaMKII KO by stimulating with pairs of pulses at multiple interstimulus intervals (ISIs: 50, 70, 100, 200, and 300 msec) using whole cell recording with 10mM EGTA in the patch pipette to inhibit post-synaptic plasticity mechanisms (Figure 4-9). PPF was not significantly different between CA3 α-CaMKII KO mice (n = 14) and controls (n = 14) over all ISIs tested ($p = 0.26$, $F_{(1,26)}$ two-way ANOVA).

We also examined facilitation at CA3-CA1 using short trains of stimuli (30 pulses instead of just 2) to see if synaptic transmission is regulated differently in CA3 α-CaMKII KO mice under repetitive stimulation conditions. The "residual calcium hypothesis" is also thought to hold for trains of stimuli [247]. Calcium enters the terminal following a pre-synaptic depolarization, remains sequestered, and combines with additional calcium influx following successive impulses. This increases p during a stimulus train. However, if stimulation persists, it can cause a depression of responses, most likely due to a decrease in the number of vesicles available for release (n) or by actively decreasing p [251]. Using whole cell recording techniques, we stimulated CA3-CA1 synapses briefly in the presence of D-APV to obtain a stable baseline and followed with a train of 30 pulses at 1Hz, 5Hz, 10Hz, and 20Hz, each train separated by a 3' recovery

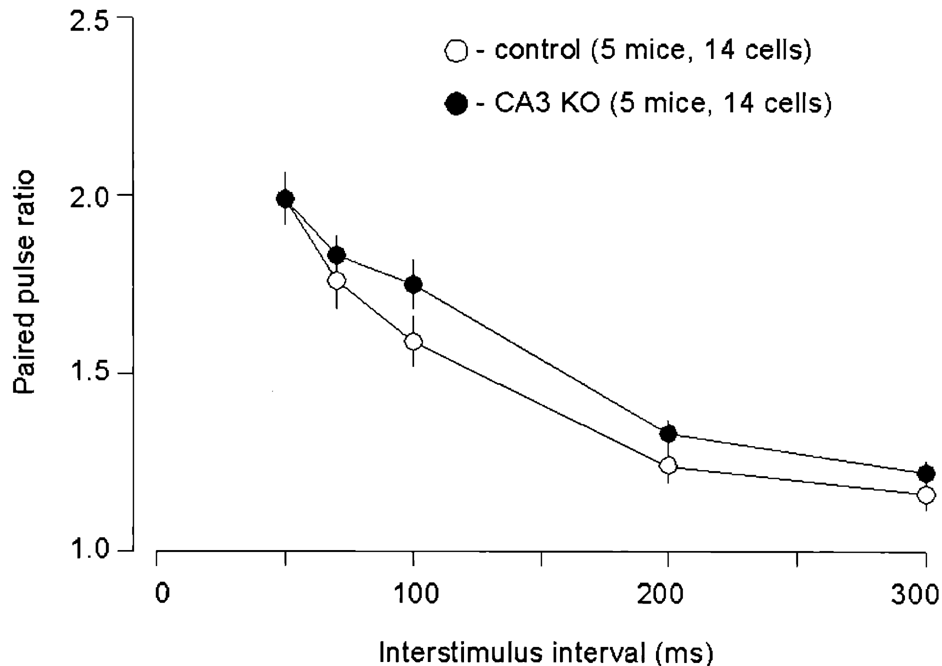


Figure 4-9: Paired pulse facilitation is normal in CA3 α-CaMKII KO mice

Pairs of presynaptic stimuli were delivered 12 times at each interstimulus interval and EPSC amplitudes were recorded and averaged for each slice. The EPSC for the second pulse was normalized to the EPSC of the first pulse. Normalized EPSCs are plotted as a function of the interstimulus interval (msec) for CA3 α-CaMKII KO and control mice. PPF is normal in CA3 α-CaMKII KO mice (n = 14) compared with controls (n = 14; p = 0.26, $F_{(1,26)}$ two-way ANOVA). Mean values are shown and error bars are ± SEM. For all whole cell short term plasticity experiments, 10mM EGTA was used in the holding pipette to block any post-synaptic plasticity mechanisms activated by repetitive stimulation.

interval. While low frequency stimulation showed no differences in facilitation between mutants and controls, CA3 α-CaMKII KO mice showed greater facilitation than controls when stimulated with higher frequency trains (Figure 4-10). This difference became more significant with increasing frequency of stimulation, with no difference between control and mutant mice at 1Hz stimulation (p = 0.86, $F_{(1,21)}$ two-way ANOVA), and increasing statistical significance at 5Hz (p = 0.26, $F_{(1,21)}$ two-way ANOVA), 10Hz (p = 0.17, $F_{(1,21)}$ two-way ANOVA), and 20Hz stimulation ($p < 0.04$, $F_{(1,19)}$ two-way ANOVA). Generally, cells with lower probabilities of release tend to show greater facilitation [247]. However, since input/output curves and PPF are relatively normal in CA3 α-CaMKII KO

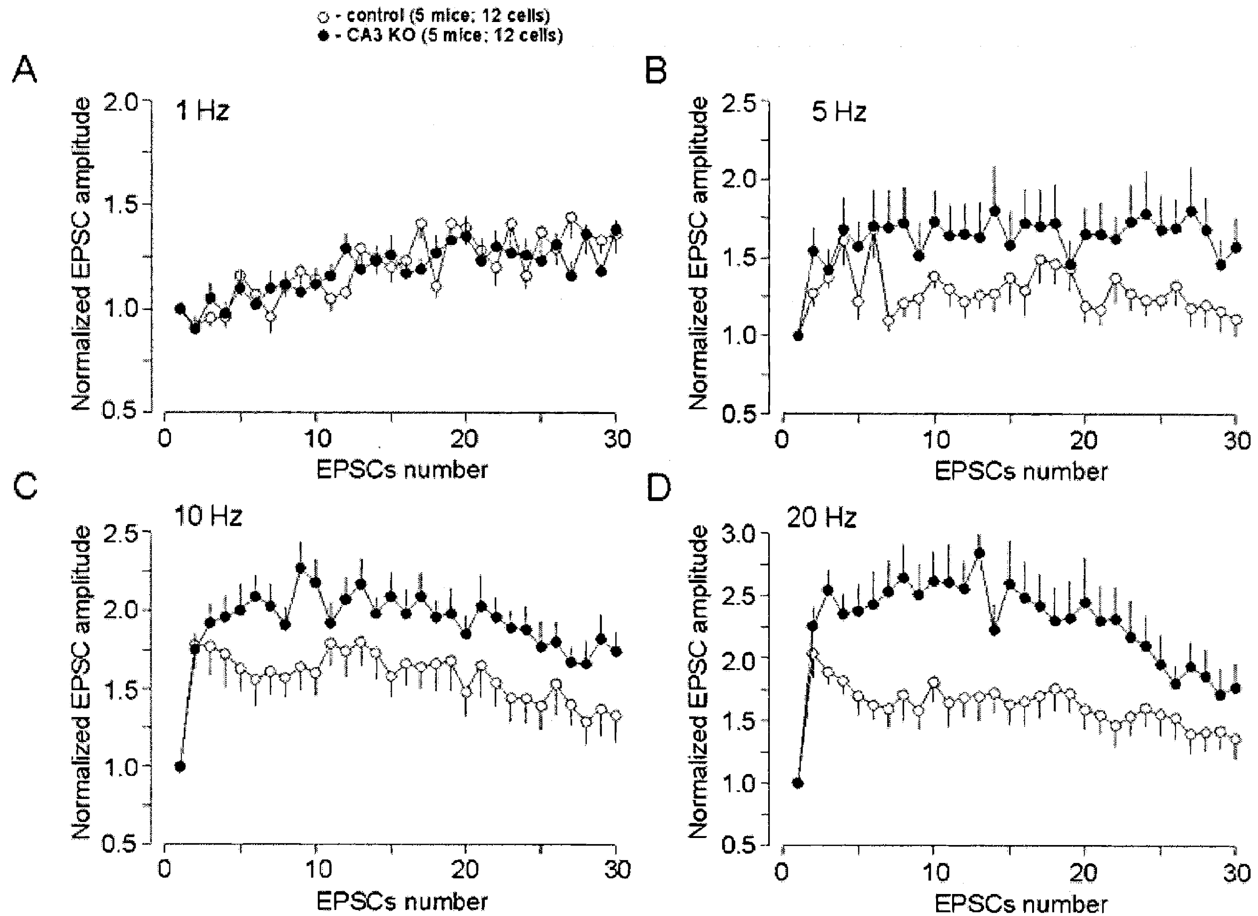


Figure 4-10: Facilitation is enhanced with increasing frequency of stimulation in CA3 α-CaMKII KO mice

After baseline EPSC recordings were obtained, 30 presynaptic stimuli were successively applied at 1Hz (A), 5Hz (B), 10Hz (C), and 20Hz (D). Normalized successive synaptic responses are plotted as a function of stimulus number for CA3 α-CaMKII KO and control mice. CA3 α-CaMKII KO mice (n = 12) show enhanced facilitation with increasing frequency of stimulation, reaching statistical significance at 20Hz compared with control mice (n = 12; $p < 0.04$, $F_{(1,19)}$ two-way ANOVA). Each genotype is normalized separately to its own initial response size. Mean values are shown and error bars indicate ± SEM.

mice, it is unlikely that this enhancement of frequency facilitation (FF) is caused by a lower baseline release probability in the mutants. Instead, it is possible that α-CaMKII could function as a negative regulator of neurotransmitter release in control mice, decreasing release probability under conditions of repetitive stimulation. The correlation between stimulation frequency (pulse number and frequency of train) and facilitation

enhancement is reminiscent of models of CaMKII as a "frequency detector" of calcium spikes. Under conditions of repetitive calcium signals, CaMKII may become highly active due to cooperative calcium/calmodulin binding, calmodulin trapping and autonomous CaMKII activity [36,39].

As mentioned, repetitive stimulation will ultimately lead to a decrease in synaptic responses, which could reflect a depletion of readily releasable vesicles and/or and an active depression of release probability [251].

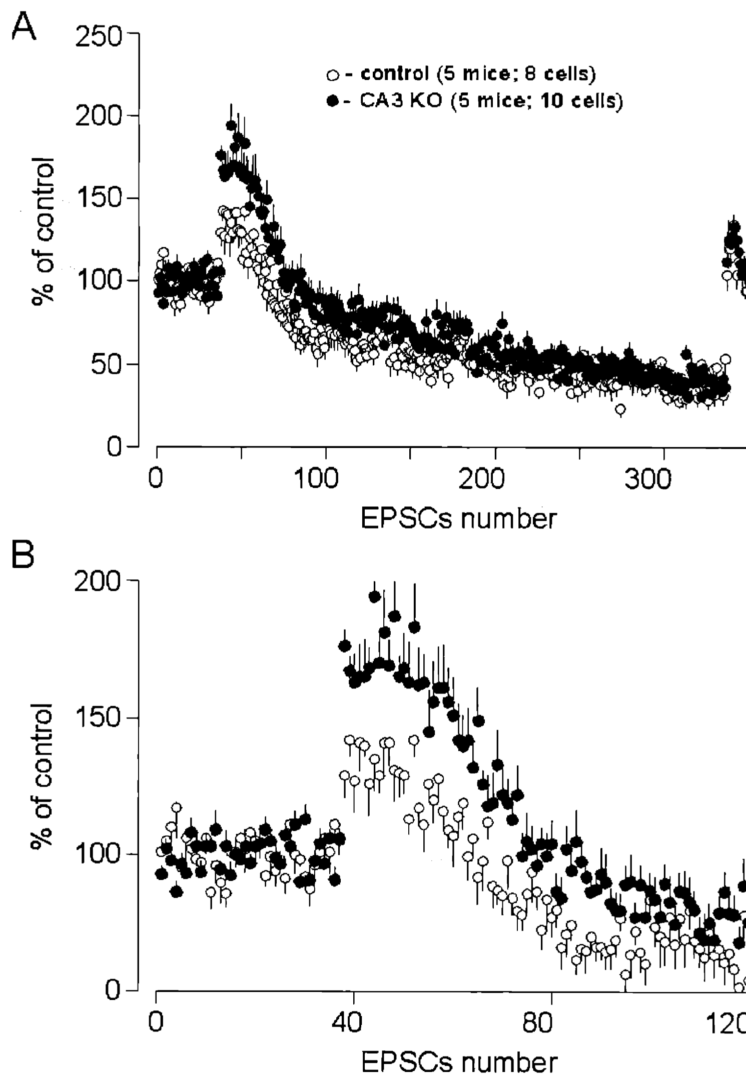


Figure 4-11: Depletion of synaptic transmission is comparable in CA3 α-CaMKII KO and control mice

(A) After baseline EPSC recordings were obtained, 300 pre-synaptic pulses were applied at 70msec intervals (14 Hz). Normalized successive synaptic responses are plotted as a function of stimulus number for CA3 α-CaMKII KO (n = 10) and control (n = 8) mice. Final depletion levels were calculated by averaging the last 30 responses of the 300 pulse depletion train. Final depletion levels are similar in CA3 α-CaMKII KO (40.9 ± 4.02%) and control mice (39.8 ± 5.6%; p = 0.87, t-test). Each genotype is normalized separately to its own initial response size. Mean values are shown and error bars are ± SEM.
(B) Facilitation phase of the depletion plot (a) on an expanded time scale.

