Role of CaMKII in Synaptic Plasticity, Learning and Disease

Geeske Marieke van Woerden

Cover: © Caroline Bruinsma, 2009

This study was performed at the Department of Neuroscience of the Erasmus MC in Rotterdam, The Netherlands. Research was supported by, The Netherlands Organisation for Scientific research (NWO-ZonMW (TOP, VIDI, VICI)), Neuro-BSIK, The European Community (EEC-SENSOPAC), Prinses Beatrix Fonds, Hersenstichting Nederland, Netherlands Epilepsy Fund and the Angelman Syndrome Foundation (ASF).

Printed by: Wöhrmann Print Service

Role of CaMKII in Synaptic Plasticity, Learning and Disease

De rol van CaMKII in synaptische plasticiteit, leren en ziekte

Proefschrift

Ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 2 december 2009 om 9:30 uur

door

Geeske Marieke van Woerden
Geboren te Utrecht

ERASMUS UNIVERSITEIT ROTTERDAM

Promotiecommissie

Promotor:	Prof.dr. Y. Elgersma
Overige leden:	Prof.dr. C.I. De Zeeuw
	Prof.dr. S.A. Kushner
	Prof.dr. B.A. Oostra

Table of contents

Chapter I

General Introduction:
Learning about CaMKII

1. The CaMKII Family

Calcium/Calmodulin-dependent protein kinase II (CaMKII), a second messenger-mediated kinase, is one of the most abundant proteins in the brain, as it accounts for up to 2% of total protein, depending on the brain region[1, 2]. It was first discovered as the Synapsin I kinase, indicating that the protein was located presynaptically and involved in neurotransmitter release[3, 4]. However, subsequent studies showed that this kinase was also present at the postsynaptic site[5, 6] where it is enriched in the postsynaptic density (PSD)[7-9].

The CaMKII family consists of four different isozymes, α, β, γ and δ, each encoded by a separate gene. The first CaMKII isozymes discovered were α- and βCaMKII[4, 10], which form holoenzymes consisting of 12 subunits[1]. Later the two other isozymes were described (γ and δ), which are much less prominent in the brain[11, 12]. Even though the different isozymes are encoded by different genes, they are highly homologous in their domain organization (as reviewed by[13]). They all contain a catalytic, an autoregulatory and an association domain (fig 1). The autoregulatory domain, containing several autophosphorylation sites, is highly conserved in all isozymes. The differences between the proteins are found in the association domain, where variable insertions reside or splicing can occur. All isozymes also have different subtypes (e.g. β' and β_M, or α_B) due to alternative splicing in the association domain.

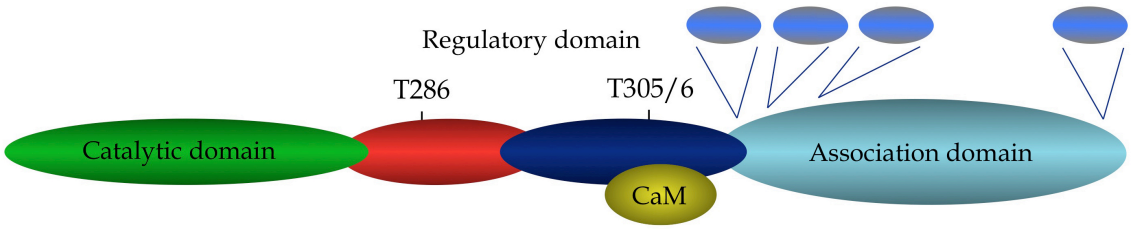


Figure 1. Domain organization of CaMKII. *All CaMKII isoforms contain a catalytic, a regulatory and an association domain. Calmodulin (CaM) binds the regulatory domain, where also the autophosphorylation sites (T286 and TT305/6) are located. The variable region consists of several variable domains (the small blue ovals around the association domain), which determine the differences between the isozymes. Adjusted from Colbran, Biochem J, 2004*

Are all these different isozymes functionally redundant, or have they evolved to fulfill specific roles? Small organisms like invertebrates (e.g. *Drosophila melanogaster* (fruit fly), *C. elegans* (nematode), *Suberites domuncula* (sponge)) only have a single *Camk2* gene (as reviewed by[14]). However, from the single *Camk2* gene in *Drosophila* at least five different isoforms are expressed, of which one during development[15] and four isoforms in the late embryonic state, all generated by alternative splicing[16]. The more evolved species (e.g. *Xenopus laevis* (frog), *Mus musculus* (mouse), *Homo sapiens* (human)) have 2 or more different *Camk2* genes. It is very likely that the different isozymes found in vertebrates are also evolved from one single ancestral *Camk2* gene. Indeed, when an evolutionary tree of the nucleotide sequence of the different isoforms throughout species is made[14], it shows that there is one ancestral *Camk2* gene (fig 2). Via multiple duplications, four *Camk2* genes evolved in mammals, with *Camk2d* being the first gene to diverge. As described above, the four different genes show a high degree of homology, but differ in the variable region, which is located in the association domain. When aligning the mouse protein sequences of the variable regions of the different genes, highest homology is seen between β- and γCaMKII, indicating that these two isozymes are closely related to each other (fig 3). αCaMKII is the most specialized isozyme, being expressed solely in the central nervous system[12, 14, 17].

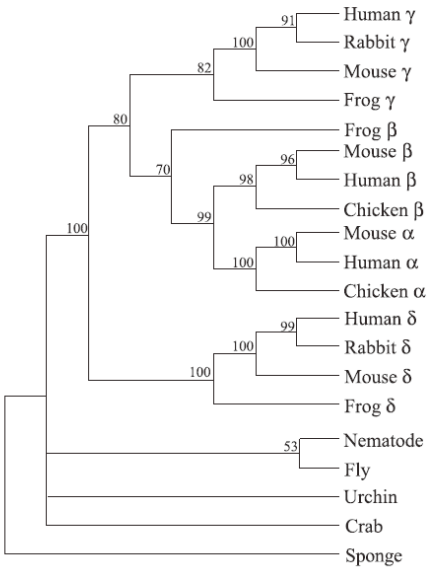


Figure 2. Phylogenic tree of the nucleotide sequences of Camk2. *Looking at the phylogenic tree, there is one single ancestral Camk2 gene, and Camk2d is the first gene to diverge. Abbreviations: γ = Camk2g; β = Camk2b; α = Camk2a; δ = Camk2d. Adapted from Tombes et al., Gene, 2003*

The phylogenic data described above indicates that indeed the different *Camk2* genes are all derived from one ancestral gene. But have they evolved further to fulfill different functions, or are they functionally redundant and just evolved through evolution without acquiring functional specializations? This question can be answered at different levels.

First, one can look at the expression patterns of the different proteins. γCaMKII and δCaMKII are expressed throughout the rat/mouse body[11, 12, 18]. βCaMKII however, is primarily expressed in the central nervous system[19], but has an isoform, β_MCaMKII, which is expressed in skeletal muscle[18]. αCaMKII is solely expressed in the central nervous system[12, 17]. These differences in expression pattern make sense, if indeed *CaMK2d* was the first gene to diverge and is expressed throughout the organism. αCaMKII, with its specific localization and not being present in invertebrates, is very likely then to fulfill another function than the other CaMKII isozymes. The other isozymes however, are also expressed in the brain, but with different regional preferences and protein expression levels. The δCaMKII and γCaMKII are much less abundant in the brain compared to αCaMKII and βCaMKII[12]. Both δCaMKII and γCaMKII are expressed throughout the brain, however not everywhere with the same expression levels. δCaMKII shows higher expression levels in the cerebellum compared to the cerebrum and brainstem[12] and is together with βCaMKII the only isozyme found in the deep cerebellar nuclei[18, 20]. γCaMKII shows lower expression levels in the cerebellum compared to the cerebrum and brainstem[11]. Both δCaMKII and γCaMKII predominate in the medulla oblongata[18]. αCaMKII and βCaMKII are the major brain specific isozymes. αCaMKII is the major forebrain isozyme, (α:β ratio of 3:1[1, 21, 22]), and βCaMKII is the main cerebellar isozyme (α:β ratio of 1:4[23]). In the olfactory bulb all four isozymes are shown to be equally abundant[18]. Thus there are regional differences, but there is also some overlap.

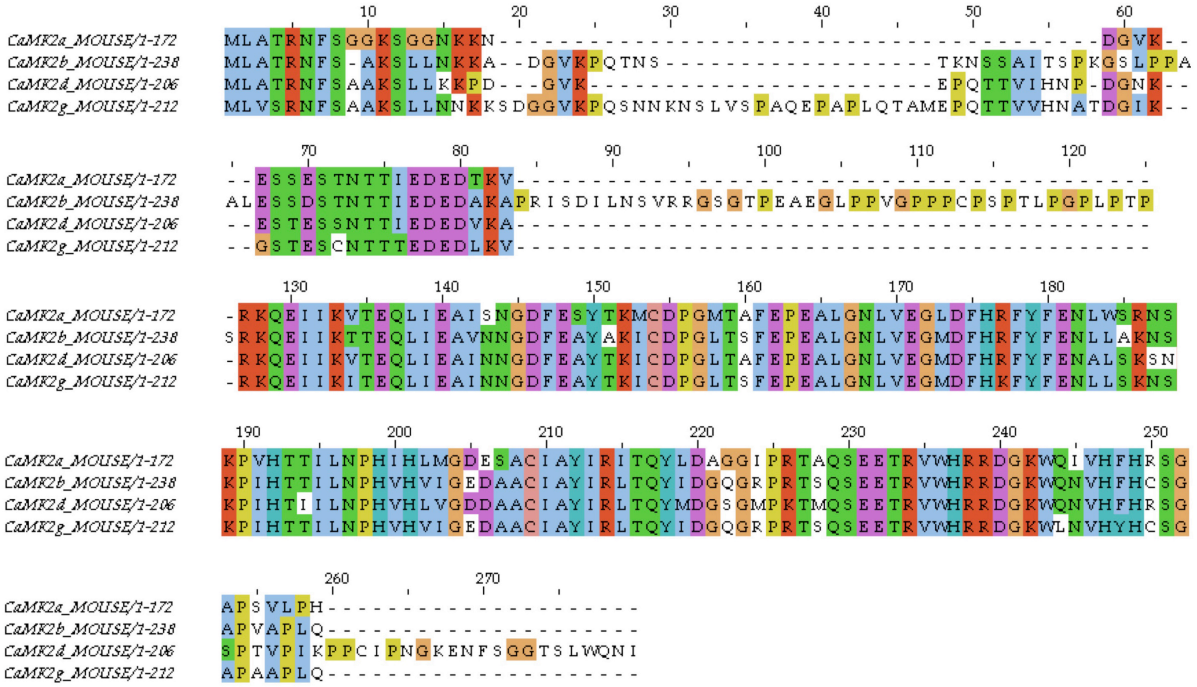


Figure 3. CaMKII variable region alignment. *Alignment of the variable region and association domain of CaMKII mouse protein sequence using MUSCLE (MUltiple Sequence Comparison by Log-Expectation). When comparing the different sequences, βCaMKII and γCaMKII show highest homology.*

Second, are there differences in the temporal expression patterns? Two isozymes are expressed during embryonic development, which are δCaMKII and γCaMKII[18]. Expression of these isozymes starts around E10 – E11 in the peripheral and the entire central nervous system, indicating that these two isozymes could play a role in the development of the nervous system. However, their expression levels increase postnatally[18]. βCaMKII is the next isozyme to be expressed. Expression starts in the late phase of embryonic development (E12.5) at low levels, which increase to a maximum at P3-5[18]. The αCaMKII isozyme however, is only expressed postnatally (starting at P5, increasing to a maximum in the adult brain[24]), in post-mitotic cells[18]. These findings indicate different roles for the different isozymes in the brain during development and post-developmentally.

What are these functional differences? This introduction will only focus on the function of the CaMKIIs in the brain, since that is the region where all four isozymes are expressed. γCaMKII and δCaMKII are less abundantly present in the brain, compared to αCaMKII and βCaMKII, and not much is known about their function, however there are a few interesting findings to mention. δCaMKII has been shown to play a role in neurite outgrowth *in vitro*, by stabilizing the actin cytoskeleton in cellular extensions[25, 26]. δCaMKII is also present in the nucleus and can, upon activation, promote BDNF gene expression[27]. The α_BCaMKII isoform and the γ_ACaMKII isoform both contain a nuclear localization signal (NLS)[11, 28], indicating they also play a role in the nucleus. However, the exact function of δCaMKII and γCaMKII and all their isoforms, remain elusive.

Extensive research has been done on the function of neuronal αCaMKII, and to a lesser extent on neuronal βCaMKII. αCaMKII mainly functions as a memory molecule in excitatory neurons in forebrain, by inducing long-lasting changes in the postsynaptic density upon transient calcium influx (as reviewed by[29] and[30]). βCaMKII contains an actin-binding domain, with which it binds to the actin cytoskeleton. Upon activation βCaMKII is

released from actin, thus it is able to regulate the cellular localization of αCaMKII[31]. Furthermore, *in vitro* studies have shown that βCaMKII can induce neurite outgrowth[32, 33], plays a role in dendritic morphology[34], can bundle F-actin and phosphorylate actin[35]. The role of βCaMKII *in vivo* is unknown and an important topic of this thesis.

The details on the functional aspects of α- and βCaMKII, and the differences between them, will be described in the rest of this introduction. But looking at these general differences between the CaMKII isozymes, one can conclude that they are not redundant at all, but have indeed evolved to fulfill specific functions in higher organisms. Also the fact that deletion of, or mutations in one of the isozymes (e.g. *Camk2a* (see review[36])) in an organism cannot be compensated for by one of the other isozymes, indicates that the different isozymes have evolved to fulfill different functions in the organism.

2. General regulation of CaMKII

All CaMKII isozymes share the same domain organization (as reviewed by[13]). They all contain a catalytic domain, an autoregulatory domain, a variable region and an association domain. Different CaMKII isozymes can either form homo-oligomers or hetero-oligomers, each consisting of 11–13 subunits[37, 38]. Crystallography studies showed that the subunits form a doughnut-like structure of two layers each containing 5-7 units, which are connected at the association domain (fig 4)[39]. The subunit composition of an oligomer determines its properties, like cellular localization and the affinity for Ca^{2+}/CaM, as will be discussed later. The autoregulatory domain is the domain that can regulate the activation state of CaMKII, and has been most extensively studied in αCaMKII (as reviewed by[40]). The N-terminal part of the autoregulatory domain binds to the catalytic domain, thereby blocking the ATP-binding site, thus inactivating the enzyme[41]. The C-terminal part of the autoregulatory domain is the part where the activated Ca^{2+}/CaM complex can bind[42].

Upon increases in Ca^{2+} levels in the cell, the following steps will occur (fig 5): a) Ca^{2+} binds to calmodulin, and this complex binds to the C-terminal part of the autoregulatory domain of CaMKII. b) Binding of this complex to the autoregulatory domain induces a conformational change. It releases the catalytic domain from the autoregulatory domain, thereby relieving the auto-inhibition. c) The conformational change now allows the catalytic domain to phosphorylate its neighboring subunit at Threonine 286 (T286 αCaMKII; T287 βCaMKII) in the autoregulatory domain. This phosphorylation increases the affinity of CaMKII for the Ca^{2+}/CaM complex more than a 1000-fold (a mechanism called "CaM trapping"[43, 44]). When eventually the concentration of Ca^{2+} in the cell is low for a longer period, the complex will dissociate from CaMKII. However, due to the T286 phosphorylation, the kinase will remain at 20-60% of its full activity[45] (dependent on the substrate), which is referred to as the autonomous active (or calcium-independent) state. It is this property that makes CaMKII a molecular memory switch. d) Upon release of the Ca^{2+}/CaM complex, the inhibitory phosphorylation sites of CaMKII (Threonine 305 and Threonine 306 (TT305/6 of αCaMKII; TT306/7 of βCaMKII) are exposed and can therefore be phosphorylated by the catalytic domain of the neighboring subunits. In this state CaMKII is still active (although less than with Ca^{2+}/CaM still bound), *i.e.* it can still fulfill its kinase function, since T286 phosphorylation keeps the catalytic domain released from the autoregulatory domain, but can no longer bind the Ca^{2+}/CaM complex due to phosphorylation of the TT305/6 residues. This therefore prevents maximum activation of the oligomer, hence the name inhibitory phosphorylation sites. e) CaMKII in the TT305/6 autophosphorylation status cannot be activated anymore upon new influx of calcium.

After dephosphorylation CaMKII returns to its resting-state (*i.e.* binding of the catalytic domain to the regulatory domain, blocking the T286 phosphorylation site). And the whole cycle can start again.

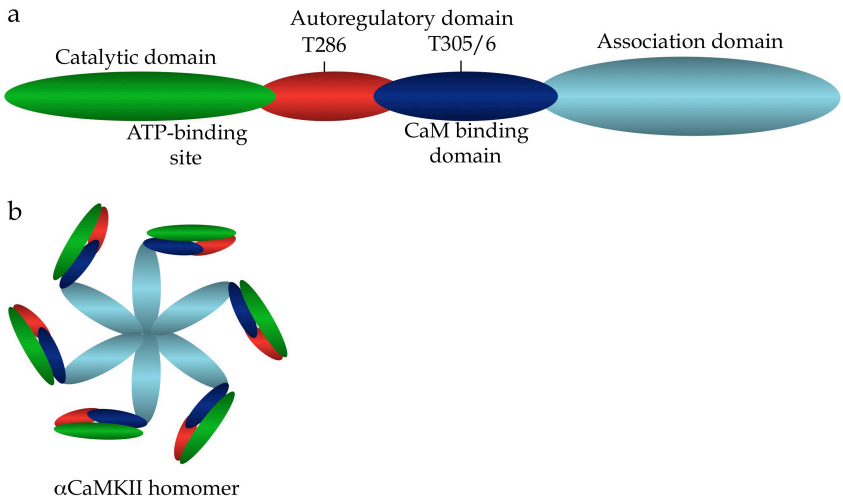


Figure 4: domain organization (a) and structure of an αCaMKII homo-oligomer (b). *The N-terminal part of the autoregulatory domain binds the catalytic domain, blocking the phosphorylation sites and the ATP-binding site.*

It has been shown that CaMKII can be directly dephosphorylated by Protein Phosphatase 1 (PP1)[46], and Protein Phosphatase 2A (PP2A), and indirectly by Protein Phosphatase 2B (PP2B or calcineurin) (as reviewed by[47]). Inhibition of PP1 activity has been shown to result in increased levels of T286 phosphorylation, therefore PP1 is suggested to "gate" the CaMKII autophosphorylation at T286[48, 49]. PP1 activity is regulated by inhibitor-1 and DARRP-32[49]. PP2B increases PP1 activity by dephosphorylating inhibitor-1 or DARRP-32[50]. PP2B is the only phosphatase that is activated via the Ca^{2+}/CaM complex, and due to its higher affinity for Ca^{2+}/CaM, PP2B is activated at lower Ca^{2+}/CaM concentrations than CaMKII[51]. PP2A and CaMKII interact directly, since CaMKII activation can reduce PP2A activity[52], and *vice versa*[53]. Also TT305/6 residues are dephosphorylated by PP1 and PP2A *in vitro*[45]. If this also occurs *in vivo* still remains elusive.

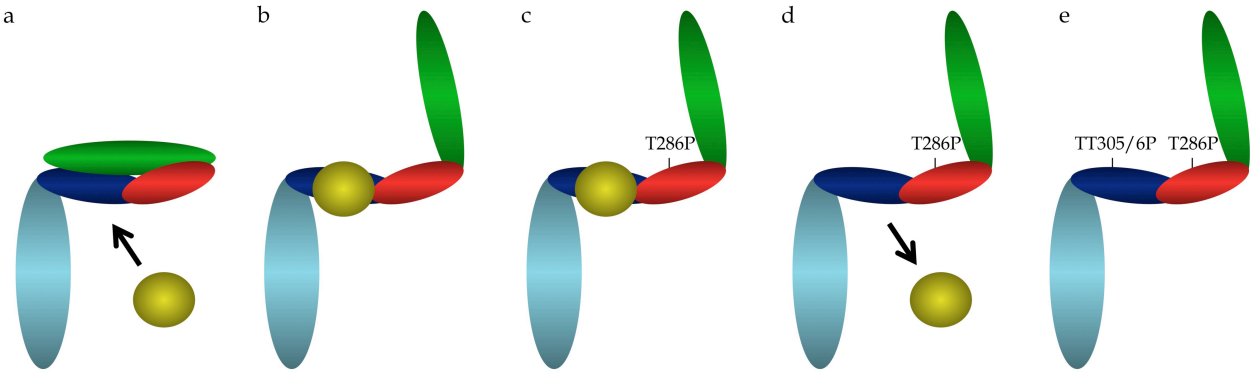


Figure 5 Schematic overview of activation and autophosphorylation of one CaMKII subunit. *(a) Ca^{2+}/CaM (yellow) binds to the CaM binding domain, resulting in release of the catalytic domain from the autoregulatory domain (b). (c) The T286 residue is now available to be phosphorylated by one of the neighboring CaMKII subunits, resulting in autonomous active CaMKII (d) When the calcium levels in the synapse are reduced again, Ca^{2+}/CaM is being released from the CaM binding site, thereby exposing the TT305/6 phosphorylation sites to be phosphorylated, again by neighboring CaMKII subunits (e). After TT305/6 phosphorylation, CaMKII can become dephosphorylated and the whole process can start again from (a).*

Using biochemical studies more phosphorylation sites have been discovered on αCaMKII *in vitro*[54-56]. Whether these sites are also phosphorylated *in vivo* is still unknown

for most of these sites, but recently evidence has been found that Threonine 253 (one of the recently discovered phosphorylation sites) is phosphorylated *in vivo* too[57]. The function of this phosphorylation site is not totally clear yet. Previously it has already been shown that phosphorylation or dephosphorylation of this site does not influence the (autonomous) kinase activity of αCaMKII and that phosphorylation at this site is Ca^{2+}-independent in *in vitro* studies[55, 56]. Recent studies show that phosphorylation at T253, like T286, also occurs during neuronal stimulation, however at a slower rate than T286 phosphorylation, and that this phosphorylation enhances the binding of αCaMKII to the PSD[57]. Phosphorylation of T253 appears to be independent of T286-phosphorylation, since a T286A or T286D mutation did not affect the levels of phosphorylated T253[57]. However, the exact physiological function of this phosphorylation site still needs to be discovered.

