# Inhibitory Autophosphorylation of CaMKII Controls PSD Association, Plasticity, and Learning

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## Summary

To investigate the function of the $\alpha$ calcium-calmodulin-dependent kinase II ($\alpha$CaMKII) inhibitory autophosphorylation at threonines 305 and/or 306, we generated knockin mice that express $\alpha$CaMKII that cannot undergo inhibitory phosphorylation. In addition, we generated mice that express the inhibited form of $\alpha$CaMKII, which resembles the persistently phosphorylated kinase at these sites. Our data demonstrate that blocking inhibitory phosphorylation increases CaMKII in the postsynaptic density (PSD), lowers the threshold for hippocampal long-term potentiation (LTP), and results in hippocampal-dependent learning that seems more rigid and less fine-tuned. Mimicking inhibitory phosphorylation dramatically decreased the association of CaMKII with the PSD and blocked both LTP and learning. These data demonstrate that inhibitory phosphorylation has a critical role in plasticity and learning.

## Introduction

CaMKII is highly abundant in the brain and a major constituent of the postsynaptic density (PSD). It assembles into hetero-oligomeric complexes consisting of $\alpha$CaMKII and $\beta$CaMKII subunits. Calcium influx through NMDA (N-methyl-D-aspartate) receptors results in activation of CaMKII and autophosphorylation of Thr286. This autophosphorylation allows the kinase to be active even in the absence of calcium (reviewed by Lisman et al., 2002). In addition, Thr286 phosphorylation promotes association with the postsynaptic density by binding to the NMDA receptor (Shen and Meyer, 1999; Shen et al., 2000; Strack et al., 1997; Strack and Colbran, 1998). Hence, a transient increase in calcium can dramatically change the activity and the localization of this kinase.

Importantly, both the induction of long-term potentiation (LTP) and behavioral training are known to trigger autophosphorylation of $\alpha$CaMKII at Thr286, suggesting that this mechanism is critical for synaptic and behavioral plasticity. Indeed, a mutation that deletes the major brain isoform of CaMKII (Silva et al., 1992a, 1992b) or prevents autophosphorylation at Thr286 (Giese et al., 1998) results in LTP and learning deficits as well as in unstable spatial maps encoded by hippocampal place cells (Cho et al., 1998). In addition, transgenic mice that express a constitutively active copy of $\alpha$CaMKII (Thr286Asp) show impaired LTP induction, unstable spatial maps, and learning deficits (Bach et al., 1995; Mayford et al., 1996, 1995; Rotenberg et al., 1996). Together, these results demonstrate that autophosphorylation of CaMKII at Thr286 is critical for the synaptic changes required to stabilize hippocampal maps used during spatial learning.

In vitro studies suggest that following autophosphorylation at Thr286, there is a second wave of CaMKII autophosphorylation (inhibitory autophosphorylation). These experiments suggest that upon dissociation of $Ca^{2+}$/CaM from the activated kinase, the CaM binding domain becomes exposed, allowing phosphorylation at Thr305 and/or Thr306 in this domain (Colbran and Soderling, 1990; Mukherji and Soderling, 1994; Patton et al., 1990). This results in a dramatic loss of $Ca^{2+}$/CaM-dependent activity caused by a decreased affinity for $Ca^{2+}$/CaM (Hashimoto et al., 1987; Kuret and Schulman, 1985; Lickteig et al., 1988; Lou and Schulman, 1989; Mukherji and Soderling, 1994). However, there is only indirect evidence that inhibitory autophosphorylation occurs in vivo. For instance, LTP induction results in maximal Thr286 phosphorylation within 15 min, but autophosphorylation at undetermined CaMKII sites continues up to 1 hr after the tetanus (Barria et al., 1997).

What is the physiological role of inhibitory autophosphorylation? It may regulate the association of the kinase with the PSD, a mechanism thought to be critical for synaptic plasticity (reviewed by Bayer and Schulman, 2001; Lisman and Zhabotinsky, 2001; Meyer and Shen, 2000). In vitro studies showed that $\alpha$CaMKII autophosphorylated at Thr305/6 has reduced affinity for the PSD (Strack et al., 1997), whereas substitution of Thr305/6 by nonphosphorylatable amino acids resulted in a >10-fold lower dissociation rate from the PSD (Shen et al., 2000). In addition, in vitro studies showed that interaction with the NMDA receptor suppresses CaMKII inhibitory autophosphorylation (Bayer et al., 2001). This suggests that phosphorylation of Thr305/6 could modulate the association of CaMKII with the PSD. Here, we demonstrate the physiological relevance of inhibitory autophosphorylation at Thr305/6. We show that it regulates association with the PSD, modulates the threshold for LTP induction, and is important for learning.

## Results

### Inhibitory Phosphorylation Occurs In Vivo
To determine if inhibitory phosphorylation occurs in vivo, we raised an antibody against a peptide corre-

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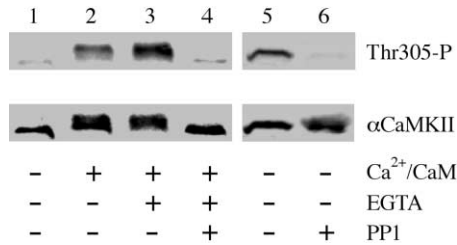
Figure 1. Western Blot Analysis of Inhibitory Autophosphorylation Using an Antibody Raised against a Thr305-Phospho Peptide

In lanes 1–4, purified αCaMKII was activated by the addition of Ca$^{2+}$/CaM (lane 2), followed by the addition of EGTA (lane 3) to induce inhibitory phosphorylation. Phosphorylation was then reversed by protein phosphatase 1 (PP1) treatment (lane 4). Lanes 5 and 6 show hippocampal lysates obtained using denaturing isolation conditions (lane 5) compared to an equivalent amount of hippocampal lysate treated with PP1 (lane 6).

sponding to amino acids 296–312 of αCaMKII, in which Thr305 was phosphorylated. The specificity of the antibody was determined using purified αCaMKII (Figure 1). Whereas a weak band was observed using untreated αCaMKII and αCaMKII treated with protein phosphatase 1 (PP1), we observed strong labeling under conditions that are known to produce inhibitory phosphorylation (activation of αCaMKII by Ca$^{2+}$/CaM followed by Ca$^{2+}$/CaM dissociation using EGTA). Interestingly, we also observed increased Thr305 phosphorylation prior to the addition of EGTA, indicating that in vitro inhibitory phosphorylation is an efficient process that can take place even in the presence of Ca$^{2+}$/CaM.

To determine whether αCaMKII phosphorylation at Thr305 occurs in vivo, a hippocampal lysate was prepared using denaturing conditions to prevent autophosphorylation during isolation. Western blot analysis using the Thr305-P antibody revealed a clear band in this lysate, which disappeared upon PP1 treatment (Figure 1). Importantly, the antibody does not recognize mutant αCaMKII in which Thr305 is mutated but phosphorylation at Thr286 is unaltered (see below; Figure 3A). Taken together, these results indicate that the antibody is specific for phosphorylated Thr305 and demonstrate that Thr305 phosphorylation occurs in vivo.

### Generation of αCaMKII Mutant Mice

To study the role of inhibitory phosphorylation, we generated three different mutants in which the endogenous αCaMKII was mutated using ES cell-mediated gene targeting (Figure 2). In the first mutant (TT305/6VA mice), we substituted αCaMKII Thr305/6 by nonphosphorylatable amino acids (respectively, Val and Ala; Figures 2A–2D) to block inhibitory autophosphorylation. In the second mutant (T305D mice), we substituted Thr305 by negatively charged Asp (Figures 2A–2D), which interferes with Ca$^{2+}$/CaM binding (Rich and Schulman, 1998) and thus mimics persistent Thr305 phosphorylated αCaMKII. As a control, we made a third mutant (αCaMKIIΔ) in which we deleted the αCaMKII exon 2. Deletion of exon 2 results in a premature translation stop due to a frameshift, making this line a αCaMKII null line. In contrast with the previously derived αCaMKII null line

(Silva et al., 1992b), the neomycin resistance gene was deleted from the genome (Figures 2E and 2F).

ES cells from the lines described above were used to derive F2 homozygous mice. Wild-type (WT) littermates from these crossings were used as controls. All mutant mice appeared healthy and showed normal (T305D and αCaMKIIΔ) or even improved (TT305/6VA) motor coordination, as assessed by an accelerating rotarod (data not shown). Nevertheless, aged, but not young, TT305/6VA mutants showed seizures that could be induced by handling. For example, 3% of 2- to 4-month-old TT305/6VA mice (1 out of 38 tested), 30% of 7- to 9-month-old TT305/6VA mice (4 out of 13 tested), and 90% of 12- to 14-month-old TT305/6VA mice (7 out of 8 tested) showed tonic and clonic seizures. Seizures were rarely observed in T305D (1 out of 52) and αCaMKIIΔ (0 out of 45) mice of any age. To avoid the confounding effects of seizures, all behavioral and electrophysiological experiments were done with mice younger than 4 months. Importantly, none of the mice used in either the electrophysiological or the behavioral experiments described here showed any signs of seizures.