Repetitive stimulation depletion experiments that drain transmitter stores could reveal differences in synapsin I dependent vesicle delivery/cycling, the size of the available synaptic release pool, or differences in the rate of recovery of vesicle stores following depletion in CA3 α-CaMKII KO mice. We stimulated CA3-CA1 synapses with 300 depolarizing pulses, one every 70msec (approximately 14Hz), in the presence of D-APV. An initial facilitation occurred, as was observed during the FF experiments, that was enhanced in CA3 α-CaMKII KO mice compared with controls. Then synaptic responses decreased to a similar steady state level over a comparable time course in both mutants (40.9 ± 4.02%) and controls (39.8 ± 5.6%; $p = 0.87$, t-test) (Figure 4-11). In addition, no differences between CA3 α-CaMKII KO and control mice were seen in the time course of recovery following depletion, demonstrated during an 11 second interval of lower stimulation frequency post-depletion ($p = 0.83$, $F_{(1,17)}$ two-way ANOVA) (Figure 4-11, 4-12).

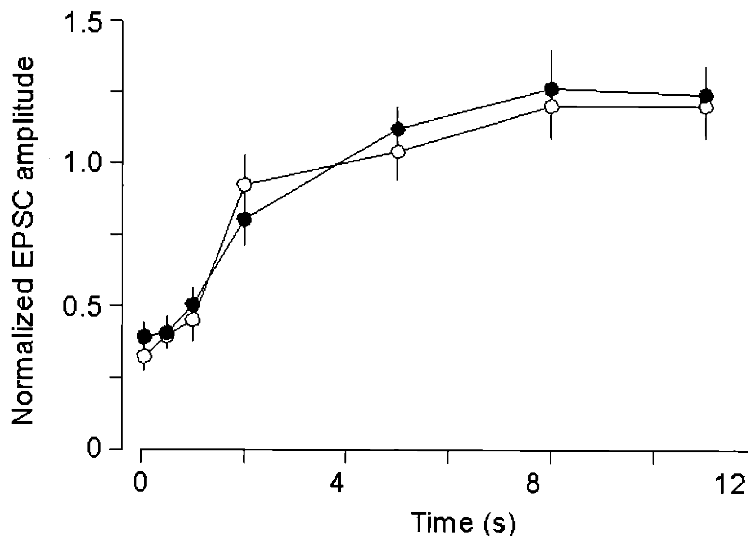


Figure 4-12: Recovery from depletion is normal in CA3 α-CaMKII KO mice

Following depletion, 7 pre-synaptic stimuli were presented at time points 50 msec, 500 msec, 1 sec, 3 sec, 5 sec, 8 sec, and 11 sec post depletion train. EPSCs were normalized to the pre-depletion baseline responses and are plotted over time (sec). No significant difference was observed between CA3 α-CaMKII KO (n = 10) and control mice (n = 8; $p = 0.83$, $F_{(1,170)}$ two-way ANOVA). Mean values are shown; error bars are ± SEM.

This suggests that α-CaMKII dependent mechanisms, such as the phosphorylation of synapsin I, are not essential for increasing the supply of synaptic vesicles to the active zone under high demand repetitive stimulation conditions, or for the calcium dependent replacement of depleted vesicles after synaptic depression [251].

Fear conditioning in CA3 α-CaMKII KO mice

As CA3-CA1 short term plasticity mechanisms have been implicated in hippocampal dependent learning [221], we examined CA3 α-CaMKII KO mice using contextual and cued fear conditioning (see Chapter 3). As described previously (Figure 3-16), we trained CA3 α-CaMKII KO and control male mice by placing them into a novel context (A) and pairing a single tone with a footshock (2 sec, 0.27mA). Mice were returned to the novel context 1 day and 7 days after training and freezing was monitored, as an indicator of memory of the conditioned association (Figure 4-12).

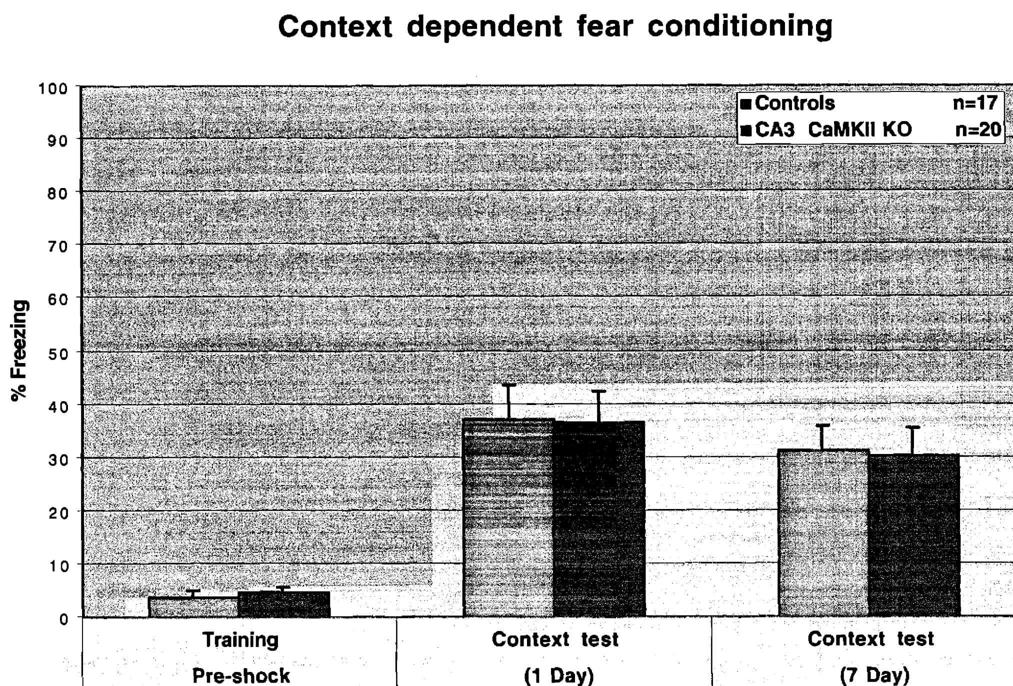


Figure 4-13: Contextual fear conditioning is normal in CA3 α-CaMKII KO mice

CA3 α-CaMKII KO mice (n = 20) and littermate controls (n = 17) were trained using a single tone/shock pairing (2 sec, 0.27mA) in a novel context. Pre-shock background freezing levels are shown. 1 day after training, CA3 α-CaMKII KO mice showed normal freezing to context (36.5 ± 5.5%) compared with controls (37 ± 6.5%; $p > 0.95$, t-test). 7 days after training, freezing was indistinguishable in mutants (30.2 ± 3.7%) compared with controls (31 ± 4.7%; $p > 0.88$, t-test). % freezing mean values are shown ± SEM.

Both CA3 α-CaMKII KO mice and controls froze in response to the conditioned context in comparable amounts at 1 day ($p > 0.95$, t-test) and 7 days ($p > 0.88$, t-test) post-training. Cued conditioning was also normal in CA3 α-CaMKII KO mice (Figure 4).

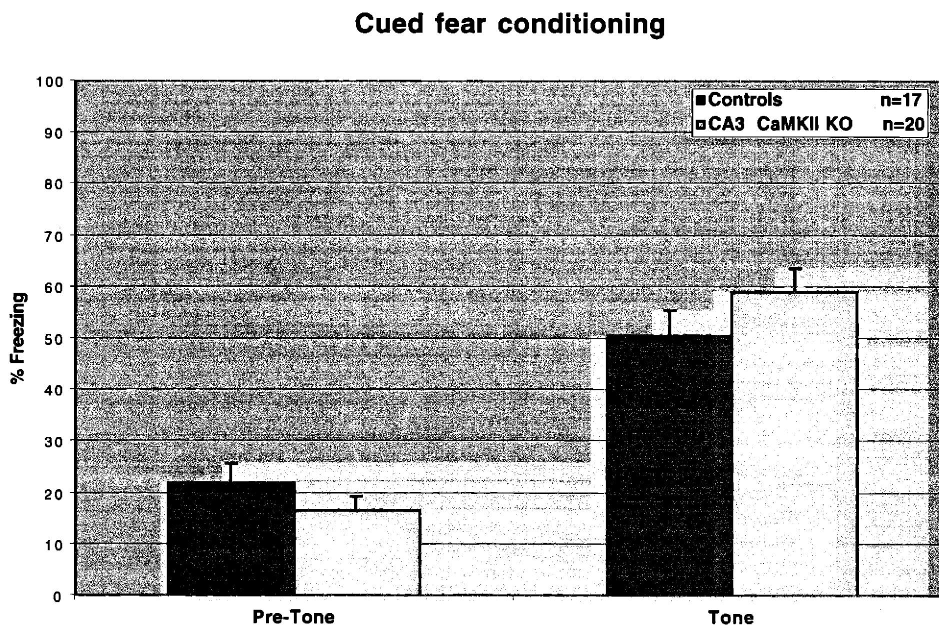


Figure 4-14: Cued fear conditioning is normal in CA3 α-CaMKII KO mice

CA3 α-CaMKII KO mice (n = 20) and littermate controls (n = 17) were trained using a single tone/shock pairing (2 sec, 0.27mA) in a novel context. 2 days after training, mice were introduced to a new context, were allowed to explore for 2 min, and the conditioning tone was played for 3 additional min. Cued freezing in mutants was not statistically different from that of controls ($p = 0.22$, t-test). % freezing mean values are shown ± SEM.

Both CA3 α-CaMKII KO mice and controls froze in response to the tone cue, and the amount of freezing was not statistically different ($p = 0.22$, t-test). These data suggest some forms of hippocampal dependent learning are intact in CA3 α-CaMKII KO mice.

Discussion

We have examined the role of CaMKII in pre-synaptic function by studying CA3-CA1 synaptic transmission and plasticity following conditional gene knockout of the dominant α-CaMKII isoform in hippocampal CA3 pyramidal cells. The basic neurotransmitter release machinery was intact at CA3-CA1 synapses in CA3 α-CaMKII KO mice, indicated by normal synaptic responses following pre-synaptic stimulation and comparable levels of spontaneous neurotransmitter release. While previous studies had implicated CaMKII phosphorylation of pre-synaptic synapsin I in LTP expression [148], LTP was normal following both tetanus and pairing induction protocols in CA3 α-CaMKII KO mice. This suggests that the CaMKII phosphorylation of synapsin I observed following LTP induction is not essential for the expression of LTP, and instead is likely an indirect consequence of plasticity induction mechanisms. However, this does not preclude other pre-synaptic signaling pathways from contributing to LTP expression, including contributions from other CaMKII isoforms. Short term plasticity mechanisms modulated by pre-synaptic calcium influx [76,87,247] were also examined, and although CaMKII has several putative substrates involved in the regulation of calcium dependent neurotransmitter release [224], post-tetanic potentiation (PTP), paired-pulse facilitation (PPF) and synaptic depletion were unaffected in CA3 α-CaMKII KO mice. Contrary to previous models [76], this suggests that CaMKII phosphorylation of synapsin I also may not underlie PTP. Interestingly, repetitive stimulation protocols using short trains of stimuli of increasing frequency revealed enhanced frequency facilitation in CA3 α-CaMKII mutants. This suggests that CaMKII may be acting pre-synaptically as a negative regulator of neurotransmitter release during certain repetitive stimulation conditions, and as a "frequency detector" of calcium spikes, reaching higher levels of activation with increasing frequency of stimulation. This could be important for

preventing synaptic terminals from depleting their vesicle stores during episodes of repetitive firing, or to maintain synaptic activity in an optimal range for information coding.