Although there are several phosphorylation sites of which the exact function is not known yet, the mechanism described in fig. 5 is the widely accepted mechanism underlying the regulation of CaMKII.

3. *Function of αCaMKII*

αCaMKII is the most extensively studied CaMKII isozyme, and presumably even one of the most studied kinases of the brain. Already since the late '80s scientists are trying to discover the exact functions of this protein, and the mechanisms by which it exerts these functions. Most of the studies have focused on the hippocampus, but lately also the role of αCaMKII in the Purkinje cells of the cerebellum has been unraveled.

3.1. The role of αCaMKII in postsynaptic hippocampal plasticity

αCaMKII is the most abundant protein in the postsynaptic density (PSD)[8], which is the active site of the postsynapse, and therefore is thought to play a crucial role in learning and memory. Indeed, pharmacological and genetic manipulations revealed that αCaMKII is necessary for hippocampal and cortical plasticity (LTP) and for learning and memory (as reviewed by[36]). But how is it involved in all these processes?

3.1.1. Translocation of αCaMKII to the post-synaptic density (PSD)

At the postsynaptic site there are three pools of CaMKII oligomers: an actin-bound pool, a soluble cytoplasmic pool and a PSD-bound pool. In basal state only a small fraction of the total CaMKII is located in the PSD, but this changes as soon as calcium enters the post-synaptic cell and binds calmodulin to form Ca^{2+}/CaM complexes[58]. The Ca^{2+}/CaM complex binds CaMKII, thereby inducing release of CaMKII oligomers from actin, T286-autophosphorylation of the subunits and translocation to the PSD[59], where αCaMKII can bind to the NR2B subunit of the NMDA receptor[60, 61]. Activation of the NMDA receptor is necessary for CaMKII autophosphorylation and activation[59, 62]. Moreover, T286-autophosphorylation is not necessary for the translocation of CaMKII to the PSD. Just the binding of Ca^{2+}/CaM is sufficient to achieve CaMKII translocation to the PSD and binding to the NR2B subunit. This has been shown *in vitro* using the *Camk2a*^{T286A} mutant, which cannot be phosphorylated at the T286 site, in which αCaMKII is still translocated to the PSD and can bind the NR2B-C subunit of the NMDA receptor, upon Ca^{2+}/CaM stimulation[59, 63]. However, *Camk2a*^{T286A} dissociates much faster from the PSD, that is as soon as the Ca^{2+} levels decrease. Binding of αCaMKII to the NR2B subunit increases the affinity of αCaMKII for CaM, which is similar to the "CaM trapping" by autophosphorylation at T286. It increases the autonomous activity of αCaMKII and additionally stabilizes the activity level, by making it less sensitive to ATP fluctuations in

the synapse[63, 64]. Furthermore, it suppresses the inhibitory phosphorylation at TT305/6. This means that αCaMKII activity can only be decreased by dissociation of αCaMKII from the NMDA receptor. Taken together, there are two different ways to generate increased autonomous activation of αCaMKII: 1) through autophosphorylation of the T286 site and 2) through interaction with the NMDA receptor. Therefore, decreasing autonomous activity can be achieved either by phosphatase activity or dissociation from the NMDA receptor. Binding of αCaMKII to the NMDA receptor also has an advantage for the NMDA receptor, since it traps the CaM, thereby reducing the inhibitory effect of CaM on the NMDA receptor[65].

3.1.2. AMPA receptor trafficking

What happens in the post-synaptic site of the synapse when αCaMKII is activated and LTP is induced? One of the major targets (next to the NMDA receptor) of αCaMKII is another ionotropic receptor present in the synapse: the AMPA receptor. Upon binding of glutamate, this receptor opens, resulting in Na^{2+}-influx. AMPA receptor channel conductance can be enhanced by phosphorylation of the GluR1 subunit[66, 67], which is shown to be important for synaptic plasticity[68] and certain forms of spatial memory[69]. Apart from the increased channel conductance of AMPA receptors, it is known that the total number of AMPA receptors present at the postsynaptic membrane largely determines the weight of the postsynaptic response to presynaptic glutamate release. Changes in the number of post-synaptic AMPA receptors has been shown to be of crucial importance for LTP[70, 71].

Phosphorylation of the GluR1 subunit of the AMPA receptor at Serine 831 (S831) has in some *in vitro* studies been attributed to activated αCaMKII[66, 72, 73], including in hippocampal cultures after glutamate stimulation[74]. However, more recently a study was published, in which it was found that not αCaMKII but PKC is responsible for the phosphorylation of GluR1 in hippocampal cultures and in neuronal lysates[75]. The role of αCaMKII in phosphorylation of the AMPA receptor is therefore not yet clearly determined, and more research is needed to clarify this further.

αCaMKII is known to play a crucial role in the trafficking of AMPA receptors and internalization in the postsynaptic membrane[76]. It has been shown that NMDA receptor activation induces a rapid translocation of AMPA receptors to the active site of the synapse, which is thought to be critical for expression of LTP[77]. This translocation of AMPA receptors does not require phosphorylation at S831[76]. How does αCaMKII exerts its role in AMPA trafficking? It has been shown that αCaMKII can activate the Ras – MAPK pathway by inhibiting SynGAP (a Ras-GTPase activating protein)[78] and that this pathway can mediate AMPA receptor trafficking during LTP induction[79]. Furthermore αCaMKII is shown to phosphorylate PSD-95 and stargazin[75], two crucial proteins involved in AMPA receptor trafficking.

PSD-95 is a scaffold protein, which is located in the PSD, close to the postsynaptic membrane, and is therefore thought to play a role in the localization and clustering of receptors at the postsynaptic membrane (as reviewed by[80]). PSD-95 has been shown to directly bind NR2B, and it can form complexes with the GluR1 subunit of the AMPA receptor via interactions with stargazin, an AMPA receptor-associated protein[81]. By interacting with stargazin, PSD-95 translocates AMPA receptors to the synapse[82].

The effect of phosphorylation of PSD-95 by αCaMKII is not yet fully unraveled, however it has been shown that phosphorylation of the *Drosophila* homologue of PSD-95 (Dgl) by αCaMKII results in more extra-synaptic localization of Dgl[83]. In addition, it is

known that phosphorylation of the NR2B subunit of the NMDA receptor by protein kinase CK2, induced by αCaMKII, results in dissociation of PSD-95 from the NR2B subunit[84]. Furthermore it has been shown that αCaMKII competes with PSD-95 to bind to the NR2B subunit in the synapse[85]. With these findings one could hypothesize that PSD-95 regulates the localization and surface expression of the NMDA receptor in synapses at the rest state, but when the synapse is stimulated and Ca^{+2} enters, αCaMKII could induce changes in the synapse, resulting in dissociation of PSD-95 from the NR2B subunit, thereby making PSD-95 available to form complexes with stargazin and translocate AMPA receptors to the activated synapse. However, more research is needed to support this hypothesis.

Stargazin can be phosphorylated by αCaMKII and PKC, since the application of either CaMKII inhibitors or PKC inhibitors blocks the phosphorylation of stargazin[86]. This phosphorylation of stargazin is required for LTP, whereas dephosphorylation of stargazin, by PP1 and PP2B, mediates LTD. When phosphorylated, stargazin promotes the trafficking of AMPA receptors to the synapse. On top of that, stargazin is even shown to be necessary for the delivery of AMPA receptors to the membrane surface, since the Stargazer mice, which lack stargazin[82], do not show any functional AMPA receptors in the synapse at all. After transfecting the mutant neurons with stargazin, hence re-expressing the protein, the AMPA receptors are translocated and delivered to the membrane surface.

3.1.3. Other αCaMKII substrates

Another binding partner for αCaMKII, which is also enriched in the PSD, is CASK. CASK is a member of the membrane associated guanylate kinases (MAGUKs), a class of proteins known to interact with the cytoskeleton, and to play a role in signaling pathways. The *Drosophila* homologue of CASK, camguk, binds to αCaMKII in the PSD resulting in an autonomously active αCaMKII. This induces conformational changes in αCaMKII favoring autophosphorylation at the T305/6 sites[87]. Eventually, this will result in direct autophosphorylation at these sites as soon as the calcium levels drop and CaM dissociates from αCaMKII[87]. αCaMKII subsequently dissociates from camguk, generating a pool of CaMKII insensitive to changes in calcium levels, until they have been dephosphorylated by phosphatases. This CaMKII/camguk (or CASK) interaction is hypothesized to be a mechanism involved in dowregulating the calcium-sensitive pool of CaMKII and thereby differentiating between active and inactive synapses[87].

As described above, αCaMKII can activate Ras by inhibiting SynGAP, and via this Ras – MAPK pathway, αCaMKII can mediate AMPA trafficking. But this activation of Ras could serve another purpose as well. Recently, it has been shown that αCaMKII is activated in single dendritic spines during LTP induction[88]. The activated αCaMKII does not spread to neighboring spines, most likely due to rapid activity decay after LTP induction and relatively slow spine-dendrite diffusion coupling[88]. Ras has also been shown to be activated in single spines, but spreads to neighboring spines upon activation[89]. Additionally, it has been shown that single spine activation, reduces the threshold for LTP of neighboring spines[90]. Therefore, it is hypothesized that the activation of Ras, through inhibition of SynGAP by αCaMKII, could regulate the threshold levels for neighboring spines, and thereby creating plasticity units on a dendritic branch[88, 90].

Apart from the PSD-associated targets of αCaMKII described above, many more targets have already been discovered, as has been described by[91-93] (see table 1 adapted from[93]). However, the exact effect of αCaMKII on all these substrates and their role in the induction or maintenance of LTP still remains unknown. Therefore, more research is

needed to fully understand the role of αCaMKII at the postsynaptic site, in the induction or maintenance of LTP.

Table 1

Substrates of CaMKII						
Receptors and Channel proteins	Scaffold proteins	Cytoskeletal and associated proteins	Motor proteins	Enzymes	Neuro-transmitter release	Others
NR2B	PSD-95-associated protein	Neurofilaments H, M and L	Myosin II B	CaMKII	Synapsin I	TOAD64
GluR1	PSD95	α-internexin	Myosin V	Dynamin 1	Synaptotagmin	TNFraf3
VDAC1	SAP97	α-tubulin	C protein	Cyclic nucleotide phosphodiesterase	Synaptophysin	Insulin receptor kinase
IP_3 receptor	SAP90	β-tubulin	Caldesmon	SynGAP		NeuroD[94]
N-type Ca^{2+} channel	Homer 1b	Clipin C		Calcineurin		LRP4[95]
$GABA_AR$	Densin-180	α-actinin		Phospholipase A2		
GABA-modulin	Liprinα1[96]	MAP2		Calpain		
$K_V4.2$[97]		Microtubule-associated protein Tau		Nitric oxide synthase		

Modified from Fink and Meyer, Curr Opinion Neurobiol, 2002. The proteins that contain a reference number were discovered later and added to the table by the author of this thesis. Abbreviations: VDAC1, voltage dependent anion Channel 1; IP_3, inositol triphosphate; TNFraf3, tumor necrosis factor receptor-associated factor 3; TOAD64, turned on after division 64kD; LRP4, LDL receptor-related protein 4.

3.1.4. αCaMKII and LTD

Whether αCaMKII at the postsynaptic site also plays a role in LTD is not clear. There is one study published where they showed that LTD was deficient in the *Camk2a*$^{-/-}$ mice[98], but subsequent studies showed it was mainly the lack of presynaptic αCaMKII that was responsible for the LTD deficit[99]. Phosphatases play an important role in LTD[100], with activation of PP1 via calcineurin (PP2B) at low [Ca^{2+}] being required for LTD induction[50]. It is known that PP1 dephosphorylates αCaMKII at low Ca^{2+}/CaM concentrations[46], but whether this occurs during LTD has never been shown. This indicates that the balance between phosphatase and kinase activity can be regulated by the [Ca^{2+}] concentration in the synapse, which then determines the direction of plasticity at the synapse (as proposed by Lisman[51]). When the [Ca^{2+}] in the synapse is high, more kinases (e.g. CaMKII) will become activated than phosphatases, resulting in AMPA receptor expression at the membrane surface, hence LTP will be induced. Low [Ca^{2+}] on the other hand, will result in more phosphatase activity compared to kinase activity, prompting AMPA receptor internalization and thereby inducing LTD.

Interestingly, αCaMKII has been found to influence excitability by regulating $GABA_A$-mediated inhibition under LTD inducing conditions. Activated αCaMKII induces a transient increase in $GABA_A$ receptor Cl^- current, which can be blocked by a CaMKII inhibitor[101]. This is probably achieved by phosphorylation of the $GABA_A$ receptor by αCaMKII since this is indeed shown to change the properties of the $GABA_A$ receptor[102]. Additionally, NMDA receptor activation, with a stimulus known to induce LTD, results in increased surface expression of $GABA_A$ receptors, which is also dependent on αCaMKII activation[103]. The same stimulus also results in internalization of AMPA receptors[103].

These findings suggest that αCaMKII could indirectly play a role in the induction of LTD, by regulating $GABA_A$ receptor properties and surface expression. The exact subcellular location of these processes is still not well known, but it is most likely that in the synapse the phosphatases regulate AMPA internalization, and in the dendritic shaft αCaMKII is transiently activated and regulates the $GABA_A$ receptor. However, this remains to be examined.

3.2. The role of αCaMKII in presynaptic hippocampal plasticity

CaMKII has first been discovered as the Synapsin I kinase[1], indicating that it is likely to play a role presynaptically. Indeed, *in vitro* studies have shown that presynaptic CaMKII is necessary for synaptic plasticity[104]. But what is the presynaptic role of CaMKII? It is known that αCaMKII is associated with presynaptic vesicles by binding to Synapsin I[105, 106]. Synapsin I is a vesicle-associated protein, that regulates the size of the reserve pool and of the readily releasable pool of vesicles at the presynapse[107]. Synapsin I in its non-phosphorylated state tethers vesicles to the actin cytoskeleton (*i.e.* the reserve pool)[106]. When the presynaptic terminal is activated, Synapsin I becomes phosphorylated, which results in dissociation of the vesicles from the actin cytoskeleton and translocation to the readily releasable pool[108-110]. Hence, more vesicles with neurotransmitter can be released (as shown by[111]). Synapsin I contains multiple phosphorylation sites, two of which are phosphorylated by αCaMKII[112]. Phosphorylation of Synapsin I by αCaMKII results in conformational changes in Synapsin I, reducing the binding affinity of Synapsin I for vesicles[106] and the binding affinity of Synapsin I for actin[113]. By showing that this phosphorylation of Synapsin I by αCaMKII also occurs during LTP induction[114], and by showing that αCaMKII co-localizes with synaptic vesicles after depolarization in high K^+[115], it can be suggested that interactions between αCaMKII and Synapsin play a role in plasticity.

Indeed, αCaMKII appears to be important in presynaptic plasticity *in vivo*, since *Camk2a*$^{-/-}$ mice showed decreased paired-pulse facilitation (PPF), a measure of presynaptic function[116], but showed enhanced post tetanic potentiation (PTP), indicating that αCaMKII actually has an inhibiting effect on vesicle release[117]. However, this latter result is somewhat in conflict with the findings that CaMKII activation can regulate vesicle transport from the reserve pool to the readily releasable pool via Synapsin I phosphorylation, as described above, and with the fact that injecting activated CaMKII into synaptosomes, enhances neurotransmitter release[118]. But Synapsin I contains more phosphorylation sites, and can also be phosphorylated by MAPK, PKA and cdk1 which prompt the same function: releasing vesicles from the actin cytoskeleton[119, 120]. Apart from that, injecting activated αCaMKII into synaptosomes reveals the functions αCaMKII could fulfill, but this does not necessarily have to be its physiological function in a living organism. Taken into account that αCaMKII is one of the most abundant proteins in the hippocampus is, it could very well be that αCaMKII does not only play an enzymatic role in the presynapse, but also exerts non-enzymatic functions. This hypothesis is confirmed in chapter II.

3.3. The role of αCaMKII in the cerebellum

The cerebellum is essential for motor control and fine-tuning of motor commands, and consists of the highly organized cerebellar cortex and the cerebellar nuclei (as reviewed by[121]). The Purkinje cells form the sole output of the cerebellar cortex, and are known to integrate the excitatory inputs originating from the mossy fiber and the climbing fiber

systems (as reviewed by[122]). The mossy fibers originate in the precerebellar nuclei and innervate the granule cells, which in turn give rise to the parallel fibers that innervate the dendritic spines of the Purkinje cells. The climbing fibers arise from the inferior olive. In the adult brain, each Purkinje cell dendritic tree is contacted by approximately 150.000 parallel fibers, whereas it receives input of just one single climbing fiber. Apart from the excitatory innervation, the Purkinje cells also receive inhibitory input from basket cells and stellate cells. All these excitatory and inhibitory synaptic inputs are integrated with the intrinsic pacemaking activity of Purkinje cells and are projected to the cerebellar nuclei.

The parallel fiber-Purkinje cell synapse is the most extensively studied synapse in the cerebellum. Similar to the hippocampus, the direction of plasticity at this synapse is shown to be dependent on the level of Ca^{2+} at the postsynapse[123]. However, the effect of the calcium concentration on the direction of plasticity at this synapse is opposite to the hippocampus. Repetitive parallel fiber stimulation leads to a moderate increase in Ca^{2+}-levels in Purkinje cells, which results in the expression of LTP[123]. Parallel fiber stimulation with additional repetitive stimulation of the climbing fiber results in high Ca^{2+} levels in the Purkinje cell, which induces LTD of the Parallel fiber input[123]. The molecular mechanisms underlying the induction of LTD have been extensively studied (as reviewed by[124]), showing that the mGluR1/PKC pathway plays a key role in the induction of LTD[125, 126]. Surprisingly, despite the fact that cerebellar αCaMKII is expressed in Purkinje cells[127], that the Ca^{2+} levels critically determine the direction of plasticity[128] and that αCaMKII plays an important role in hippocampal plasticity, the role of αCaMKII in parallel fiber-Purkinje cell plasticity was never properly assessed. This is surprising, especially since it has been shown that CaMKII inhibitors can potentiate glutamate response at the Purkinje cell synapse[129], indicating that CaMKII is also likely to play a role in cerebellar plasticity. The first full study on the direct involvement of αCaMKII in cerebellar plasticity and learning showed that CaMKII indeed plays an essential role in cerebellar LTD[130]; *Camk2a*$^{-/-}$ mice do not show LTD when measured at a young age and, surprisingly, even show LTP when LTD is induced at an older age. Additionally, the application of a CaMKII inhibitor during a LTD induction protocol also results in the expression of LTP. No differences were found in LTP at any age. Plasticity at the parallel fiber-Purkinje cell synapse, is proposed to be necessary for cerebellar motor learning (as reviewed by[131]). One of the most commonly used tasks to test this hypothesis in mice is to measure adaptation of compensatory eye movements during visuo-vestibular training. The baseline performance in this motor task was normal in *Camk2a*$^{-/-}$ mice, but the mutants showed impaired learning in a gain-increase training paradigm, indicating that αCaMKII indeed plays a role in cerebellar motor learning[130].

These data show that αCaMKII is essential for LTD and not for LTP in the parallel fiber-Purkinje cell synapse, thus that αCaMKII is essential during conditions where the calcium level in the cell is high. In the hippocampus αCaMKII is also activated when the calcium levels are high, resulting in LTP (as described previously). Thus, the final response induced by αCaMKII activity in Purkinje cells compared to hippocampal pyramidal cells is completely opposite, which is quite surprising, since both types of neurons require the insertion or internalization of AMPA receptors for the induction of LTP or LTD respectively. In pyramidal cells, high calcium levels result in AMPA receptor insertion in the synaptic membrane (as reviewed by[132]), whereas in Purkinje cells, high calcium levels result in AMPA receptor internalization[133]. Both require αCaMKII, therefore

it would be very interesting to unravel the exact mechanisms underlying αCaMKII-dependent plasticity in both cell types.