### CaMKII Expression and Phosphorylation

The expression level and phosphorylation state of the mutated proteins was analyzed by Western blot analysis of hippocampal lysates (Figure 3A). TT305/6VA mice as well as T305D mice expressed the mutated αCaMKII protein, although the expression level was approximately 2-fold reduced as compared to WT mice (46% ± 10% for both mutants). As expected, αCaMKIIΔ mice expressed no αCaMKII at all. No changes were observed in the expression level of βCaMKII in any of the mutants (Figure 3A).

Western blot analysis using the Thr305-P antibody revealed a clear band in a lysate obtained from WT mice, whereas no band was observed in lysates from any of the mutants, confirming the presence of the desired mutations in these mutants (Figure 3A). Importantly, the TT305/6VA mutation did not affect phosphorylation at Thr286, since the relative amount of αCaMKII phosphorylated at Thr286 was unaltered in the TT305/6VA mice (Figure 3A), and calcium-independent activity was comparable to WT mice (15% ± 2% and 15% ± 1% of total Ca$^{2+}$/CaM dependent activity for TT305/6VA mutants and WT, respectively). The T305D mutation should mimic the Thr305 phosphorylated kinase and prevent Ca$^{2+}$/CaM binding and subsequent autophosphorylation at Thr286. Indeed, Thr286 phosphorylation was severely reduced in T305D mice (Figure 3A), indicating that the T305D mutation mimics inhibitory phosphorylated kinase.

### Inhibitory Autophosphorylation Affects
### CaMKII Localization

Elegant studies using cultured neurons showed that a mutation that blocks phosphorylation at Thr305/6 reduced the dissociation of GFP-tagged CaMKII from the PSD (Shen and Meyer, 1999). To determine the effect of the TT305/6VA mutation on the localization of αCaMKII in vivo, we measured the levels of αCamKII and βCaMKII in PSD-enriched hippocampal fractions from TT305/6VA and WT mice (Figures 3B–3E). Despite the
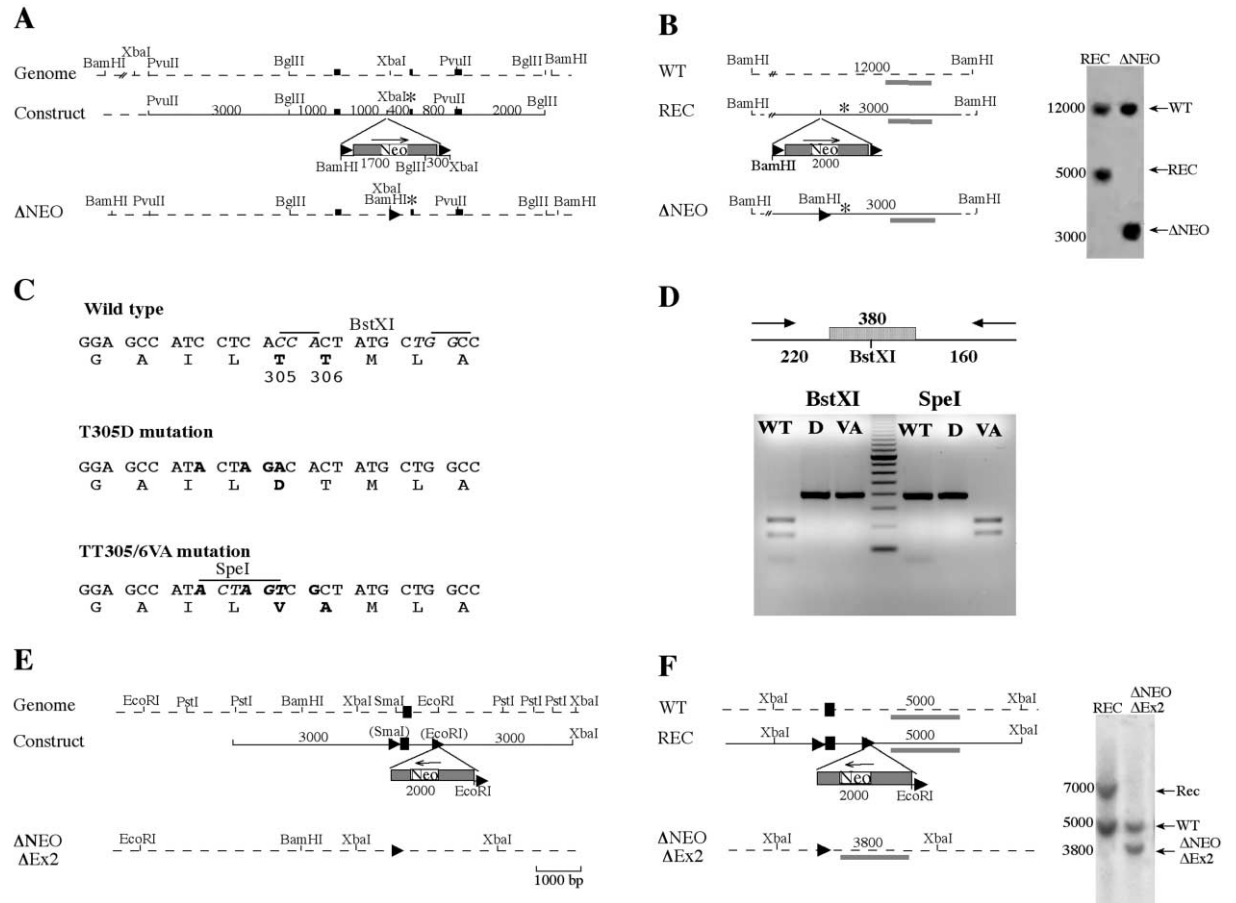
**Figure 2. The Generation of Thr305/6 and αCaMKIIΔ Mutant Mice**

(A) Schematic diagram of the procedure for generating the Thr305/6 mutant ES clones. (Top) WT αCaMKII locus with the location of the exons around Thr305/6 depicted as filled boxes. (Middle) Targeting construct used for the generation of the Thr305/6 mutations. The asterisk indicates the mutated exon encoding Thr305/6. The LoxP sites flanking the neomycin gene are depicted as triangles. (Bottom) Mutant αCaMKII locus after homologous recombination and deletion of the neomycin gene by transient transfection of Cre recombinase.

(B) Southern blot analysis of the targeted ES cells. (Left) Shown is the position of the BamHI sites that were used to digest the genomic DNA. The position of the probe used for the analysis is depicted as a gray bar. (Right) Southern blot obtained after BamHI digestion of mutant DNA. It yielded a homologous recombination band (REC) and a recombinant DNA band denoting deletion of the neomycin gene (ΔNeo). The annotation of the bands obtained is indicated with the arrows. The numbers refer to the approximate sizes of the bands in base pairs after BamHI digestion.

(C) αCaMKII DNA sequence of the exon encoding Thr305/306. (Upper) WT DNA indicating the presence of the Thr residues at 305 and 306 (bold), and the position of the BstXI restriction enzyme site (italics). (Middle) DNA of the T305D mutant. Base pair and amino acid substitutions are in bold. Note that the BstXI site is no longer present. (Bottom) DNA of the TT305/6VA mutant. Note that the BstXI site is no longer present and that a SpeI site (italics) has been introduced.

(D) Analysis of wild-type (WT) mice, T305D (D), and homozygous TT305/6VA (VA) mice. A 380 base pair fragment encoding the mutated exon was amplified by PCR, and the obtained products were digested with either BstXI or SpeI to reveal the presence of the mutations.

(E) Schematic diagram of the procedure for generating the αCaMKIIΔ mutant ES clones. (Top) WT αCaMKII locus with the location of the exon 2 depicted as a filled box. (Middle) Targeting construct used for the generation of the αCaMKIIΔ mutation. The LoxP sites flanking the neomycin gene and the exon 2 are depicted as triangles. (Bottom) Mutant αCaMKII locus after homologous recombination and after the deletion of the exon 2 and neomycine gene by Cre mediated recombination.

(F) Southern blot analysis of the targeted ES cells. (Left) Shown is the position of the XbaI sites that were used to digest the genomic DNA. The position of the probe used for the analysis is depicted as a gray bar. (Right) Southern blot obtained after XbaI digestion of mutant DNA. Shown is the DNA obtained after homologous recombination (REC) and after deletion of the neomycin cassette and exon 2 (ΔNeo + ΔEx2). The numbers refer to the approximate sizes in base pairs.

