### Kinase activity is not required for αCaMKII-dependent presynaptic plasticity at CA3-CA1 synapses

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Using targeted mouse mutants and pharmacologic inhibition of  $\alpha$ CaMKII, we demonstrate that the  $\alpha$ CaMKII protein, but not its activation, autophosphorylation or its ability to phosphorylate synapsin I, is required for normal short-term presynaptic plasticity. Furthermore, aCaMKII regulates the number of docked vesicles independent of its ability to be activated. These results indicate that a CaMKII has a nonenzymatic role in short-term presynaptic plasticity at hippocampal CA3-CA1 synapses.

The  $\alpha$  isoform of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II ( $\alpha$ CaMKII) was originally identified as synapsin I kinase<sup>1</sup>. Subsequent studies showed that a CaMKII is abundantly associated with presynaptic vesicles by binding to synapsin I (ref. 2). Together with the observation that aCaMKII is one of the most abundant proteins of the hippocampus<sup>3</sup>, these results suggest that aCaMKII also has a nonenzymatic function, but such a function has not directly been demonstrated yet. Analysis of aCaMKII knockout (KO) mice confirmed a presynaptic role for aCaMKII in short-term presynaptic plasticity<sup>4,5</sup>, but these experiments did not address whether this role is mediated by αCaMKII as a kinase, as a structural protein, or as both.

To study the enzymatic requirements of aCaMKII in presynaptic plasticity, we used four different lines of aCaMKII mutants. Autophosphorylation at the threonine 286 (T286) and T305/T306 sites was prevented by using aCaMKII-T286A (T286 is mutated to alanine) mice, which lack a CaMKII autonomous (Ca2+/CaM independent) activity<sup>6</sup>, and αCaMKII-T305V/T306A (T305 and T306 are mutated to valine and alanine, respectively) mice, which lack a CaMKII inhibitory autophosphorylation<sup>7</sup>. Furthermore, we used aCaMKII-T305D (T305 is mutated to an aspartic acid) mice, in which constitutive autophosphorylation at the T305 site in the Ca<sup>2+</sup>/CaM domain is mimicked, preventing aCaMKII in these mice from becoming activated<sup>7</sup>. The fourth line (αCaMKII-KO) lacks the entire αCaMKII protein<sup>7</sup> (for an overview of all phenotypes see<sup>8</sup>).

Because these mutants were backcrossed in C57BL/6, we first tested whether the originally reported long-term potentiation (LTP) deficits

(in hybrid 129Sv/C57BL/6 mice) were still present<sup>6,7,9</sup>. We confirmed that a CaMKII activation and its subsequent autophosphorylation at T286 are absolute requirements for LTP, but that loss of αCaMKII can partially be compensated for (Supplementary Fig. 1 online). In contrast, loss of inhibitory phosphorylation in aCaMKII-T305V/ T306A mice reduced the threshold for LTP induction as reported previously7 (Supplementary Fig. 1). Western blots of isolated synaptosomes of all the mutants did not reveal changes in the levels of the  $\beta$ ,  $\gamma$ and  $\delta$  CaMKII isoforms, nor was the amount of calmodulin affected in these mutants (Supplementary Fig. 2 online).

We looked at the ability of these mutants to phosphorylate synapsin I at serine 603 (S603, site 3), which is an exclusive CaMKII site. aCaMKII-KO mice showed a marked decrease of synapsin I phosphorylation compared to wild-type mice (P < 0.001, ANOVA, Fig. 1), suggesting that none of the other CaMKII isoforms could efficiently compensate for the loss of aCaMKII phosphorylation of synapsin I in vivo. Notably, steady-state levels of phosphorylated synapsin I were not affected in  $\alpha$ CaMKII-T286A (P = 0.2) mice and in  $\alpha$ CaMKII-T305V/306A mice (P = 0.3), indicating that loss of autonomous activity or self-inhibition does not have a large effect on synapsin I phosphorylation in vivo. In contrast, activation of aCaMKII is an absolute requirement for synapsin I phosphorylation, as S603 phosphorylation in the aCaMKII-T305D mutant was not significantly