Very little is known about putative pre-synaptic mechanisms that underlie plastic changes, such as short term and long term potentiation. Such mechanisms could increase or decrease the probability of neurotransmitter release or the amount of neurotransmitter released per fusion event [62,63]. To regulate neurotransmitter release pre-synaptically, there are several steps that protein phosphorylation events could regulate [224]. First, protein kinases could phosphorylate ion channels, such as voltage dependent calcium channels, and their associated proteins. This could regulate the amount of calcium entry into the pre-synaptic terminal following an action potential, and it is this calcium signal that is critical for initiating the release mechanism. It is known that CaMKII can phosphorylate the N-type voltage dependent calcium channel synprint site *in vitro*, which causes dissociation of calcium channels from the SNARE proteins/synaptic vesicles complex [225]. CaMKII can also phosphorylate the putative calcium sensor, synaptotagmin [226], which increases the interaction of synaptotagmin with the vesicle associated SNARE complex [252]. Second, protein kinases can modify proteins involved in the multiple stages of synaptic vesicle traffic, including recruitment from the reserve pool, mobilization to active zones, priming, and endocytic recycling. The phosphorylation of synapsin I by CaMKII is thought to mobilize vesicles from the reserve pool [233]. CaMKII can also phosphorylate the synaptic vesicle proteins synaptophysin [230], synaptobrevin [229], and Rabphilin 3A [231], although the significance of these phosphorylation events is still unknown. According to the SNARE hypothesis, synaptobrevin forms a heterotrimeric core complex with the synaptic membrane proteins syntaxin and SNAP-25 [224], which are also phosphorylated by CaMKII *in vitro* [227]. The SNARE complex forms a receptor for the soluble proteins αSNAP and NSF, and it is thought that the dissolution of this complex following SNAP stimulated ATP hydrolysis

by NSF, is the biochemical correlate of vesicle priming [224]. αSNAP and NSF are also phosphorylated by CaMKII, though the biochemical effects of these modifications are unknown [228]. Finally, the dynamics and control of the final fusion and neurotransmitter release event could be modified by kinase activity, and recent models suggest a role for PKC and not CaMKII [74,75].

Despite the innumerable putative CaMKII interactions in the pre-synaptic terminal, we observed that deletion of α-CaMKII in the pre-synaptic side of the CA3-CA1 synapse had a very specific effect on the frequency facilitation of neurotransmitter release, with no effect on LTP or other mechanisms. Previous arguments for a pre-synaptic CaMKII contribution to LTP were drawn from work on the vesicle associated protein synapsin I. Synapsin I is phosphorylated at its C-terminal by CaMKII, although the highly homologous synapsin II isoform is not, and this phosphorylation is increased following LTP induction [148,240]. However, it looks like neither synapsin I nor α-CaMKII are essential via this putative LTP expression pathway. Global knockouts of synapsin I and double knockouts of synapsin I and II also show normal LTP at the CA3-CA1 synapse [219,242]. It is possible that redundancy due to additional synapsin isoforms [244], or by other CaMKII isoforms [36] could compensate in knockout mice, yet there is no evidence that these homologous isoforms perform similar functions *in vivo*. Knock-in studies targeting the synapsin I CaMKII phosphorylation site could address the possibility of CaMKII redundancy, but would be inconclusive if other synapsin isoforms could compensate, as suggested by the global synapsin I knockout data. It is also possible that synapsin I phosphorylation by CaMKII is simply not significant for modulating synaptic vesicle dynamics in mammalian systems. This does not preclude other pre-synaptic signaling pathways from involvement in pre-synaptic LTP expression mechanisms. The observation that increased phosphorylation of a pre-synaptic CaMKII substrate occurs with LTP induction still argues for the existence of retrograde signaling mechanism,

regardless of whether this change was involved in LTP itself or was merely an indirect consequence.

The global α-CaMKII knockout mouse had abnormal long term and short term plasticity at the CA3-CA1 synapse [111,117,118,137]. LTP and LTD were both deficient, and PPF was decreased [111,117] while PTP was enhanced, in one study [117], and normal in another [118]. Our studies indicate that a post-synaptic CaMKII requirement could sufficiently explain the LTP deficit (see Chapter 3). It is still unclear what role CaMKII plays in LTD; increased autophosphorylation of CaMKII has been reported following both LTP [148] and LTD [253]. As PTP and PPF are both thought to be short term synaptic enhancements caused by pre-synaptic changes [76], it is surprising that they were altered in the global knockout, but normal in the CA3 α-CaMKII KO mouse. While it is possible that the post-synaptic loss of α-CaMKII in combination with a pre-synaptic deficiency led to these changes, developmental deficits could also be contributing to the global knockout phenotype. The regional restriction of the CA3 α-CaMKII KO that occurred following normal development in the adult mouse in a pure C57Bl/6 genetic background is the optimal system for studying the role of CaMKII in presynaptic function.

Very little is known about the role of CaMKII in regulating pre-synaptic neurotransmitter release, despite the numerous CaMKII substrates present in axon terminals. We observed that under certain repetitive stimulation conditions, which may elevate pre-synaptic calcium concentrations via temporal summation, there is greater facilitation of synaptic responses in the CA3 mutants. Recent studies argue that the simple incremental calcium summation itself may not be as important as persistent activity of downstream calcium targets that could be reinforced with subsequent signals [87]. Our results suggest that the persistent activity of α-CaMKII could act pre-synaptically as a negative regulator of neurotransmitter release and/or a calcium buffer during repetitive stimulation. This is somewhat unexpected, as early experiments injecting CaMKII into the squid giant axon [238,239] and introducing constitutively active CaMKII into

synaptosomes [254] suggested that CaMKII promotes neurotransmitter release. Several studies have demonstrated that some forms of short term synaptic enhancement occur normally in the presence of CaMKII inhibitors, including PPF at *Aplysia* central synapses [255] and PPF/FF at hippocampal CA3 association/commissural synapses [256], augmentation at the crayfish neuromuscular junction [257] and PTP in dorsal molecular layer of the electrosensory lateral line lobe (ELL) of the fish brain [258]. However, CaMKII inhibitors have also been shown to reduce PPF/FF at mossy fiber synapses [256], block PTP in the ventral molecular layer of the ELL of the fish brain [258], and to impair facilitation at the Drosophila neuromuscular junction [259]. No studies have demonstrated an increase in synaptic enhancement in the presence of CaMKII inhibitors [87], although this has not been specifically addressed at the CA3-CA1 synapse. In addition, no inhibitor or mutant mouse studies have identified an enhancement of facilitation in the absence of other synaptic transmission abnormalities.

One possible mechanism for CaMKII-mediated suppression of neurotransmitter release during frequency facilitation could involve the highly efficient calcium buffering capacity of CaMKII. With repetitive stimulation and sequential episodes of calcium influx, CaMKII binds calcium/calmodulin in a cooperative manner and can become an efficient calcium/calmodulin sink [42] (Figure 4-15). It is possible that in control mice, CaMKII passively traps calcium/calmodulin and consequently competes effectively with molecules in the neurotransmitter release pathway that also require calcium or calmodulin. Computational models suggest that CaMKII competition for calcium could become significant at synaptic release sites (J. Lisman, personal communication). Consequently, it is possible that such calcium buffering by CaMKII could establish a facilitation ceiling, which would be a function of stimulation frequency.

While the calcium buffer hypothesis is a simple passive model to explain FF enhancement in CA3 α-CaMKII KO mice, it is also possible that CaMKII actively suppresses neurotransmitter release by modifying substrates at release sites

Recent studies have shown that the CaMKII substrate synapsin I may negatively regulate release of transmitter at active zones [233,260], in addition to maintaining the reserve pool. Interestingly, synapsin I global knockout mice demonstrated normal synaptic physiology except for enhanced paired pulse facilitation (PPF) [219]. Recent *in vitro* studies have shown that the N-type voltage dependent calcium channel synprint site is a CaMKII substrate [225]. Phosphorylation at synprint causes dissociation of calcium channels from the SNARE proteins/synaptotagmin complex. Therefore, it is possible that when CaMKII is activated following repetitive stimulation, it phosphorylates the synprint site and causes synaptic vesicles to dissociate from calcium channels, decreasing the probability of neurotransmitter release. Unfortunately, no phospho-specific antibody for the synprint site exists, and even if it were produced, it would be difficult to monitor increased phosphorylation and dissociation of the calcium channel complex specifically at stimulated, CA3 pre-synaptic terminals. It would be interesting to create a knock-in mouse in which the CaMKII phosphorylate synprint site in the N-type calcium channel gene locus was removed to see if enhanced facilitation is the consequence. There is evidence that PKC can phosphorylate this synprint site as well [225], but it is possible that CaMKII is a more efficient kinase for this reaction under repetitive stimulation conditions of increasing frequency due to its biochemistry (Figure 4-15).

CaMKII is a holoenzyme made up of homologous subunits that can phosphorylate each other following calcium/calmodulin binding and enzyme activation [36]. Autophosphorylation of CaMKII at individual subunits causes a dramatic increase in calmodulin affinity ("calmodulin trapping"), as well as an ability to remain active ("autonomous") even after calcium levels fall and calmodulin dissociates from the enzyme [42]. Since subunits cannot autophosphorylate their subunit neighbors unless both "kinase" and "substrate" subunit are calmodulin bound, total activity of the holoenzyme is limited by the number of bound calmodulin molecules per holoenzyme.

If calcium signals occur in close temporal proximity, it can lead to increased cooperative calmodulin binding, which further stimulates autophosphorylation and trapping and increases total holoenzyme activity. This has led to proposals that CaMKII may function as a "frequency detector" of calcium spikes, becoming highly active under conditions of repetitive calcium signals [39] (Figure 4-15).

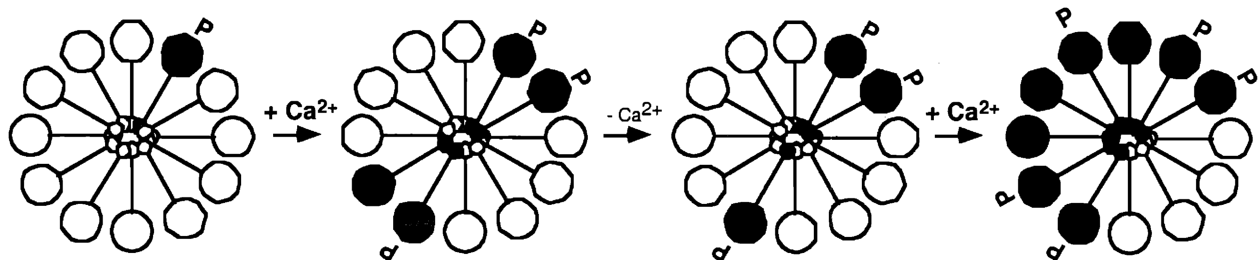


Figure 4-15: CaMKII functions as a frequency detector of calcium spikes

The CaMKII holoenzyme is depicted as an assembly of 12 subunits. As each subunit binds calcium/calmodulin and becomes active (filled circle) it can autophosphorylate an adjacent subunit (P) that is also calcium/calmodulin bound. Autophosphorylation leads to calmodulin trapping at the phosphorylated subunit. As calcium levels decrease, calcium/calmodulin will eventually diffuse off subunits that are not autophosphorylated, while trapped subunits will retain full activity for a substantially longer period. If another calcium signal occurs in close proximity to the first, additional subunits will become activated by calcium/calmodulin binding, and a higher combined level of CaMKII activity will be reached that reflects the frequency of calcium spikes. The cooperativity of calmodulin trapping may allow the kinase to be a frequency detector with a threshold frequency beyond which it becomes highly active [36].

While it is unclear what levels of CaMKII activity actually occur at the pre-synaptic terminal *in vivo*, either during steady-state, low- frequency or high-frequency stimulation conditions, it is possible that CaMKII sensitivity to stimulus frequency allows it to maximize activity under increasing stimulus frequency conditions. This could explain why the enhancement of frequency facilitation appears to increase with increasing stimulation frequency and number in CA3 α-CaMKII KO mice, with little effect on PPF (only two pulses). In the wild-type mouse, CaMKII would normally become highly active with increasing stimulation frequency, leading to greater suppression of

neurotransmitter release during the stimulus train. In other words, it could increase its activity in response to increasing demand. Alternatively, cooperative calmodulin binding with increased presynaptic activity could simply deplete pre-synaptic calcium/calmodulin stores. CaMKII could passively serve as a calcium/calmodulin sink with increasing stimulation, indirectly causing a decrease in neurotransmitter release.

While the paucity of information regarding pre-synaptic CaMKII substrates *in vivo* makes it difficult to establish the precise mechanism for the negative regulation of neurotransmitter release, it would be possible to examine whether CaMKII mediated inhibition is generally due to an active or passive process. If CaMKII is acting primarily as a calcium/calmodulin buffer during repetitive stimulation in control animals, increasing pre-synaptic calcium buffering with EGTA-AM, a cell-permeable, cleavable ester of the calcium chelator EGTA, in mutant hippocampal slices may suppress the facilitation enhancement. Alternatively, a bath applied CaMKII inhibitor (*e.g.* KN-93) should enhance frequency facilitation in control mice to similar levels seen in CA3 α-CaMKII KO mice if an active CaMKII phosphorylation step is required. It would also be interesting to perform quantal analysis during facilitation to determine whether the increased release is due to an increase in the probability of release or an increase in the number of release sites. We assume that changes in presynaptic function underlie the phenotype observed in CA3 α-CaMKII KO mice, but it is possible that changes in p or n could reflect indirect post-synaptic changes subsequent to CaMKII deletion.