4. *Function of βCaMKII*

βCaMKII is the second CaMKII isoform that is highly expressed in the brain. In contrast to αCaMKII, it is expressed throughout the mammalian brain. There are a number of important differences between αCaMKII and βCaMKII that need to be mentioned here.

First, the ratio between αCaMKII and βCaMKII subunits in hetero-oligomers differ between forebrain and cerebellum, with α/β ratios of 3:1 versus 1:4[22, 23] respectively. This difference in subunit ratio is mainly caused by the lack of αCaMKII subunits in cerebellar granule cells[127, 130]. The physiological relevance of the difference in subunit composition between cerebellum and forebrain is still unknown, but it is likely to be of great importance since αCaMKII and βCaMKII are not only differentially expressed in a spatial manner (*i.e.* with respect to cell-type), but also in a temporal manner: βCaMKII expression starts at E12.5 and reaches its maximum level at P3, whereas αCaMKII expression starts at P5 and reaches maximum levels in mature mice[18, 134]. Furthermore, the expression of the subunits is also regulated by neuronal activity: increased activity results in high α/β ratio's and vice versa[135].

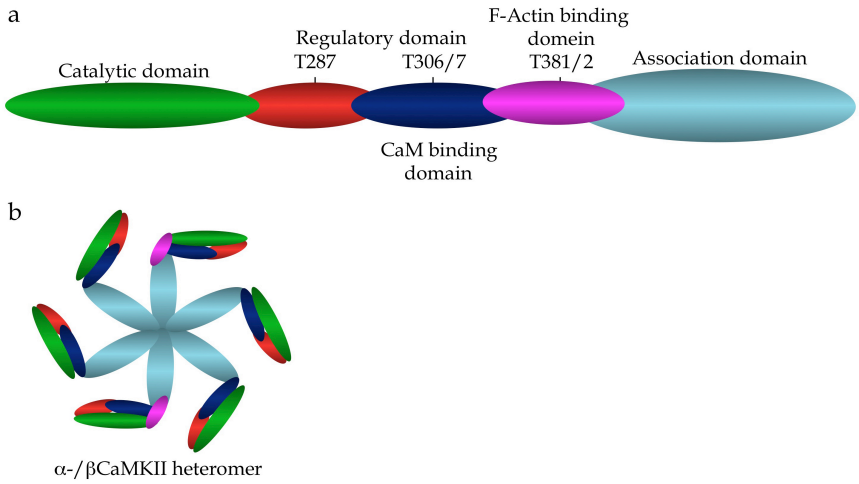


Figure 6: Domain organization of βCaMKII and structure of an α-/βCaMKII heteromer. *(a) Unlike αCaMKII, βCaMKII has a F-actin binding domain, containing two extra phosphorylation sites, of which the function is unknown. (b) Two βCaMKII subunits are enough to tether the α-/βCaMKII heteromer to actin.*

Second, βCaMKII has a higher affinity for Ca^{2+}/CaM[22]. Half-maximal autophosphorylation levels are achieved at 130 nM calmodulin for αCaMKII and at 15 nM calmodulin for βCaMKII. Accordingly, the sensitivity range of the heteromeric holoenzyme is dependent on the ratio of α to β subunits[22, 136].

Third, βCaMKII has an extra domain in the variable region[137, 138] (fig 6). This domain allows βCaMKII to bind to F-actin in an activity-controlled manner[31, 59]. It has been shown that only two βCaMKII subunits per holoenzyme are sufficient to change the localization of the entire holoenzyme[31]. Mainly due to these different actin-binding properties αCaMKII and βCaMKII have opposing effects on synaptic strength[135], and on the degree of dendritic arborization[34]. Additional evidence that the actin binding domain in the variable region in βCaMKII probably plays a critical role in synaptic function, comes from the observation that exons in this domain are controlled at the transcriptional level by alternative splicing[13, 35]. Indeed, the presence of just one single exon in the variable region determines the interaction with F-actin[35] (fig 7).

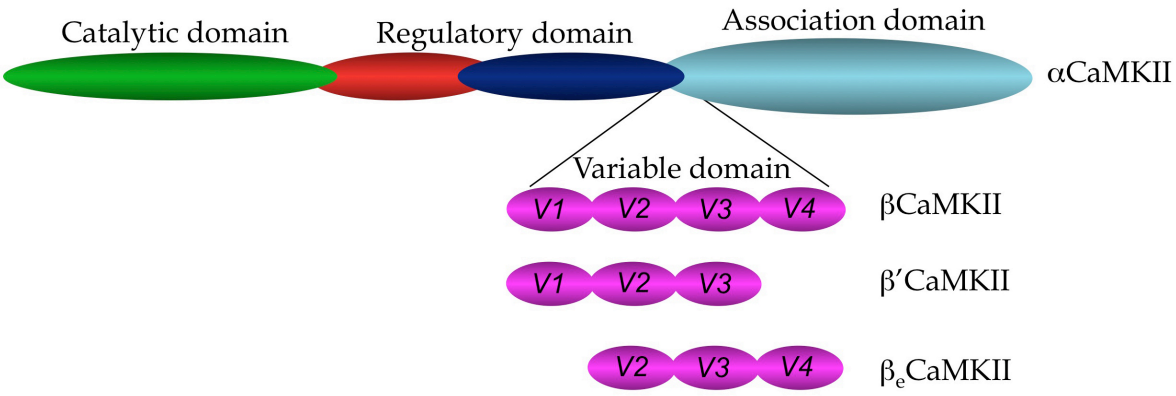


Figure 7: Splice variants of βCaMKII. *Only βCaMKII and β'CaMKII can bind to F-actin, indicating that alternative splicing of exon V1 regulates the F-actin binding properties of βCaMKII. V1-4 indicate exons in the variable domain. Adjusted from O'leary et al., Mol. Biol Cell, 2006.*

β_eCaMKII is an isoform that is highly expressed during embryonic development, but the expression declines after birth and is absent from P4[28]. This specific isoform does not have the F-actin binding domain, which makes it highly similar to αCaMKII. As αCaMKII is not expressed during embryonic development, it seems likely that β_eCaMKII functions as a replacement of αCaMKII.

At the posttranslational level the actin binding domain is likely to be regulated by phosphorylation at Thr382[137]. However, the precise function of this phosphorylation site is currently unknown.

4.1. The role of βCaMKII in hippocampal plasticity

In contrast to αCaMKII, there were no *Camk2b* mouse mutants generated until recently, therefore little is known on the function of βCaMKII in the hippocampus *in vivo*. However, many studies have been published on the role of βCaMKII in hippocampal neurons *in vitro*. This section will give a short overview of what is known about βCaMKII *in vitro*.

Overexpression of βCaMKII or reduction of βCaMKII levels has been shown to have several effects on hippocampal neurons. First, it is shown that in developing hippocampal neurons, overexpression of βCaMKII enhances dendritic arborization and increases the number of synapses, whereas knock-down of *Camk2b* by siRNA results in exactly the opposite phenotype[32, 34], indicating that βCaMKII plays an important role during development. Second, it is hypothesized that βCaMKII plays a role in synaptic homeostasis. As βCaMKII has a higher affinity for CaM compared to αCaMKII[22], it is able to detect low levels of calcium influx. When the calcium influx increases, the levels of βCaMKII in the spine decrease[135], thereby changing the sensitivity of the α-/βCaMKII holoenzyme for calcium. Additionally, the decrease in βCaMKII levels also reduces the number of synapses on a dendrite[34], thereby preventing overactivation of the neuron. However, these latter findings are not supported in other studies, in which it is shown that LTP induction results in increased CaMKII activity together with synaptogenesis[139].

Manipulating *Camk2b* affects mostly the localization and function of αCaMKII and the bundling and organization of actin. βCaMKII has been suggested to play a structural role, since it tethers the holoenzyme to the actin cytoskeleton, thereby regulating the subcellular localization of αCaMKII[31, 140]. Indeed, it has been shown that when there is no βCaMKII

present, αCaMKII is uniformly expressed throughout the neuron[31, 141]. Upon calcium influx (*e.g.* by glutamate stimulation), Ca^{2+}/CaM binds to βCaMKII and βCaMKII dissociates from actin, thereby releasing the heteromeric holoenzyme from the actin cytoskeleton and allowing it to translocate to the PSD[59].

Binding of βCaMKII to actin has implications for actin polymerization and for the structure of the actin cytoskeleton. βCaMKII has been shown to bind and bundle the F-actin filaments[35], but it also binds the G-actin monomers, thereby inhibiting actin polymerization[142]. Hence, βCaMKII is not only able to stabilize actin, but also to regulate the density of the F-actin bundles.

Until recently only one study was published on the role of βCaMKII *in vivo*, in which it was shown that overexpression of βCaMKII in the dentate gyrus of the hippocampus, results in decreased LTP in the perforant path, and impaired consolidation of long-term memory in the contextual fear-conditioning task[143]. However, the exact mechanisms underlying these phenotypes were not deciphered.

In chapter IV of this thesis we assessed the role of βCaMKII in the hippocampus, using a *Camk2b*$^{-/-}$ mouse.

5. *Overview of Camk2a and Camk2b mutants*

Using mutant mice as a tool to study the role of specific proteins in the mechanisms underlying learning and memory has become a complete subfield within neuroscience. To study the exact roles of αCaMKII and βCaMKII approximately 15 different mutants have been generated over time, starting with the *Camk2a*$^{-/-}$[144, 145]. Table 2 and 3 give an overview of the mouse mutants currently available to study the role of αCaMKII and/or βCaMKII, with their molecular, morphological, plasticity and behavioral phenotypes in hippocampus/cortex an cerebellum respectively (adjusted from[36]).

Table 2.

Overview of *Camk2* mutants and their hippocampal or cortical phenotypes

Mutant#	**Purpose**	**Molecular and morphological characterization##**	**Plasticity##**	**Behavioral Phenotype**
Camk2a-null (-/-)[144-146]	Elimination of αCaMKII protein	* Expression level of βCaMKII is unaltered[144-146] but targeting of βCaMKII to PSD is increased[146] * Ca^{2+} (in)dependent activity 60% reduced in forebrain.	* HC-LTP reduced[144, 146, 147] * NC-LTP reduced in adult animals[148-150] * NC-LTP normal in young animals[148, 149] * Decreased LTD[98] * Decreased PPF[144] * Normal I/O[146] * Normal PTP[98] * HC VGCC-dependent LTP at 200Hz decreased (Borgesius et al., unpublished)	* Hippocampus dependent learning severely impaired but learning in some animals is observed after extended training[145, 146, 150] * increased seizure susceptibility[151] (background dependent?[146])
Camk2a-null (+/-)[144, 146]	Reduction of αCaMKII protein	* αCaMKII 50% reduced, βCaMKII levels normal * Ca^{2+} (in)dependent activity 30% reduced in forebrain [152] * Immature dentate gyrus[153]	* HC-LTP normal [146, 154, 155] * NC-LTP reduced[155] * Decreased PPF[154] * Increased augmentation[154]	* Normal learning[146] or reduced hippocampus dependent learning[154] (background and protocol dependent) * Severely impaired remote memory[156] * Decreased fear and increased aggressive behavior[157]
Camk2a-Floxed + CA3-cre[117]	Elimination of αCaMKII protein in CA3 area	αCaMKII is absent from CA3 area after 4 months of age	* Increased frequency facilitation * normal PPF and basal P_r	* Not tested

Camk2a^{T286A} [158]	Blocks T286 phosphorylation (no autonomous activity)	* Normal expression level of α/βCaMKII. Ca^{2+} independent activity reduced by 60% * Increased phosphorylation of βCaMKII in dentate-gyrus after LTP induction[159]	* HC-LTP and NC-LTP impaired[158, 160-162] * Normal I/O[158] * Normal NMDA receptor-dependent LTP at the MMP[159] * Impaired Schaffer commissural-CA1 LTP[159] * Increased excitability of CA1 pyramidal neurons[163]	* Severely impaired learning in watermaze[158, 164] * Unstable place cells[165]
Tg-*Camk2a*^{T286D} [166]	Mimics constitutive T286 phosphorylation	Ca^{2+} independent activity 2 fold increased	* Favors LTD[166] * Normal NC-LTP[167] * Normal PPF[166]	* Impaired learning in Barnes maze[168] * Impaired olfactory based spatial learning[169] * Normal cued and contextual conditioning[168] * Unstable place cells[170]
Tg-*Camk2a*^{T286D} [171] (Tet inducible)	Mimics constitutive T286 phosphorylation in an inducible fashion	Ca^{2+} independent activity 6 fold increased at max. induction	* Favors LTP at low expression level[172] * Favors LTD at high expression level[171, 172] * NC-LTP and LTD normal[167]	* Impaired learning in Barnes maze[171] * Impaired learning in watermaze[172] * Impaired cued and contextual conditioning[171, 172]
Tg-*Camk2a*^{T286D} EC [173] (Tet inducible)	Mimics constitutive T286 phosphorylation in the medial enthorinal cortex in an inducible fashion	Not tested	Not tested	* Impaired learning in watermaze * Impaired recognition memory in a visual paired comparison task * Normal cued and contextual fear memory * Normal anxiety-related responses and locomotion in open field and light/dark transition test * Disruption of previously established memory in watermaze
Camk2a$^{TT305/6VA}$ [146]	Blocks T305/T306 (inhibitory) phosphorylation	Increased levels of CaMKII in PSD	* Favors LTP * Normal LTD and I/O * Reduced metaplasticity in the lateral perforant path[174]	* Impaired watermaze learning (initial learning OK) * Impaired reversal learning * Impaired context discrimination * Old mice get seizures
Camk2a^{T305D} [146]	Mimics constitutive T305 (inhibitory) phosphorylation	* Severely reduced levels of α/βCaMKII in PSD * Reduced NR2A subunits in the synapse and cytoplasm of the spine[175]	* Favors LTD * No LTP * Normal LTD and I/O	* Severely impaired in watermaze and cued conditioning. (Can not learn after overtraining)
Camk2a$^{A3'UTR}$ [176]	Impairs dendritic targeting of mRNA	Reduced amount of PSD associated CaMKII	* Normal (early) LTP * Reduced late-phase LTP	* Impaired learning in watermaze * Normal STM but impaired LTM after cued and contextual conditioning
Tg-*Camk2a*^{F89G} [177]	Inducible system allowing rapid (8 min) reversible decrease of αCaMKII transgene activity	Ca^{2+} (in)dependent activity 2-3 fold increased without inhibitor	* Normal I/O and PPF * Enhanced LTD at 3Hz * Enhanced LTP at > 10Hz * Enhanced LTP at 100Hz when CaMKII is overexpressed during the first 10 min after tetanization[178]	* Impaired memory consolidation of contextual and cued conditioning when CaMKII activity is changed at the first week after training. * impaired memory retrieval of object recognition and fear memory when CaMKII is overexpressed at the time of recall, restricted to the memory being retrieved[179] *impaired STM when CaMKII is overexpressed within the first 10 min after training[178]
Camk2a^{K42R} [152]	Blocks kinase activity	* 20% decrease in levels of αCaMKII in forebrain * 40% decrease in levels of phospho-T286 in forebrain * Ca^{2+} (in)dependent activity 60-70% decreased * Faster activity-dependent postsynaptic translocation	* Normal I/O, PPF and PTP * Reduced NMDA receptor-dependent LTP at 100Hz	* Severely impaired one-trial inhibitory avoidance learning

Tg-*Camk2b*^{F90G} [143]	Inducible system allowing rapid reversible decrease of βCaMKII transgene activity in dentate gyrus	Ca^{2+} (in)dependent activity 2-3 fold increased without inhibitor	* Normal I/O and PPF * Normal LTP at the Schaffer collateral pathway * Reduced LTP at the perforant path	* Impaired LTM consolidation of contextual conditioning when βCaMKII is overexpressed the initial day after training
Camk2b-null (-/-) (Borgesius et al., unpublished)	Elimination of the βCaMKII protein	* Normal αCaMKII expression * Normal T286 phosphorylation in total HC lysates * No gross morphological pathogenesis	* Normal I/O, increased PPF * Decreased LTP at 100Hz and 10Hz * Normal LTD at 1Hz * Normal VGCC-dependent LTP at 200Hz	* impaired learning in fear conditioning * impaired motor performance and coordination[180]
Camk2b^{A303R} (Borgesius et al., unpublished)	Blocks the Calmodulin binding site	* Normal α/βCaMKII expression * Normal T286 phosphorylation, absent T287 phosphorylation in total HC lysates * No gross morphological pathogenesis	* Normal I/O and PPF * Normal LTP at 100Hz and 10Hz * Normal LTD at 1Hz	* normal fear conditioning learning * impaired motor performance and coordination
Ube3A$^{m-/p+}$ [181, 182]	Encodes E6-AP ubiquitin ligase. Mouse model for Angelman syndrome.	* Increased T286-P and T305-P[183] * Decreased αCaMKII in PSD[183] * Decreased PP1/PP2a activity[183]	* Impaired LTP[181] * Normal I/O[181]	* Impaired spatial learning[181, 182] * Impaired motor coordination[181, 182] * Inducible seizures[181, 182]

 # References given in this column refer to primary papers describing the generation and analysis of the mutant.
Molecular characterization and plasticity refers to hippocampus unless otherwise specified
Abbreviations: NC= neocortex; I/O= input/output (a parameter used to assess synaptic transmission); HC=hippocampus, MMP= Medial perforant path; LTM= long-term memory (24 hours); STM= short-term memory (1 hour); PSD= postsynaptic density; PPF = paired pulse facilitation; PTP=post-tetanic potentiation; P_r = probability of release; STM= short-term memory (30 minutes); Tg= transgenic; VGCC= voltage-gated calcium channel.

Table 3.

Overview of Camk2 mutants and their cerebellar phenotypes

Mutant#	Purpose	Molecular and morphological characterization##	Plasticity##	Behavioral Phenotype
Camk2a-null (-/-)[130]	Elimination of αCaMKII protein	* Normal Purkinje cell morphology and maturation * Delayed climbing fiber elimination * No up-regulation of βCaMKII	* Normal PPF * Normal basal synaptic transmission * Impaired LTD at young age * LTP after LTD induction at adult age * Normal LTP at all ages	* Impaired cerebellar learning in visuo-vestibular training * Normal saccade performance
Camk2b-null (-/-)[180]	Elimination of the βCaMKII protein	* Normal Purkinje cell morphology and maturation * Normal climbing fiber elimination * No up-regulation of αCaMKII * Ca^{2+} (in)dependent activity 50% decreased	* Normal PPF and PPD * Normal basal properties of Purkinje cells * Normal synaptic transmission at the climbing fiber-Purkinje cell synapse * Reversed bidirectional plasticity at the parallel fiber-Purkinje cell synapse	* Severe ataxia * Impaired motor performance and coordination on rotarod and balance beam * Normal motivation to move in open field and phenotyper

 # References given in this column refer to primary papers describing the cerebellar analysis of the mutants
Molecular characterization and plasticity refers to cerebellum
Abbreviations: PPF = paired pulse facilitation; PPD= paired pulse depression (specific for the climbing fiber-Purkinje cell synapse)

6. *CaMKII and Disease*

Given its important role in plasticity it is not surprising that CaMKII is found to be, one way or another, involved in neurological and psychiatric disorders. These include Alzheimer's disease, epilepsy, schizophrenia and Angelman syndrome. This section will discuss what is known so far about the role of CaMKII in these diseases.

6.1. CaMKII and Alzheimer's disease

Alzheimer's disease is the most common form of dementia and one of the most costly diseases in western society. It is a neurodegenerative disease characterized by senile plaques, neurofibrillary tangles, neurotransmitter deficiencies and neuronal loss, which result in memory loss in patients. Unfortunately, the exact pathogenesis underlying Alzheimer's disease is still not completely clarified. There are two biochemical processes that characterize the disease: 1) abnormal elevation of Aβ peptide and 2) abnormal hyperphosphorylation of the tau protein. Eventually these processes lead to synaptic loss and cell death (as discussed by[184]). Studies in rats have shown that elevation of the Aβ levels cause impairment of LTP[185]. Additionally, it has been shown that increased levels of Aβ result in decreased expression of AMPA receptors at the synapse[186]. CaMKII is one of the key proteins involved in AMPA receptor translocation, and recently it has been shown that increasing levels of Aβ does not change the activity of CaMKII, but selectively changes the subcellular localization of CaMKII[187]. The amount of CaMKII located at synapses was decreased, whereas no difference in total amount of CaMKII was found. Moreover, this study showed that this mislocalization of CaMKII was the main cause for reduced expression of AMPA receptors at the membrane[187], indicating that CaMKII is likely to play a role in the memory loss seen in Alzheimer patients. These molecular processes are thought to occur already before neurodegeneration starts. At this time point, no changes are observed in NMDA receptor properties. However at later stages, as seen in brains of deceased Alzheimer patients, there is a reduction in NMDA receptor subunits[188, 189], together with a reduction of CaMKII autophosphorylation at T286, in both frontal cortex and hippocampus[188], indicating that also the NMDA receptor is involved, but how exactly is still unknown. Thus, mislocalization of CaMKII could underlie the early memory loss seen in patients with Alzheimer's disease, by reducing the number of AMPA receptors at the membrane, and at a later stage by losing its enzymatic properties. The exact mechanisms by which the increased levels of Aβ cause a different distribution of CaMKII, or how CaMKII loses its enzymatic properties still remain elusive and require more research.