2-fold decrease in total expression level of αCaMKII in TT305/6VA mice, we found the same level of PSD-associated αCaMKII in this mutant as in WT (Figures 3B and 3C), suggesting that the TT305/6VA mutant kinase has an increased affinity for the PSD. Since the subunit composition of the holoenzyme is dependent upon availability of the αCaMKII and βCaMKII subunits (Brocke et al., 1999), the reduced expression of αCaMKII increases the relative number of βCaMKII subunits per CaMKII holoenzyme. Therefore, the increased affinity of αCaM-KII-TT305/6VA for the PSD should also result in an increased amount of PSD-associated βCaMKII. Indeed, we found a >3-fold increase in the amount of PSD-associated βCaMKII in TT305/6VA mice (Figure 3D) even
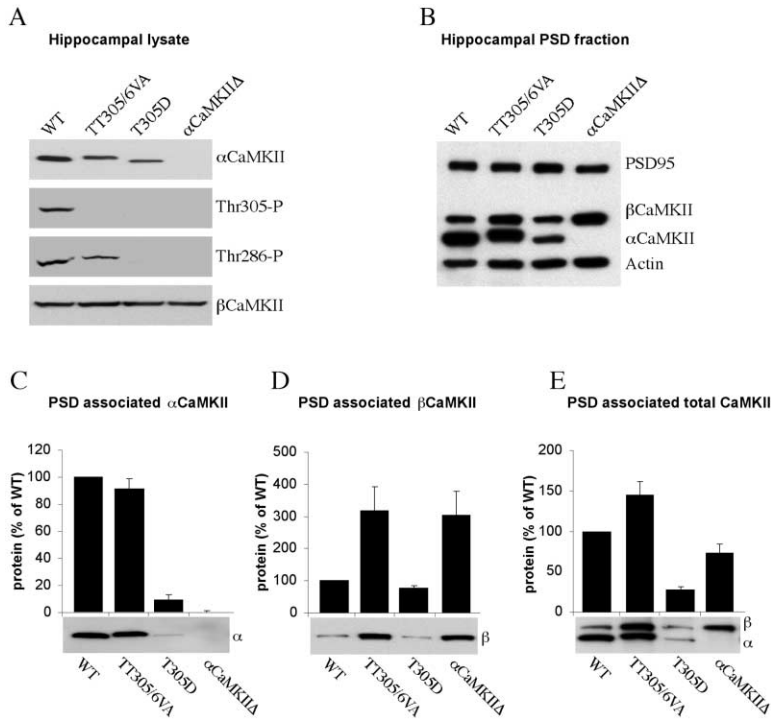
Figure 3. Analysis of CaMKII Expression and Localization

(A) Western blot analysis of CaMKII expression and phosphorylation in WT and homozygous mutant mice.

(B–E) Western blot analysis and quantification of PSD-enriched fractions isolated from WT and homozygous mutant mice. (B) Blot showing PSD95, αCaMKII and βCaMKII, and actin. (C) αCaMKII, (D) βCaMKII, and (E) total αCaMKII and βCaMKII. Graphs are based on the average of five independent experiments.

though the total expression level of βCaMKII is normal (Figure 3A). Taken together, we found that the total levels of αCaMKII and βCaMKII associated with the PSD were 1.5-fold higher in TT305/6VA mutants than in WT controls (146% + 17% of WT levels, Figure 3E).

We also tested the levels of PSD-associated αCaMKII in the T305D mutant and found that these mice have a 10-fold reduction of PSD-associated αCaMKII as compared to WT mice (9% + 4% of WT levels, Figures 3B and 3C). Moreover, the combined levels of both αCaMKII and βCaMKII associated with the PSD were 4-fold lower in T305D mutants than in WT controls (28% + 4% of WT levels; Figures 3B and 3E). This decrease cannot be caused simply by the reduced overall expression level of αCaMKII in T305D mice because in αCaMKIIΔ mice, which have no αCaMKII, we found significantly more CaMKII associated with the PSD (73% + 11% of WT) than in the T305D mutant. This was due to a 3-fold increase in the levels of PSD-associated βCaMKII in αCaMKIIΔ mice (Figures 3D and 3E). Since increased levels of PSD-associated βCaMKII were not observed in the T305D mutant (Figure 3D), the T305D mutation in αCaMKII also affects the association of βCaMKII with the PSD, consistent with the observation that these βCaMKII subunits are in a holoenzyme with αCaMKII (Brocke et al., 1999). Taken together, these data demonstrate that phosphorylation at Thr305/6 regulates the amount of CaMKII associated with the PSD in vivo.

### The Loss of Inhibitory Autophosphorylation Lowers the Threshold for LTP Induction
We used extracellular (field) recordings of Schaffer collateral-CA1 synapses in hippocampal slices to determine the importance of inhibitory phosphorylation on synaptic plasticity. All mutants showed similar synaptic

transmission ($F3,34 = 1.54$, $p > 0.05$; Figure 4A). However, a weak stimulus of four pulses delivered at 100 Hz (100 Hz/0.04 s), which does not induce LTP in WT slices (101% ± 1%, two out of eight slices showed >105% potentiation), induced significant levels of LTP in TT305/6VA mice (107% ± 1%, eight of nine slices showed >105% potentiation; Figure 4B). Similarly, a 10 Hz/10 s tetanus also revealed significant LTP in TT305/6VA mice (114% ± 4%, eight of ten slices showed >110% potentiation), but not in WT mice (102% ± 4%, four of twelve slices showed >110% potentiation; Figure 4C). The LTP observed in TT305/6VA mice is NMDA-receptor dependent, since it is blocked by APV, an NMDA-receptor antagonist (WT, 97% ± 2%; TT305/6VA, 101% ± 3%; $F1,10 = 2.6$, $p > 0.05$; Figure 4D).

Does the lack of inhibitory phosphorylation result in a general enhancement in synaptic plasticity? We addressed this question by measuring LTP induced by several different tetanic protocols. A "two-theta" protocol (two bursts of 100 Hz/0.04 s, separated by 200 ms) triggered a similar level of LTP in slices obtained from either WT or TT305/6VA mice (WT, 114% ± 3%; TT305/6VA, 115% ± 2%; Figure 4E). Similarly, a 100 Hz/1 s tetanus induced equivalent levels of LTP in both groups of mice (WT, 148% ± 7%; TT305/6VA, 147% ± 6%; Figure 4F). Taken together, these results demonstrate that the loss of inhibitory phosphorylation and the concomitant increase in PSD-associated CaMKII does not result in an overall enhancement in LTP. Instead, this mutation lowers the threshold for LTP induction.

### Inhibitory Autophosphorylation Does Not Affect Responses to Low-Frequency Stimulation
Kinetic modeling studies have suggested that inhibitory phoshorylation limits the levels of CaMKII activation dur-
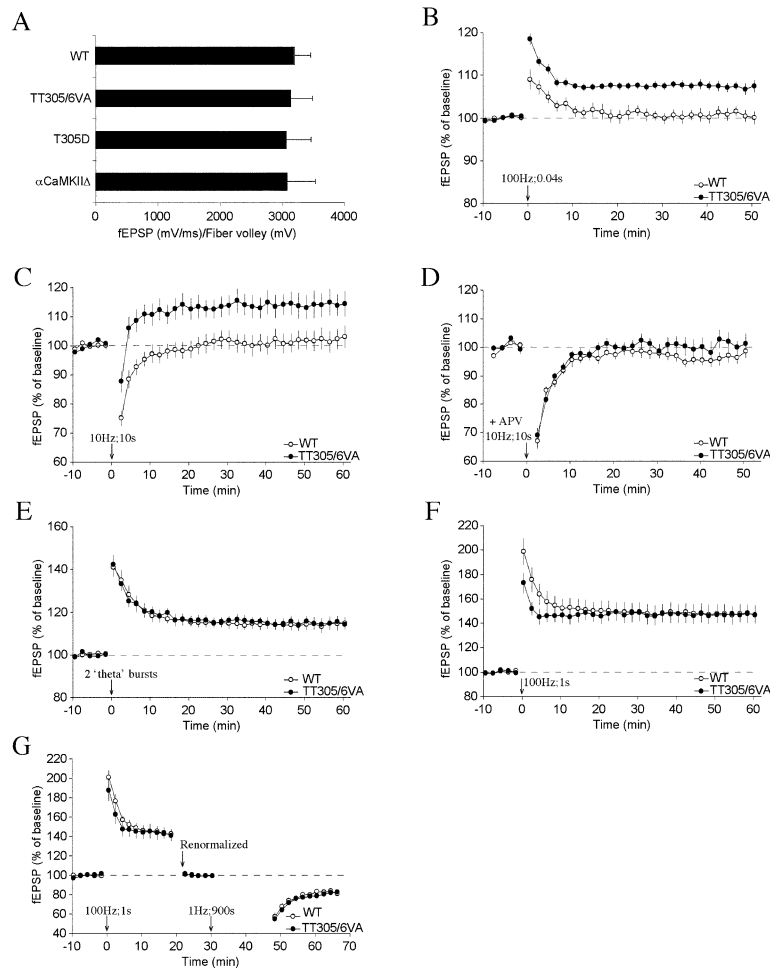
Figure 4. LTP and LTD at the Schaffer Collateral-CA1 Synapse of the TT305/6VA Mutant

(A) Synaptic transmission in WT and mutant mice. Slices were stimulated at 40 μA, and synaptic transmission was calculated by measuring the relation between the amplitude of the evoked presynaptic fiber volley and the slope of the field excitatory postsynaptic potential (fEPSP).