Little is known about the function of short term modulations of neurotransmitter release *in vivo*. CaMKII may function presynaptically during frequency facilitation to monitor levels of neurotransmitter release to prevent synapses from being drained of transmitter during periods of increased activity. It seems likely that synapses need to maintain their probability of release in an optimal range for efficient information storage. Some computational models of information processing propose a role for short term plasticity in transforming temporal information into a spatial code [261] and in some forms

of short term memory, such as working memory [262]. Studies have shown that short term changes in synaptic efficacy are critical for habituation in *Aplysia* [263], associative conditioning in Drosophila [259], and learning and memory in mice [221]. CA3 α-CaMKII KO mice performed normally in both contextual and cued versions of the fear conditioning paradigm. Interestingly, synapsin I mutant mice, which demonstrated enhanced PPF, also performed normally in this task. However, synapsin II mutant mice and synapsin I/II double mutants, which show a decrease in PTP, and global α-CaMKII (+/-) mice, which have a decrease in PPF and an increase in PTP, showed severe behavioral deficits [221].

While the study of learning and memory in CA3 α-CaMKII KO mice is less complicated than in the CA1 α-CaMKII KO mice because of the specificity of the targeted deletion and the decreased incidence of seizure, it should be kept in mind that there are multiple synapses that include CA3 pyramidal cells. We observed that CA3 α-CaMKII KO mice showed normal LTP at the CA3-CA1 synapse and normal behavior in a hippocampus dependent learning task. Our electrophysiological studies focused on CA3-CA1 synapses where CA3 is the presynaptic cell, but CA3 cells also receive inputs from the mossy fiber inputs from the dentate gyrus granule cells, perforant path inputs from the stellate cells in the superficial layers of the entorhinal cortex, and recurrent commissural/associational (C/A) collaterals of the CA3 pyramidal cells themselves [264]. It is possible that deletion of α-CaMKII in "post-synaptic" CA3 could have an effect on synaptic transmission and plasticity at those synapses, as well as on dependent behaviors. However, NMDA receptor independent mossy fiber – CA3 LTP does not appear to be dependent upon CaMKII in post-synaptic CA3 cells [53,80]. It is not known if CaMKII plays a role in NMDA receptor dependent perforant path-CA3 LTP [265] or C/A-CA3 recurrent collateral LTP [80], which are less well characterized. Interestingly, recent studies have shown that CA3 specific conditional knockout of the NMDAR subunit 1 (CA3 NR1 KO mice) causes a deficiency in C/A -CA3 recurrent collateral LTP and in

associative memory recall and CA1 place cell activity when performing tasks requiring pattern completion [136].

Materials and Methods

Generation of conditional knock-out mice

Recombinant floxed α-CaMKII (*f*α-CaMKII) mice were produced as described in Chapter 3 *Materials and Methods.* One line of floxed mice (CF) was crossed with G32-4 Cre transgenic mice to produce CA3 conditional knock-out mice.

Mice

Genotyping and maintenance of the mouse colony was as described in Chapter 3. In brief, mating pairs of (*f*α-CaMKII/+, +/+) x (*f*α-CaMKII /+, *Cre* /+) mice were set up to produce (*f*α-CaMKII/ *f*α-CaMKII, *Cre*/+) mice (CA3 α-CaMKII KO) and three types of littermate controls: wild-type (+/+, +/+), CW2 (+/+, *Cre*/+), and homozygous *f*α-CaMKII (*f*α-CaMKII/*f*α-CaMKII). Mating pairs of (*f*α-CaMKII/*f*α-CaMKII, *Cre*/+) x (*f*α-CaMKII/*f*α-CaMKII, +/+) were also used to facilitate the expansion process. When available, male (*f*α-CaMKII/*f*α-CaMKII, *Cre*/+) mice were used in these mating pairs. Electrophysiology and behavior experiments were performed on male mice between the ages of 4-5.5 months. *f*α-CaMKII littermates were used as controls and all experiments were performed blind to genotype.

In situ hybridization

As described in Chapter 3.

Immunohistochemistry

As described in Chapter 3.

Extracellular recordings

As described in Chapter 3. All experiments were performed on male littermates 4-5 months old, and data was collected and analyzed blind to genotype.

Intracellular recordings

Hippocampal slices were prepared as described for extracellular recordings in Chapter 3. Whole cell recordings were obtained from CA1 pyramidal cells under visual guidance (DIC/infrared optics) using an EPC-9 amplifier and Pulse v8.09 software (HEKA Elektronik). EPSCs were evoked by field stimulation of the Schaffer collateral/commissural fiber – CA1 pathway with bipolar tungsten electrodes using brief current pulses. The patch electrodes (3-5mOhm) contained (in mM) 5 NaCl, 120 K-gluconate, 10 EGTA, 5 NaCl, 1 $MgCl_2$, 10 Hepes (pH 7.2 with KOH titration), 2 Mg-ATP, and 0.1 Na-GTP. Series resistance was monitored throughout each experiment and was in the range of 10-25mOhms. The EPSCs were filtered at 1-2 kHz and digitized at 2.5-5.0 kHz. EPSC amplitudes were measured as the difference between the mean current during a prestimulus baseline and the mean current over a 2ms window at the peak of the response. The holding potential was –70 mV. All recordings were done in 50μM picrotoxin. In PPF experiments, 12 pairs of stimuli were recorded and averaged at each interstimulus interval (50, 70, 100, 200 and 300 msec). In FF experiments, 30 pulses were given at 1Hz, 5 Hz, 10 Hz, and 20 Hz, with each train separated by 3 min to allow the synapses to recover. In synaptic depletion experiments, a train of 300 depolarizing pulses was given at 70msec intervals (approximately 14Hz). Final depletion levels were calculated by averaging the last 30 responses of the 300 pulse depletion train. Following depletion, synapses recovered during a short series of 7 pulses at time points 50 msec, 500 msec, 1 sec, 3 sec, 5 sec, 8 sec, and 11 sec post train. In LTP pairing experiments, patch electrodes contained 0.2mM EGTA. For LTP

induction, the patched neuron was voltage clamped at +30mV and Schaffer collateral fibers were stimulated with 80 pulses at 2Hz. The magnitude of LTP was measured by comparing the average of responses over a 5 min interval 30-35 min post pairing with the average of responses over a 5 min interval preceding the pairing. Spontaneous miniature EPSCs were recorded on videotape for off-line analysis. Data was analyzed with the Mini Analysis Program v4.3.0 (Synaptosoft Inc., Leonia, NJ, USA). For the cumulative graphs, responses were normalized to the median amplitude of baseline mEPSCs. 1 μM of tetrodotoxin was included to the bathing solution in these experiments. All experiments were performed on male littermates (4-5 months old) and data was collected and analyzed blind to genotype.

Analysis of locomotor behavior

As described in Chapter 3. All mice analyzed were male littermates 4.5-5.5 months old. Experiments were performed blind to genotype.

Fear conditioning

As described in Chapter 3. All mice analyzed were male littermates 4.5-5.5 months old. Experiments were performed blind to genotype.

Acknowledgements

Kazutoshi Nakazawa constructed the G32-4 Cre transgenic mouse. The electrophysiology experiments were performed by Ivan Goussakov in the laboratory of Vadim Bolshakov. We are grateful to Chanel Lovett, Frank Bushard, Jayson Derwin, Wenjiang Yu, and Xiao-ning Zhuo for excellent technical assistance at various stages of this project.

Chapter 5

Short communication

Conditional expression of the endogenous CaMKII inhibitor, KII-IN, in transgenic mice

Introduction

Conditional gene knockout is a powerful approach for inhibiting a specific molecule of interest, but it is inefficient in situations where multiple redundant isoforms of the molecule exist. For example, many of the pre-synaptic proteins involved in the neurotransmitter release mechanism exist in multiple, highly homologous isoforms encoded by separate genes [266], making gene targeting an impractical approach for studying the role of these types of molecules in the release pathway. Such targeting would require making targeting constructs and recombinant mice for each gene of interest, and crossing these mice until the appropriate multiple gene knockout genotype is obtained. This can be extremely time consuming and expensive, and the final mating process is very inefficient. An alternative approach is to "target" multiple isoforms of the gene of interest by overexpressing an inhibitor specific for the genes of interest. This dominant-negative transgenic approach is appealing because (a) a suitable inhibitor may interfere with multiple target genes, (b) regionally restricted promoters can allow expression in a temporally and/or regionally restricted pattern, (c) transgenic constructs and mice can be produced relatively quickly, compared with recombinant mice, (d) the production of multiple transgenic lines can lead to unpredictable, fortuitous expression patterns, (e) novel application of the Cre/loxP system can give additional temporal and spatial restriction of transgene expression (Figure 5-1, Figure 5-2), and (f) recent developments using inducible transgenic gene expression systems offer the possibilities

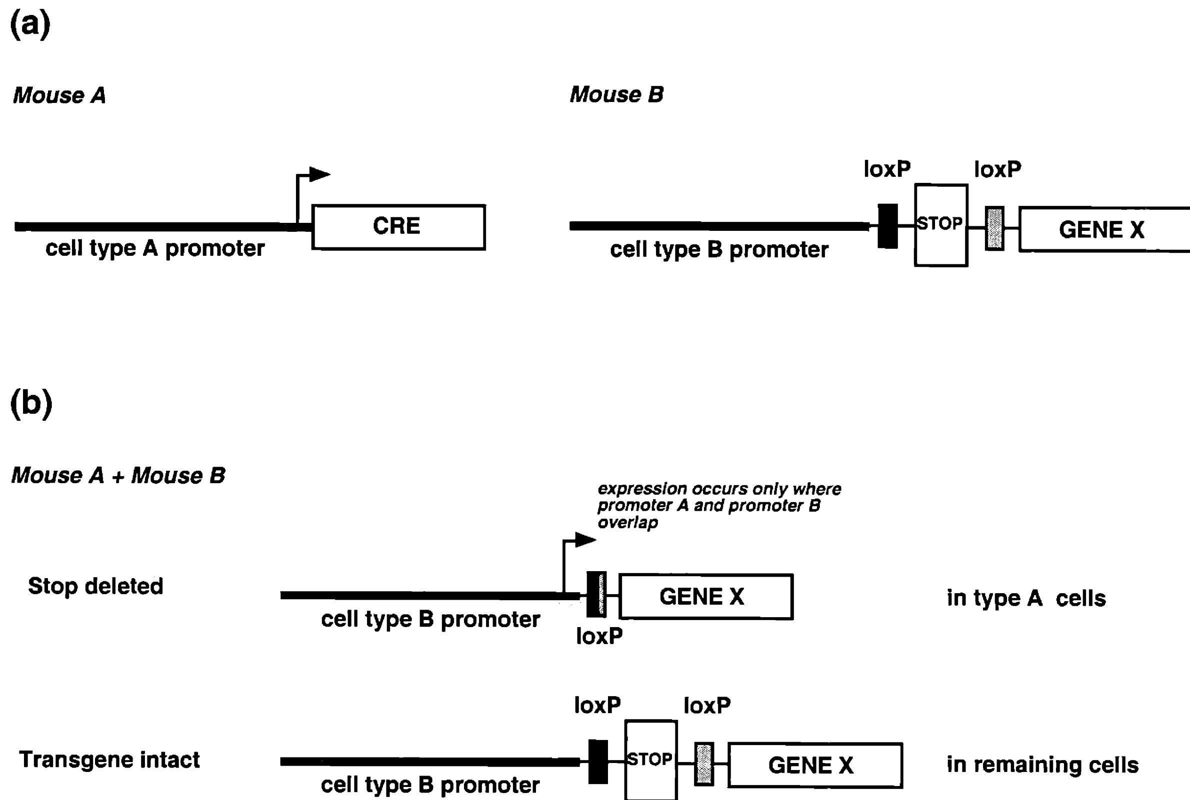


Figure 5-1: Conditional transgene expression using the Cre/loxP system

(a) Two different mouse lines are required for conditional transgene expression using the Cre/loxP system. The first line is a transgenic mouse that expresses Cre in the desired regional/temporal specific pattern. The second line is a transgenic mouse in which loxP sequences have been inserted flanking a transcription/translation *Stop* sequence (Gibco BRL), positioned between a promoter of choice and the gene of interest.
(b) When the two lines are crossed, Cre recombination will delete the *Stop* sequence in cells where Cre is expressed. This will allow expression of gene X in cells where Cre recombination has occurred (promoter A) and where promoter B is expressed. The combinatorial effects of combining multiple transgene promoters can potentially create very specific patterns of transgene expression.

of turning transgene expression “on and off” – a powerful tool for the study of learning and temporally defined stages of memory encoding, consolidation and recall, while avoiding the detrimental effects of transgene expression during development (see Figure 1-5, Figure 5-2).