CaMKII has also been shown to phosphorylate tau protein[190], however, whether this also contributes to the disease remains elusive.

6.2. CaMKII and epilepsy

Epilepsy is a chronic neurological disease, characterized by spontaneous recurrent seizures, which result from excessive or synchronous discharges of a population of neurons[191]. The process responsible for the transformation of a population of normal neurons into neurons that show this neurophysiological abnormal behavior is called epileptogenesis. The molecular and cellular mechanisms underlying epileptogenesis are still not fully understood, but in the last few years more and more evidence became available showing that CaMKII is involved in this process. The first evidence for the involvement of CaMKII in seizures came from kindling experiments. Kindling is a model for epilepsy, in which repeated stimulation of certain brain regions results in a progressive build-up of seizure activity and could eventually result in spontaneous epileptic seizures[192]. It has been shown that kindling results in reduction of αCaMKII activity and expression levels (as reviewed by[193]). Subsequent studies showed that direct induction of seizures in neuronal cultures, also result in decreased autophosphorylation of both αCaMKII and βCaMKII[194]. This down-regulation of CaMKII activity and expression level, is shown to be the result of changes in intracellular calcium levels, induced by prolonged

NMDA receptor activity[195]. Additionally, it has also been shown *vice versa*, that downregulation of αCaMKII, as in the *Camk2a*$^{-/-}$ null mutant, results in increased neuronal excitability and seizures[151], and that the percentage of αCaMKII downregulation correlates with the increase in neuronal excitability[196]. However, not only reducing the amount of CaMKII, but also manipulating its activity level is already enough to induce seizures, as *Camk2a*$^{TT305/6VA}$ homozygous mutant mice are shown to have seizures[146]. The most direct evidence for CaMKII playing a significant role in epilepsy comes from the mouse model for Angelman Syndrome (AS). Both AS mice and patients suffer from severe epilepsy. In AS mice, CaMKII activity is found to be decreased, due to increased CaMKII TT305/6 phosphorylation[183]. When these mice are crossed with mice that cannot be phosphorylated anymore on the TT305/6 residues (*Camk2a*$^{TT305/6VA}$), thereby reducing the amount of inhibitory phosphorylated CaMKII molecules in AS mice, the epileptic phenotype is largely rescued, showing a direct involvement of CaMKII in epileptogenesis[197].

But what mechanism can explain the link between decreased CaMKII activity and enhanced excitability of neurons? It has been shown that during the initial phase of seizure activity, CaMKII translocates to the synapses, and the expression level of extra-synaptic CaMKII decreases[198]. This is in correspondence with the finding that there is a large increase in intracellular calcium levels. After prolonged elevated levels of calcium, CaMKII is likely to prevent excessive activation, by TT305/6 phosphorylation[199], thereby reducing its activity. αCaMKII is known to be able to positively regulate $GABA_A$ receptor function[102]. With both reduced expression and reduced activation of αCaMKII during epileptogenesis, this effect on $GABA_A$ receptors is gone; inhibition of neurons is reduced, which could lead to over-excitation and eventually epileptic activity. On the other hand, the same should also account for AMPA receptor insertion, which is also regulated by αCaMKII. In conclusion, more research is needed to fully understand the exact mechanism underlying epilepsy and the role of αCaMKII in enhanced neuronal excitability.

6.3. CaMKII and schizophrenia

Schizophrenia is a chronic, severe mental disorder. The three main characteristics of the disorder are (1) positive symptoms, *i.e.* hallucinations and delusions; (2) negative symptoms, *i.e.* apathy and poverty of speech; (3) cognitive dysfunction, *i.e.* impaired working memory and conceptual disorganization. More and more evidence becomes available confirming the existence of both genetic and environmental factors in the etiology of schizophrenia (as reviewed by[200]). So far, different research groups identified several genes, which carry the title 'schizophrenia genes'[201]. However, how these genes are involved in the disease and how they all contribute exactly to the characteristics of the disease is still hypothetical.

Even though CaMKII has never been shown to be involved in the etiology of the disease, there is evidence that CaMKII does play a role in the disorder. Using postmortem frontal cerebral cortex tissues from schizophrenic patients, it was found that *Camk2b* mRNA levels are elevated compared to those in healthy subjects in this brain region[202, 203]. This was an interesting finding, since expression of βCaMKII in the brain starts already during development, indicating that it is likely to play a role in brain development[18]. Overactivation of CaMKII in young neurons has been shown to slow down dendritic growth[31]. Therefore it could be hypothesized that βCaMKII expression is already elevated during development, causing abnormal neurodevelopment and subsequent predisposition to schizophrenia. However, this remains to be investigated.

Since there are many genes involved in schizophrenia, it is difficult to make a good animal model to study the mechanisms underlying the disease. Nevertheless, withdrawal from repeated phencyclidine (PCP) treatment (a noncompetitive NMDA receptor antagonist) has been found to result in schizophrenia-like symptoms in humans[204] and animals[205]. Using these animal models, it has been shown recently that the NMDA-CaMKII signaling pathway in the prefrontal cortex is disturbed after PCP treatment[206] (*i.e.* learning induced phosphorylation of CaMKII is absent in PCP treated mice), and that this is associated with dopaminergic hypofunction[207]. Whether it is the αCaMKII or the βCaMKII isoform that is affected is not specified in these studies. However, αCaMKII has also been suggested to play a role in schizophrenia, since *Camk2a*$^{+/-}$ mice show schizophrenia-like behavioral phenotypes, and show abnormal neurogenesis in the dentate gyrus, a brain region involved in working memory[153].

Taken together, even though CaMKII is not one of the 'schizophrenia genes', several studies now have provided evidence for a significant role for CaMKII in the disease. However, more research is needed to fully understand the exact involvement of CaMKII in schizophrenia.

6.4. CaMKII and Angelman Syndrome

Angelman Syndrome (AS) is a severe genetic neurological disorder, first described by Harry Angelman in 1965[208]. He wrote a report on three children, which he called 'Puppet Children', with the same clinical features: severe mental retardation and epilepsy, ataxia, excessive laughter, absence of speech and dysmorphic facial features[208].

Angelman Syndrome is a classical example of genetic imprinting disorders. It is caused by loss of function of the maternal *UBE3A* gene located on chromosome 15q11-13, which encodes the ubiquitin ligase E6AP[209, 210]. It is shown that in the brain there is a specific imprinting pattern of the *UBE3A* gene[211, 212], with only the maternal copy being expressed. The paternal allele shows expression of an antisense *UBE3A* transcript in the brain[213](fig 10).

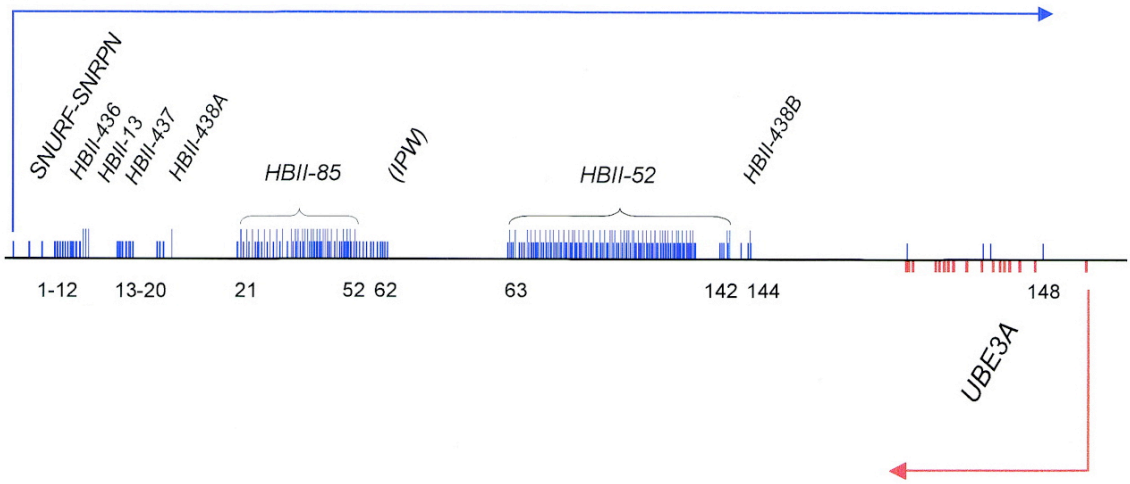


Figure 10. Detailed map of the SNURF-SNRPN/UBE3A region on chromosome 15q11-13. *The paternally expressed SNURF-SNRPN/UBE3A antisense transcript with exons is indicated with the blue horizontal and vertical lines. The maternally expressed UBE3A sense transcript with exons is indicated with the red horizontal and vertical lines. (Adapted from Runte et al., Hum Mol Genet, 2001)*

Imprinting defects account for only 3-5% of the AS patients, so what genetic defects do the other AS patient show then? Most of the Angelman Syndrome patients have large *de*

novo deletions of the 15q11-13, which are always of maternal origin. Approximately 5-10% of the AS patients have maternally inherited point mutations in the *UBE3A* gene, and 1-2% of the patients have paternal uniparental disomy (UPD) of chromosome 15 (as reviewed by[214]). However, even though the *UBE3A* gene was identified as the 'Angelman Syndrome gene' already 16 years ago, little progress has been made providing insight in the role of E6AP in neuronal function. E6AP is an E3 ubiquitin ligase, involved in the degradation of certain proteins. Over the years several targets have been found for E6AP (as shown in table 5), but the critical target(s) explaining the characteristics of the disease is (are) still unknown.

Table 5

Targets of E6AP	***Function of the protein***
p53[215]	Tumor suppressor protein
HHR23A[216]	DNA repair protein
Mcm 7 substrate[217]	Involved in initiation of DNA replication
E6AP[218]	Protein degradation
Blk[219]	Tyrosine kinase
PTPN3[220]	Tyrosine phosphatase
PML[221]	Tumor suppressor protein
Trihydrophobin 1[222]	Part of the negative transcription elongation factor complex
Pbl/ECT2[223]	Rho guanine nucleotide exchange factor (Rho-GEF)
AIB1[224]	Steroid receptor coactivator
p27[225]	Cyclin-dependent kinase inhibitor
TSC2[226]	Tumor suppressor protein
Annexin A1[227]	Ca^{2+}- and phospholipid-binding protein

Targets of E6AP.

When the *UBE3A* gene was discovered as the AS gene, a mouse model was generated to study the mechanisms underlying the syndrome[181]. These *Ube3a*$^{m-/p+}$ mice (in which the maternal copy of the gene is deleted), showed motor deficits, impaired learning and memory and epilepsy[181], as seen in patients. Later studies found that CaMKII activity was reduced in these mice, and that this was due to increased phosphorylation at the TT305/6 autophosphorylation sites on CaMKII[183]. Since CaMKII is known to play a pivotal role in neuronal function and since the phenotype of the CaMKII^{T305D} mice resembles the phenotype seen in the *Ube3a*$^{m-/p+}$ mice (AS mice)[146], it was hypothesized that inhibition of CaMKII is responsible for the phenotypes seen in AS mice. In chapter V of this thesis we tested this hypothesis.

7. Scope of this thesis

The general scope of this thesis is to further elucidate the role of αCaMKII and βCaMKII in neurological processes in different regions of the brain. Chapter II focuses on the role of αCaMKII in presynaptic hippocampal plasticity, showing that αCaMKII plays a structural, instead of an enzymatic role at the presynaptic site. Chapter III and IV focus on the function of βCaMKII in cerebellar plasticity (chapter III) and in hippocampal plasticity (chapter IV). There it is shown that in the cerebellum βCaMKII is responsible for the direction of plasticity at the parallel fiber – Purkinje cell synapse, both enzymatically and structurally, whereas in the hippocampus βCaMKII is more likely to play a structural role in plasticity. Finally, chapter V focuses on the role of αCaMKII in Angelman Syndrome, showing that reducing the amount of auto-inhibited CaMKII in AS mice rescues the neurological problems seen in AS mice.

References

1. Bennett, M.K., Erondu, N.E. & Kennedy, M.B. Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. *J Biol Chem* **258**, 12735-12744 (1983).
2. Erondu, N.E. & Kennedy, M.B. Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain. *J Neurosci* **5**, 3270-3277 (1985).
3. Kennedy, M.B. & Greengard, P. Two calcium/calmodulin-dependent protein kinases, which are highly concentrated in brain, phosphorylate protein I at distinct sites. *Proc Natl Acad Sci U S A* **78**, 1293-1297 (1981).
4. Kennedy, M.B., McGuinness, T. & Greengard, P. A calcium/calmodulin-dependent protein kinase from mammalian brain that phosphorylates Synapsin I: partial purification and characterization. *J Neurosci* **3**, 818-831 (1983).
5. Sieghart, W., Schulman, H. & Greengard, P. Neuronal localization of Ca2+-dependent protein phosphorylation in brain. *J Neurochem* **34**, 548-553 (1980).
6. Yamauchi, T. & Fujisawa, H. A calmodulin-dependent protein kinase that is involved in the activation of tryptophan 5-monooxygenase is specifically distributed in brain tissues. *FEBS Lett* **129**, 117-119 (1981).
7. Carlin, R.K., Grab, D.J. & Siekevitz, P. Function of a calmodulin in postsynaptic densities. III. Calmodulin-binding proteins of the postsynaptic density. *J Cell Biol* **89**, 449-455 (1981).
8. Kennedy, M.B., Bennett, M.K. & Erondu, N.E. Biochemical and immunochemical evidence that the "major postsynaptic density protein" is a subunit of a calmodulin-dependent protein kinase. *Proc Natl Acad Sci U S A* **80**, 7357-7361 (1983).
9. Kelly, P.T., McGuinness, T.L. & Greengard, P. Evidence that the major postsynaptic density protein is a component of a Ca2+/calmodulin-dependent protein kinase. *Proc Natl Acad Sci U S A* **81**, 945-949 (1984).
10. Yamauchi, T. & Fujisawa, H. Purification and characterization of the brain calmodulin-dependent protein kinase (kinase II), which is involved in the activation of tryptophan 5-monooxygenase. *Eur J Biochem* **132**, 15-21 (1983).
11. Tobimatsu, T., Kameshita, I. & Fujisawa, H. Molecular cloning of the cDNA encoding the third polypeptide (gamma) of brain calmodulin-dependent protein kinase II. *J Biol Chem* **263**, 16082-16086 (1988).
12. Tobimatsu, T. & Fujisawa, H. Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. *J Biol Chem* **264**, 17907-17912 (1989).
13. Hudmon, A. & Schulman, H. Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. *Biochem J* **364**, 593-611 (2002).
14. Tombes, R.M., Faison, M.O. & Turbeville, J.M. Organization and evolution of multifunctional Ca(2+)/CaM-dependent protein kinase genes. *Gene* **322**, 17-31 (2003).
15. Takamatsu, Y., Ohsako, S., Nishida, Y. & Yamauchi, T. Identification of an alternative form of the Drosophila Ca2+/calmodulin-dependent protein kinase II that is maternally derived. *Biochim Biophys Acta* **1220**, 188-192 (1994).
16. Ohsako, S., Nishida, Y., Ryo, H. & Yamauchi, T. Molecular characterization and expression of the Drosophila Ca2+/calmodulin-dependent protein kinase II gene. Identification of four forms of the enzyme generated from a single gene by alternative splicing. *J Biol Chem* **268**, 2052-2062 (1993).
17. Lin, C.R., *et al.* Molecular cloning of a brain-specific calcium/calmodulin-dependent protein kinase. *Proc Natl Acad Sci U S A* **84**, 5962-5966 (1987).

18. Bayer, K.U., Lohler, J., Schulman, H. & Harbers, K. Developmental expression of the CaM kinase II isoforms: ubiquitous gamma- and delta-CaM kinase II are the early isoforms and most abundant in the developing nervous system. *Brain Res Mol Brain Res* **70**, 147-154 (1999).
19. Karls, U., *et al.* Structure, expression, and chromosome location of the gene for the beta subunit of brain-specific Ca2+/calmodulin-dependent protein kinase II identified by transgene integration in an embryonic lethal mouse mutant. *Mol Cell Biol* **12**, 3644-3652 (1992).
20. Conlee, J.W., Shapiro, S.M. & Churn, S.B. Expression of the alpha and beta subunits of Ca2+/calmodulin kinase II in the cerebellum of jaundiced Gunn rats during development: a quantitative light microscopic analysis. *Acta Neuropathol* **99**, 393-401 (2000).
21. Vallano, M.L. Separation of isozymic forms of type II calcium/calmodulin-dependent protein kinase using cation-exchange chromatography. *J Neurosci Methods* **30**, 1-9 (1989).
22. Brocke, L., Chiang, L.W., Wagner, P.D. & Schulman, H. Functional implications of the subunit composition of neuronal CaM kinase II. *J Biol Chem* **274**, 22713-22722 (1999).
23. Miller, S.G. & Kennedy, M.B. Distinct forebrain and cerebellar isozymes of type II Ca2+/calmodulin-dependent protein kinase associate differently with the postsynaptic density fraction. *J Biol Chem* **260**, 9039-9046 (1985).
24. Hanley, R.M., *et al.* Functional analysis of a complementary DNA for the 50-kilodalton subunit of calmodulin kinase II. *Science* **237**, 293-297 (1987).
25. Johnson, L.D., *et al.* delta Ca(2+)/Calmodulin-dependent protein kinase II isozyme-specific induction of neurite outgrowth in P19 embryonal carcinoma cells. *J Neurochem* **75**, 2380-2391 (2000).
26. Caran, N., Johnson, L.D., Jenkins, K.J. & Tombes, R.M. Cytosolic targeting domains of gamma and delta calmodulin-dependent protein kinase II. *J Biol Chem* **276**, 42514-42519 (2001).
27. Takeuchi, Y., Fukunaga, K. & Miyamoto, E. Activation of nuclear Ca(2+)/calmodulin-dependent protein kinase II and brain-derived neurotrophic factor gene expression by stimulation of dopamine D2 receptor in transfected NG108-15 cells. *J Neurochem* **82**, 316-328 (2002).
28. Brocke, L., Srinivasan, M. & Schulman, H. Developmental and regional expression of multifunctional Ca2+/calmodulin-dependent protein kinase isoforms in rat brain. *J Neurosci* **15**, 6797-6808 (1995).
29. Elgersma, Y. & Silva, A.J. Molecular mechanisms of synaptic plasticity and memory. *Curr Opin Neurobiol* **9**, 209-213 (1999).
30. Lisman, J.E. & McIntyre, C.C. Synaptic plasticity: a molecular memory switch. *Curr Biol* **11**, R788-791 (2001).
31. Shen, K., Teruel, M.N., Subramanian, K. & Meyer, T. CaMKIIbeta functions as an F-actin targeting module that localizes CaMKIIalpha/beta heterooligomers to dendritic spines. *Neuron* **21**, 593-606 (1998).
32. Nomura, T., Kumatoriya, K., Yoshimura, Y. & Yamauchi, T. Overexpression of alpha and beta isoforms of Ca2+/calmodulin-dependent protein kinase II in neuroblastoma cells -- H-7 promotes neurite outgrowth. *Brain Res* **766**, 129-141 (1997).
33. Yamauchi, T., Yoshimura, Y., Nomura, T., Fujii, M. & Sugiura, H. Neurite outgrowth of neuroblastoma cells overexpressing alpha and beta isoforms of Ca2+/calmodulin-dependent protein kinase II-effects of protein kinase inhibitors. *Brain Res Brain Res Protoc* **2**, 250-258 (1998).