Figure 1 Phosphorylation of synapsin I and CaMKII-T286/T287 in synaptosomes obtained from aCaMKII mutants. (a) Phosphorylation of synapsin I S603 was not affected by impaired aCaMKII autophosphorylation, but required  $\alpha$ CaMKII protein and its activation by Ca<sup>2+</sup>/calmodulin. (b) Phosphorylation of αCaMKII-T286 and βCaMKII-T287 was absent in αCaMKII-T305D mice. Graph represents data from βCaMKII-T287 only. Error bars indicate s.e.m. Each sample contains pooled fractions from four independent isolations.

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above background level (P = 0.2; **Fig. 1a**). The dominant-negative nature of the  $\alpha$ CaMKII-T305D mutation was further illustrated by the fact that this was also the only mutant in which autophosphorylation at both  $\alpha$ CaMKII-T286 and  $\beta$ CaMKII-T287 was indistinguishable from background level (both P > 0.8; **Fig. 1b**), further suggesting that  $\alpha$ CaMKII is inactive in this mutant. Taken together, these results show that these mutants provide an ideal tool for dissecting the requirements of  $\alpha$ CaMKII activation,  $\alpha$ CaMKII autophosphorylation and synapsin I S603 phosphorylation in short-term presynaptic plasticity.

Previous studies from multiple laboratories<sup>4,5,8</sup>, using independently generated targeted deletions of  $\alpha$ CaMKII<sup>5,9</sup>, have demonstrated that the loss of aCaMKII results in enhanced augmentation and decreased synaptic fatigue, which are both measures of presynaptic plasticity. Augmentation is an increase in the evoked postsynaptic response that is observed several seconds after high-frequency afferent stimulation, caused by facilitated exocytosis<sup>10</sup>. We measured augmentation at hippocampal CA3-CA1 synapses using extracellular field recording (Supplementary Methods online). aCaMKII-KO mice showed a striking increase in synaptic augmentation (during 3-11 s:  $F_{4,92} = 13, P < 0.0001$ , repeated measures ANOVA; Fig. 2), confirming that  $\alpha$ CaMKII critically regulates this form of presynaptic plasticity<sup>4</sup>. Activation of presynaptic aCaMKII may be important in synaptic augmentation, as transiently elevated presynaptic calcium is thought to be a major factor underlying this process. However, although αCaMKII activity is regulated by autophosphorylation, augmentation was not affected in the T286A and T305V/T306A autophosphorylation-deficient mutants (Fig. 2c). The lack of a phenotype in these mutants could reflect the short-term nature of this kind of plasticity, and/or the fact that phosphorylation of synapsin I S603 was unaffected in these mutants (Fig. 1). Unexpectedly however, augmentation was also unaffected in  $\alpha$ CaMKII-T305D mutants (P = 0.5, ANOVA; Fig. 2b,c), where  $\alpha$ CaMKII activation was blocked and synapsin I phosphorylation was absent (Fig. 1). These results strongly suggest that synaptic augmentation does not depend on the ability of  $\alpha$ CaMKII to phosphorylate synapsin I, nor on the activation of  $\alpha$ CaMKII.

Because activation of both  $\alpha$ CaMKII and  $\beta$ CaMKII seem to be impaired in the  $\alpha$ CaMKII-T305D mutant (**Fig. 1b**), we would expect that a similar result should be obtained if the augmentation experiment is carried out in the presence of the membrane-permeable CaMKII inhibitor KN-93 (ref. 11). This inhibitor competes with Ca<sup>2+</sup>/calmodulin binding and therefore prevents activation of both  $\alpha$ CaMKII and  $\beta$ CaMKII. LTP experiments in the presence of this inhibitor showed that KN-93 was indeed able to block LTP (**Supplementary Fig. 1**). In contrast, we observed similar levels of augmentation in the presence of KN-93 or its inactive analog KN-92 (P = 0.5, ANOVA; **Fig. 2c**), confirming that CaMKII activation is not required for normal augmentation.