While the dominant-negative transgenic approach is appealing for its potential flexibility, it has many technical limitations. First, a suitable inhibitor is required that

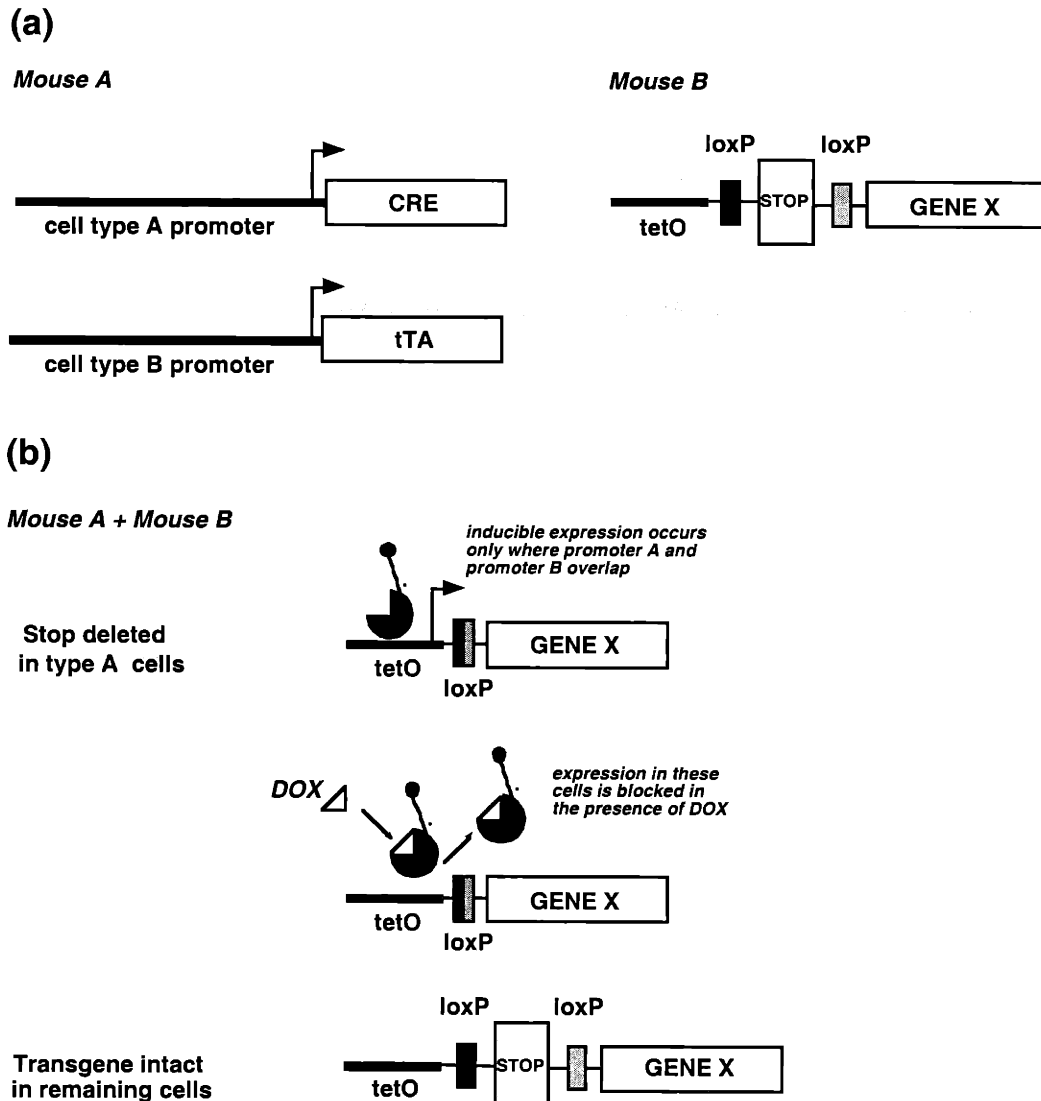


Figure 5-2: Conditional transgene expression using the Cre/loxP and tTA system

(a) Three different transgenes are required for the production of inducible, tissue-specific conditional transgene expression. In the above figure, two transgenes shown are co-expressed in Mouse A, for clarity. The tTA system functions as previously described in Figure 1-5. The lox *Stop* lox sequence is added upstream of the gene of interest, Gene X, to allow tissue specific transgene expression in a pattern reflecting the combinatorial effects of promoters A and B.
(b) When these three transgenes are combined in one mouse, the *Stop* cassette is excised and Gene X is expressed only in those cells where tTA is also expressed. This expression is blocked in the presence of Dox.

inhibits efficiently and specifically. Second, suitable promoters and regulatory sequences must be obtained to express the inhibitor in the desired pattern within the

brain, and to target the inhibitor within the cell. Third, since transgenic promoters do not always recapitulate their expected expression patterns due to positional effects upon transgene insertion, many independent lines of transgenic mice need to be produced and characterized to find ones with suitable expression patterns. Of course, unexpected expression patterns are occasionally stumbled upon that can become incredibly useful. Fourth, the production of double and triple transgenics is somewhat unwieldy. And finally, inducible gene expression systems are still in need of optimization, to ensure reliable and reproducible induction with minimal background expression in the absence of inducer.

While we have used conditional gene knockout approaches for studying CaMKII function in synaptic plasticity and learning and memory, a dominant negative transgenic approach would complement these studies. α-CaMKII is the dominant CaMKII isoform expressed in the brain, but the moderately expressed highly homologous β isoform is also present [37]. The global α-CaMKII knockout mouse showed significant residual CaMKII activity [111], and the observed LTP deficiency was not complete [111,137], perhaps due to the presence of other CaMKII isoforms. We saw similar levels of residual LTP in CA1 α-CaMKII KO mice (Chapter 3), suggesting that some CaMKII function may persist specifically in post-synaptic CA1. It should be noted that we do not know if the β isoform of CaMKII shares substrates with the α isoform *in vivo*, and they are known to have different preferred subcellular localizations [36,172]. However, they are able to co-assemble into functional holoenzymes both *in vitro* and possibly, *in vivo* [36,267]. Using conditional transgene expression, it may be possible to inhibit all CaMKII isoforms in a spatially and temporally regulated fashion to avoid developmental effects of transgene expression, while targeting the dominant-negative transgene to the desired hippocampal region(s).

Multiple inhibitors of CaMKII exist, including small peptides, organic molecules, and recently cloned endogenous gene products. Early studies of LTP mechanisms using CaMKII inhibitors introduced through patch pipettes used small peptides encoding

segments of the pseudosubstrate autoinhibitory domain of α-CaMKII [51]. These peptides likely inhibited all CaMKII isoforms since the autoinhibitory domains of CaMKII isoforms are almost identical [184], and one such peptide was found to disrupt associative and nonassociative learning when expressed as a transgene in flies [181]. However, there is some evidence that these inhibitors are not truly specific for CaMKII, and instead may also inhibit protein kinase C (PKC) [268]. The membrane-permeant organic inhibitor KN-62 blocks CaMKII activity by binding to the molecule and interfering with calmodulin binding [241], and has been shown to cause retrograde amnesia when infused into the hippocampus of rats [182]. Again, KN-62 has been shown to be less than specific for CaMKII; it is also a potent inhibitor of CaMKIV [269]. Recently, a novel endogenous CaMKII inhibitor, CaMKII-IN (KII-IN), was cloned which binds to the catalytic domain of both the α and β isoforms of CaMKII and is a potent (IC_{50} = 50nM) and inhibitor of activated CaMKII [270]. KII-IN appears to be highly specific for CaMKII vs. other kinases (CaMKIV, PKC, PKA) and has no other known functions. Using this inhibitor, we produced conditional transgenic mice using the Cre/loxP and tTA systems to explore the utility of a dominant negative transgenic approach to specifically inhibiting CaMKII function.

Results

Expression of the endogenous KII-IN transgene

Initial characterization of endogenous KII-IN expression patterns by Northern blot showed broad expression throughout the brain and in testis [270]. We performed *in situ* hybridization analysis of endogenous CaMKII-IN to determine wild type expression patterns in the adult mouse, which had not yet been described in detail. *In situ* hybridization was performed using a ^{33}P labeled cRNA probe specific for the CaMKII-IN 3' untranslated region (3'UTR). This was compared with expression of the dominant α-

CaMKII isoform using a ^{33}P labeled cRNA probe specific for the 3' untranslated region of α-CaMKII. We found that endogenous KII-IN is expressed at low levels in the adult forebrain, and is selectively reduced in the CA1 region of the hippocampus, implicating an important role for active CaMKII in this hippocampal region (Figure 5-3).

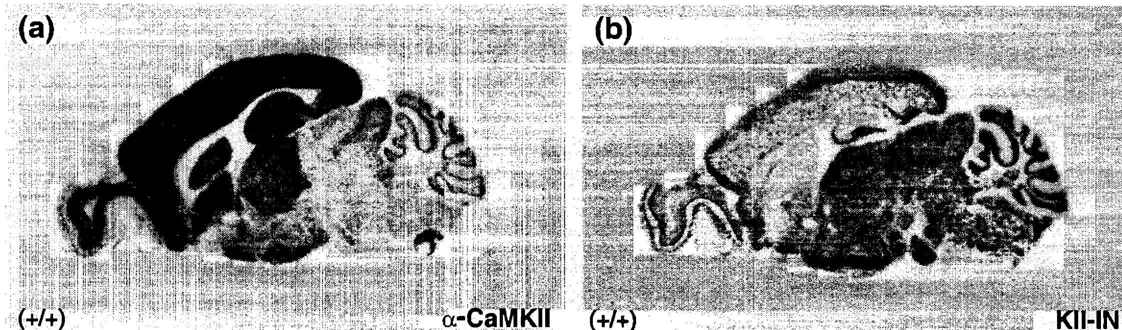


Figure 5-3: Expression of the endogenous CaMKII inhibitor, CaMKII-IN, in the wild-type adult mouse

Scanned images of sagital brain sections from wild-type (+/+) adult mice hybridized with cRNA probes specific for either α-CaMKII (a) or KII-IN (b). Silver grains representing mRNA signals are in black. KII-IN is expressed at low levels relative to α-CaMKII in the forebrain, but is highly expressed in the midbrain and cerebellum.

Interestingly, KII-IN is expressed in a pattern that complements α-CaMKII; it is expressed at low levels in the forebrain where α-CaMKII predominates, and is expressed in higher levels in the mid/hindbrain, where α-CaMKII levels are restricted. KII-IN mRNA does not appear to be present in dendritic regions of the hippocampus, unlike α-CaMKII mRNA.

Production of KII-IN transgenic mice

To create conditional KII-IN transgenic mice, we prepared four constructs that express the inhibitor as a transgene using traditional methods (#1), using the Cre/loxP system for control of spatial expression (#2), using the tTA system for temporal control (#3), and

using the Cre/loxP system in combination with the tTA system for inducible regionally restricted transgene expression (#4) (Figure 5-4).