(B) Stimulation at 100 Hz/0.04 s reveals larger LTP in TT305/6VA slices. LTP in WT, 101% ± 1% (ten slices/eight mice); TT305/6VA, 107% ± 1% (nine slices/five mice).

(C) Stimulation at 10 Hz/10 s reveals larger LTP in TT305/6VA slices. LTP in WT, 102% ± 4% (12 slices/12 mice); TT305/6VA, 114% ± 4% (ten slices/ten mice).

(D) Stimulation at 10 Hz/10 s in the presence of APV. LTP in WT, 97% ± 2% (six slices/four mice); TT305/6VA, 101% ± 3% (six slices/five mice).

(E) Two-theta burst stimulation. LTP: WT, 114% ± 3% (eight slices/eight mice); TT305/6VA, 115% ± 2% (six slices/six mice).

(F) Stimulation at 100 Hz/1 s. LTP: WT, 148% ± 7% (nine slices/eight mice); TT305/6VA, 147% ± 6% (nine slices/eight mice).

(G) Depotentiation following LTP induction. Slices were first stimulated at 100 Hz/1 s and, after 30 min, depotentiated with 1 H/900 s stimulation. Values from the final 10 min of LTP prior to the depotentiaton were renormalized to baseline. Depotentiation in WT, 82% ± 2% (seven slices/seven mice); depotentiaton in TT305/6VA, 81% ± 2% (nine slices/nine mice).

ing prolonged low-frequency stimulation (Coomber, 1998; Dosemeci and Albers, 1996). Thus, the TT305/6VA mutation could change the responses to low-frequency stimulation. However, a 1 Hz/900 s tetanus did not reveal any differences between WT and TT305/6VA mutants (WT, 95% ± 4%; TT305/6VA, 97% ± 4%; F1,18 = 0.08, p > 0.05; data not shown). Thus, blocking αCaMKII inhibitory phosphorylation does not affect responses to low-frequency stimulation.

Inhibitory phosphorylation in vitro is typically observed after prior activation of CaMKII. Therefore, we induced LTP and then examined whether low-frequency stimulation would reveal a difference between the depotentiation levels of TT305/6VA and WT mice. CA1 synapses were first potentiated with a 100 Hz/1 s tetanus, and after 30 min they were depotentiated with low-frequency stimulation (1 Hz/900 s). Again, no differences were observed between WT and TT305/6VA mutant slices after either LTP induction (30 min after tetanus: WT, 142% ± 5%; TT305/6VA, 140% ± 5%) or depotentiation (WT, 82% ± 2%; TT305/6VA, 81% ± 2%; Figure 4G), confirming that inhibitory autophosphorylation does not change the responses to low-frequency stimulation.

## The T305D Mutation Blocks LTP Induction

The data presented above showed that the TT305/6VA mutation increased the levels of PSD-associated CaMKII and facilitated LTP induction. The T305D mutation dramatically decreased the levels of PSD-associated CaMKII and, therefore, would be expected to block induction of LTP. Indeed, a two-theta burst tetanus induced LTP in WT, but not in T305D mutants (T305D, 101% ± 3%; WT, 114% ± 3%; F1,11 = 11, p < 0.05; Figure 5A). Similarly, a stronger tetanus (100 Hz/1 s) also failed to induce LTP in the T305D mice (T305D, 102% ± 2%; WT, 148% ± 7%; F1,14 = 29, p < 0.05; Figure 5B). These results demonstrate that the T305D mutation results in a block of hippocampal CA1 LTP.

## The T305D Mutation Shifts the Balance between LTP and LTD

The balance between LTP and LTD is thought to be determined by competition between postsynaptic phosphatases and kinases (Lisman, 1989). Therefore, the lower levels of PSD-associated CaMKII in T305D mutants might have shifted this balance toward LTD. To test this, we used a 10 Hz/10 s stimulation protocol, which did not change synaptic efficacy of WT slices. In
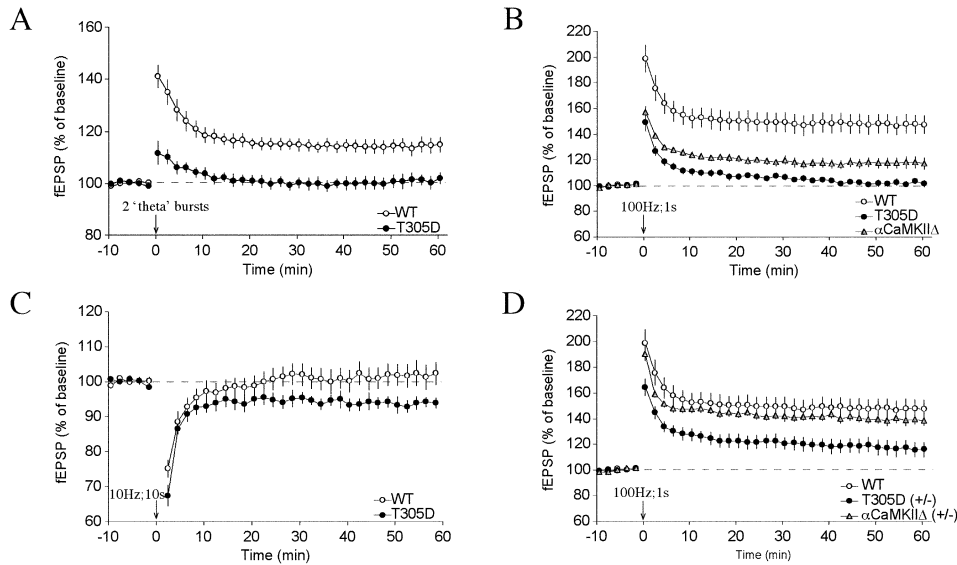
Figure 5. LTP at the Schaffer Collateral-CA1 Synapse in T305D and αCaMKIIΔ Mice

(A) Impaired two-theta burst LTP in T305D mice. LTP in WT, 114% ± 3% (same slices as in Figure 4E; see Experimental Procedures); T305D, 101% ± 3% (five slices/five mice).

(B) Impaired 100 Hz/1 s LTP in T305D and αCaMKIIΔ mice. LTP in WT, 148% ± 7% (nine slices/eight mice); T305D, 102% ± 2% (seven slices/seven mice); αCaMKIIΔ, 118% ± 4% (eight slices/eight mice).

(C) Stimulation at 10 Hz/1 s induces LTD in the 305D mutant. LTP in WT, 102% ± 4% (12 slices/12 mice); T305D, 93% ± 2% (nine slices/eight mice).

(D) Impaired 100 Hz/1 s LTP in heterozygous T305D mutants, but not in heterozygous αCaMKIIΔ mutants. LTP in T305D heterozygous mice, 117% ± 6% (nine slices/nine mice); αCaMKIIΔ heterozygous mice, 139% ± 4% (seven slices/six mice).

contrast, we observed a modest, but significant, depression in T305D mutant slices (T305D, 93% ± 2%; WT, 102% ± 4%; $F_{1,19}$ = 4.5, p < 0.05; Figure 5C).

## The T305D Mutation Has a Dominant-Negative Effect on LTP Induction

Our biochemical studies demonstrated that PSD-associated βCaMKII levels are lower in T305D mice than in αCaMKIIΔ mutants, suggesting that the T305D mutant kinase actually prevents association of βCaMKII with the PSD. In agreement, the LTP deficit in T305D mice is significantly more pronounced than in αCaMKIIΔ mutants. For example, a 100 Hz/1 s tetanus failed to induce LTP in T305D mutants but induced modest levels of LTP in αCaMKIIΔ mice (T305D, 102% ± 2%; αCaMKIIΔ, 118% ± 4%; $F_{1,14}$ = 9.6, p < 0.05; Figure 5B). The different effects of these mutations on LTP is also apparent in heterozygotes. The potentiation induced by a 100 Hz/1 s tetanus in heterozygous T305D mice was almost 3-fold lower than in WT mice (T305D-Het, 117% ± 6; WT, 148% ± 7%; $F_{1,16}$ = 11.2, p < 0.05), whereas LTP was normal in αCaMKIIΔ heterozygous mice (139% ± 4; $F_{1,14}$ = 0.93, p > 0.05; Figure 5D). These results suggest that the residual LTP observed in αCaMKII null mutants (this study and Hinds et al., 1998; Silva et al., 1992b) is mediated by PSD-associated βCaMKII and that the T305D mutation blocks this residual LTP by interfering with the association of βCaMKII with the PSD.