34. Fink, C.C., *et al.* Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CaMKII. *Neuron* **39**, 283-297 (2003).
35. O'Leary, H., Lasda, E. & Bayer, K.U. CaMKIIbeta association with the actin cytoskeleton is regulated by alternative splicing. *Mol Biol Cell* **17**, 4656-4665 (2006).
36. Elgersma, Y., Sweatt, J.D. & Giese, K.P. Mouse genetic approaches to investigating calcium/calmodulin-dependent protein kinase II function in plasticity and cognition. *J Neurosci* **24**, 8410-8415 (2004).
37. Morris, E.P. & Torok, K. Oligomeric structure of alpha-calmodulin-dependent protein kinase II. *J Mol Biol* **308**, 1-8 (2001).
38. Hudmon, A., Kim, S.A., Kolb, S.J., Stoops, J.K. & Waxham, M.N. Light scattering and transmission electron microscopy studies reveal a mechanism for calcium/calmodulin-dependent protein kinase II self-association. *J Neurochem* **76**, 1364-1375 (2001).
39. Rosenberg, O.S., Deindl, S., Sung, R.J., Nairn, A.C. & Kuriyan, J. Structure of the autoinhibited kinase domain of CaMKII and SAXS analysis of the holoenzyme. *Cell* **123**, 849-860 (2005).
40. Griffith, L.C. Calcium/calmodulin-dependent protein kinase II: an unforgettable kinase. *J Neurosci* **24**, 8391-8393 (2004).
41. Colbran, R.J., Smith, M.K., Schworer, C.M., Fong, Y.L. & Soderling, T.R. Regulatory domain of calcium/calmodulin-dependent protein kinase II. Mechanism of inhibition and regulation by phosphorylation. *J Biol Chem* **264**, 4800-4804 (1989).
42. Payne, M.E., *et al.* Calcium/calmodulin-dependent protein kinase II. Characterization of distinct calmodulin binding and inhibitory domains. *J Biol Chem* **263**, 7190-7195 (1988).
43. Meyer, T., Hanson, P.I., Stryer, L. & Schulman, H. Calmodulin trapping by calcium-calmodulin-dependent protein kinase. *Science* **256**, 1199-1202 (1992).
44. Singla, S.I., Hudmon, A., Goldberg, J.M., Smith, J.L. & Schulman, H. Molecular characterization of calmodulin trapping by calcium/calmodulin-dependent protein kinase II. *J Biol Chem* **276**, 29353-29360 (2001).
45. Patton, B.L., Miller, S.G. & Kennedy, M.B. Activation of type II calcium/calmodulin-dependent protein kinase by Ca2+/calmodulin is inhibited by autophosphorylation of threonine within the calmodulin-binding domain. *J Biol Chem* **265**, 11204-11212 (1990).
46. Shields, S.M., Ingebritsen, T.S. & Kelly, P.T. Identification of protein phosphatase 1 in synaptic junctions: dephosphorylation of endogenous calmodulin-dependent kinase II and synapse-enriched phosphoproteins. *J Neurosci* **5**, 3414-3422 (1985).
47. Colbran, R.J. & Brown, A.M. Calcium/calmodulin-dependent protein kinase II and synaptic plasticity. *Curr Opin Neurobiol* **14**, 318-327 (2004).
48. Brown, G.P., *et al.* Long-term potentiation induced by theta frequency stimulation is regulated by a protein phosphatase-1-operated gate. *J Neurosci* **20**, 7880-7887 (2000).
49. Blitzer, R.D., *et al.* Gating of CaMKII by cAMP-regulated protein phosphatase activity during LTP. *Science* **280**, 1940-1942 (1998).
50. Mulkey, R.M., Endo, S., Shenolikar, S. & Malenka, R.C. Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature* **369**, 486-488 (1994).
51. Lisman, J. A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory. *Proc Natl Acad Sci U S A* **86**, 9574-9578 (1989).

52. Fukunaga, K., *et al.* Decreased protein phosphatase 2A activity in hippocampal long-term potentiation. *J Neurochem* **74**, 807-817 (2000).
53. Yamashita, T., *et al.* Regulation of CaMKII by alpha4/PP2Ac contributes to learning and memory. *Brain Res* **1082**, 1-10 (2006).
54. Lou, L.L., Lloyd, S.J. & Schulman, H. Activation of the multifunctional Ca2+/calmodulin-dependent protein kinase by autophosphorylation: ATP modulates production of an autonomous enzyme. *Proc Natl Acad Sci U S A* **83**, 9497-9501 (1986).
55. Hanson, P.I., Kapiloff, M.S., Lou, L.L., Rosenfeld, M.G. & Schulman, H. Expression of a multifunctional Ca2+/calmodulin-dependent protein kinase and mutational analysis of its autoregulation. *Neuron* **3**, 59-70 (1989).
56. Dosemeci, A., Gollop, N. & Jaffe, H. Identification of a major autophosphorylation site on postsynaptic density-associated Ca2+/calmodulin-dependent protein kinase. *J Biol Chem* **269**, 31330-31333 (1994).
57. Migues, P.V., *et al.* Phosphorylation of CaMKII at Thr253 occurs in vivo and enhances binding to isolated postsynaptic densities. *J Neurochem* **98**, 289-299 (2006).
58. Zhang, Y.P., Holbro, N. & Oertner, T.G. Optical induction of plasticity at single synapses reveals input-specific accumulation of alphaCaMKII. *Proc Natl Acad Sci U S A* **105**, 12039-12044 (2008).
59. Shen, K. & Meyer, T. Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. *Science* **284**, 162-166 (1999).
60. Strack, S. & Colbran, R.J. Autophosphorylation-dependent targeting of calcium/ calmodulin-dependent protein kinase II by the NR2B subunit of the N-methyl- D-aspartate receptor. *J Biol Chem* **273**, 20689-20692 (1998).
61. Strack, S., McNeill, R.B. & Colbran, R.J. Mechanism and regulation of calcium/calmodulin-dependent protein kinase II targeting to the NR2B subunit of the N-methyl-D-aspartate receptor. *J Biol Chem* **275**, 23798-23806 (2000).
62. Fukunaga, K., Soderling, T.R. & Miyamoto, E. Activation of Ca2+/calmodulin-dependent protein kinase II and protein kinase C by glutamate in cultured rat hippocampal neurons. *J Biol Chem* **267**, 22527-22533 (1992).
63. Bayer, K.U., De Koninck, P., Leonard, A.S., Hell, J.W. & Schulman, H. Interaction with the NMDA receptor locks CaMKII in an active conformation. *Nature* **411**, 801-805 (2001).
64. Pradeep, K.K., *et al.* Regulation of Ca2+/calmodulin-dependent protein kinase II catalysis by N-methyl-D-aspartate receptor subunit 2B. *Biochem J* **419**, 123-132, 124 p following 132 (2009).
65. Ehlers, M.D., Zhang, S., Bernhadt, J.P. & Huganir, R.L. Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* **84**, 745-755 (1996).
66. Barria, A., Derkach, V. & Soderling, T. Identification of the Ca2+/calmodulin-dependent protein kinase II regulatory phosphorylation site in the alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate-type glutamate receptor. *J Biol Chem* **272**, 32727-32730 (1997).
67. Roche, K.W., O'Brien, R.J., Mammen, A.L., Bernhardt, J. & Huganir, R.L. Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. *Neuron* **16**, 1179-1188 (1996).
68. Lee, H.K., Barbarosie, M., Kameyama, K., Bear, M.F. & Huganir, R.L. Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. *Nature* **405**, 955-959 (2000).

69. Lee, H.K., *et al.* Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* **112**, 631-643 (2003).
70. Liao, D., Hessler, N.A. & Malinow, R. Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature* **375**, 400-404 (1995).
71. Zamanillo, D., *et al.* Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. *Science* **284**, 1805-1811 (1999).
72. McGlade-McCulloh, E., Yamamoto, H., Tan, S.E., Brickey, D.A. & Soderling, T.R. Phosphorylation and regulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II. *Nature* **362**, 640-642 (1993).
73. Barria, A., Muller, D., Derkach, V., Griffith, L.C. & Soderling, T.R. Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. *Science* **276**, 2042-2045 (1997).
74. Tan, S.E., Wenthold, R.J. & Soderling, T.R. Phosphorylation of AMPA-type glutamate receptors by calcium/calmodulin-dependent protein kinase II and protein kinase C in cultured hippocampal neurons. *J Neurosci* **14**, 1123-1129 (1994).
75. Tsui, J. & Malenka, R.C. Substrate localization creates specificity in calcium/calmodulin-dependent protein kinase II signaling at synapses. *J Biol Chem* **281**, 13794-13804 (2006).
76. Hayashi, Y., *et al.* Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science* **287**, 2262-2267 (2000).
77. Shi, S.H., *et al.* Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science* **284**, 1811-1816 (1999).
78. Chen, H.J., Rojas-Soto, M., Oguni, A. & Kennedy, M.B. A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II. *Neuron* **20**, 895-904 (1998).
79. Zhu, J.J., Qin, Y., Zhao, M., Van Aelst, L. & Malinow, R. Ras and Rap control AMPA receptor trafficking during synaptic plasticity. *Cell* **110**, 443-455 (2002).
80. Kim, E. & Sheng, M. PDZ domain proteins of synapses. *Nat Rev Neurosci* **5**, 771-781 (2004).
81. Schnell, E., *et al.* Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number. *Proc Natl Acad Sci U S A* **99**, 13902-13907 (2002).
82. Chen, L., *et al.* Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* **408**, 936-943 (2000).
83. Koh, Y.H., Popova, E., Thomas, U., Griffith, L.C. & Budnik, V. Regulation of DLG localization at synapses by CaMKII-dependent phosphorylation. *Cell* **98**, 353-363 (1999).
84. Chung, H.J., Huang, Y.H., Lau, L.F. & Huganir, R.L. Regulation of the NMDA receptor complex and trafficking by activity-dependent phosphorylation of the NR2B subunit PDZ ligand. *J Neurosci* **24**, 10248-10259 (2004).
85. Meng, F.J., Guo, J., Song, B., Yan, X.B. & Zhang, G.Y. Competitive binding of postsynaptic density 95 and Ca2+-calmodulin dependent protein kinase II to N-methyl-D-aspartate receptor subunit 2B in rat brain. *Acta Pharmacol Sin* **25**, 176-180 (2004).
86. Tomita, S., Stein, V., Stocker, T.J., Nicoll, R.A. & Bredt, D.S. Bidirectional synaptic plasticity regulated by phosphorylation of stargazin-like TARPs. *Neuron* **45**, 269-277 (2005).
87. Lu, C.S., Hodge, J.J., Mehren, J., Sun, X.X. & Griffith, L.C. Regulation of the Ca2+/CaM-responsive pool of CaMKII by scaffold-dependent autophosphorylation. *Neuron* **40**, 1185-1197 (2003).
88. Lee, S.J., Escobedo-Lozoya, Y., Szatmari, E.M. & Yasuda, R. Activation of CaMKII in single dendritic spines during long-term potentiation. *Nature* **458**, 299-304 (2009).

89. Harvey, C.D., Yasuda, R., Zhong, H. & Svoboda, K. The spread of Ras activity triggered by activation of a single dendritic spine. *Science* **321**, 136-140 (2008).
90. Harvey, C.D. & Svoboda, K. Locally dynamic synaptic learning rules in pyramidal neuron dendrites. *Nature* **450**, 1195-1200 (2007).
91. Yoshimura, Y., Aoi, C. & Yamauchi, T. Investigation of protein substrates of Ca(2+)/calmodulin-dependent protein kinase II translocated to the postsynaptic density. *Brain Res Mol Brain Res* **81**, 118-128 (2000).
92. Skelding, K.A. & Rostas, J.A. Regulation of CaMKII In vivo: The Importance of Targeting and the Intracellular Microenvironment. *Neurochem Res* (2009).
93. Fink, C.C. & Meyer, T. Molecular mechanisms of CaMKII activation in neuronal plasticity. *Curr Opin Neurobiol* **12**, 293-299 (2002).
94. Gaudilliere, B., Konishi, Y., de la Iglesia, N., Yao, G. & Bonni, A. A CaMKII-NeuroD signaling pathway specifies dendritic morphogenesis. *Neuron* **41**, 229-241 (2004).
95. Tian, Q.B., *et al.* Interaction of LDL receptor-related protein 4 (LRP4) with postsynaptic scaffold proteins via its C-terminal PDZ domain-binding motif, and its regulation by Ca/calmodulin-dependent protein kinase II. *Eur J Neurosci* **23**, 2864-2876 (2006).
96. Hoogenraad, C.C., *et al.* Liprinalpha1 degradation by calcium/calmodulin-dependent protein kinase II regulates LAR receptor tyrosine phosphatase distribution and dendrite development. *Dev Cell* **12**, 587-602 (2007).
97. Varga, A.W., *et al.* Calcium-calmodulin-dependent kinase II modulates Kv4.2 channel expression and upregulates neuronal A-type potassium currents. *J Neurosci* **24**, 3643-3654 (2004).
98. Stevens, C.F., Tonegawa, S. & Wang, Y. The role of calcium-calmodulin kinase II in three forms of synaptic plasticity. *Curr Biol* **4**, 687-693 (1994).
99. Stanton, P.K. & Gage, A.T. Distinct synaptic loci of Ca2+/calmodulin-dependent protein kinase II necessary for long-term potentiation and depression. *J Neurophysiol* **76**, 2097-2101 (1996).
100. Mulkey, R.M., Herron, C.E. & Malenka, R.C. An essential role for protein phosphatases in hippocampal long-term depression. *Science* **261**, 1051-1055 (1993).
101. Aguayo, L.G., Espinoza, F., Kunos, G. & Satin, L.S. Effects of intracellular calcium on GABAA receptors in mouse cortical neurons. *Pflugers Arch* **435**, 382-387 (1998).
102. Churn, S.B., *et al.* Calcium/calmodulin-dependent kinase II phosphorylation of the GABAA receptor alpha1 subunit modulates benzodiazepine binding. *J Neurochem* **82**, 1065-1076 (2002).
103. Marsden, K.C., Beattie, J.B., Friedenthal, J. & Carroll, R.C. NMDA receptor activation potentiates inhibitory transmission through GABA receptor-associated protein-dependent exocytosis of GABA(A) receptors. *J Neurosci* **27**, 14326-14337 (2007).
104. Ninan, I. & Arancio, O. Presynaptic CaMKII is necessary for synaptic plasticity in cultured hippocampal neurons. *Neuron* **42**, 129-141 (2004).
105. Benfenati, F., *et al.* Synaptic vesicle-associated Ca2+/calmodulin-dependent protein kinase II is a binding protein for Synapsin I. *Nature* **359**, 417-420 (1992).
106. Greengard, P., Valtorta, F., Czernik, A.J. & Benfenati, F. Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* **259**, 780-785 (1993).
107. Pieribone, V.A., *et al.* Distinct pools of synaptic vesicles in neurotransmitter release. *Nature* **375**, 493-497 (1995).

108. Yamagata, Y., Obata, K., Greengard, P. & Czernik, A.J. Increase in Synapsin I phosphorylation implicates a presynaptic component in septal kindling. *Neuroscience* **64**, 1-4 (1995).
109. Yamagata, Y., Jovanovic, J.N., Czernik, A.J., Greengard, P. & Obata, K. Bidirectional changes in Synapsin I phosphorylation at MAP kinase-dependent sites by acute neuronal excitation in vivo. *J Neurochem* **80**, 835-842 (2002).
110. Jovanovic, J.N., Czernik, A.J., Fienberg, A.A., Greengard, P. & Sihra, T.S. Synapsins as mediators of BDNF-enhanced neurotransmitter release. *Nat Neurosci* **3**, 323-329 (2000).
111. Kushner, S.A., *et al.* Modulation of presynaptic plasticity and learning by the H-ras/extracellular signal-regulated kinase/Synapsin I signaling pathway. *J Neurosci* **25**, 9721-9734 (2005).
112. Schiebler, W., Jahn, R., Doucet, J.P., Rothlein, J. & Greengard, P. Characterization of Synapsin I binding to small synaptic vesicles. *J Biol Chem* **261**, 8383-8390 (1986).
113. Valtorta, F., Greengard, P., Fesce, R., Chieregatti, E. & Benfenati, F. Effects of the neuronal phosphoprotein Synapsin I on actin polymerization. I. Evidence for a phosphorylation-dependent nucleating effect. *J Biol Chem* **267**, 11281-11288 (1992).
114. Nayak, A.S., Moore, C.I. & Browning, M.D. Ca2+/calmodulin-dependent protein kinase II phosphorylation of the presynaptic protein Synapsin I is persistently increased during long-term potentiation. *Proc Natl Acad Sci U S A* **93**, 15451-15456 (1996).
115. Tao-Cheng, J.H., Dosemeci, A., Winters, C.A. & Reese, T.S. Changes in the distribution of calcium calmodulin-dependent protein kinase II at the presynaptic bouton after depolarization. *Brain Cell Biol* **35**, 117-124 (2006).
116. Chapman, P.F., Frenguelli, B.G., Smith, A., Chen, C.M. & Silva, A.J. The alpha-Ca2+/calmodulin kinase II: a bidirectional modulator of presynaptic plasticity. *Neuron* **14**, 591-597 (1995).
117. Hinds, H.L., Goussakov, I., Nakazawa, K., Tonegawa, S. & Bolshakov, V.Y. Essential function of alpha-calcium/calmodulin-dependent protein kinase II in neurotransmitter release at a glutamatergic central synapse. *Proc Natl Acad Sci U S A* **100**, 4275-4280 (2003).
118. Nichols, R.A., Sihra, T.S., Czernik, A.J., Nairn, A.C. & Greengard, P. Calcium/calmodulin-dependent protein kinase II increases glutamate and noradrenaline release from synaptosomes. *Nature* **343**, 647-651 (1990).
119. Jovanovic, J.N., *et al.* Neurotrophins stimulate phosphorylation of Synapsin I by MAP kinase and regulate Synapsin I-actin interactions. *Proc Natl Acad Sci U S A* **93**, 3679-3683 (1996).
120. Jovanovic, J.N., *et al.* Opposing changes in phosphorylation of specific sites in Synapsin I during Ca2+-dependent glutamate release in isolated nerve terminals. *J Neurosci* **21**, 7944-7953 (2001).
121. Apps, R. & Garwicz, M. Anatomical and physiological foundations of cerebellar information processing. *Nat Rev Neurosci* **6**, 297-311 (2005).
122. Ito, M. The molecular organization of cerebellar long-term depression. *Nat Rev Neurosci* **3**, 896-902 (2002).
123. Coesmans, M., Weber, J.T., De Zeeuw, C.I. & Hansel, C. Bidirectional parallel fiber plasticity in the cerebellum under climbing fiber control. *Neuron* **44**, 691-700 (2004).
124. Ito, M. Cerebellar long-term depression: characterization, signal transduction, and functional roles. *Physiol Rev* **81**, 1143-1195 (2001).
125. Linden, D.J. & Connor, J.A. Participation of postsynaptic PKC in cerebellar long-term depression in culture. *Science* **254**, 1656-1659 (1991).

126. Hansel, C., Linden, D.J. & D'Angelo, E. Beyond parallel fiber LTD: the diversity of synaptic and non-synaptic plasticity in the cerebellum. *Nat Neurosci* **4**, 467-475 (2001).
127. Walaas, S.I., *et al.* Cell-specific localization of the alpha-subunit of calcium/calmodulin-dependent protein kinase II in Purkinje cells in rodent cerebellum. *Brain Res* **464**, 233-242 (1988).
128. Bienenstock, E.L., Cooper, L.N. & Munro, P.W. Theory for the development of neuron selectivity: orientation specificity and binocular interaction in visual cortex. *J Neurosci* **2**, 32-48 (1982).
129. Kasahara, J. & Sugiyama, H. Modulation of glutamate sensitivities by inhibitors of a protein kinase and a protein phosphatase in cultured rat Purkinje cells. *Neurosci Lett* **247**, 139-142 (1998).
130. Hansel, C., *et al.* alphaCaMKII Is essential for cerebellar LTD and motor learning. *Neuron* **51**, 835-843 (2006).
131. De Zeeuw, C.I. & Yeo, C.H. Time and tide in cerebellar memory formation. *Curr Opin Neurobiol* **15**, 667-674 (2005).
132. Malinow, R. & Malenka, R.C. AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci* **25**, 103-126 (2002).
133. Wang, Y.T. & Linden, D.J. Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. *Neuron* **25**, 635-647 (2000).
134. Sahyoun, N., *et al.* Early postnatal development of calmodulin-dependent protein kinase II in rat brain. *Biochem Biophys Res Commun* **132**, 878-884 (1985).
135. Thiagarajan, T.C., Piedras-Renteria, E.S. & Tsien, R.W. alpha- and betaCaMKII. Inverse regulation by neuronal activity and opposing effects on synaptic strength. *Neuron* **36**, 1103-1114 (2002).
136. De Koninck, P. & Schulman, H. Sensitivity of CaM kinase II to the frequency of Ca2+ oscillations. *Science* **279**, 227-230 (1998).
137. Miller, S.G., Patton, B.L. & Kennedy, M.B. Sequences of autophosphorylation sites in neuronal type II CaM kinase that control Ca2(+)-independent activity. *Neuron* **1**, 593-604 (1988).
138. Bulleit, R.F., Bennett, M.K., Molloy, S.S., Hurley, J.B. & Kennedy, M.B. Conserved and variable regions in the subunits of brain type II Ca2+/calmodulin-dependent protein kinase. *Neuron* **1**, 63-72 (1988).
139. Jourdain, P., Fukunaga, K. & Muller, D. Calcium/calmodulin-dependent protein kinase II contributes to activity-dependent filopodia growth and spine formation. *J Neurosci* **23**, 10645-10649 (2003).
140. Schulman, H. The multifunctional Ca2+/calmodulin-dependent protein kinase. *Adv Second Messenger Phosphoprotein Res* **22**, 39-112 (1988).
141. Allison, D.W., Chervin, A.S., Gelfand, V.I. & Craig, A.M. Postsynaptic scaffolds of excitatory and inhibitory synapses in hippocampal neurons: maintenance of core components independent of actin filaments and microtubules. *J Neurosci* **20**, 4545-4554 (2000).
142. Sanabria, H., Swulius, M.T., Kolodziej, S.J., Liu, J. & Waxham, M.N. {beta}CaMKII regulates actin assembly and structure. *J Biol Chem* **284**, 9770-9780 (2009).
143. Cho, M.H., Cao, X., Wang, D. & Tsien, J.Z. Dentate gyrus-specific manipulation of beta-Ca2+/calmodulin-dependent kinase II disrupts memory consolidation. *Proc Natl Acad Sci U S A* **104**, 16317-16322 (2007).