Our biochemical analyses (**Fig. 1**) indicated that CaMKII activity in the  $\alpha$ CaMKII-T305D mutant was reduced to such an extent that phosphorylation of CaMKII substrates was undetectable. If CaMKII activity is indeed not required for augmentation, the potent CaMKII inhibitor AIP-II (autocamtide-2–related inhibitory peptide II) should not affect augmentation either. This inhibitor is 500 times more potent than KN-93, and it is noncompetitive for Ca<sup>2+</sup>/calmodulin and exogenous substrates, thus also blocking basal and autonomously active CaMKII<sup>12</sup>. Efficient penetration was achieved by making use of AIP that was fused to the antennapedia transport peptide, and by preincubating slices for 1 h with AIP (see **Supplementary Methods**). Indeed, LTP was fully blocked, indicating that the drug was able to penetrate the slice (**Supplementary Fig. 1**), and notably, Ant–AIP-II showed no



**Figure 2** Presynaptic short-term plasticity requires  $\alpha$ CaMKII protein, but not its autophosphorylation, activation or activity. (**a**–**c**) Increased synaptic augmentation in  $\alpha$ CaMKII-KO mutant mice was not caused by the lack of CaMKII kinase activity. fEPSP responses (normalized to pretetanus baseline) of CaMKII-KO (**a**) and CaMKII-T305D (**b**) mice were recorded at the indicated time after a 10 theta-burst tetanus. Traces are from baseline response (gray) and the response 5 s post-tetanization (black). Augmentation summary of responses obtained 5 s post-tetanus normalized to baseline is shown in **c**. Black bars represent mutants or drug-treated slices, white bars represent control slices as indicated. (**d**–**f**). Decreased synaptic fatigue during repetitive stimulation in  $\alpha$ CaMKII-KO mice was not caused by the lack of  $\alpha$ CaMKII kinase activity. (**d**,**e**) fEPSP responses (normalized against baseline) of CaMKII-KO (**d**) and CaMKII-KO mice was not caused by the lack of  $\alpha$ CaMKII kinase activity. (**d**,**e**) fEPSP responses (normalized against baseline) of CaMKII-KO (**d**) and CaMKII-T305D (**e**) mice were recorded during a 10-Hz tetanus. Only the first and even numbered stimuli are shown for clarity. Traces are from wild-type (gray) and  $\alpha$ CaMKII-KO slices (black) recorded from stimulus number 21–30. Depletion summary of the last (100) stimulus of the 10-Hz train is shown in **f**. Black bars represent mutant or drug-treated slices, normalized against the controls as indicated (white bars, set at 100%). Numbers between brackets indicate the number of slices. Error bars indicate s.e.m.

discernable effect on synaptic transmission (**Supplementary Fig. 3** online), which makes it suitable for use in these experiments. However, like KN-93, Ant–AIP-II did not affect augmentation (P = 0.1, ANOVA; **Fig. 2c**). Taken together, these results indicate that  $\alpha$ CaMKII protein, but not  $\alpha$ CaMKII activity, is required for normal augmentation.

Previous whole-cell patch-clamp recordings in CA3-restricted αCaMKII-KO mice showed a substantial enhancement of the excitatory postsynaptic current amplitude during repetitive stimulation of CA3-CA1 synapses<sup>5</sup>, suggesting that the fatigue rate of neurotransmitter release is regulated by a CaMKII. Likewise, extracellular field recordings from our global a CaMKII-KO mice also demonstrated reduced synaptic fatigue (Fig. 2d). The responses to repetitive 10-Hz stimulation reveal the competing processes of facilitation, vesicle depletion and vesicle mobilization<sup>10</sup>. aCaMKII-KO mutants had a similarly shaped curve as wild-type mice; however, there was a significant effect of genotype  $(F_{1,45} = 4.5, P < 0.05)$  and a significant interaction between genotype and stimulus number (F<sub>99,4455</sub> = 4.5, P < 0.0001). αCaMKII-KO mutants were only significantly different from wild-type mice after 20 stimuli (first 20:  $F_{19,855} = 1.2$ , P = 0.3; last 80:  $F_{79,3555} = 2.6$ , P < 0.001), suggesting a differential rate of vesicle depletion and/or mobilization, the cellular processes primarily responsible for the maintenance of excitatory postsynaptic potential (EPSP) amplitude during prolonged stimulation<sup>10</sup>.