## Loss of Inhibitory Phosphorylation Impairs Spatial Learning

Mutations that disrupt CaMKII function lead to spatial learning deficits in the Morris water maze task (Giese et al., 1998; Silva et al., 1992a), and training in this hippocampus-dependent task results in the activation of CaMKII (Tan and Liang, 1996). To determine whether the loss of inhibitory phosphorylation and concomitant change in the threshold for LTP induction affects spatial learning, we tested TT305/6VA mice in the hidden-platform version of the Morris water maze, in which mice are trained to search for a submerged platform in a pool of water. After 8 and 14 days of training, spatial learning was assessed with probe trials in which the platform was removed from the pool, and the mice were allowed to search for it for 60 s. In the probe trial given at day 8, we found that both TT305/6VA and WT mice spent significantly more time searching in the quadrant where the platform was during training (target quadrant: TQ) than in the other three quadrants (WT, TQ = 43%, t8 = 2.5, p < 0.05; TT305/6VA, TQ = 38%, t16 = 2.6, p < 0.05; Figure 6B). There were no significant differences between TT305/6VA mutants and WT mice in the time spent in the target quadrant ($F_{1,24}$ = 0.06, p > 0.05) or the number of crossings over the location where the platform had been ($F_{1,24}$ = 0.02, p > 0.05), suggesting that initial spatial learning was neither disrupted nor enhanced by the TT305/6VA mutation. In contrast, the probe trial given after 14 days of training revealed a difference between WT and TT305/6VA mutants. WT mice searched significantly more in the target quadrant than TT305/6VA mice (WT, TQ = 54%; TT305/6VA, TQ = 37%; $F_{1,23}$ = 6.8, p < 0.05) and crossed the position where the platform had been significantly more often than TT305/6VA mice (WT, average number of crossings = 5.3; TT305/6VA, crossings = 2.0; $F_{1,23}$ = 15, p < 0.05). It is interesting to note that in the probe trial
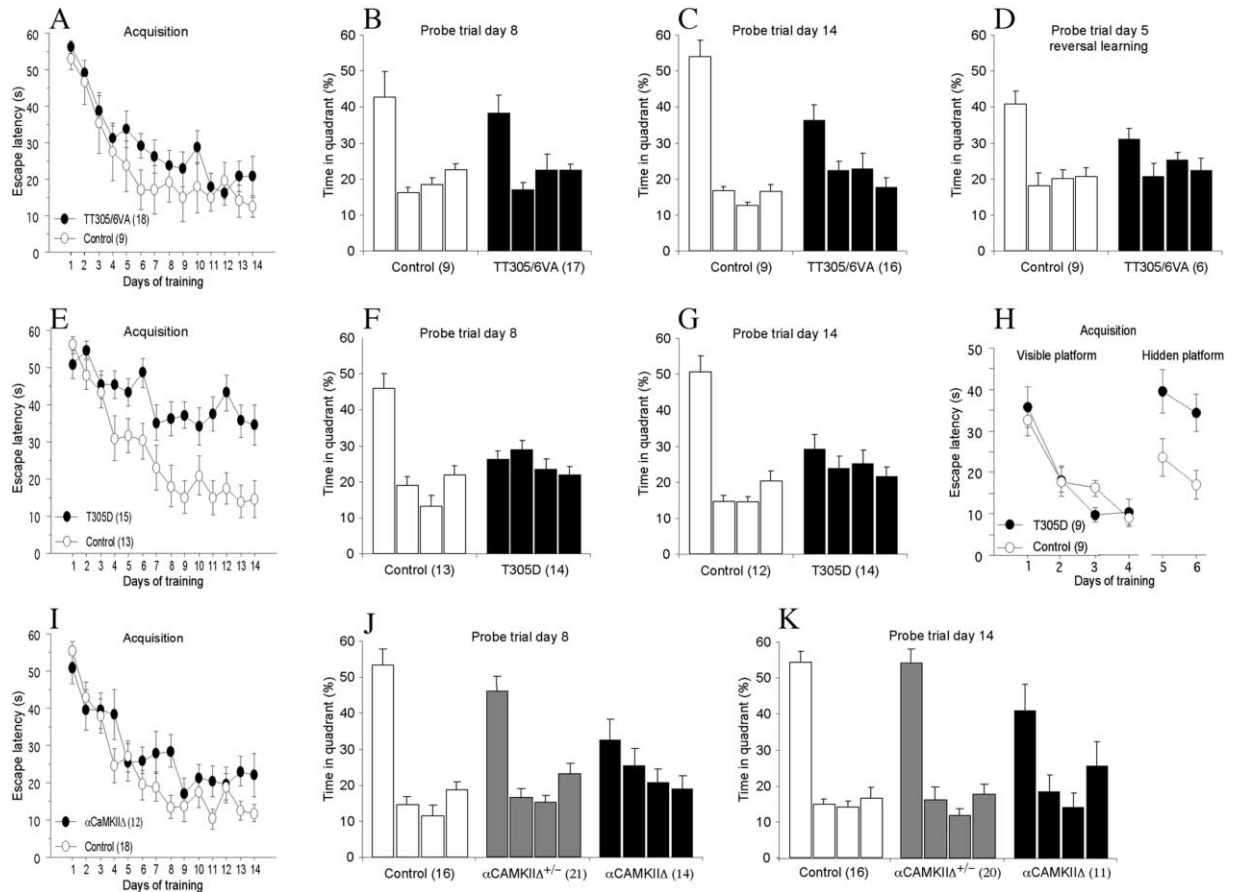
Figure 6. Learning Deficits in the Morris Water Maze

(A, E, and I) Time to find the hidden platform plotted versus training day. Numbers between parentheses indicate the number of animals used for the experiment.

(B, F, and J) Probe trial given after 8 days of training. The platform was removed, and the mice were given 60 s to search for it. Bars indicate from left to right: time spent in target quadrant, adjacent right quadrant, opposite quadrant, and adjacent left quadrant.

(C, G, and K) Probe trial given after 14 days of training. The platform was removed and the mice were given 60 s to search for it.

(D) Probe trial given after 5 days of training at a novel platform position. Mice were trained for 14 days with the platform at a given position. Mice that spent more than 35% in target quadrant were then trained for an additional 5 days with the platform in the opposite position and tested in a probe trial with the platform removed.

(H) Time to find the platform plotted versus training day. The platform was marked with a visible cue for the first 4 days. At days 5 and 6 the platform was hidden.

at day 8, approximately half of the animals in both groups did not search selectively (less than 35% of time in TQ: TT305/6VA, 8 out of 17 mice; WT, five out of nine mice). During the probe trial at the end of the fourteenth day of training, all but one of the WT mice searched selectively in the target quadrant, suggesting a clear improvement in their performances. In contrast, in the day 14 probe trial still 6 of 16 of the mutants failed to search selectively in the target quadrant, and the other mutant animals had not significantly improved their performance either.

Although the TT305/6VA mutation affected spatial learning, it did not affect general performance in this task. For example, across days, both groups of mice decreased the time to find the platform ($F_{13,25} = 16$, $p < 0.05$), and there was no significant difference between TT305/6VA mice and WT mice in this measure of learning ($F_{1,25} = 1.9$, $p > 0.05$; Figure 6A). Similarly, swim speed in each of the two probe trials was not significantly

different between TT305/6VA mutants and WT mice (day 8, $F_{1,24} = 1.1$, $p > 0.05$; day 14, $F_{1,24} = 0.08$, $p > 0.05$). These data demonstrate that the TT305/6VA mutation affects spatial learning and suggest that although initially TT305/6VA mice learn as well as controls, their performance does not improve with additional training.

**Loss of Inhibitory Phosphorylation Blocks Reversal Learning**

The water maze results described above demonstrate that the TT305/6VA mutation affects spatial learning. To further explore this deficit, we tested the mice in a reversal spatial learning task, which is thought to examine the flexibility of learned information. A new group of mice was trained for 14 days, as described above. Again, we found that in the probe trial given after 14 days of training, all WT mice, but only half the number of mutants (6 out of 11), spent more than 35% of the time searching for the platform in the target quadrant. The poor learners

(<35% in TQ) were then excluded so that the performance of the two selected groups of mice was similar (WT, TQ = 56%; TT305VA, TQ = 59%), This avoids the confounding effects of initial learning deficits on the analysis of the reversal-training task. The good learners were trained for an additional 5 days (two trials/day) with the platform at the opposite site of the pool. A probe trial given at the end of reversal training showed that WT, but not TT305/6VA, mice searched selectively for the platform in its novel platform location (WT, TQ = 42%, t8 = 8.6, p < 0.05; TT305/6VA, TQ = 32%, t5 = 1.0, p > 0.05; Figure 6D). Thus, despite being initially matched for performance, the TT305/6VA mice showed clear deficits in reversal learning, suggesting that the information acquired by these mice is less flexible than in WT controls.