144. Silva, A.J., Stevens, C.F., Tonegawa, S. & Wang, Y. Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. *Science* **257**, 201-206 (1992).
145. Silva, A.J., Paylor, R., Wehner, J.M. & Tonegawa, S. Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. *Science* **257**, 206-211 (1992).
146. Elgersma, Y., *et al.* Inhibitory autophosphorylation of CaMKII controls PSD association, plasticity, and learning. *Neuron* **36**, 493-505 (2002).
147. Hinds, H.L., Tonegawa, S. & Malinow, R. CA1 long-term potentiation is diminished but present in hippocampal slices from alpha-CaMKII mutant mice. *Learn Mem* **5**, 344-354 (1998).
148. Glazewski, S., Chen, C.M., Silva, A. & Fox, K. Requirement for alpha-CaMKII in experience-dependent plasticity of the barrel cortex. *Science* **272**, 421-423 (1996).
149. Kirkwood, A., Silva, A. & Bear, M.F. Age-dependent decrease of synaptic plasticity in the neocortex of alphaCaMKII mutant mice. *Proc Natl Acad Sci U S A* **94**, 3380-3383 (1997).
150. Gordon, J.A., Cioffi, D., Silva, A.J. & Stryker, M.P. Deficient plasticity in the primary visual cortex of alpha-calcium/calmodulin-dependent protein kinase II mutant mice. *Neuron* **17**, 491-499 (1996).
151. Butler, L.S., *et al.* Limbic epilepsy in transgenic mice carrying a Ca2+/calmodulin-dependent kinase II alpha-subunit mutation. *Proc Natl Acad Sci U S A* **92**, 6852-6855 (1995).
152. Yamagata, Y., *et al.* Kinase-dead knock-in mouse reveals an essential role of kinase activity of Ca2+/calmodulin-dependent protein kinase IIalpha in dendritic spine enlargement, long-term potentiation, and learning. *J Neurosci* **29**, 7607-7618 (2009).
153. Yamasaki, N., *et al.* Alpha-CaMKII deficiency causes immature dentate gyrus, a novel candidate endophenotype of psychiatric disorders. *Mol Brain* **1**, 6 (2008).
154. Silva, A.J., *et al.* Impaired learning in mice with abnormal short-lived plasticity. *Curr Biol* **6**, 1509-1518 (1996).
155. Frankland, P.W., O'Brien, C., Ohno, M., Kirkwood, A. & Silva, A.J. Alpha-CaMKII-dependent plasticity in the cortex is required for permanent memory. *Nature* **411**, 309-313 (2001).
156. Frankland, P.W., Bontempi, B., Talton, L.E., Kaczmarek, L. & Silva, A.J. The involvement of the anterior cingulate cortex in remote contextual fear memory. *Science* **304**, 881-883 (2004).
157. Chen, C., Rainnie, D.G., Greene, R.W. & Tonegawa, S. Abnormal fear response and aggressive behavior in mutant mice deficient for alpha-calcium-calmodulin kinase II. *Science* **266**, 291-294 (1994).
158. Giese, K.P., Fedorov, N.B., Filipkowski, R.K. & Silva, A.J. Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. *Science* **279**, 870-873 (1998).
159. Cooke, S.F., *et al.* Autophosphorylation of alphaCaMKII is not a general requirement for NMDA receptor-dependent LTP in the adult mouse. *J Physiol* **574**, 805-818 (2006).
160. Glazewski, S., Giese, K.P., Silva, A. & Fox, K. The role of alpha-CaMKII autophosphorylation in neocortical experience-dependent plasticity. *Nat Neurosci* **3**, 911-918 (2000).
161. Hardingham, N., *et al.* Neocortical long-term potentiation and experience-dependent synaptic plasticity require alpha-calcium/calmodulin-dependent protein kinase II autophosphorylation. *J Neurosci* **23**, 4428-4436 (2003).

162. Taha, S., Hanover, J.L., Silva, A.J. & Stryker, M.P. Autophosphorylation of alphaCaMKII is required for ocular dominance plasticity. *Neuron* **36**, 483-491 (2002).
163. Sametsky, E.A., Disterhoft, J.F. & Ohno, M. Autophosphorylation of alphaCaMKII downregulates excitability of CA1 pyramidal neurons following synaptic stimulation. *Neurobiol Learn Mem* **92**, 120-123 (2009).
164. Need, A.C. & Giese, K.P. Handling and environmental enrichment do not rescue learning and memory impairments in alphaCamKII(T286A) mutant mice. *Genes Brain Behav* **2**, 132-139 (2003).
165. Cho, Y.H., Giese, K.P., Tanila, H., Silva, A.J. & Eichenbaum, H. Abnormal hippocampal spatial representations in alphaCaMKIIT286A and CREBalphaDelta- mice. *Science* **279**, 867-869 (1998).
166. Mayford, M., Wang, J., Kandel, E.R. & O'Dell, T.J. CaMKII regulates the frequency-response function of hippocampal synapses for the production of both LTD and LTP. *Cell* **81**, 891-904 (1995).
167. Glazewski, S., Bejar, R., Mayford, M. & Fox, K. The effect of autonomous alpha-CaMKII expression on sensory responses and experience-dependent plasticity in mouse barrel cortex. *Neuropharmacology* **41**, 771-778 (2001).
168. Bach, M.E., Hawkins, R.D., Osman, M., Kandel, E.R. & Mayford, M. Impairment of spatial but not contextual memory in CaMKII mutant mice with a selective loss of hippocampal LTP in the range of the theta frequency. *Cell* **81**, 905-915 (1995).
169. Wiedenmayer, C.P., Myers, M.M., Mayford, M. & Barr, G.A. Olfactory based spatial learning in neonatal mice and its dependence on CaMKII. *Neuroreport* **11**, 1051-1055 (2000).
170. Rotenberg, A., Mayford, M., Hawkins, R.D., Kandel, E.R. & Muller, R.U. Mice expressing activated CaMKII lack low frequency LTP and do not form stable place cells in the CA1 region of the hippocampus. *Cell* **87**, 1351-1361 (1996).
171. Mayford, M., *et al.* Control of memory formation through regulated expression of a CaMKII transgene. *Science* **274**, 1678-1683 (1996).
172. Bejar, R., Yasuda, R., Krugers, H., Hood, K. & Mayford, M. Transgenic calmodulin-dependent protein kinase II activation: dose-dependent effects on synaptic plasticity, learning, and memory. *J Neurosci* **22**, 5719-5726 (2002).
173. Yasuda, M. & Mayford, M.R. CaMKII activation in the entorhinal cortex disrupts previously encoded spatial memory. *Neuron* **50**, 309-318 (2006).
174. Zhang, L., *et al.* Hippocampal synaptic metaplasticity requires inhibitory autophosphorylation of Ca2+/calmodulin-dependent kinase II. *J Neurosci* **25**, 7697-7707 (2005).
175. Park, C.S., Elgersma, Y., Grant, S.G. & Morrison, J.H. alpha-Isoform of calcium-calmodulin-dependent protein kinase II and postsynaptic density protein 95 differentially regulate synaptic expression of NR2A- and NR2B-containing N-methyl-d-aspartate receptors in hippocampus. *Neuroscience* **151**, 43-55 (2008).
176. Miller, S., *et al.* Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. *Neuron* **36**, 507-519 (2002).
177. Wang, H., *et al.* Inducible protein knockout reveals temporal requirement of CaMKII reactivation for memory consolidation in the brain. *Proc Natl Acad Sci U S A* **100**, 4287-4292 (2003).
178. Wang, Z.W. Regulation of synaptic transmission by presynaptic CaMKII and BK channels. *Mol Neurobiol* **38**, 153-166 (2008).
179. Cao, X., *et al.* Inducible and selective erasure of memories in the mouse brain via chemical-genetic manipulation. *Neuron* **60**, 353-366 (2008).

180. van Woerden, G.M., *et al.* betaCaMKII controls the direction of plasticity at parallel fiber-Purkinje cell synapses. *Nat Neurosci* **12**, 823-825 (2009).
181. Jiang, Y.H., *et al.* Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron* **21**, 799-811 (1998).
182. Miura, K., *et al.* Neurobehavioral and electroencephalographic abnormalities in Ube3a maternal-deficient mice. *Neurobiol Dis* **9**, 149-159 (2002).
183. Weeber, E.J., *et al.* Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *J Neurosci* **23**, 2634-2644 (2003).
184. Small, S.A. & Duff, K. Linking Abeta and tau in late-onset Alzheimer's disease: a dual pathway hypothesis. *Neuron* **60**, 534-542 (2008).
185. Walsh, D.M., *et al.* Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. *Nature* **416**, 535-539 (2002).
186. Hsieh, H., *et al.* AMPAR removal underlies Abeta-induced synaptic depression and dendritic spine loss. *Neuron* **52**, 831-843 (2006).
187. Gu, Z., Liu, W. & Yan, Z. {beta}-Amyloid impairs AMPA receptor trafficking and function by reducing Ca2+/calmodulin-dependent protein kinase II synaptic distribution. *J Biol Chem* **284**, 10639-10649 (2009).
188. Amada, N., Aihara, K., Ravid, R. & Horie, M. Reduction of NR1 and phosphorylated Ca2+/calmodulin-dependent protein kinase II levels in Alzheimer's disease. *Neuroreport* **16**, 1809-1813 (2005).
189. Bi, H. & Sze, C.I. N-methyl-D-aspartate receptor subunit NR2A and NR2B messenger RNA levels are altered in the hippocampus and entorhinal cortex in Alzheimer's disease. *J Neurol Sci* **200**, 11-18 (2002).
190. Yoshimura, Y., Ichinose, T. & Yamauchi, T. Phosphorylation of tau protein to sites found in Alzheimer's disease brain is catalyzed by Ca2+/calmodulin-dependent protein kinase II as demonstrated tandem mass spectrometry. *Neurosci Lett* **353**, 185-188 (2003).
191. Lothman, E.W., Bertram, E.H., 3rd & Stringer, J.L. Functional anatomy of hippocampal seizures. *Prog Neurobiol* **37**, 1-82 (1991).
192. Goddard, G.V., McIntyre, D.C. & Leech, C.K. A permanent change in brain function resulting from daily electrical stimulation. *Exp Neurol* **25**, 295-330 (1969).
193. Bronstein, J.M., Farber, D.B. & Wasterlain, C.G. Regulation of type-II calmodulin kinase: functional implications. *Brain Res Brain Res Rev* **18**, 135-147 (1993).
194. Blair, R.E., Churn, S.B., Sombati, S., Lou, J.K. & DeLorenzo, R.J. Long-lasting decrease in neuronal Ca2+/calmodulin-dependent protein kinase II activity in a hippocampal neuronal culture model of spontaneous recurrent seizures. *Brain Res* **851**, 54-65 (1999).
195. Blair, R.E., Sombati, S., Churn, S.B. & Delorenzo, R.J. Epileptogenesis causes an N-methyl-d-aspartate receptor/Ca2+-dependent decrease in Ca2+/calmodulin-dependent protein kinase II activity in a hippocampal neuronal culture model of spontaneous recurrent epileptiform discharges. *Eur J Pharmacol* **588**, 64-71 (2008).
196. Churn, S.B., Sombati, S., Jakoi, E.R., Severt, L. & DeLorenzo, R.J. Inhibition of calcium/calmodulin kinase II alpha subunit expression results in epileptiform activity in cultured hippocampal neurons. *Proc Natl Acad Sci U S A* **97**, 5604-5609 (2000).
197. van Woerden, G.M., *et al.* Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of alphaCaMKII inhibitory phosphorylation. *Nat Neurosci* **10**, 280-282 (2007).

198. Singleton, M.W., Holbert, W.H., 2nd, Lee, A.T., Bracey, J.M. & Churn, S.B. Modulation of CaM kinase II activity is coincident with induction of status epilepticus in the rat pilocarpine model. *Epilepsia* **46**, 1389-1400 (2005).
199. Yamagata, Y., Imoto, K. & Obata, K. A mechanism for the inactivation of Ca2+/calmodulin-dependent protein kinase II during prolonged seizure activity and its consequence after the recovery from seizure activity in rats in vivo. *Neuroscience* **140**, 981-992 (2006).
200. Roth, T.L., Lubin, F.D., Sodhi, M. & Kleinman, J.E. Epigenetic mechanisms in schizophrenia. *Biochim Biophys Acta* (2009).
201. Harrison, P.J. & Owen, M.J. Genes for schizophrenia? Recent findings and their pathophysiological implications. *Lancet* **361**, 417-419 (2003).
202. Novak, G., Seeman, P. & Tallerico, T. Schizophrenia: elevated mRNA for calcium-calmodulin-dependent protein kinase IIbeta in frontal cortex. *Brain Res Mol Brain Res* **82**, 95-100 (2000).
203. Novak, G., Seeman, P. & Tallerico, T. Increased expression of calcium/calmodulin-dependent protein kinase IIbeta in frontal cortex in schizophrenia and depression. *Synapse* **59**, 61-68 (2006).
204. Javitt, D.C. & Zukin, S.R. Recent advances in the phencyclidine model of schizophrenia. *Am J Psychiatry* **148**, 1301-1308 (1991).
205. Nagai, T., *et al.* Effect of AD-5423 on animal models of schizophrenia: phencyclidine-induced behavioral changes in mice. *Neuroreport* **14**, 269-272 (2003).
206. Nabeshima, T., Mouri, A., Murai, R. & Noda, Y. Animal model of schizophrenia: dysfunction of NMDA receptor-signaling in mice following withdrawal from repeated administration of phencyclidine. *Ann N Y Acad Sci* **1086**, 160-168 (2006).
207. Mouri, A., *et al.* Involvement of a dysfunctional dopamine-D1/N-methyl-d-aspartate-NR1 and Ca2+/calmodulin-dependent protein kinase II pathway in the impairment of latent learning in a model of schizophrenia induced by phencyclidine. *Mol Pharmacol* **71**, 1598-1609 (2007).
208. Angelman, H. "Puppet" children: A report on three cases. *Dev Med Child neurol* **7**, 681-688 (1965).
209. Kishino, T., Lalande, M. & Wagstaff, J. UBE3A/E6-AP mutations cause Angelman syndrome. *Nat Genet* **15**, 70-73 (1997).
210. Matsuura, T., *et al.* De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. *Nat Genet* **15**, 74-77 (1997).
211. Rougeulle, C., Glatt, H. & Lalande, M. The Angelman syndrome candidate gene, UBE3A/E6-AP, is imprinted in brain. *Nat Genet* **17**, 14-15 (1997).
212. Vu, T.H. & Hoffman, A.R. Imprinting of the Angelman syndrome gene, UBE3A, is restricted to brain. *Nat Genet* **17**, 12-13 (1997).
213. Rougeulle, C., Cardoso, C., Fontes, M., Colleaux, L. & Lalande, M. An imprinted antisense RNA overlaps UBE3A and a second maternally expressed transcript. *Nat Genet* **19**, 15-16 (1998).
214. Horsthemke, B. & Wagstaff, J. Mechanisms of imprinting of the Prader-Willi/Angelman region. *Am J Med Genet A* **146A**, 2041-2052 (2008).
215. Scheffner, M. Ubiquitin, E6-AP, and their role in p53 inactivation. *Pharmacol Ther* **78**, 129-139 (1998).
216. Kumar, S., Talis, A.L. & Howley, P.M. Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination. *J Biol Chem* **274**, 18785-18792 (1999).

217. Kuhne, C. & Banks, L. E3-ubiquitin ligase/E6-AP links multicopy maintenance protein 7 to the ubiquitination pathway by a novel motif, the L2G box. *J Biol Chem* **273**, 34302-34309 (1998).
218. Nuber, U., Schwarz, S.E. & Scheffner, M. The ubiquitin-protein ligase E6-associated protein (E6-AP) serves as its own substrate. *Eur J Biochem* **254**, 643-649 (1998).
219. Oda, H., Kumar, S. & Howley, P.M. Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination. *Proc Natl Acad Sci U S A* **96**, 9557-9562 (1999).
220. Jing, M., Bohl, J., Brimer, N., Kinter, M. & Vande Pol, S.B. Degradation of tyrosine phosphatase PTPN3 (PTPH1) by association with oncogenic human papillomavirus E6 proteins. *J Virol* **81**, 2231-2239 (2007).
221. Louria-Hayon, I., *et al.* E6AP promotes the degradation of the PML tumor suppressor. *Cell Death Differ* **16**, 1156-1166 (2009).
222. Yang, Y., *et al.* Ubiquitin-dependent proteolysis of trihydrophobin 1 (TH1) by the human papilloma virus E6-associated protein (E6-AP). *J Cell Biochem* **101**, 167-180 (2007).
223. Reiter, L.T., Seagroves, T.N., Bowers, M. & Bier, E. Expression of the Rho-GEF Pbl/ECT2 is regulated by the UBE3A E3 ubiquitin ligase. *Hum Mol Genet* **15**, 2825-2835 (2006).
224. Mani, A., *et al.* E6AP mediates regulated proteasomal degradation of the nuclear receptor coactivator amplified in breast cancer 1 in immortalized cells. *Cancer Res* **66**, 8680-8686 (2006).
225. Mishra, A., Godavarthi, S.K. & Jana, N.R. UBE3A/E6-AP regulates cell proliferation by promoting proteasomal degradation of p27. *Neurobiol Dis* (2009).
226. Zheng, L., *et al.* E3 ubiquitin ligase E6AP-mediated TSC2 turnover in the presence and absence of HPV16 E6. *Genes Cells* **13**, 285-294 (2008).
227. Shimoji, T., *et al.* Identification of annexin A1 as a novel substrate for E6AP-mediated ubiquitylation. *J Cell Biochem* **106**, 1123-1135 (2009).

Chapter II

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

Mohammad Reza Hojjati, Geeske M. van Woerden, William J. Tyler, Karl Peter Giese, Alcino J. Silva, Lucas Pozzo-Miller and Ype Elgersma

Published in Nat Neurosci. 2007 Sept;10(9):1125-7

Using targeted mouse mutants and pharmacologic inhibition of αCaMKII, we demonstrate that the αCaMKII protein, but not its activation, autophosphorylation or its ability to phosphorylate Synapsin I, is required for normal short-term presynaptic plasticity. Furthermore we show that αCaMKII regulates the number of docked vesicles independent of its ability to be activated. These results indicate that αCaMKII plays a non-enzymatic role in short-term presynaptic plasticity at hippocampal CA3-CA1 synapses.

The alpha isoform of Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII) was originally identified as Synapsin I kinase[1]. Subsequent studies showed that αCaMKII is abundantly associated with presynaptic vesicles by binding to Synapsin I[2]. Together with the observation that αCaMKII is one of the most abundant proteins of the hippocampus[3], these results may suggest that αCaMKII also has a non-enzymatic function, but such a function has not directly been demonstrated yet. Analysis of *Camk2a* knock-out (*Camk2a*$^{-/-}$) mice confirmed a presynaptic role of αCaMKII in short-term presynaptic plasticity[4, 5], but these experiments did not address whether this role is mediated by αCaMKII as a kinase, as a structural protein, or both.

To study the enzymatic requirements of αCaMKII in presynaptic plasticity, we made use of four different lines of αCaMKII mutants. Autophosphorylation at the T286 and T305/T306 site was prevented by using *Camk2a*^{T286A} mice, which lack αCaMKII autonomous (Ca^{2+}/CaM independent) activity[6] and by *Camk2a*$^{T305/306VA}$ mice, which lack αCaMKII inhibitory autophosphorylation[7]. Furthermore, we made use of *Camk2a*^{T305D} mice, in which constitutive autophosphorylation at the T305 site in the Ca^{2+}/CaM domain is mimicked. Hence, αCaMKII in these mice can not be activated[7]. The fourth line lacks the entire αCaMKII protein (*Camk2a*$^{-/-}$)[7] (for an overview of all phenotypes see [8]).

Since these mutants were backcrossed in C57BL6, we first tested whether the originally reported LTP deficits (in hybrid 129Sv/C57BL6 mice) were still present[6, 7, 9]. Indeed, we confirmed that αCaMKII 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)**. In contrast, loss of inhibitory phosphorylation in *Camk2a*$^{TT305/6VA}$ mice, reduced the threshold for LTP induction as reported previously[7] (**Supplementary Fig. 1).** Western blots on isolated synaptosomes of all the mutants did not reveal changes in the levels of the β, γ, δCaMKII isoforms, nor was the amount of calmodulin affected in these mutants (**Supplementary Fig. 2**).

We looked at the ability of these mutants to phosphorylate Synapsin I at S603 (site 3), which is an exclusive CaMKII site. As shown in **Fig.1**, *Camk2a*$^{-/-}$ mice showed a marked decrease of Synapsin I phosphorylation as compared to wild-type mice (p<0.001; ANOVA), suggesting that none of the other CaMKII isoforms can efficiently compensate for the loss of αCaMKII phosphorylation of Synapsin I *in vivo.* Notably, steady-state levels of phosphorylated Synapsin I were not affected in *Camk2a*^{T286A} (p=0.2) mice and in *Camk2a*$^{TT305/306VA}$ mice (p=0.3), indicating that loss of autonomous activity or self-inhibition is not important for Synapsin I phosphorylation *in vivo*. In contrast, activation of αCaMKII is an absolute requirement, since phosphorylation of Synapsin I in the *Camk2a*^{T305D} mutant was not significantly above background level (p=0.2; **Fig. 1a**). The dominant negative nature of the *Camk2a*^{T305D} mutation is further illustrated by the fact that this is also the only mutant in which autophosphorylation at both αCaMKII-T286 and βCaMKII-T287 is indistinguishable from background level (both p>0.8; **Fig. 1b),** further suggesting that

αCaMKII in this mutant is inactive. Taken together, these results show that these mutants provide an ideal tool to dissect the requirements for αCaMKII activation, αCaMKII autophosphorylation, and Synapsin I S603 phosphorylation in short-term presynaptic plasticity.