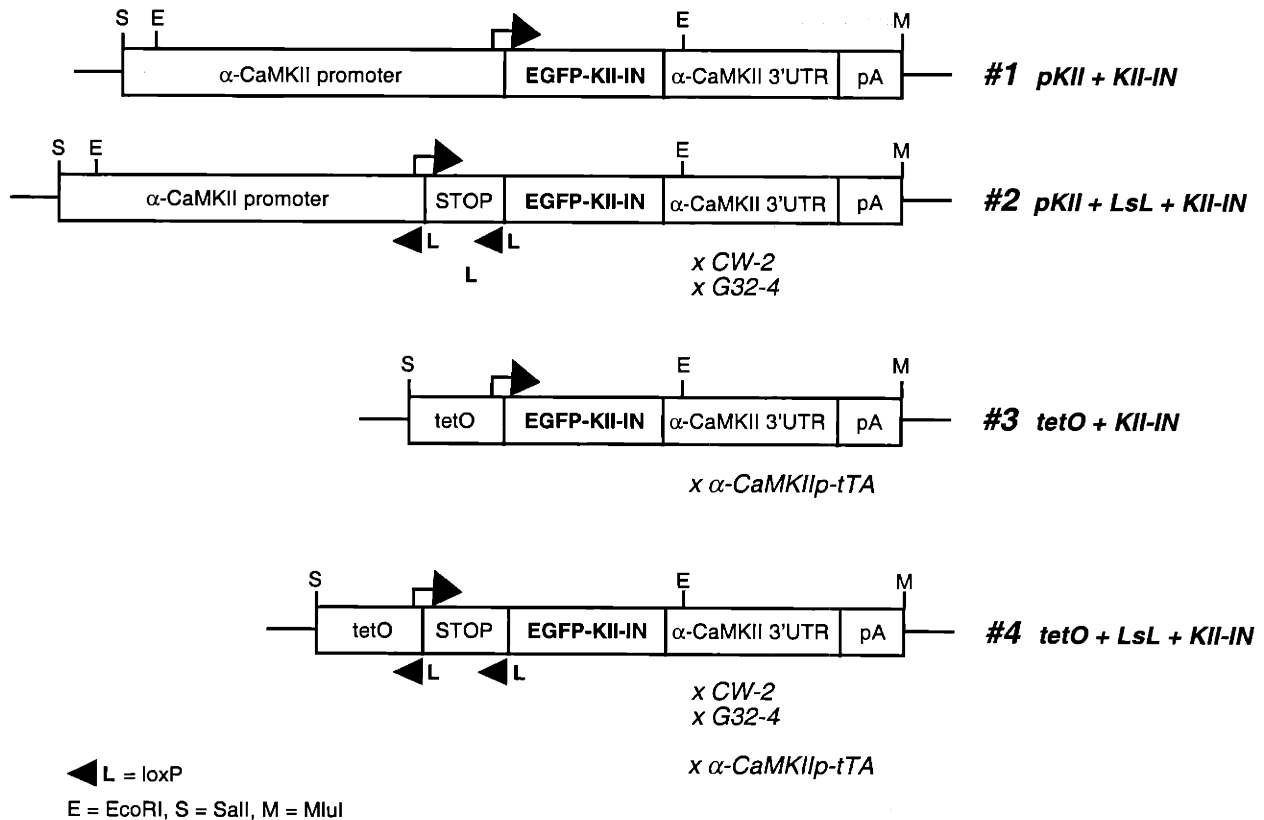


Figure 5-4: Transgenic constructs for conditional expression of KII-IN

Four different transgenic constructs were designed. The core of all constructs was the KII-IN coding region fused with the EGFP reporter gene. The α-CaMKII 3'UTR was cloned downstream to target the transgenic mRNA to dendrites, and an SV40 polyadenylation site and intronic sequence were also added (pA). In construct #1, the α-CaMKII promoter was cloned upstream to drive transgene expression in the forebrain. In construct #2, the loxP-*Stop*-loxP cassette (LsL) was cloned just upstream of EGFP-KII-IN to inhibit transcription/translation. In the presence of a second transgene expressing Cre recombinase, the *Stop* cassette will be excised and transcription/translation will proceed. Possible Cre transgenic mice include the CW-2 and G32-4 Cre lines mentioned in previous chapters 3 and 4. In construct #3, the tetO promoter region was cloned upstream of the core construct, to allow temporal control of transgene expression in the presence of a second transgene expressing the tetracycline transactivator, tTA. Possible tTA transgenic mice include a tTA line driven by the α-CaMKII promoter [135]. In construct #4, the *Stop* cassette was added to allow spatial restriction of transgene expression in the presence of Cre, and additional temporal restriction in the presence of tTA.

The coding region of CaMKII-IN was cloned downstream of the enhanced green fluorescent protein cDNA (EGFP – Clonetech), a useful marker for monitoring transgene expression, to ultimately produce an EGFP-KII-IN fusion protein. The lengthy 3'UTR of KII-IN (1.1kb of a total 1.4kb cDNA) was removed, as the function of this region was not known and there was some concern that it might regulate gene expression and/or targeting. It was replaced with the 3'UTR from α-CaMKII, a sequence known to target mRNAs to dendrites [125], and an SV40 polyadenylation signal. CaMKII-IN is not found to be highly expressed in dendrites in wild type mice [270]. It was thought that such dendritic targeting may be required to inhibit the significant quantities of CaMKII that are highly localized at the post-synaptic density in dendrites [57]. For the first transgenic construct (construct #1), this modified EGFP-KII-IN fusion was cloned downstream of the α-CaMKII promoter [125,134]. This promoter has been shown to drive transgene expression strongly throughout the forebrain postnatally, in a pattern reminiscent of endogenous α-CaMKII. While transgenic recapitulation of endogenous promoter expression patterns is often inconsistent, we chose this promoter in hopes of increasing our chances of efficiently inhibiting endogenous CaMKII. To obtain spatially restricted transgenic expression, we inserted a lox-*Stop*-lox cassette (LsL, Gibco BRL) immediately upstream of the coding region (construct #2). When a transgenic mouse harboring this construct as a transgene is crossed to a Cre transgenic mouse, the transcription/translation*Stop* sequences will be excised and the fusion protein will be expressed in a pattern reflecting the combinatorial effects of Cre excision and α-CaMKII promoted expression. To obtain temporally restricted transgenic expression using the tetracycline transactivator system, we substituted the tetO promoter - which consists of multiple repeats of the tet operator linked to a minimal eukaryotic promoter element – for the α-CaMKII promoter in the above constructs (construct #3 and #4). When transgenic mice expressing the eukaryotic transcription activator tTA are crossed to these tetO mice, tTA will drive expression of the fusion protein in a pattern reflecting tTA expression, and Cre

excision of the *Stop* cassette (if relevant, construct #4). In the presence of doxycycline, a more efficient tetracycline derivative, tTA driven transgene expression is blocked [131].

Linearized transgenic constructs were diluted to a concentration of 5-10ng/μl and were injected into 1 day old embryos of C57Bl/6 mice, as previously described [123]. Founder mice were identified by screening pups with a probe specific for the SV40 polyA signal or the LsL sequence from the transgenic constructs, and transgene copy number was approximated (Figure 5-5). For construct #1(pKII + KII-IN), 12 founders were produced and were crossed to C57Bl/6 mice to produce F1 progeny, and 7 transmitted the transgene to the germline. For construct #2 (pKII + LsL+KII-IN), 3 founders were produced and were crossed to C57Bl/6 mice to produce F1 progeny, and all 3 were successfully expanded. F1 animals carrying construct #2 were then crossed to the CW-2 Cre expressing line, as previously described, to restrict expression of the transgene mRNA in a tissue specific manner. For construct #3 (tetO + KII-IN), 9 founders were produced and 4 transmitted the tetO driven transgene to F1 progeny. For construct #4 (tetO + LsL + KII-IN), only 4 founders were produced, but all 4 transmitted the transgene through the germline. F1 tetO transgenic animals carrying either construct #3 or #4 were crossed to tTA transgenic mice driven by the α-CaMKII promoter [135]. This tTA line (B13; pKII-tTA) has been shown to induce tetO mediated gene expression widely in the forebrain, with weak expression specifically in hippocampal CA3 [135]. Transgene copy number was estimated by Southern hybridization, and all lines that transmitted the transgene through the germline were maintained for further analysis.

Expression studies of transgenic lines

Using *in situ* hybridization, we examined the expression patterns of the KII-IN transgenes in adult mice (> 5 weeks). Transgenic mice constructed using the α-CaMKII promoter (constructs #1 and #2) were found to have very poor levels of gene

Construct	Founder	Sex	Copy #	Mating?	Germline?	Cross	Expression
#1	53	female	1	Yes			
	57	female	1				
	58	male	20-30	Yes	Yes		
	108	male	1	Yes	Yes		
	113	female	1				
	114	female	3-4	Yes	Yes		low?
	115	female	5				
	121	female	1-2	Yes	Yes		low?
	122	female	1-2				
	123	female	5-7	Yes	Yes		low?
	124	female	5	Yes	Yes		
	125	male	10	Yes	Yes		
#2	5	male	1-3	Yes	Yes	x CW-2	very low
	41	male	2-5	Yes	Yes	x CW-2	very low
	47	male	1-2	Yes	Yes	x CW-2	
#3	59	male	1	Yes			
	61	male	1	Yes	Yes	x pKII-tTA	moderate
	72	female	20-30	Yes	Yes	x pKII-tTA	moderate
	80	male	10	Yes	Yes	x pKII-tTA	moderate
	84	female	1	Yes			
	98	female	1				
	99	male	25-30	Yes	Yes	x pKII-tTA	moderate
	103	female	1				
	104	female	2-3				
#4	16	male	25-30	Yes	Yes	x pKII-tTA	moderate
	19	male	5-7	Yes	Yes	x pKII-tTA	moderate
	21	male	2-3	Yes	Yes	x pKII-tTA	moderate
	22	male	3-5	Yes	Yes	x pKII-tTA	moderate

Figure 5-5: Characterization of KII-IN transgenic founders

Table describing transgenic founders produced via pronuclear injection of transgenes (Figure 5-4). Copy number was estimated by Southern hybridization. Founders that mated successfully and that transmitted their transgene through the germline are indicated. KII-IN Transgenic mice that were crossed to other transgenic mice lines before analysis are also indicated. See Figure 5-4 for construct descriptions.

expression broadly in the forebrain (data not shown). Expression levels did not correlate with copy number and mRNA was not targeted to dendritic regions. Because Cre recombination occurs broadly throughout the forebrain in CW-2 transgenic mice, it did not offer dramatically improved localization of transgene expression when crossed to mice harboring the *Stop* cassette (construct #2). As we suspect that high levels of KII-IN expression would be required for adequate inhibition of all CaMKII isoforms, these transgenic lines were not sufficient for our purposes, and were set aside.

tetO transgenic lines crossed to the pKII-tTA transgenic line [135] were also analyzed using *in situ* hybridization to see if tTA promoted expression. *In situ* hybridization was performed using a ^{33}P labeled EGFP cRNA probe and transgenic expression was observed in scattered cells in the cortex, the CA1 region of the hippocampus and the subiculum, and at very high levels in the striatum (Figure 5-6a). Again, mRNA appeared to be restricted to the pyramidal cell body layer in CA1, despite the presence of the α-CaMKII 3'UTR that is thought to target mRNA to dendrites [125]. This expression pattern was surprisingly consistent among transgenic lines. One line of tetO-KII-IN (line 72), tTA+ mice was examined for expression of the KII-IN-GFP transgene in the presence or absence of doxycycline for 1 or 2 weeks duration (approximately 20μg/day in the mouse chow). Expression of the transgene was turned off completely following 1 week Dox treatment, although some striatal transgene expression remained in the mouse treated with Dox for 2 weeks (Figure 5-6). It has been reported previously that Dox suppression of tTA-promoted transcription can be variable from mouse to mouse (Bujard, H., personal communication; Hayashi, M., McHugh, T., personal communication). No background KII-IN transgene expression was observed in mice that did not co-express the pKII-tTA transgene (data not shown).

To create tissue specific inducible transgene expression, transgenic mice carrying the tetO-LsL-KII-IN construct (construct #4) were first crossed to tTA mice and inducible expression of the *Stop* cassette was monitored by *in situ* hybridization. Expression of

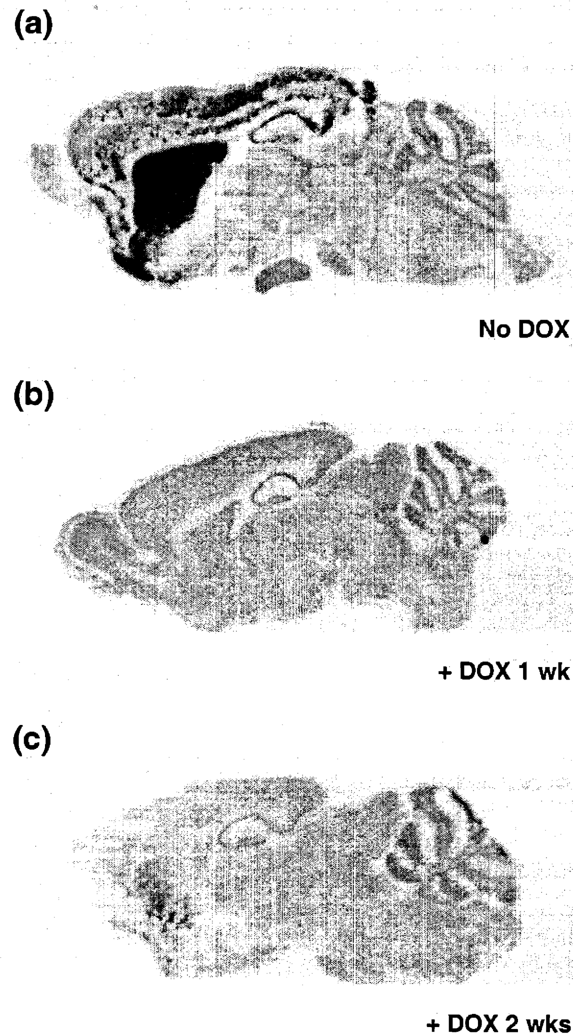


Figure 5-6: tTA promoted expression of a KII-IN transgene can be regulated by the administration of doxycycline

Line 72 (tetO-KII-IN, construct #3) was crossed to pKII-tTA transgenic mice [125] and induced transgene expression was monitored via *in situ* hybridization. (a) KII-IN transgene expression was observed in forebrain regions, including scattered cells of the cortex, hippocampal CA1, and subiculum, with saturating expression in the striatum.
(b) Following 1 week administration of Dox in the mouse chow (approx. 20μg/day), tTA induction of KII-IN transcription was completely blocked.
(c) Following 2 weeks administration of Dox, tTA induction of KII-IN transcription was strongly inhibited, though some residual expression is still observed in the striatum. All mice examined in this experiment were littermates.

the KII-IN is inhibited unless the *Stop* cassette is excised via Cre recombinase, but a cRNA probe for the *Stop* cassette can detect partial expression of this sequence. All of the lines examined showed LsL expression patterns reminiscent of those seen for construct #3 (Figure 5-6a), including scattered cortical expression, high striatal expression and selective expression in hippocampal CA1 and subiculum (Figure 5.7).