## Spatial Learning Is Severely Impaired in T305D Mice

The T305D mutation mimics persistent inhibitory phosphorylated CaMKII, blocks αCaMKII activation, and reduces the amount of PSD-associated CaMKII. Additionally, our LTP findings demonstrate that the deficits of the T305D mutants are more severe than those of αCaMKIIΔ mice. Is their spatial learning also more impaired than that of αCaMKIIΔ mice? To address this question, we trained T305D and αCaMKIIΔ mice and their littermate controls in the Morris water maze as described above. Although all groups of mice needed less time to find the platform during training (T305D and control, F13,26 = 14.6, p < 0.05; αCaMKIIΔ and control, F13,28 = 20.0, p < 0.05; Figures 6E and 6I), there was a dramatic difference between T305D mice and their control littermates (F1,26 = 13, p < 0.05; Figure 6E). In contrast, escape latencies during training were not significantly different between αCaMKIIΔ mice and control littermates (F1,28 = 2.3, p > 0.05; Figure 6I).

Spatial learning was also assessed by a probe trial given at the end of the eighth day of training. The control groups of both mutants spent significantly more time searching for the platform in the target quadrant than in the other three quadrants (WT-T305D, TQ = 46%, t12 = 5, p <0.05; WT-αCaMKIIΔ, TQ = 54%, t15 = 6.5, p < 0.05; Figures 6F and 6J). However, neither the T305D nor αCaMKIIΔ mice searched selectively in the target quadrant (T305D, TQ = 26%, t13 = 0.5, p > 0.05; αCaMKIIΔ, TQ = 33%, t13 = 1.5, p > 0.05 Figures 6F and 6J), and both mutant groups spent significantly less time searching in the target quadrant than their control littermates (T305D, F1,25 = 17, p < 0.05; αCaMKIIΔ, F1,28 = 8.4 p < 0.05) and crossed the location where the platform had been during training significantly less times (T305D, F1,25 = 14, p < 0.05; αCaMKIIΔ, F1,28 = 7.0, p < 0.05). There were no significant differences between either mutant and their control mice in swim speed (305D, F1,25 = 2.4, p > 0.05; αCaMKIIΔ, F1,28 = 3.1, p > 0.05). In contrast, it is important to note that heterozygous αCaMKIIΔ mice, which have comparable expression levels of αCaMKII as T305D mice and TT305/6VA mice, were not statistically different from littermate controls in the time spent in the target quadrant, the number of platform crossings, and in the number of animals that searched selectively during probe trials

(Figures 6J and 6K αCaMKIIΔ+/− and data not shown). Thus, a reduction of 50% of αCaMKII levels does not affect hippocampal learning per se, which is consistent with the observation that these animals have normal hippocampal LTP (Figure 5D).

To determine whether T305D and αCaMKIIΔ mutant mice could learn the location of the platform after additional training, another probe trial was given at day 14. With extended training, 8 out of 11 αCaMKIIΔ mice learned to search selectively in the target quadrant, although their searches were still less selective than their controls (WT-αCaMKIIΔ, TQ = 54%; αCaMKIIΔ, TQ = 41%, F1,26 = 4.5, p < 0.05; Figure 6K). In contrast, despite the additional training, 10 out 14 T305D mice still failed to search selectively in the target quadrant (TQ = 29%, t14 = 0.98, p > 0.05; Figure 6G). Thus, just as LTP, spatial learning is more severely affected by the T305D than the αCaMKIIΔ mutation.

The profound learning deficit of the T305D mutant could be due to deficits in behavioral determinants other than spatial learning, such as motivation, coordination, or vision. Therefore, we tested the T305D mice in the visible-platform task of the water maze, a nonspatial learning task known to be independent of hippocampal function. In this task, the platform that has been marked by a visible cue. A new group of T305D mice and controls were trained for 4 days (eight trials/day) with the visible-platform on a fixed location, but with a pseudo-random start position. Both T305D and WT mice improved with training (F3,14 = 26, p < 0.05), and their performances were not significantly different (F1,14 = 0.03, p = 0.9; Figure 6H), indicating that their spatial learning impairments were not caused by deficits in motivation, motor coordination, or vision. At the end of this training, we removed the platform and trained the mice for two additional days (eight trials/day). Now, with the visible cue removed, T305D mutants took significantly longer to find the platform (F1,14 = 12, p < 0.05; Figure 6H). In a probe trial given immediately after the end of training, WT mice, but not the mutants, searched selectively for the platform, and they spent significantly more time in the target quadrant than the T305D mutants (WT, TQ = 35%; T305D TQ = 19%; F1,15 = 22, p < 0.05; data not shown). Taken together, these results indicate that impairments in spatial learning account for the Morris water maze deficits of the T305D mice.

## Loss of Inhibitory Phosphorylation Results in Generalization of Contextual Conditioning

To test hippocampal-dependent learning in a different paradigm, we used contextual fear conditioning. In this task mice learn to associate the context in which they are trained with a foot shock. 24 hr after training, the mice were placed back in the training chamber, and conditioning was assessed by measuring freezing. Freezing is a natural response to fear and it reflects the cessation of all movement except for respiration. Exposure to the shock clearly increased freezing in TT305/6VA mice (t28 = 7.4, p < 0.05; Figure 7C) and they were not significant different from littermate controls (F1,54 = 0.8, p > 0.05). In contrast, both the T305D and αCaMKIIΔ mutations severely disrupted contextual conditioning, as they showed significantly less freezing
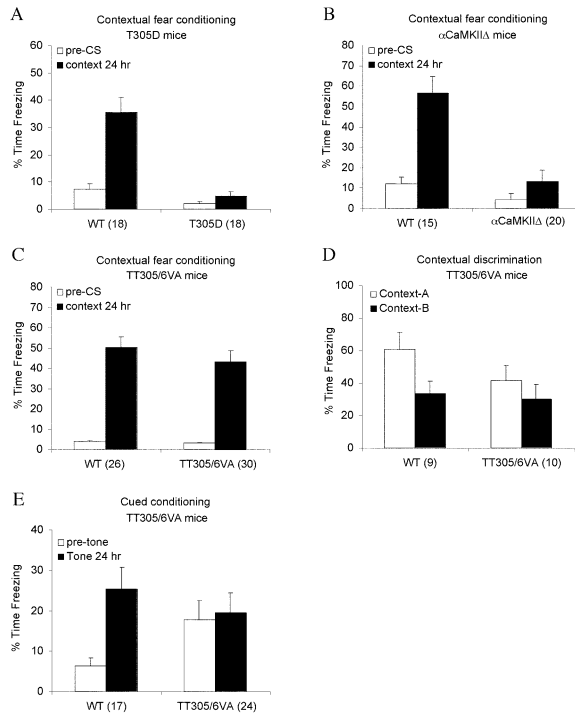
Figure 7. Generalization of Context during Fear Conditioning in TT305/6VA Mutants

(A–C) Contextual fear conditioning. Percentage of time spent freezing during training before the foot shock (pre-US, white bars) and 24 hr after conditioning (black bars). Numbers between parentheses indicate the number of animals used for each experiment.

(D) Contextual discrimination. Percentage of time spent freezing in the conditioning chamber (context A, white bars) and in a similar chamber (context B, black bars).

(E) Cued conditioning. Percentage of time spent freezing before the onset of the tone (pre-tone, white bars) and after the onset of the tone (black bars).

than littermate controls (T305D, $F_{1,34}$ = 28, p < 0.05; αCaMKIIΔ, $F_{1,33}$ = 21, p < 0.05; Figures 7A and 7B).

Previous studies demonstrated that mice can use hippocampal-independent strategies to recognize the context in which they are trained. For example, hippocampal lesioned mice can learn to recognize the context in which they are trained by associating a prominent cue in that context (i.e., shock grid) with the shock (unimodal strategy). To address this possibility, we also tested the TT305/6VA mice in contextual discrimination, a task known to be very sensitive to hippocampal lesions (Frankland et al., 1998). In this task, mice are tested for their ability to discriminate between two similar chambers, one in which they receive a foot shock (chamber A) and another in which they do not (chamber B). Since both chambers share many cues, a unimodal strategy is not effective at distinguishing between the chambers. 24 hrs after being shocked in chamber A, the mice were tested in both chambers A and B. Unlike WT mice, TT305/6VA mice showed similar freezing responses in both chambers (WT freezing A, 61% and B, 33%; t8 = 2.7, p < 0.05; TT305/6VA freezing A, 41% and B, 31%; t9 = 1.0, p > 0.05; Figure 7D).

We also tested tone (cued) conditioning in the TT305/

6VA mice. In this test, the foot shock coterminates with a 30 s tone. 24 hr after training, the mice were tested in a modified chamber that differs from the conditioning chamber. Unlike WT mice, which displayed a significant increase in freezing after the onset of the tone, the TT305/6VA mice showed high levels of freezing both before and after the onset of the tone (WT, before 8%, after 35%, t7 = 2.7, p < 0.05; TT305/6VA, before 30%, after 31%, t8 = 0.28, p > 0.05; Figure 7E). This result confirms that the TT305/6VA mice can be conditioned but that they are unable to discriminate between conditioning and testing chambers. Importantly, this could not be caused by decreased expression level of αCaMKII, since heterozygous αCaMKIIΔ mice, which have a similar amount of αCaMKII, were normal in both contextual and cued conditioning (data not shown). Taken together, the fear conditioning data corroborates the importance of inhibitory phosphorylation for hippocampal-dependent learning.