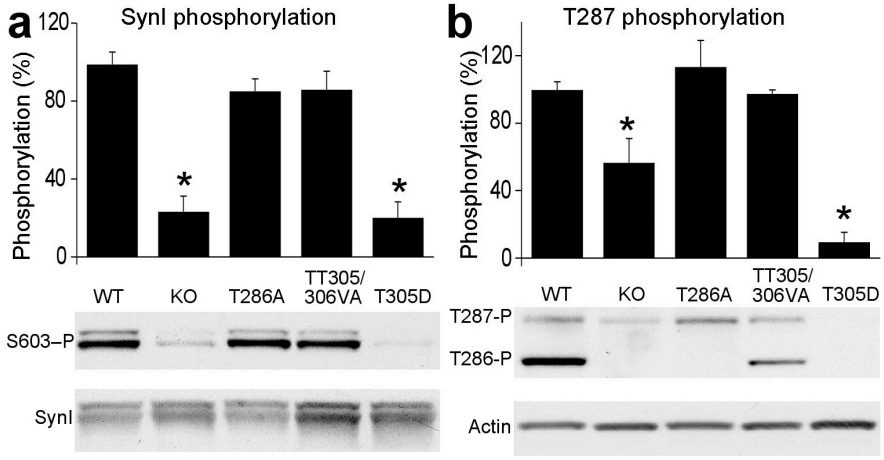


Figure 1. *Phosphorylation of Synapsin I and CaMKII-T286/T287 in synaptosomes obtained from Camk2a mutants.* ***(a)*** *Phosphorylation of Synapsin I S603 is not affected by impaired αCaMKII autophosphorylation, but requires αCaMKII protein and its activation by Ca^{2+}/Calmodulin.* ***(b)*** *Phosphorylation of αCaMKII-T286 and βCaMKII-T287 is absent in $Camk2a^{T305D}$ mice. Graph represents data of βCaMKII-T287 only. Error bars indicate SEM. Each sample contains pooled fractions from 4 independent isolations.*

Previous studies from multiple laboratories[4, 5, 8], using independently generated targeted deletions of αCaMKII[5, 9], have demonstrated that loss of αCaMKII results in enhanced augmentation and decreased synaptic fatigue, which are both measures of presynaptic plasticity. Augmentation is an increase in the evoked postsynaptic response observed several seconds after high-frequency afferent stimulation, caused by facilitated exocytosis[10]. We measured augmentation at hippocampal CA3-CA1 synapses using extracellular field recording (**Supplementary methods**). As shown in **Fig. 2a,c** $Camk2a^{-/-}$ mice showed a striking increase in synaptic augmentation (during 3-11s: $F_{(4,92)}=13$, $p<0.0001$ repeated measures ANOVA), confirming that αCaMKII critically regulates this form of presynaptic plasticity[4]. Since transiently elevated presynaptic calcium is thought to be an important factor underlying synaptic augmentation, activation of presynaptic αCaMKII activity may play an important role. However, although αCaMKII activity is regulated by autophosphorylation, augmentation was not affected in the $Camk2a^{T286A}$ and $Camk2a^{TT305/6VA}$ 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 is unaffected in these mutants **(Fig. 1).** Unexpectedly however, augmentation was also unaffected in $Camk2a^{T305D}$ mutants ($p=0.5$ ANOVA; **Fig. 2b,c)** in which αCaMKII activation is blocked and Synapsin I phosphorylation is absent **(Fig. 1)**. These results strongly suggest that synaptic augmentation does not depend on the ability to phosphorylate Synapsin I, nor on the activation of αCaMKII.

Since activation of both αCaMKII and βCaMKII seem to be impaired in the $Camk2a^{T305D}$ mutant **(Fig. 1b)** a similar result should be obtained if the augmentation experiment is performed in the presence of the membrane-permeable CaMKII inhibitor KN-93[11]. This inhibitor competes with Ca^{2+}/Calmodulin binding and therefore prevents activation of both αCaMKII and βCaMKII. LTP experiments in the presence of this inhibitor showed that KN-93 is indeed able to block LTP (**Supplementary Fig. 1f**). In

contrast, augmentation was similar in the presence of KN-93 as compared to its inactive analogue KN-92 (p=0.5 ANOVA; **Fig. 2c)**, confirming that CaMKII activation is not required for normal augmentation.

The biochemical experiments of **Fig.1** indicate that CaMKII activity in the *Camk2a*^{T305D} mutant is reduced to such extend, that phosphorylation of CaMKII substrates is 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 as well. This inhibitor is 500 times more potent than KN-93 and more importantly, it is non-competitive for Ca^{2+}/Calmodulin and exogenous substrates, thus also blocking basal and autonomously active CaMKII[12]. Efficient penetration was achieved by making use of AIP fused to the Antennapedia transport peptide, and by preincubating the slices for 1 hour (see **Supplementary Methods**). Indeed, LTP was fully blocked, indicating that the drug is able to penetrate the slice (**Supplementary Fig. 1g**), and importantly, Ant-AIP-II showed no discernable effect on synaptic transmission (**Supplementary Fig. 3**), which makes it suitable to be used 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 αCaMKII protein, but not αCaMKII activity, is required for normal augmentation.

Previous whole-cell patch-clamp recordings in CA3-restricted *Camk2a*$^{-/-}$ mice, showed a significant enhancement of the EPSC amplitude during repetitive stimulation of CA3-CA1 synapses[5], suggesting that the fatigue rate of neurotransmitter release is regulated by αCaMKII. Likewise, extracellular field recordings from our global *Camk2a*$^{-/-}$ 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[10]. *Camk2a*$^{-/-}$ 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 ($F_{(99,4455)}$=4,5, p<0.0001): *Camk2a*$^{-/-}$ 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 EPSP amplitude during prolonged stimulation[10].

To test the αCaMKII autophosphorylation and Synapsin I phosphorylation requirements in this presynaptic measure, we repeated the experiment in the αCaMKII point mutants. No significant effect of genotype was observed in either *Camk2a*^{T286A} or *Camk2a*$^{TT305/6VA}$ autophosphorylation defective mutants **(**both p>0.3 ANOVA at stimulus number 100, **Fig 2f**). Notably, depletion rate was also not affected in *Camk2a*^{T305D} mutants (p>0.7; **Fig. 2e,f**), in which activation of αCaMKII is prevented and phosphorylation of Synapsin I is 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, this strongly suggests that the αCaMKII protein plays a structural role rather than an enzymatic role during this form of short-term presynaptic plasticity.

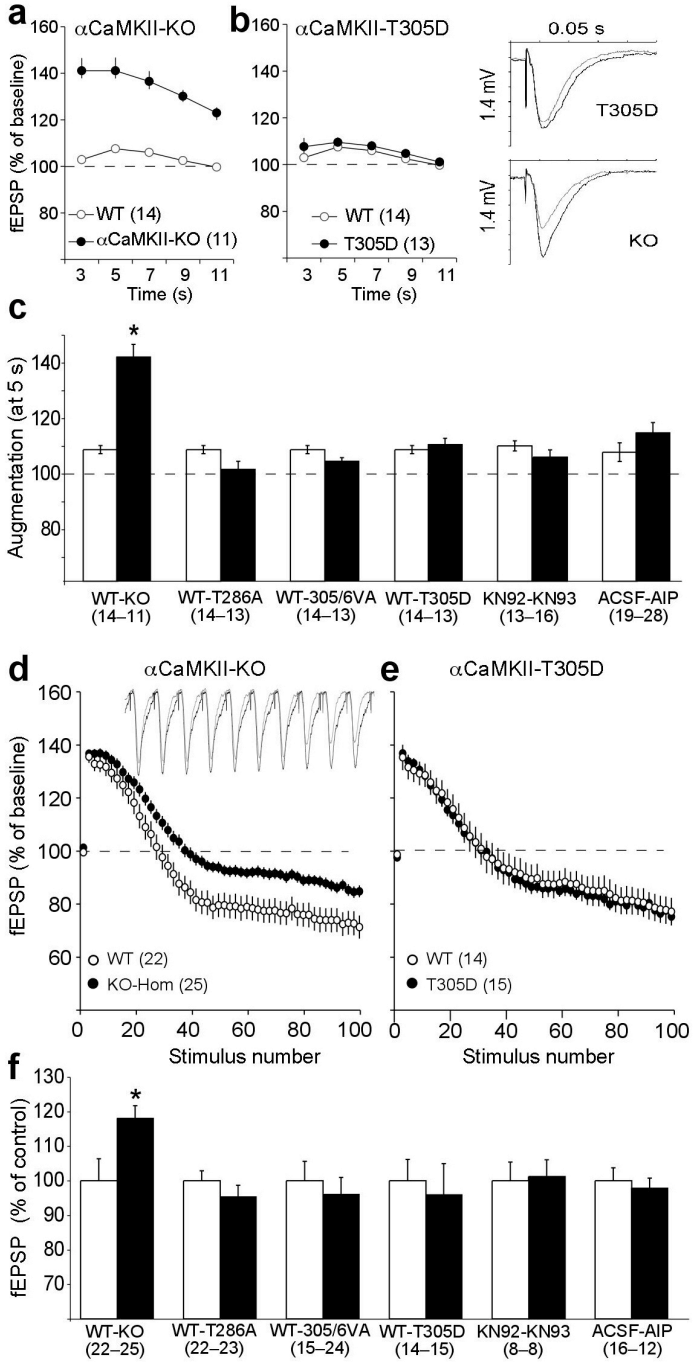


Figure 2. *Presynaptic short-term plasticity requires αCaMKII protein but not its autophosphorylation, activation or activity.* ***(a-c)*** *Increased synaptic augmentation in Camk2a$^{-/-}$ mutant mice is not caused by the lack of CaMKII kinase activity.* ***(a,b)*** *fEPSP responses (normalized to pre-tetanus baseline) were recorded at the indicated time after a 10 theta-burst tetanus. Traces are from baseline response (grey) and the response 5s post tetanization (black).* ***(c)*** *Augmentation summary of responses obtained 5 seconds post-tetanus normalized to baseline. Black bars represent mutants or drug treated slices, white bars represent control slices as indicated.* ***(d-f).*** *Decreased synaptic fatigue during repetitive stimulation in Camk2a$^{-/-}$ mice is not caused by the lack of αCaMKII kinase activity.* ***(d,e)*** *fEPSP responses (normalized against baseline) were recorded during a 10Hz tetanus. Only the first and even numbered stimuli are shown for clarity. Traces are from wild-type (grey) and Camk2a$^{-/-}$ slices (black) recorded from stimulus number 21-30.* ***(f)*** *Depletion summary of the last (100th) stimulus of the 10Hz train. Black bars represent mutant or drug treated slices, normalized against the controls as indicated (white bars, set at 100%). Numbers between brackets indicate number of slices. Error bars indicate SEM.*

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 RRP[13, 14]. We obtained measurements at the active zone of excitatory synapses on CA1 spines of wild-type, *Camk2a*$^{-/-}$, and *Camk2a*^{T305D} mice. Indeed, there was a significant effect of genotype ($F_{(2,41)}$=4.7, p<0.05), with synapses in *Camk2*$^{-/-}$ mice showing a 20% increase in the total number of docked vesicles compared to wild-type (Fisher's PLSD p<0.05) or *Camk2a*^{T305D} mice (p<0.01) (**Fig. 3a).** In contrast, no significant difference in the number of docked vesicles was observed between the *Camk2a*^{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 not different between the mutants and wild-type mice (all measures p>0.2; **Supplementary Fig. 4**). Thus, the absence of αCaMKII protein results in an increased number of docked vesicles whereas the loss of αCaMKII activation and Synapsin I S603 phosphorylation does not affect vesicle docking.

Together, these results suggest a model in which αCaMKII functions non-enzymatically 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 *Camk2a*$^{-/-}$ mice is indeed mediated by a larger RRP, then presynaptic function should be normal under conditions that minimize depletion of the RRP. Accordingly, we decreased the extracellular calcium concentration, which limits the rate of depletion from the RRP[15]. Indeed, decreasing the extracellular calcium concentration from 2.5 to 0.8 mM normalized the responses during 10 Hz stimulation in *Camk2a*$^{-/-}$ mice ($F_{(1,31)}$< 0.0001, p= 1, **Fig. 3b**), supporting a model that αCaMKII limits the available pool of readily-releasable neurotransmitter vesicles.

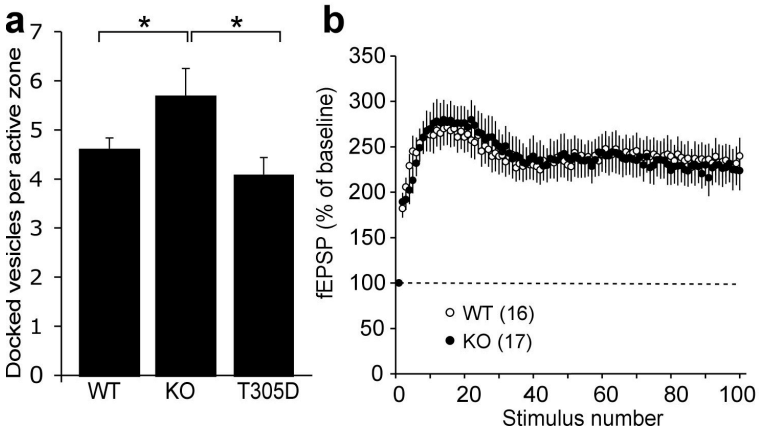


Figure 3: *αCaMKII protein regulates the number of docked vesicles.* ***(a)*** *Quantitative electron microscopy of asymmetric synapses on dendritic spines of CA1 pyramidal neurons, show a 20% increase in the number of docked vesicles in Camk2a*$^{-/-}$ *mice.* ***(b)*** *Decreasing the depletion rate by lowering extracellular [Ca*$^{2+}$*], reverses the phenotype of the Camk2a*$^{-/-}$ *mice during repetitive stimulation. Error bars indicate SEM.*

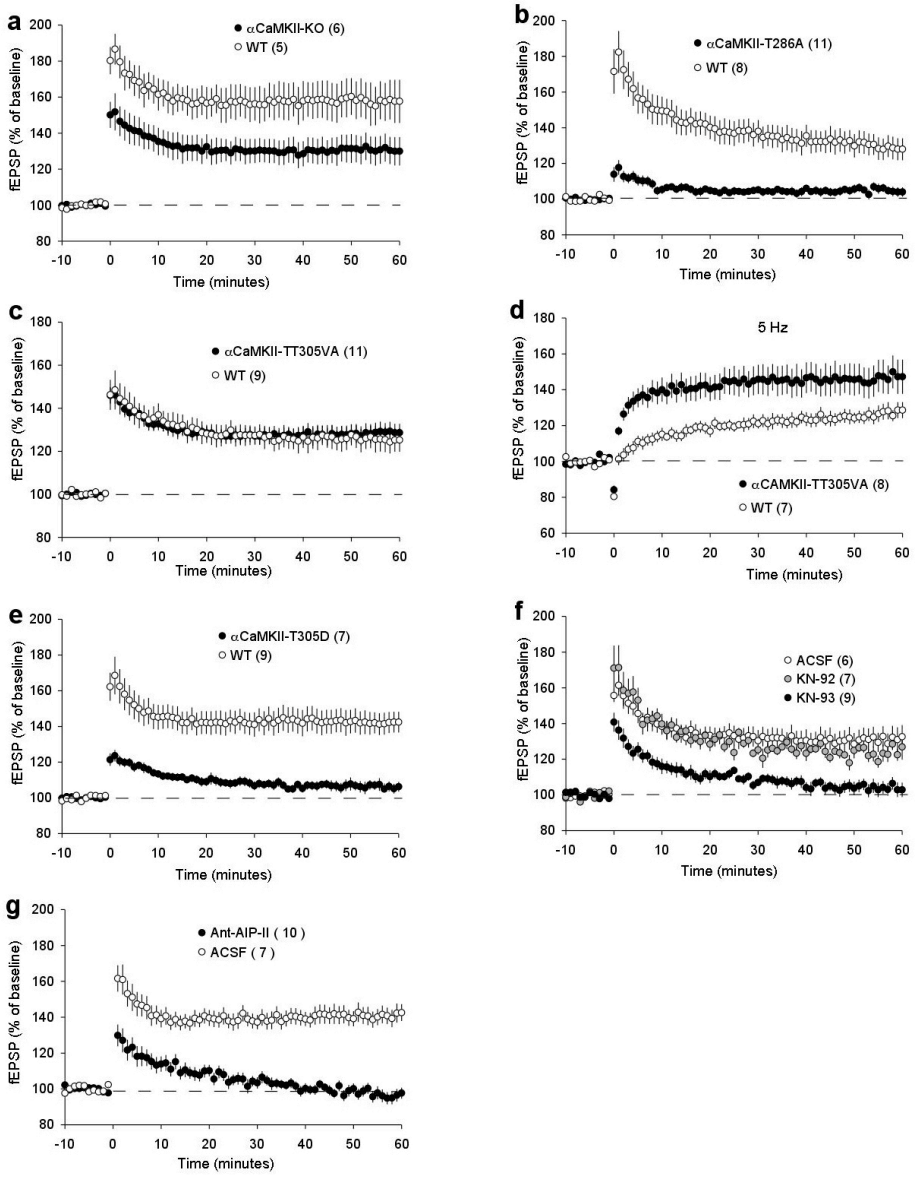
Taken together, our experiments suggest that Synapsin S603 phosphorylation and αCaMKII activity are not required for short-term presynaptic plasticity measures such as augmentation and synaptic fatigue during repetitive stimulation. Specifically, we have demonstrated that at hippocampal CA3-CA1 synapses, αCaMKII functions independent of its kinase activity to modulate short-term presynaptic plasticity by limiting the number of presynaptic docked neurotransmitter vesicles. Therefore, this is the first demonstration that αCaMKII also plays a non-enzymatic role in the synapse.

Acknowledgements
We greatly appreciate the help from Heinz Beck, Malte Merkens, Roger Anwyl, Kevin Wang and Roger Colbran for their (hands-on) advice on the use of CaMKII inhibitors, and from Gerard Borst and Steven Kushner for stimulating discussions and critically reading the manuscript. We thank Minetta Elgersma, Hans van der Burg (ErasmusMC, Neuroscience) and Edward Phillips (Neurobiology, UAB) for technical support. This work was supported by grants from NWO-ZonMW (TOP, VIDI) and Neuro-BSIK to YE. We also thank the assistance of the UAB Neuroscience Cores (P30-NS47466, P30-HD38985, P30-NS57098).

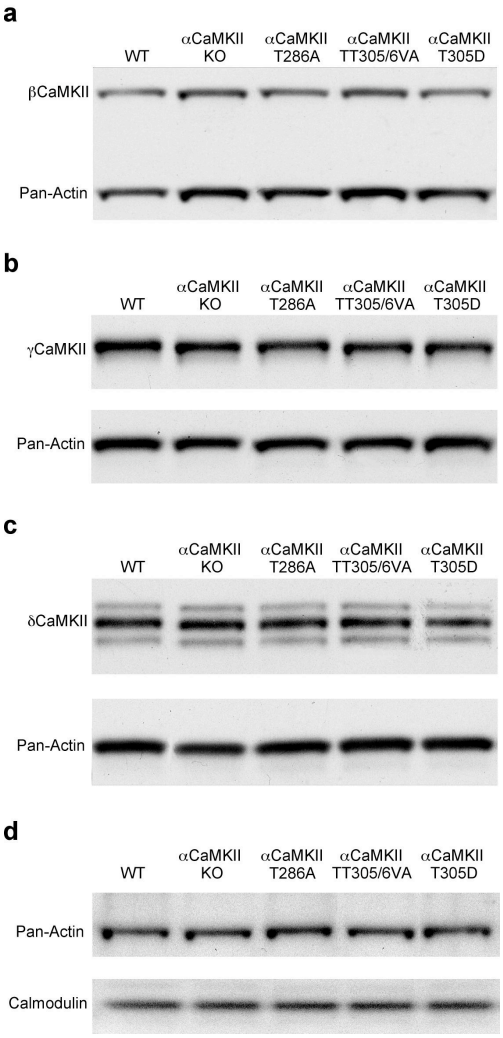
References

1. Bennett, M.K., Erondu, N.E. & Kennedy, M.B. Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. *J. Biol. Chem.* **258**, 12735-12744 (1983).
2. Benfenati, F., *et al.* Synaptic vesicle-associated Ca2+/calmodulin-dependent protein kinase II is a binding protein for synapsin I. *Nature* **359**, 417-420 (1992).
3. Erondu, N.E. & Kennedy, M.B. Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain. *J Neurosci* **5**, 3270-3277 (1985).
4. Chapman, P.F., Frenguelli, B.G., Smith, A., Chen, C.M. & Silva, A.J. The alpha-Ca2+/calmodulin kinase II: a bidirectional modulator of presynaptic plasticity. *Neuron* **14**, 591-597 (1995).
5. Hinds, H.L., Goussakov, I., Nakazawa, K., Tonegawa, S. & Bolshakov, V.Y. Essential function of alpha-calcium/calmodulin-dependent protein kinase II in neurotransmitter release at a glutamatergic central synapse. *Proc Natl Acad Sci U S A* **100**, 4275-4280 (2003).
6. Giese, K.P., Fedorov, N.B., Filipkowski, R.K. & Silva, A.J. Autophosphorylation at Thr286 of the a calcium-calmodulin kinase II in LTP and learning. *Science* **279**, 870-873 (1998).
7. Elgersma, Y., *et al.* Inhibitory autophosphorylation of CaMKII controls PSD association, plasticity, and learning. *Neuron* **36**, 493-505 (2002).
8. Elgersma, Y., Sweatt, J.D. & Giese, K.P. Mouse genetic approaches to investigating calcium/calmodulin-dependent protein kinase II function in plasticity and cognition. *J Neurosci* **24**, 8410-8415 (2004).
9. Silva, A.J., Stevens, C.F., Tonegawa, S. & Wang, Y. Deficient hippocampal long-term potentiation in alpha-calcium- calmodulin kinase II mutant mice. *Science* **257**, 201-206 (1992).
10. Zucker, R.S. & Regehr, W.G. Short-term synaptic plasticity. *Annu Rev Physiol* **64**, 355-405 (2002).
11. Sumi, M., *et al.* The newly synthesized selective Ca2+/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. *Biochem Biophys Res Commun* **181**, 968-975 (1991).
12. Ishida, A., Kameshita, I., Okuno, S., Kitani, T. & Fujisawa, H. A novel highly specific and potent inhibitor of calmodulin-dependent protein kinase II. *Biochem Biophys Res Commun* **212**, 806-812 (1995).
13. Schikorski, T. & Stevens, C.F. Morphological correlates of functionally defined synaptic vesicle populations. *Nat Neurosci* **4**, 391-395 (2001).
14. Tyler, W.J. & Pozzo-Miller, L.D. BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. *J Neurosci* **21**, 4249-4258 (2001).
15. Borst, J.G. & Sakmann, B. Calcium influx and transmitter release in a fast CNS synapse. *Nature* **383**, 431-434 (1996).