To test the  $\alpha$ CaMKII autophosphorylation and synapsin I phosphorylation requirements in this presynaptic measure, we repeated this experiment in the  $\alpha$ CaMKII point mutants. No significant effect of genotype was observed in either  $\alpha$ CaMKII-T286A or  $\alpha$ CaMKII-T305V/T306A autophosphorylation-defective mutants (both P > 0.3, ANOVA at stimulus number 100; **Fig. 2f**). Notably, the depletion rate was also not affected in  $\alpha$ CaMKII-T305D mutants (P > 0.7; **Fig. 2e,f**), in which activation of  $\alpha$ CaMKII was prevented and phosphorylation of synapsin I was absent. In addition, there was no discernable effect on depression in slices treated with KN-93 or Ant–AIP-II (P = 0.9 and P = 0.7, respectively; **Fig. 2f**). Taken together, these data strongly suggest that the  $\alpha$ CaMKII protein plays a structural role rather than an enzymatic role, during this form of short-term presynaptic plasticity.

Mechanistically, enhanced synaptic augmentation and reduced synaptic depression could reflect changes in the pool sizes of the synaptic vesicles, in particular the size of the readily releasable pool (RRP). Therefore, we used electron microscopy to measure the number of docked vesicles, a morphological correlate of the RRP13,14. We obtained measurements at the active zone of excitatory synapses on CA1 spines of wild-type, aCaMKII-KO and aCaMKII-T305D mice. Indeed, there was a significant effect of genotype ( $F_{2,41} = 4.7, P < 0.05$ ), with synapses in αCaMKII-KO mice showing a 20% increase in the total number of docked vesicles compared with wild-type (Fisher's PLSD, P < 0.05) or  $\alpha$ CaMKII-T305D mice (P < 0.01) (Fig. 3a). In contrast, no significant difference in the number of docked vesicles was observed between the aCaMKII-T305D mutant and wild-type mice (Fisher's PLSD, P = 0.24). Additional measurements of the number of reserve pool vesicles, active zone length and presynaptic terminal area were similar between the mutants and wild-type mice (all measures P > 0.2; Supplementary Fig. 4 online). Thus, the absence of a CaMKII protein results in an increased number of docked vesicles, whereas the loss of aCaMKII activation and synapsin I S603 phosphorylation does not affect vesicle docking.

Together, these results suggest a model in which  $\alpha$ CaMKII functions nonenzymatically to limit the size of the RRP, thereby modulating short-term presynaptic plasticity. If the observed increase in the size of EPSPs during repetitive stimulation in  $\alpha$ CaMKII-KO mice is indeed mediated by a larger RRP, then presynaptic function should be normal



**Figure 3**  $\alpha$ CaMKII protein regulates the number of docked vesicles. (a) Quantitative electron microscopy of asymmetric synapses on dendritic spines of CA1 pyramidal neurons showed a 20% increase in the number of docked vesicles in  $\alpha$ CaMKII-KO mice. (b) Decreasing the depletion rate by lowering extracellular calcium reversed the phenotype of the  $\alpha$ CaMKII-KO mice during repetitive stimulation. Error bars indicate s.e.m.

under conditions that minimize depletion of the RRP. Accordingly, we decreased the extracellular calcium concentration, which limits the rate of depletion from the RRP<sup>15</sup>. Decreasing the extracellular calcium concentration from 2.5 to 0.8 mM normalized the responses during 10-Hz stimulation in  $\alpha$ CaMKII-KO mice ( $F_{1,31} < 0.0001$ , P = 1, **Fig. 3b**), supporting the idea that  $\alpha$ CaMKII limits the available pool of readily-releasable neurotransmitter vesicles.