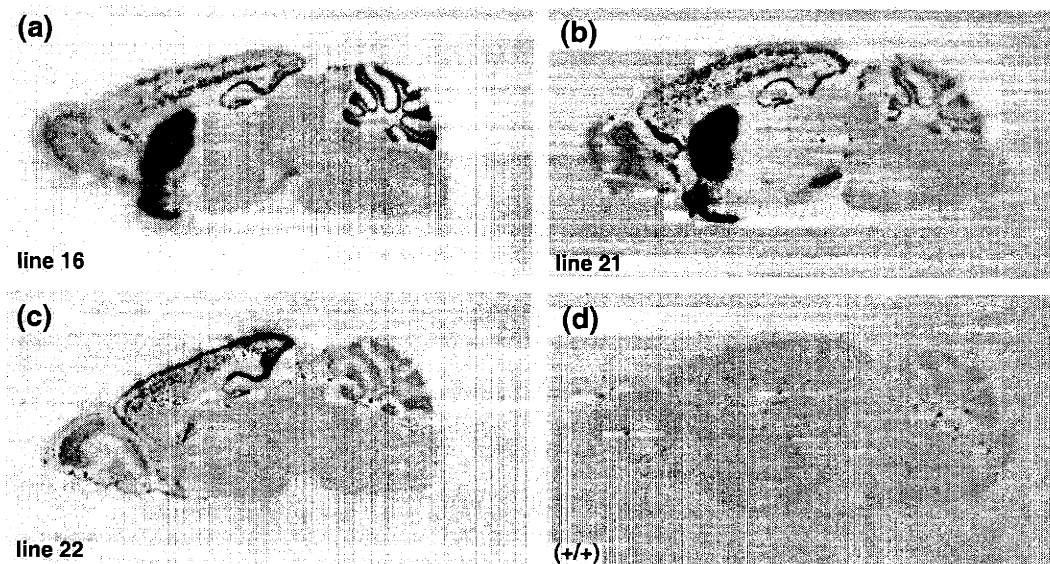


Figure 5-7: tTA promoted expression of the *Stop* cassette in tetO-LsL-KII-IN transgenic mice

tetO-LsL-KII-IN transgenic mice (construct #4) were crossed to pKII-tTA transgenic mice and induced expression of the *Stop* cassette was monitored via *in situ* hybridization. tTA induced expression is similar in multiple forebrain regions in lines 16 (a), 21 (b) and 22 (c). Hybridization to a wild-type (+/+) section is shown as a negative control (d).

For tissue specific expression of the KII-IN transgene, double transgenic tetO+, tTA+ transgenic lines (Figure 5-7) must be crossed to a Cre expressing transgenic mouse. Unfortunately, our CW-2 and G32-4 Cre transgenic lines are not of much help for further spatial restriction of transgene expression. The *Stop* sequence is not expressed in CA3 in tetO+, tTA+ double transgenics, so CA3 specific transgene expression using the

G32-4 Cre transgenic mouse cannot be obtained using these tTA+ transgenic lines. As CW-2 mice have not proven to be truly CA1 specific, and instead promote recombination widely in the forebrain, these are also of limited utility. Since producing triple transgenic mice for analysis is somewhat unwieldy, we will delay further analysis of inducible tissue specific KII-IN expression until more specific Cre mice are available.

Discussion

We have explored a few possible methods for inhibiting CaMKII function *in vivo* using conditional transgene expression. A dominant negative transgenic approach toward studying CaMKII function has the advantage of inhibiting multiple CaMKII isoforms in a highly specific manner. The recent cloning of a novel CaMKII inhibitor, KII-IN [270], has supplied the highly specific inhibitor that had been lacking in previous studies [182,271]. We have constructed several types of KII-IN transgenic mice that utilize novel applications of the Cre/loxP and tetracycline transactivator systems for the temporal and spatial control of transgene expression. Several lines of transgenic mice were created that express the KII-IN transgene in an inducible manner in the presence of tTA, and this expression can be regulated by the administration of the tetracycline derivative, doxycycline. Additional promoters and Cre expressing transgenic mice need to be characterized to allow more regionally restricted transgene expression using this combinatorial multi-transgenic approach. It is still to be determined whether over-expression of the KII-IN gene is sufficient to inhibit CaMKII at the synapse. However, the conditional transgenic approach has exciting potential for dissecting the molecular pathways underlying physiology and behavior using spatially targeted gene expression and temporal control to preserve normal development, and reversible transgenic manipulation for the study of the different stages of learning and memory formation.

We pursued several different types of transgenic constructs because it was unclear which approach would be productive. Our basic approach was to simply reiterate "normal" α-CaMKII expression patterns in a KII-IN transgenic mouse by taking advantage of both 5' and 3' α-CaMKII regulatory regions that were available. Surprisingly, constructs driven by the α-CaMKII promoter did not give high levels of expression compared with examples published previously [134,135], and observed in our laboratory (Kang, H., personal communication). The 3' UTR of α-CaMKII also did not appear to target mRNA efficiently to dendrites, although it is possible that a small amount of targeting occurred and was difficult to detect. This is also contrary to previously published reports [134,135]. It is possible that this simply reflects the variability in transgene expression that is often observed among different lines expressing the same transgene due to positional effects at the site of insertion and copy number. Alternatively, it is possible that our unique combination of transgene, regulatory regions, and polyadenylation and splicing sequences may have produced a much more restricted expression pattern in our α-CaMKII inspired transgenic constructs. It would be useful to produce additional transgenic lines with these constructs, as well as pursue other neural specific, high-expressing promoters, to try to obtain more useful expression patterns. Unfortunately, isolating and characterizing such promoters has been a rate limiting step in the production of neural-specific transgenic mice, and inter-line variability in transgenic mouse production can be quite unpredictable for producing useful patterns of transgene expression.

Conditional regional and temporal control of gene expression is also dependent upon the expression patterns of the respective Cre and tTA transgenic lines that are crossed with the line of interest (Figures 5-1, 5-2). While we were able to obtain tTA induced transgene expression restricted to the CA1 side of the CA3-CA1 synapse without combinatorial transgenics (Figure 5-6), expression was also scattered in the cortex, among other regions. Additional Cre and tTA transgenic lines are needed to

increase the range of possibilities for limited regional expression. For example, it would be useful to create a CA3 specific tTA line using the recently characterized KA-1 promoter [136], and a truly CA1 specific line, perhaps by creating more tTA lines driven by the α-CaMKII promoter [123]. To study the function of different brain regions during each stage of memory formation, encoding and recall, it would be particularly useful to have combinatorial expression patterns limited to the hippocampus, cortex, and amygdala.

One of the remaining problems for conditional transgenics is to optimize a system for reproducible and robust temporal control of transgene expression. While the tTA system has been shown to be effective in the brain [130,135,272], it is somewhat limited by slow kinetics of induction control in the presence or absence of Dox, and inter-animal variability. This is likely due to a combination of poor doxycycline transport across the blood-brain barrier and variable Dox dosage due to self-administration. The tTA system also requires a constant dose of doxycycline to be administered throughout development to block transgene expression until the desired time point. This can be problematic, as inconsistent doxycycline dosage could lead to leaky transgene expression at undesired early time points, and doxycycline itself has been shown to be detrimental to learning when chronically administered [135]. An optimal system for temporal control would leave the transgene silent until an appropriate inducing agent is administered. This is possible, in theory, using the reverse tetracycline transactivator system (rtTA) [131]. This system is similar in design to the tTA system, except expression downstream of the tetO element is mediated by rtTA only when Dox is administered. While it has been used in the brain in one study [273], recent developments of rtTA mutants by Bujard and coworkers show promise with higher, more rapid induction capabilities. Conveniently, the same tetO transgenic mice could be potentially used with either the tTA or rtTA system.

Materials and Methods

Generation of transgenic mice

A 237bp fragment consisting of the coding region of the CaMKII inhibitor (KII-IN) was prepared using the polymerase chain reaction (PCR; *pfu* Taq polymerase, Promega). This fragment did not include the 5' UTR, the initial 'ATG' codon, or the 3' UTR found in the original 1.4kb KII-IN cDNA [270]. The inhibitor PCR product was cloned in-frame, downstream of the EGFP cDNA (pEGFP-C1 vector, Clonetech) to allow the production of an EGFP-KII-IN fusion protein. The 3.2kb 3'UTR from the α-CaMKII cDNA (gift from M. Mayford) was cloned at the 3' end of the fusion construct, followed by an additional 850bp fragment that included an SV40 early splice region and an SV40 polyadenylation signal (XhoI-BamHI fragment, pMSG vector, Pharmacia Biotech). This EGFP-KII-IN fusion plus downstream regulatory sequences formed the core of subsequent transgenic constructs. In construct #1, the core was cloned downstream of the 8.5kb α-CaMKII promoter region (gift from M. Mayford and E. Kandel). In construct #2, the core was cloned downstream of the α-CaMKII promoter and an intervening 1.6kb loxP – *Stop* – loxP cassette (NotI fragment, pBS302 vector, Gibco BRL). In construct #3, the core was cloned downstream of the 475bp tetracycline responsive promoter $P_{hCMV*-1}$ (pTRE vector, Clonetech), referred to as tetO in this chapter. In construct #4, the core was also cloned downstream of the $P_{hCMV*-1}$ promoter and an intervening 1.6kb loxP – *Stop* –loxP cassette. The KII-IN PCR product and all cloning junctions were confirmed by sequencing.

Mice

All transgenic mice were created in a C57Bl/6 genetic background and founders were mated to C57Bl/6 mice for expansion. F1 and F2 transgenic animals were crossed to the tTA line (mixed genetic background) to produce double transgenic mice. Initial

genotyping of founders and F1 progeny was done by Southern hybridization of tail DNA to determine copy number of the transgene and to ensure that the transgene was stable as it was transmitted through the germline. Subsequently, PCR primers were designed for the polyadenylation signal (pA) to allow rapid genotyping of stable lines. All transgenic mice were maintained as heterozygotes. The pKII-tTA transgenic line was constructed by Mark Mayford [135], was purchased from Jackson Laboratory {TgN (CamK2tTA)1Mmay}, and was the only line in a mixed genetic background.

In situ hybridization

As described in Chapter 3. For studies of endogenous gene expression patterns, ^{33}P labeled cRNA probes included a 650bp SmaI fragment specific for the 3' end of the CaMKII-IN 3'UTR, and a 550bp EcoRI fragment specific for the α-CaMKII 3'UTR to detect endogenous expression patterns of CaMKII-IN and α-CaMKII, respectively. For detection of transgenic DNA, ^{33}P labeled cRNA probes included a 725bp fragment specific for the EGFP marker sequence (constructs #1 and #3) and a 600bp specific for the 5' end of the loxP–*Stop*-loxP sequence (constructs #2 and #4).

Dox administration

To inhibit expression of the tetO-KII-IN transgene in lines co-expressing tTA, doxycycline (Dox) was administered orally in the mouse chow (Bio-Serve Inc., 40mg Dox / kg chow). Mice were fed *ad libitum* for a duration of 1 or 2 weeks, as indicated. Average chow intake was 5g / day, for an average daily dose of 200μg Dox daily. Efficiency of Dox transport across the blood-brain barrier is low, with approximately 14% of Dox reaching the cerebral spinal fluid [274].