## Discussion

Previous findings demonstrated that autophosphorylation at Thr286 is critical for the induction of LTP and for learning (Giese et al., 1998). However, the function of the inhibitory phosphorylation at Thr305/306 was unknown. Our results unveiled a critical role for inhibitory phosphorylation in synaptic plasticity and learning that is fundamentally distinct from the role of phosphorylation at Thr286: blocking inhibitory phosphorylation lowers the thresholds for LTP induction, while blocking Thr286 phosphorylation impairs LTP. In addition, blocking inhibitory phosphorylation results in less fine-tuned spatial learning and contextual discrimination, as well as in impaired reversal learning in the watermaze task, while preventing Thr286 phosphorylation simply blocks learning. Mimicking inhibitory phosphorylation dramatically decreased association of CaMKII with the PSD and blocked both LTP and learning. These data demonstrate that inhibitory phosphorylation has a critical modulatory role in plasticity and learning.

### Inhibitory Autophosphorylation Regulates PSD Association of CaMKII

Even though in vitro experiments demonstrated CaMKII inhibitory autophosphorylation more than a decade ago, it was unknown whether this occurred in vivo and its functional relevance remained elusive. The more recent findings that the affinity of CaMKII for the PSD is decreased by disrupting $Ca^{2+}$/CaM binding (Shen and Meyer, 1999; Strack et al., 1997) and increased by preventing Thr305/306 phosphorylation (Shen et al., 2000) provided new insights into the possible function of inhibitory phosphorylation. In agreement with these important findings, we demonstrated in vivo that a mutation that prevented inhibitory phosphorylation results in increased levels of PSD-associated CaMKII. In addition, we showed that the CaMKII T305D mutation, which mimics persistent inhibitory phosphorylation, results in decreased levels of PSD-associated CaMKII.

## Inhibitory Phosphorylation Regulates the Thresholds for LTP Induction

Since CaMKII has a pivotal role in synaptic plasticity, we tested how the loss of inhibitory phosphorylation and the concomitant increase of PSD-associated CaMKII affected LTP induction. We found that LTP could be induced in the TT305/6VA mutant under conditions that are ineffective in controls. Under these conditions at the threshold for LTP/LTD induction, we observed that half the number of WT slices showed some potentiation (11 out of 22) and the other half showed some depression. However, only 1 out of 19 slices obtained from TT305/6VA mice showed some depression under these weak stimulation conditions, indicating a lower threshold for LTP induction and concomitant higher threshold for LTD induction. Importantly, this could not be due to increased CaMKII activity, since TT305/6VA mutants have reduced $Ca^{2+}$/CaM-dependent activity and relatively normal $Ca^{2+}$/CaM-independent activity.

Surprisingly, the TT305/6VA mutation did not result in larger potentiation under conditions that induce nonsaturated LTP in WT slices. For example, a two-theta burst tetanus, which results in nonsaturated LTP in WT slices, did not reveal enhanced LTP in the mutants. In addition, loss of inhibitory phosphorylation did not change the level of LTD or depotentiation obtained after prolonged low-frequency stimulation. This suggests that inhibitory phosphorylation does not result in wholesale changes in synaptic weights per se, but changes preferentially the threshold at which synapses undergo potentiation.

What could be the underlying mechanism for these observations? It is possible that CaMKII occupancy of binding sites in the PSD (i.e., NMDARs, etc.), as well as other factors (AMPA receptors at the synapse) determine whether the PSD is either in a "LTP-on" or "LTP-off" state. Partial occupancy keeps the PSD in the LTP-off mode, while full occupation of those sites may trigger a change within the PSD that switches signaling into the LTP-on mode. Once in the LTP-on mode, the number of activated subunits within the CaMKII holoenzymes may determine the levels of potentiation in that synapse. The TT305/6VA mutants have more PSD-associated CaMKII, suggesting that in these mice a larger proportion of the CaMKII binding sites in the PSD are already occupied. Therefore, stimulation that is not sufficiently strong to switch the PSD into the LTP-on mode in WT mice may do so in TT305/6VA mutants and, therefore, result in LTP. Under conditions that induce LTP in WT synapses, all PSD CaMKII binding sites may become occupied in both TT305/6VA mutants and WT mice. Once this threshold is crossed, the level of potentiation is very likely determined by the autonomous activity of PSD-associated holoenzyme, which is similar in WT and TT305/6VA mutants.

## Reduction of PSD-Associated CaMKII Results in Impaired LTP Induction

Our data suggest that hippocampal LTP and learning is very sensitive to changes in the amount of PSD-associated CaMKII, but much less so to the overall expression level of αCaMKII. T305D mice have a 4-fold reduction of PSD-associated CaMKII and showed profound LTP and learning deficits. In contrast, homozygous αCaM-KIIΔ mice, which have no αCaMKII at all, showed only a moderate reduction of PSD-associated CaMKII. These mice showed residual levels of LTP and some spatial learning after extended training. The moderate reduction of PSD-associated CaMKII in these mice was due to a 3-fold increase in the levels of PSD-associated βCaMKII. This indicates that even in the absence of αCaMKII, βCaMKII can associate with the PSD, occupy available CaMKII binding sites in the PSD, and mediate plasticity and learning. Importantly, the difference in LTP between αCaMKIIΔ and T305D mutants cannot be explained by lower kinase activity in the T305D mice, since the $Ca^{2+}$/CaM-dependent as well as the relative $Ca^{2+}$/CaM independent activity is 1.4-fold higher in T305D mutants than in αCaMKIIΔ mutants (data not shown).

We found that weak tetanic stimulation (10 Hz/10 s) that did not change the synaptic efficacy of WT slices resulted in modest but stable LTD in the T305D mutant slices. This observation supports the model that the threshold for LTP/LTD induction is determined by the balance and competition between activated kinases and phosphatases (Lisman, 1989). Interestingly, a shift toward LTD specifically at low-stimulation frequencies (i.e., 10 Hz) has also been observed in mice that express a transgene encoding an activated form of αCaMKII (T286D) (Mayford et al., 1995). In light of our findings, it would be interesting to test how much of the CaMKII is associated with the PSD in these transgenic mice, since the persistent activation of the T286D kinase in the absence of $Ca^{2+}$ influx could result in increased inhibitory phosphorylation and a concomitant reduced affinity for binding to the PSD. This possibility is supported by the observation that CaMKII mutant proteins with high autonomous activity, including the T286D mutant protein, show clear reduction in $Ca^{2+}$/CaM dependent activity in COS cells (Yang and Schulman, 1999), suggesting increased inhibitory phosphorylation.

## Basal Inhibitory Autophosphorylation and Excitability

Inhibitory phosphorylation can also occur at basal conditions in the absence of prior CaMKII activation and results specifically in phosphorylation of αCaMKII Thr306 (Hanson and Schulman, 1992; Colbran 1993). Therefore, unstimulated synapses could lose their PSD-associated CaMKII due to progressive basal inhibitory phosphorylation. Recently, it was proposed that PSD-associated CaMKII could serve as a regulated linkage between an AMPA-receptor anchoring unit and the PSD (Lisman and Zhabotinsky, 2001). Thus, synaptic inactivity could lead to slowly rising levels of Thr306 phosphorylation, to reduced PSD-associated CaMKII, and consequently to PSDs with reduced levels of AMPA receptors (depressed or silent synapses). Thus, inhibitory phosphorylation may also serve to downregulate the excitability of networks. This hypothesis may explain the increased occurrence of seizures in older TT305/6VA mice. Interestingly, induction of status epilepticus leads to a 50% reduction in CaMKII kinase activity in rats without any accompanying change in protein expression levels (Kochan et al., 2000). Thus, it is possible that inhibitory phosphorylation is induced after seizures occur to downregulate excitability.

## LTP Thresholds and Spatial Learning

Improved learning has previously been observed in mutant mice with moderate but general enhancements in LTP (Manabe et al., 1998; Tang et al., 1999). Although the TT305/6VA mutation lowered the threshold for LTP induction, this mutation did not result in general increases in LTP or in improved learning. Instead, while the performance of controls continued to improve, performance of TT305/6VA mice seemed to stabilize at a lower level. Similarly, TT305/6VA mutants were unable to distinguish as well between contexts as controls, a result consistent with the idea that this mutation resulted in deficient hippocampal spatial representations. Additionally, the TT305/6VA mutation seemed also to result in more rigid, less flexible spatial learning, since unlike WT mice, they were unable to find the platform in a new location.

This intriguing pattern of spatial learning deficits could be due to the LTP abnormalities found in these mutants. The lower thresholds for synaptic weight changes in the TT305/6VA mutants should lead to larger spatial representations. This higher synaptic permissiveness should also result in increases in the number of spurious associations stored in mutant networks. This increased noise may account for the inability of TT305/6VA mutants to reach the performance levels of WT mice, and could explain the deficits in contextual discrimination. Moreover, the presumably larger spatial representations in TT305/6VA mutants may be behind their deficits in the reversal spatial learning task: the larger and noisier spatial representations of the mutants could be harder to modify than the smaller and more focused representations of WT mice.