Taken together, our experiments suggest that synapsin I S603 phosphorylation and  $\alpha$ CaMKII activity are not required for short-term presynaptic plasticity measures such as augmentation and synaptic fatigue during repetitive stimulation. Specifically, we have demonstrated that, at hippocampal CA3-CA1 synapses,  $\alpha$ CaMKII functions independently of its kinase activity to modulate short-term presynaptic plasticity by limiting the number of presynaptic docked neurotransmitter vesicles.

Note: Supplementary information is available on the Nature Neuroscience website.

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#### COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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### **Supplementary Data**

# Kinase activity is not required for CaMKII-dependent presynaptic plasticity at hippocampal CA3-CA1 synapses

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### **Supplementary Figure 1**

**Supplementary Fig. 1.** Long-term potentiation at Schaffer Collateral CA1 synapses in αCaMKII mutant mice after back-crossing in C57BL6 (a-e) and in pharmacologically treated wild-type slices (f,g). LTP in all experiments was induced by four bursts of high frequency stimulation (four stimuli at 100 Hz), each burst separated by 200 ms, except in (d) in which LTP was induced by 150 stimuli at 5 Hz.

(a) LTP is reduced but not absent in  $\alpha$ CaMKII-KO mice, as observed previously<sup>(7,9,19)</sup>.

(b,e) LTP is absent in  $\alpha$ CaMKII-T286A and T305D mice, as reported previously<sup>(6,7)</sup>.

(c,d) LTP in  $\alpha$ CaMKII-TT305/6VA mice is normal upon strong stimulation (4 theta burst LTP) but increased upon weak stimulation (5Hz), consistent with previous findings<sup>(7,20)</sup>.

(f,g) LTP is blocked in wild-type slices in presence of CaMKII inhibitor KN93 (f) and Ant-AIP-II (g).

Error bars represent SEM. Numbers between brackets refer to the number of slices used. See Supplementary methods for cited references.

# **Supplementary Figure 2**



Supplementary Fig. 2. No changes in synaptic  $\beta$ CaMKII,  $\gamma$ CaMKII,  $\delta$ CaMKII and Calmodulin in  $\alpha$ CaMKII-KO mutants or point mutants. Synaptosomes were isolated as described (**Supplementary methods**), and analyzed by Western blotting. Each sample contains pooled fractions from 4 independent isolations.

## **Supplementary Figure 3**





(a) Presynaptic excitability is not significantly affected by Ant-AIP-II

(F1,55=1.4; p=0.24 Repeated Measures ANOVA) .

- (b) Postsynaptic fEPSP responses are not affected by Ant-AIP-II (F1,60=0.9; p=0.34).
- (c) Overall synaptic transmission (fEPSP as function of the presynaptic fiber volley) is unaffected by Ant-AIP-II, in agreement with previous findings<sup>(21)</sup>.

Error bars represent SEM. See Supplementary methods for cited references.

### **Supplementary Figure 4**



Measure	Wild-Type	αCaMKII-KO	αCaMKII-T305D
Active zone (AZ) length (nm)	234±10	242±26	190±15
Terminal area	138±15	158±18	112±19
Reserve pool vesicles	40±4	47±5	37±5
Reserve pool vesicles/area	309±21	306±15	365±40
Docked vesicles/AZ	4.6±0.2	5.6±0.5	4.0±0.3
Docked vesicles µm	20±0.7	24±1.3	21±1.2

**Supplementary Fig. 4**. Quantitative electron microscopy of asymmetric synapses on dendritic spines of CA1 pyramidal neurons.  $\alpha$ CaMKII-KO have more docked vesicles at the active zone than wild-type mice and  $\alpha$ CaMKII-T305D mice. Arrowheads denote the lateral boundaries of the postsynaptic density, which was used to define the active zone across the synaptic cleft. Arrows show examples of vesicles counted as docked (see Methods for their definition). Statistical differences (*p*<0.05 compared to wild-type mice) are printed in bold. All values are mean ± SEM.