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Chapter 6

Conclusions

Conditional gene knockout and transgene expression is a powerful approach for studying gene function in the brain *in vivo*. We examined the role of α-CaMKII in hippocampal synaptic plasticity by selectively targeting gene deletion at either the pre-synaptic or post-synaptic side of the CA3 pyramidal cell -CA1 pyramidal cell hippocampal synapse. This synapse is particularly important in the study of synaptic plasticity mechanisms, such as LTP, and their association with learning and memory because (a) it is the most widely studied excitatory synapse in the brain, (b) it expresses associative NMDA receptor dependent LTP of a type present at many excitatory synapses, (c) it is the main output pathway of the hippocampus [264], a brain region known to be critical for the formation of new memories [19], (d) cells at this synapse fire at rates that vary with the location of an animal in space ("place fields") and disruption of place cell activity is associated with abnormal spatial learning [126,136] (e) hypoxic [17,18] lesions at this synapse disrupt learning and memory (f) and several pharmacology and mouse genetic experiments have identified a correlation between synaptic plasticity at this synapse and spatial/contextual learning [23,100,111]. We chose to clarify the role of CaMKII in CA3-CA1 plasticity mechanisms and associated behaviors because this molecule has been the target of intense study in the field because of its putative role in LTP [171,253], its optimal localization [55-57] and interesting biochemical properties [36] (Figure 1-3, Figure 4-15). Conditional deletion of α-CaMKII in either the pre- or post-synaptic cell at the CA3-CA1 could clarify potential "pre- vs. post-" LTP expression mechanisms, as CaMKII substrates have been found to be modified on both sides of the synapse following LTP induction [64,66,148]. In addition, the role of CaMKII in pre-synaptic regulation of neurotransmitter release is poorly defined [224], so such studies could provide insight into

CaMKII contributions to neurotransmitter release. Our conditional gene-deletion experiments will improve upon previous studies of LTP and learning in genetically altered mice by avoiding many of the confounding variables of the global α-CaMKII knockout, including developmental abnormalities and associated compensatory changes due to early onset of gene knockout, ubiquitous gene deletion, and analysis in a mixed genetic background. Finally, it will complement previous conditional gene targeting of NMDAR1 in CA1 pyramidal cells by producing abnormal synaptic plasticity due to downstream changes while leaving NMDAR synaptic transmission and consequent calcium influx intact. Such studies could contribute to the debate as to whether hippocampal CA3-CA1 plastic changes contribute to learning and memory in mammalian systems.

Conditional deletion of α-CaMKII using the Cre transgenic mouse, CW-2 [194], was specific to CA1 pyramidal cells at the CA3-CA1 synapse, but deletion was widespread in other forebrain regions, including the cortex and amygdala. However, this deletion was still restricted compared with global deletion, sparing hippocampal CA3, the striatum and the thalamus, and it did not begin until after early development and synaptogenesis was complete. We discovered that post-synaptic knockout of α-CaMKII led to a deficiency in LTP at the CA3-CA1 synapse, quite similar quantitatively and qualitatively to that we observed in the global α-CaMKII mouse (Figure 6-1). The mean value of potentiation we obtained in CA1 mutants and global knockout mice was approximately 50-60% of that observed in control slices. This deficiency was not as complete as the deficiency observed in experiments using CaMKII inhibitors in the post-synaptic cell [51,52,213], and was different from the bimodal LTP deficit observed in initial experiments of α-CaMKII in an outbred background [111,118]. CaMKII peptide inhibitors are not truly specific, and are known to inhibit PKC [268]. It is possible that this contributed to the more complete LTP deficit observed in studies using peptide inhibitors. Differences between the conditional and global CaMKII knockout mice could be due to

	Global α-CaMKII KO	CA1 α-CaMKII KO	CA3 α-CaMKII KO
LTP (tetanus)	-	-	NL
LTP (pairing)	-	*n.d.*	NL
LTD	-	*n.d.*	*n.d.*
PTP	+	NL	NL
PPF	-	*n.d.*	NL
FF	*n.d.*	*n.d.*	+
SD	*n.d.*	*n.d.*	NL
Spatial memory	-	*n.d.*	*n.d.*
Contextual memory	-	-	NL
Cued memory	-	-	NL
Freezing response	-	-	NL
Shock sensitivity	+	+	*n.d.*
Motor seizures	+	+	NL
Mating behavior	-	-	NL
Activity	+	+	NL

Figure 6-1: Synaptic plasticity and behavior in CaMKII knockout mice

Summary of synaptic plasticity and behavioral phenotypes observed in the α-CaMKII global knockout and in CA1 and CA3 conditional α-CaMKII knockout mice. All physiologically assays were performed at CA3-CA1 hippocampal synapses. LTP = long term potentiation; LTD = long term depression; PTP = post-tetanic potentiation; PPF = paired-pulse facilitation; FF = frequency facilitation; SD = synaptic depletion. +, increased; -, decreased / impaired; NL = normal; *n.d.* = not determined.

developmental abnormalities and/or compensatory pathways, but our follow-up studies on these mice suggest that differences in genetic background could be responsible. For that reason, it is important for the field to standardize genetic backgrounds used for studies of physiology and behavior, as modifier genes can clearly confound comparative studies [165]. While we did not observe upregulation of the β-CaMKII isoform in CA1 α-CaMKII KO mice, it would be useful to examine a "complete" post-synaptic CaMKII

knockout by mutating both the α and β isoforms or by overexpressing a specific CaMKII inhibitor (see Chapter 5).

CA1 α-CaMKII KO mice demonstrated several abnormal behaviors, including hyperactivity and abnormal contextual and cued fear conditioning. These abnormal behaviors could reflect changes in synaptic physiology in the hippocampus, as abnormal hippocampal function is known to cause hyperactivity [201] and impaired contextual learning [203-205]. However, abnormalities in cued conditioning and fear induced freezing behavior suggest that CaMKII deficiency in the amygdala may also be contributing to poor performance [203,204]. To study the association of a specific behavior with physiology using a genetic approach, it is critical to produce as specific a knockout as possible, both temporally and spatially. Because CaMKII is widely expressed, and may be involved in plasticity mechanisms at many synapses throughout the brain [275], it would be useful to have regional knockouts of CaMKII in multiple areas of the brain to examine different types of learning, such as hippocampal dependent spatial learning and amygdala dependent conditioning. Spatial resolution in combination with temporal control would also permit more specific questions to be addressed in the stages of memory formation (hippocampus), memory consolidation (hippocampus vs. cortex) and recall. Preliminary experiments in conditional transgenic mice suggest that such temporal and spatial control of gene expression is possible, although it is dependent upon the characterization of the required tissue specific promoters and/or more lines of Cre transgenic mice and tTA/rtTA mice.

While the evidence for a post-synaptic contribution of CaMKII to LTP is weighty, it would be interesting to characterize which putative post-synaptic LTP expression mechanisms may be altered in CA1 α-CaMKII KO mice. GluR1 phosphorylation has been implicated as a potential expression mechanism [64], and CaMKII phosphorylation is known to increase channel conductance [65]. It would be interesting to look at the phosphorylation levels of GluR1 in mutant mice vs. controls, especially considering that

input/output curves are slightly decreased in CA1 α-CaMKII KO mice. It is also possible that CaMKII is involved in delivering GluR1 receptors to the post-synaptic membrane following LTP induction [66], and that this may be reflected in an increased proportion of "silent synapses" [72,73] in CA1 CaMKII KO mice. Alternatively, LTP has been associated with changes in spine morphology and dynamics, which may involve CaMKII [178-180]. Using transgenic mice that overexpress the CaMKII inhibitor, KII-IN, specifically in CA1 at the CA3-CA1 synapse, it would be interesting to induce LTP in cultured hippocampal slices and follow changes in spine morphology using 2-photon microscopy. EGFP expression from the KII-IN-EGFP fusion protein would allow neurons positive for the transgene to be identified, and decreased spine dynamics may be observed in transgenic mice.

Conditional deletion of α-CaMKII using the Cre transgenic mouse, G32-4 [136] was highly specific in CA3 pyramidal cells in adult mice, with minimal recombination identified in other regions of the brain. This allowed us to address the role of CaMKII in pre-synaptic function at the CA3-CA1 synapse. We found that LTP was normal, using both pairing and tetanus induction protocols, in CA3 α-CaMKII KO mice (Figure 6-1). While this does not preclude pre-synaptic mechanisms from contributing to LTP expression, it suggests that CaMKII phosphorylation of synapsin I to mobilize vesicles from a reserve pool is not a likely mechanism. CaMKII phosphorylation of synapsin I was also proposed to play a role in pre-synaptic short term plasticity mechanisms, like post-tetanic potentiation (PTP) [76]. However, PTP was normal in CA3 α-CaMKII KO mice, as were paired pulse facilitation (PPF) and synaptic depletion (SD). This contrasts with studies in the global α-CaMKII KO that showed enhanced PTP and decreased PPF. While PTP and PPF are thought to be transduced by pre-synaptic changes in release probability, it is possible that post-synaptic deletion of α-CaMKII in combination with a pre-synaptic deletion could have produced these abnormal short term plasticity phenotypes in global α-CaMKII KO mice.

CaMKII has several putative pre-synaptic substrates [224], suggesting that it contributes to multiple functions in synaptic terminals, and yet neurotransmitter release and pre-synaptic plasticity were generally intact in CA3 α-CaMKII KO mice. However, while facilitation was normal at CA3-CA1 synapses in mutant mice when pairs of pulses where given, trains of pulses (30 stimuli) at increasing frequencies of stimulation (1Hz, 5Hz, 10Hz, 20Hz) revealed higher levels of facilitation in CA3 mutant mice, which became more significant with increasing stimulation frequency. These data suggest that CaMKII actually suppresses neurotransmitter release in control mice, and that this suppression becomes more significant with increased frequency of stimulation. This fits nicely with models of CaMKII as a "frequency detector" of calcium spikes. CaMKII cooperatively binds calcium/calmodulin, leading to autophosphorylation and calmodulin trapping, and total holoenzyme activity increases depending upon the timing of subsequent calcium spikes [36,39]. This allows CaMKII to efficiently modulate its activity levels depending upon demand. While CaMKII mediated inhibition could be due to a passive buffering of calcium/calmodulin, it is possible that CaMKII modifies a molecule involved in the release pathway to decrease the probability of neurotransmitter release. This could include molecules that regulate calcium influx, detect calcium influx, control vesicle trafficking, or promote the actual vesicular fusion mechanism. CaMKII does phosphorylate molecules involved in each of those steps *in vitro*, but the functions of such modifications are not well characterized. Previous experiments with CaMKII had suggested that it promoted neurotransmitter release, rather than inhibited it [238,254]. One intriguing possibility is that CaMKII phosphorylation of the synprint site of N-type calcium channels, which leads to dissociation of the channel from the SNARE protein/synaptic vesicle complex [225], could decrease the probability of release by decreasing accessibility of the SNARE/synaptotagmin complex to calcium influx. While it is difficult to dissect out the mechanism for CaMKII pre-synaptic inhibition using molecular approaches in hippocampal slices, it would be interesting to mutate the CaMKII synprint

phosphorylation site using conditional "knock-in" technology to see if it is the essential target for this active suppression.

CA3 α-CaMKII KO mice demonstrated normal behavior, particularly compared with CA1 α-CaMKII KO and α-CaMKII global KO mice. The specific loss of CaMKII in CA3 was not associated with hyperactivity, seizures, or contextual and cued conditioning deficits. Consequently, normal hippocampal dependent learning correlated with normal CA3-CA1 LTP in CA3 α-CaMKII KO mice. However, it is possible that enhanced frequency facilitation may be reflected in some behavioral change, particularly in behavior that requires rapid temporal decoding of signals or short term memory changes, like working memory. It should also be kept in mind that although the CA3 deletion is sufficiently restricted within the hippocampus, the CA3-CA1 synapse is not the only synapse affected by this mutation. CA3 cells receive multiple inputs, including projections from the dentate gyrus, entorhinal cortex, and from the recurrent collaterals of the CA3 cells themselves [264]. At these synapses "post-synaptic" CaMKII levels are affected, though at CA3-CA3 synapses both sides are affected. Plasticity mechanisms are at play at each of these synapses, and it is possible that their disruption could be reflected in behavioral changes. CA3-CA3 recurrent connections are particularly intriguing because they have been proposed to function in hippocampal learning models as autoassociative networks [276]. Recently, LTP at CA3-CA3 synapses was founded to be nearly absent in conditional CA3 NMDAR1 knockout mice [136], and this deficit was correlated with impairments in hippocampal dependent memory tasks requiring pattern completion, an ability thought to be provided by recurrent autoassociative networks [277,278]. Pattern completion refers to an ability to recall a memory when presented with an incomplete set of reference cues. Interestingly, CA3 NMDAR1 KO mice also showed a reduction in several place cell characteristics (place field size, firing rate, burst frequency) during behavior that required successful pattern completion [136]. While it is not known what role CaMKII plays at CA3-CA3 synapses, CaMKII is generally involved

in NMDA receptor dependent LTP mechanisms [8,155,275], and could be contributing to plasticity at these synapses. It would be informative to examine both CA3 and CA1 α-CaMKII KO mice using *in vivo* recording to see if the absence of CaMKII has affected basic firing properties and place field dynamics in CA1, and how such changes may be reflected in behavioral deficits. Such analysis could also give some insight into the hyperexcitability phenotype observed in CaMKII mutant mice. Behavioral analysis may reveal that CA1 α-CaMKII KO mice have difficulty with normal spatial reference memory formation [23,126], while CA3 α-CaMKII KO mice show deficits only when forced to recall a spatial memory in the absence of the full set of original cues [136]. This holistic approach of studying correlated changes in electrophysiological firing properties, synaptic plasticity and behavior in genetically modified animals is a powerful method to dissect mechanisms that underlie different forms of memory, and different stages of the mnemonic process, in the mammalian brain.

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