The data presented in this study demonstrate that inhibitory phosphorylation modulates PSD-associated CaMKII levels, the thresholds for hippocampal LTP and hippocampal-dependent learning. These results illustrate the complexly rich web of interactions that link molecular, cellular, and cognitive mechanisms.

### Experimental Procedures

#### Generation of αCaMKII Mutant Mice

αCaMKII Thr305/6 mutant ES cells were generated as follows: a genomic PvuII-BglII fragment of 8.2 Kb encoding the αCaMKII exon with Thr 305 and 306 (Figure 2A) was subcloned from the 14.4 cosmid (Silva et al., 1992b). The two mutants (T305D and TT305/6VA) were made by replacing the 42 bp ApaI/BstXI fragment with oligonucleotides encoding the mutations as indicated in Figure 2C. A PGK neomycin cassette flanked by LoxP sites was inserted in the XbaI 1200 bp upstream of the targeted exon (Figure 2A). After transfection of R1 embryonic stem cells (derived from the 129 genetic background; Nagy et al., 1993), targeted clones (approximately 30%) were identified by Southern blot analysis. The presence of the mutation was verified, and the PGK neo cassette was removed by transient expression of Cre recombinase (pBS185, Gibco)

αCaMKIIΔ mutant ES cells were generated as follows: a genomic clone of approximately 8 Kb encoding the αCaMKII exon 2 (amino acids 22–53) was isolated by screening a mouse library. The targeting construct was made by inserting a PGK neomycin cassette flanked by LoxP sites into the EcoRI site approximately 1.2 Kbp downstream of exon 2. Another LoxP site was inserted into the SmaI site 60 bp upstream of exon 2. Targeted ES clones (61 out of 130) were identified by Southern blot analysis. PCR analysis revealed that 20 out of these 61 clones contained the additional 5′ LoxP site. Deletion of the neomycin gene was achieved as described above. Approximately 30% of the analyzed clones had a deletion of the

neomycin gene as well as a deletion of exon 2. This results in a frame-shift of the sequence downstream of exon 1. Therefore, mice derived from these cells are null mutants.

F1 heterozygous mice were obtained by injection of the ES cells into C57BL/6 blastocysts, and crossing the obtained chimeras with C57/BL6tac mice. F2 homozygous offspring and WT controls used for behavioral analysis were obtained by crossing the F1 heterozygous mice. All experiments were done with the experimenter blind to genotype.

#### Production and Characterization of an Antibody against the Thr305-Phospho Peptide

A rabbit polyclonal antibody (PB60) was raised against the purified peptide (CRRKLKGAIL-phosphoT-TMLATRN-(NH2)) (custom made by Covance) and used at 1:2000 dilution for Western blotting. The specificity was tested using purified αCaMKII (Sigma) (2 ng/μl in a buffer containing 0.05 mM ATP, 50 mM PIPES (pH 7.0), 0.2 mg/ml BSA, and 20 mM MgCl$_2$). αCaMKII was activated by adding 1 mM CaCl$_2$ and 0.02 mg/ml CaM, followed by 10 min incubation at 30°C. Inhibitory phosphorylation was subsequently induced by the addition of 1 mM EGTA (pH 7.0) and another 10 min incubation at 30°C. Dephosphorylation was achieved by 30 min incubation at 30°C in 50 mM Tris-HCl, 0.1 mM Na$_2$EDTA, 5 mM dithiothreitol, 0.01% Brij 35 (pH 7.0), and 1.5 mM MnCl2 buffer containing 2.5 U PP1 (NEB). To determine if inhibitory phosphorylation occurred in vivo, the hippocampus of an adult mouse was quickly removed; heated at 80°C in denaturing lysis buffer containing 100 mM TRIS-HCl 6.8, 2% SDS, and 1 mM EDTA; and homogenized. This was compared to the same amount of PP1 treated lysate that was obtained by homogenization in nondenaturing lysis buffer (1% Triton X-100, 25 mM Hepes [pH 7.5], 150 mM NaCl, 10% glycerol, 2 mM EDTA, and protease inhibitors). Blots were probed with the Thr305-P antibody and stripped and reprobed with αCaMKII (C6974, 1:2000; Sigma) antibody.

#### Protein Analysis

For expression studies, lysates were prepared by quickly removing the hippocampus from adult mice and by homogenation in lysis buffer containing 10 mM TRIS-HCl 6.8, 2.5% SDS, 2 mM EDTA, protease and phosphates inhibitor coctails (Sigma), and 1 μM Microcystin. PSD-enriched fractions were obtained by rapidly dissecting the hippocampus and by homogenization in 0.32 M Sucrose, 1 mM NaHCO$_3$, 1 mM MgCl$_2$, 1 mM HEPES (pH 7.4), and 1 μM Microcystin. This extract was immediately used for the isolation of P2 synaptosomes (Carlin et al., 1980). The concentration of the synaptosomes was adjusted to 1 mg/ml, and PSDs were obtained from 100 μg synaptosomes by extraction with 1% (v/v) Triton X-100 (15 min on ice), followed by centrifugation. Western blots were probed with antibodies against αCamKII (6G9, 1:10,000; Chemicon), βCaMKII (CB-β1, 1:2000; Zymed Laboratories), Thr286P-αCaMKII (22B1, 1:2000; Affinity Bioreagents), PSD95 (7E3-1B8, 1:2000; Affinity Bioreagents), Actin (C4, 1:2000; Chemicon), or Thr305-P-αCaMKII (see above). For the Western blot presented in Figure 3B, we modified antibody concentrations to visualize all four proteins on one blot.

Western blots were quantified by using serial dilutions and by using NIH Image software. A mixture of the antibodies against αCaMKII and βCaMKII resulted in approximately a 3:1 signal ratio when used on hippocampal lysates (data not shown) or PSD fractions (Figure 3E) obtained from WT cells. This is in agreement with the published literature, therefore allowing us to determine the total amount of PSD-associated CaMKII by determining the sum of the intensity of both bands (Figure 3E).

#### Electrophysiology

Field recordings were made from transverse hippocampal slices in a submerged recording chamber as described (Giese et al., 1998). EPSPs were evoked alternatively in separate pathways (control and tetanized) within the CA1 Schaffer collateral/commissural afferents at 1/3 of the maximum fEPSP amplitude. All recordings were done using the same setup and in the same time span. Wild-type animals were semirandomly taken from all three mutant groups (which are in the same genetic background) for the synaptic transmission measurements and for the two-theta and 100 Hz/1 s stimulation protocols. Since there were no significant differences between the physio-

logical results of different groups of WT mice, their data were combined. For the 10 Hz/10 s experiment, we combined the wild-type data of the T305D and TT305/6VA controls. For the LTD, depotentiation, and 100 Hz/0.04 s measurements, we used TT305/6VA littermate controls only.

## Water Maze

Our Morris water maze has been described previously (Bourtchuladze et al., 1994). The mice were given two trials every day (30 s inter-trial interval [ITI]) for 14 days, and the starting position was varied from trial to trial. Probe trials were given at training days 8, 11, and 14. During probe trials, the platform was removed, and the mice were allowed to search for it for 60 s. For the visible platform task, the platform was marked with a visible cue (colored ball). Mice were given two blocks (1 hr ITI) of four trials (30 s ITI) per day, using a fixed platform position and with changes of the starting position in each new trial.

## Fear Conditioning

Chambers A and B were largely similar but modified to have some unique features (geometry, background noise, odor) (Anagnostaras et al., 2000). Chamber C, which was used for cued conditioning, shared no obvious cues with chambers A and B. For cued and context conditioning, mice were placed in chamber A for 3 min and received a mild footshock (0.75 mA for 2 s) after 150 s. For cued conditioning, a 30 s tone (2800 Hz at 85 dB) coterminated with the shock. Conditioning was tested 24 hr later in chamber A (contextual conditioning, test for 5 min) or in chamber C (cued conditioning, test 2 min without tone, 3 min with tone). For contextual discrimination, each mouse was habituated to chamber A and B for 3 min. The next day, animals were placed in chambers A and B for 3 min (the order was balanced). After 150 s a foot shock (0.75 mA for 2 s) was delivered in chamber A, but not in B. After 24 hr, the mice were tested in both chambers A and B (the order was balanced) for 5 min. Freezing was assessed using an automated procedure (Anagnostaras et al., 2000).

## Statistical Analysis

A two-way ANOVA with repeated measures was used to analyze the acquisition data from the water maze. A single-factor ANOVA was used to analyze differences between genotypes for water maze probe trials (time in quadrant, swimspeed, platform crossings) and for LTP/LTD induction (based on average of last 10 min of recoding); Post hoc comparisons (Fisher's PLSD) between genotypes were carried out when appropriate. Planned comparisons using a paired t test were used to analyze whether mice searched selectively in the water maze (comparison between target quadrant and the average of other quadrants), and for the analysis of fear conditioning data.

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