### **Supplementary Materials and Methods**

#### Animals

To study the role of presynaptic  $\alpha$ CaMKII, we made use of a  $\alpha$ CaMKII mutant, in which  $\alpha$ CaMKII exon 2 is deleted<sup>7</sup>. Exon 2 encodes the catalytic site, and deletion of this exon results in a premature translation stop due to a frame shift, thus effectively resulting in a  $\alpha$ CaMKII knock-out (KO) line<sup>7</sup>. To study the role of autophosphorylation and activity of  $\alpha$ CaMKII, we made use of three other different mutants in which the endogenous  $\alpha$ CaMKII was mutated using ES cell-mediated gene targeting. In the first mutant (T286A),  $\alpha$ CaMKII Thr286 is substituted by alanine<sup>6</sup>. This mutation results in a kinase that is unable to switch to its Ca<sup>2+</sup>/CaM-independent state. In the  $\alpha$ CaMKII TT305/306VA mutant,  $\alpha$ CaMKII Thr305 and Thr306 are substituted by non-phosphorylation<sup>7</sup>. In the  $\alpha$ CaMKII-T305D mutant, Thr305 is substituted by negative charged Aspartate. This mimics persistent Thr305 phosphorylated  $\alpha$ CaMKII, which interferes with Ca<sup>2+</sup>/CaM binding, thus blocking  $\alpha$ CaMKII activation in this mutant<sup>7</sup>. For a review of previous reported phenotypes see<sup>9</sup>.

All mice were obtained by breeding heterozygous parents. Littermate control animals were used for every experiment except for augmentation. For augmentation we combined the littermate wild-type animals of the various genotypes since they were indistinguishable. All mutants described in this paper were homozygous for the mutation unless specified otherwise. To allow for direct comparison with previously published experiments, we made use of hybrid F2, 129SvJ-C57BL/6 animals for augmentation experiments and C57BL/6 (backcrossed at least 8 times) animals for all other experiments. For experiments using CaMKII inhibitors we used wild-type mice in the aforementioned background. All experiments were approved by a Dutch ethical committee (DEC) for animal research.

### Slice preparation and electrophysiological recordings

Electrophysiology experiments were performed on mice between the ages of 2 and 5 months and all experiments were performed blind to genotype. After the animals had been sacrificed, hippocampi were removed in ice-cold artificial cerebrospinal fluid (ACSF), and saggital hippocampal slices (400 µm) were prepared using a vibratome. Normal hippocampal slices and hippocampal minislices (from which the CA3 area was removed) were maintained in artificial cerebrospinal fluid (ACSF) at room temperature for at least 1.5 hour to recover before experiments were initiated. Then they were transferred to a submerged chamber field recording set-up and perfused continuously at a rate of 2mL/minute with ACSF equilibrated with 95% O<sub>2</sub>, 5% CO<sub>2</sub> at 30°C. ACSF contained (in mM) 120 NaCl, 3.5 KCl, 2.5 CaCl<sub>2</sub>, 1.3 MgSO<sub>4</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub> and 10 D-glucose. For the low-calcium experiment (Fig. 3), the calcium concentration was reduced to 0.8 mM. Extracellular recording of field excitatory postsynaptic potentials (fEPSPs) were started 20 minutes after transfer to the recording chamber (except for the CaMKII inhibitor studies, see below), and made in CA1 striatum radiatum area with a Pt/Ir recording electrode (FHC, Bowdoinham, ME). A bipolar Pt/Ir electrode was used to stimulate Schaffer collateral/commissural afferents with a stimulus duration of 100µs. Stimulus response curves were obtained at the beginning of each experiment and these were not different between mutants. Subsequent stimulation was performed at 1/3 of the maximum response. A stable baseline was recorded before the onset of each experiment. Similar to the previous study<sup>4</sup>, we used a 10 theta-burst stimulation protocol in the presence of D-APV (2-amino-5-phosphonovalerate, 50 µM, Tocris) to measure synaptic augmentation. The 10 Hz experiments were done in the presence of D-APV and picrotoxin (100µM, Tocris), and on mini-slices in which CA3 area was removed to prevent epileptic discharges. The input/output curves of Supplementary fig. 3 were obtained in the presence of D-APV, prior to recording augmentation.

KN92, KN93 (10  $\mu$ M) and Ant-AIP-II (4  $\mu$ M) were obtained from Calbiochem. Experiments using CaMKII inhibitors were performed at 32°C with a perfusion rate of 5–6 mL/minute. These slices were pre-incubated with the drug under these conditions in the chamber for at least 1 hour before the experiment was started. Control slices were treated in the same way, except that either no drug or KN92 was present. The efficacy of KN93 and AIP was tested by their ability to impair LTP induction (**Supplementary figure 1**). All LTP experiments were performed at a stimulation strength of 2/3 of maximum.

### Analysis of presynaptic vesicle distribution

The size of the readily releasable pool (RRP) was estimated by quantitative electron microscopy of asymmetric synapses on dendritic spines of CA1 pyramidal neurons. We quantified the number of clear, round synaptic vesicles in contact and within one vesicle diameter from the presynaptic active zone. This morphologically defined set of vesicles is thought to correlate with the readily-releasable pool of quanta<sup>13,16,17</sup>. We used a random sampling method from single thin sections as described<sup>14</sup>, since we previously showed that data obtained with this method correlates well with an unbiased serial sectioning approach<sup>18</sup>. Only complete profiles of nonperforated asymmetric synapses on dendritic spines within striatum radiatum of area CA1 were photographed. The parameters measured in each synapse were: (1) the length of the active zone; (2) presynaptic terminal area, (3) total number of small (~50 nm) clear and round synaptic vesicles per terminal; and (4) the number of docked vesicles per active zone. The docked vesicles are defined as those up to onevesicle-diameter (~50 nm) distance from the active zone, according to criteria developed by Reese and colleagues<sup>16</sup>, because these vesicles have been shown to be depleted during sustained, repetitive activity. For the synaptic sizes (active zone length, terminal bouton area) we quantified 70 wt, 53 CaMKII-KO and 54 T305D synapses (from 2 mice of every genotype). For the vesicle distribution analysis we quantified 21/13/10 synapses respectively. This method was previously validated by comparing it to serial sectioning<sup>18</sup>.

#### Western blot analysis

Synaptosomes were isolated as described previously<sup>7</sup>. The concentration of the synaptosomes was adjusted to 1 mg/ml. Western blots were probed with antibodies directed against Thr286/287- $\alpha/\beta$ -CaMKII (#06-881, 1:5000; Upstate Cell Signaling Solutions), Anti-Phospho-Ser603 Synapsin I (p1560-603, 1:1000; PhosphoSolutions), Synapsin I (AB1543P, 1:10.000; Chemicon),  $\beta$ CaMKII (CB- $\beta$ 1, 1:2000; Zymed),  $\delta$ CaMKII (sc-5392, 1:200; Santa Cruz),  $\gamma$ CaMKII (sc-1541, 1:1000; Santa Cruz) Calmodulin (#465, 1:500, Swant) and Actin (MAB1501R, 1:2000; Chemicon). Blots were first probed with the antibody of interest and after stripping (Restore western blot stripping buffer; Pierce) re-probed with the loading control antibody. Western blots were quantified using NIH-Image.

### Data analysis and statistics

The experimenter was blind to the genotype until the experiment and analysis were completed. Error bars in graph indicate the standard error of mean. Sample size numbers in the graphs indicate the number of slices, and this number was also used for statistical comparisons. ANOVA's were used for morphological studies and for determining the augmentation (5 seconds after the tetanus) and for determining the depletion (at the 100<sup>th</sup> stimulus). Repeated measures ANOVA were used for statistical analysis of the synaptic transmission curves, and for the entire augmentation and 10Hz curves, followed by a posthoc Fisher's PLSD analysis if necessary.

Compromise Power Analyses was performed to determine the statistical power of the morphologcal studies using G\*Power (Erdfelder E, et al (1996) GPOWER: A general power analysis program. Behavior Research Methods, Instruments, and Computers 28:1-11). This analysis gave values of statistical power larger than 0.95 (i.e. 95% confidence of accepting the null hypothesis when it is true.

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