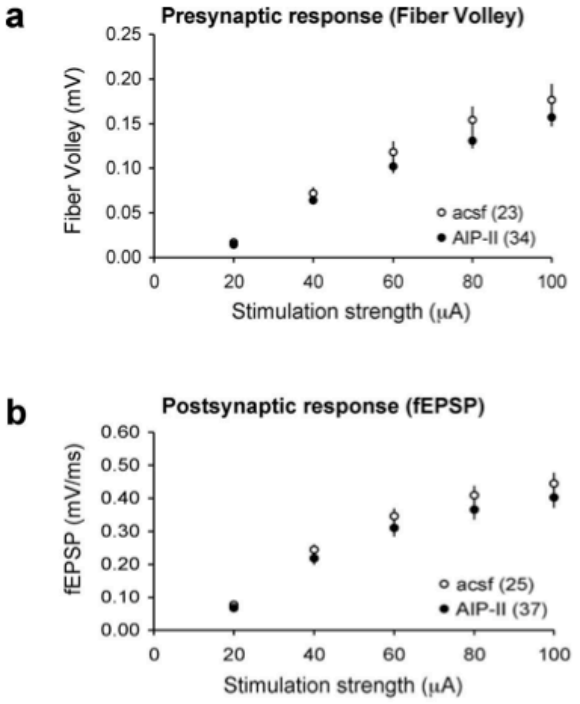
Supplementary Figures



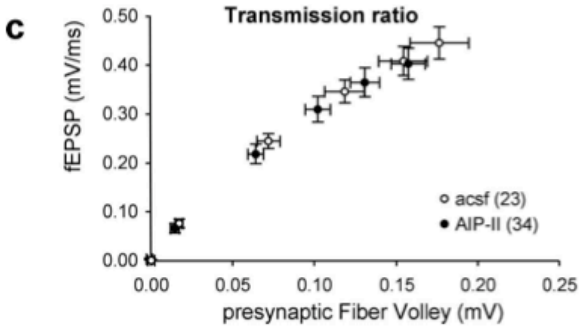
Supplementary Figure 1. *Long-term potentiation at Schaffer Collateral CA1 synapses in Camk2a mutant mice after back-crossing in C57BL6* ***(a-e)*** *and in pharmacologically treated wild-type slices* ***(f,g).*** *LTP in all experiments was induced by four bursts of high frequency stimulation (four 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 Camk2a$^{-/-}$ mice, as observed previously[1-3].*
(b,e) *LTP is absent in Camk2a^{T286A} and Camk2a^{T305D} mice, as reported previously[1, 4].*
(c,d) *LTP in Camk2a$^{TT305/6VA}$ mice is normal upon strong stimulation (4 theta burst LTP) but increased upon weak stimulation (5Hz), consistent with previous findings[1, 5].*
(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.*

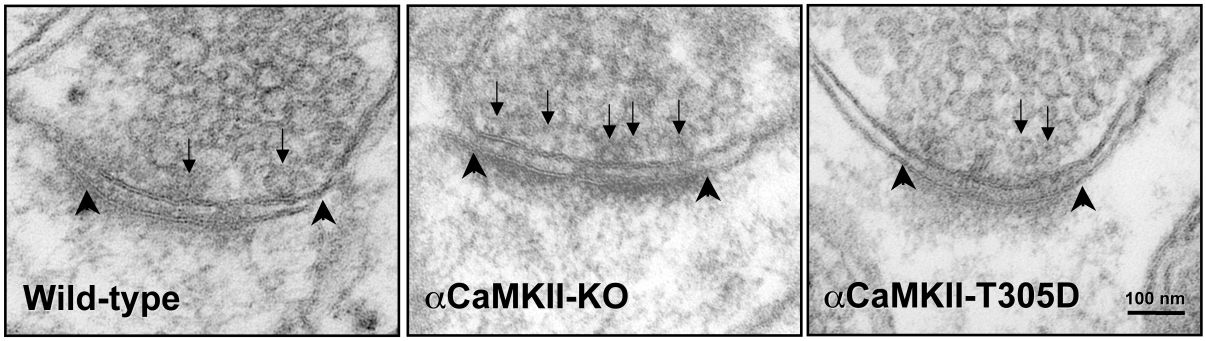


Supplementary Figure 2*. No changes in synaptic βCaMKII, γCaMKII, δCaMKII and Calmodulin in Camk2a mutants or point mutants. Synaptosomes were isolated as described (**Supplementary methods**), and analyzed by Western blotting. Each sample contains pooled fractions from 4 independent isolations.*



Supplementary Figure 3. *Synaptic transmission is not affected by Ant-AIP-II.* ***(a)*** *Presynaptic excitability is not significantly affected by Ant-AIP-II ($F_{1,55}$=1.4; p=0.24 Repeated Measures ANOVA).* ***(b)*** *Postsynaptic fEPSP responses are not affected by Ant-AIP-II ($F_{1,60}$=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[6]. Error bars represent SEM. See **Supplementary methods** for cited references.*





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. Camk2a$^{-/-}$ have more docked vesicles at the active zone than wild-type mice and Camk2a^{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 αCaMKII, we made use of a *Camk2a* mutant, in which *Camk2a* exon 2 is deleted[1]. 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 *Camk2a* knock-out (*Camk2a*$^{-/-}$) line[1]. To study the role of autophosphorylation and activity of αCaMKII, we made use of three different mutants in which the endogenous *Camk2a* was mutated using ES cell-mediated gene targeting. In the first mutant (T286A), *Camk2a* Thr286 is substituted by alanine[4]. This mutation results in a kinase that is unable to switch to its Ca^{2+}/CaM-independent state. In the *Camk2a*$^{TT305/306VA}$ mutant, *Camk2a* Thr305 and Thr306 are substituted by non-phosphorylatable amino acids (respectively, Valine and Alanine) to block inhibitory autophosphorylation[1]. In the *Camk2a*^{T305D} mutant, Thr305 is substituted by negative charged Aspartate. This mimics persistent Thr305 phosphorylated αCaMKII, which interferes with Ca^{2+}/CaM binding, thus blocking αCaMKII activation in this mutant[1]. For a review of previous reported phenotypes see[3].

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 appropriate (see above) 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 and analysis 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_2, 5% CO_2 at 30°C. ACSF contained (in mM) 120 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ 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[7], 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 μM) and Ant-AIP-II (4 μM) 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 recording 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 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[8-10]. We used a random sampling method from single thin sections as described[11], since we previously showed that data obtained with this method correlates well with an unbiased serial sectioning approach[12]. 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 one-vesicle-diameter (~50 nm) distance from the active zone, according to criteria developed by Reese and colleagues[8], 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 *Camk2a*$^{-/-}$ and 54 *Camk2a*^{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[12].

Western blot analysis

Synaptosomes were isolated as described previously[1]. The concentration of the synaptosomes was adjusted to 1 mg/ml. Western blots were probed with antibodies directed against Thr286/287-α/β-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), βCaMKII (CB-β1, 1:2000; Zymed), δCaMKII (sc-5392, 1:200; Santa Cruz), γCaMKII (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 100th 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 post-hoc 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.

References

1. Elgersma, Y., *et al.* Inhibitory autophosphorylation of CaMKII controls PSD association, plasticity, and learning. *Neuron* **36**, 493-505 (2002).
2. Hinds, H.L., Tonegawa, S. & Malinow, R. CA1 long-term potentiation is diminished but present in hippocampal slices from alpha-CaMKII mutant mice. *Learn Mem* **5**, 344-354 (1998).
3. Elgersma, Y., Sweatt, J.D. & Giese, K.P. Mouse genetic approaches to investigating calcium/calmodulin-dependent protein kinase II function in plasticity and cognition. *J Neurosci* **24**, 8410-8415 (2004).
4. Giese, K.P., Fedorov, N.B., Filipkowski, R.K. & Silva, A.J. Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. *Science* **279**, 870-873 (1998).
5. Zhang, L., *et al.* Hippocampal synaptic metaplasticity requires inhibitory autophosphorylation of Ca2+/calmodulin-dependent kinase II. *J Neurosci* **25**, 7697-7707 (2005).
6. Lengyel, I., *et al.* Autonomous activity of CaMKII is only transiently increased following the induction of long-term potentiation in the rat hippocampus. *Eur J Neurosci* **20**, 3063-3072 (2004).
7. Chapman, P.F., Frenguelli, B.G., Smith, A., Chen, C.M. & Silva, A.J. The alpha-Ca2+/calmodulin kinase II: a bidirectional modulator of presynaptic plasticity. *Neuron* **14**, 591-597 (1995).
8. Dickinson-Nelson, A. & Reese, T.S. Structural changes during transmitter release at synapses in the frog sympathetic ganglion. *J Neurosci* **3**, 42-52 (1983).
9. Pozzo-Miller, L.D., *et al.* Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. *J Neurosci* **19**, 4972-4983 (1999).
10. Schikorski, T. & Stevens, C.F. Morphological correlates of functionally defined synaptic vesicle populations. *Nat Neurosci* **4**, 391-395 (2001).
11. Tyler, W.J. & Pozzo-Miller, L.D. BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. *J Neurosci* **21**, 4249-4258 (2001).
12. Kushner, S.A., *et al.* Modulation of presynaptic plasticity and learning by the H-ras/extracellular signal-regulated kinase/synapsin I signaling pathway. *J Neurosci* **25**, 9721-9734 (2005).

Chapter III

βCaMKII controls the direction of plasticity at parallel fiber – Purkinje cell synapses.

Geeske M. van Woerden*, Freek E. Hoebeek*, Zhenyu Gao, Raghavendra Y. Nagaraja, Casper C. Hoogenraad, Steven A. Kushner, Christian Hansel, Chris I. De Zeeuw and Ype Elgersma

Published in Nat Neurosci. 2009 July;12(7):823-5

* These authors contributed equally

Here we show that βCaMKII, the predominant CaMKII isoform of the cerebellum, plays an essential role in controlling the direction of plasticity at the parallel fiber–Purkinje cell synapse: a protocol that induces synaptic depression in wild–type mice, results in synaptic potentiation in *Camk2b* knock–out mice, and *vice versa.* These findings provide us with unique experimental insight into the mechanisms that transduce graded calcium signals into either synaptic depression or potentiation.

The ability to strengthen and weaken synaptic connections is essential for memory formation. According to the Bienenstock, Cooper, and Munro model of synaptic plasticity, the magnitude and direction of synaptic weight changes is not only affected by the temporal pattern of calcium influx, but also by the ability of the synapse to change the modification threshold for synaptic depression and potentiation[1]. However, the precise mechanisms by which a synapse controls the direction of synaptic weight change are still poorly understood.

Calcium–calmodulin dependent kinase type II (CaMKII) plays an essential role in transducing neuronal calcium signals. The CaMKII holoenzyme is a heteromeric protein complex, composed of a cell–type dependent ratio of αCaMKII to βCaMKII isoforms. In the adult hippocampus and neocortex, αCaMKII is the predominant isoform[2], where its quantity and autophosphorylation status determine the amplitude and induction threshold of long–term potentiation (LTP)[3-5]. In contrast, αCaMKII is required for long–term depression (LTD), but not LTP, at cerebellar parallel fiber–Purkinje cell synapses[6]. Notably, although βCaMKII is the most prominent cerebellar isoform[2], the function of βCaMKII at this synapse is unknown. In addition, although *in vitro* studies show that changes in the α– to βCaMKII ratio have opposing effects on unitary synaptic strength[7], it is unknown to what extent changes in the subunit ratio would affect synaptic plasticity *in vivo*.

To elucidate the role of βCaMKII in synaptic plasticity, we generated a *Camk2b* knock–out mouse by inserting the neomycin resistance gene in the βCaMKII regulatory domain at exon 11 (**Supplementary Fig. 1**). *In situ* hybridization and semi–quantitative RT–PCR analysis of these mice showed loss of full–length *Camk2b* mRNA (**Fig. 1** and **Supplementary Fig. 2**). In addition, immunostaining and Western blot analysis revealed a total loss of βCaMKII immunoreactivity and no presence of a truncated βCaMKII protein in these mutants (**Fig. 1c**,**e**). The loss of βCaMKII did not cause up–regulation of *Camk2a* mRNA or protein (**Fig. 1b** and **Supplementary Fig. 2**). Consequently, total CaMKII activity was significantly reduced, particularly in the cerebellum (reduction of 51% ($P<0.0001$) for Ca^{2+}/CaM–dependent activity and 41% ($P<0.005$) for Ca^{2+}/CaM–independent activity; **Supplementary Fig. 1b**), altogether suggesting a successful knock–out of the *Camk2b* gene.

Even though adult *Camk2b*$^{-/-}$ mice showed no differences in survival (measured from weaning till 8 months of age) or general health, they showed pronounced ataxia (**Supplementary Movie 1**), and severe deficits on the accelerating rotarod test and balance beam test **(Fig. 1g**, **Supplementary Fig. 3**). Ataxia commonly arises from cerebellar dysfunction, and is often observed in mutants with immature or degenerated Purkinje cells. However, Thionin staining of brain slices from adult mice revealed no gross differences in overall brain development between wild–type mice and *Camk2b*$^{-/-}$ mutants at the light microscopy level (**Fig. 1a**). Moreover, Purkinje cells of *Camk2b*$^{-/-}$ mutants did not show significant changes in the complexity of dendritic branching, and the density of spines, nor in Purkinje cell spine maturity (spine neck length and head width), indicating

that the loss of βCaMKII does not affect gross brain development or the maturation of Purkinje cells *in vivo* (**Fig. 1f**, **Supplementary Fig. 4**).

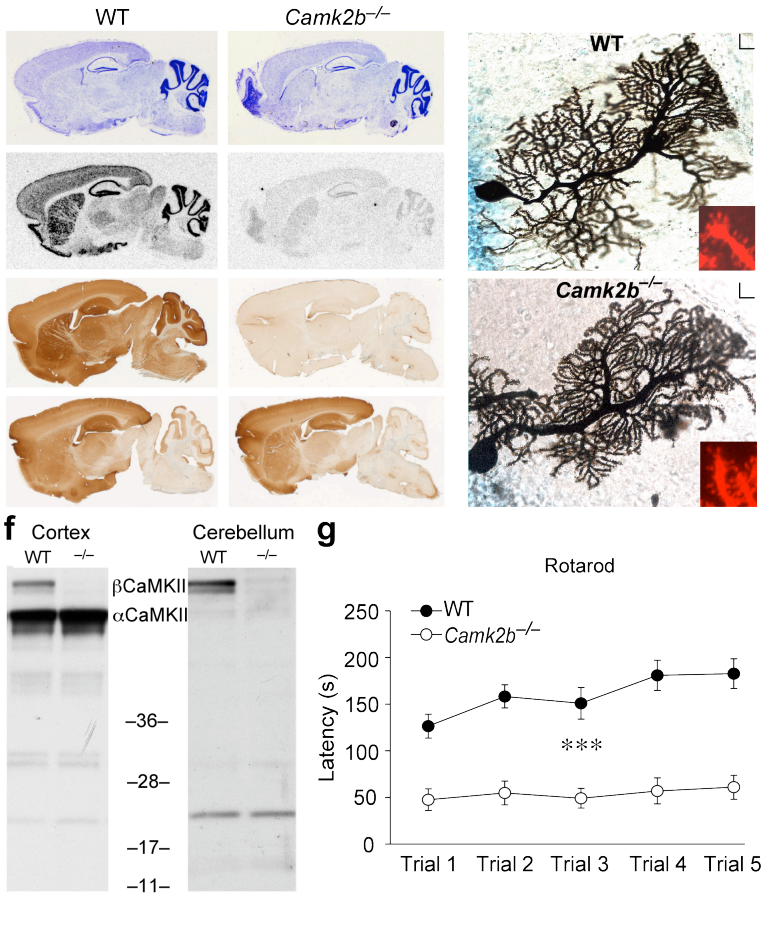


Fig. 1. Basal characterization of the Camk2b$^{-/-}$ mutant. ***(a)*** *Thionin staining shows no apparent morphological changes in Camk2b$^{-/-}$ brains.* ***(b)*** *In situ analysis shows decreased levels of 3' UTR Camk2b mRNA in Camk2b$^{-/-}$ mutant mice, indicating a successful gene disruption.* ***(c,d)*** *Immunocytochemistry using α– and βCaMKII–isoform specific antibodies, shows complete loss of βCaMKII immunoreactivity in Camk2b$^{-/-}$ brains* ***(c)*** *which is not compensated by up–regulation of αCaMKII immunoreactivity* ***(d)****.* ***(e)*** *Western blot analysis of wild–type and mutant cortex and cerebellum using a N–terminal pan–CaMKII antibody revels no truncated βCaMKII product in Camk2b$^{-/-}$ mutants.* ***(f)*** *Analysis of Purkinje cell morphology using Golgi–Cox staining and diOlistic labeling (inlay), showed no differences in arborization and spine density, neck length and head width (see for quantification* ***Supplementary Fig. 4****). Scale bar: 10mM.* ***(g)*** *Motor performance assessed by an accelerating rotarod. Camk2b$^{-/-}$ mice (n=16) stay on the rod significantly shorter than wild–type littermates (n=16) and do not increase their performance upon training (effect of genotype: P<0.005; interaction training x genotype P<0.05; Repeated measures ANOVA). Depicted is the latency until the mice fell from the rod. All animal procedures were approved by a Dutch ethical committee for animal experiments (DEC).*

Purkinje cells are the sole output of the cerebellar cortex, and plasticity at the parallel fiber–Purkinje cell synapse is generally believed to mediate cerebellar motor learning. To investigate the role of βCaMKII in parallel fiber–Purkinje cell plasticity, we performed *in vitro* whole–cell recordings of Purkinje cells from adult wild–type and *Camk2b$^{-/-}$* mice. The ability to induce LTD was tested by paired parallel fiber and climbing fiber stimulation at 1Hz for 5 minutes[8,9]. This stimulation protocol indeed resulted in significant LTD in wild–type mice (83 ± 5%; $P < 0.01$), but failed to induce LTD in *Camk2b$^{-/-}$* mice. In fact, it resulted in significant LTP (112 ± 3%; $P< 0.01$) in these mutants (**Fig. 2a**), similar to what has been reported previously for *Camk2a$^{-/-}$* mice[6]. These results suggest that a critical amount of both αCaMKII and βCaMKII is required for the induction of cerebellar LTD.

We next tested the role of βCaMKII in cerebellar LTP, by applying the same parallel fiber stimulus, but now in the absence of climbing fiber stimulation[10]. This postsynaptic form of LTP requires lower calcium transients than LTD induction[9,10]. Using this protocol, we indeed obtained significant LTP in wild–type Purkinje cells (120 ± 3%; $P< 0.001$), but surprisingly *Camk2b$^{-/-}$* Purkinje cells now showed robust LTD (84 ± 4%; $P< 0.01$; **Fig. 2b**). To our knowledge, this is the first report of a genetic or pharmacological manipulation that bidirectionally inverts the polarity of changes in synaptic strength.

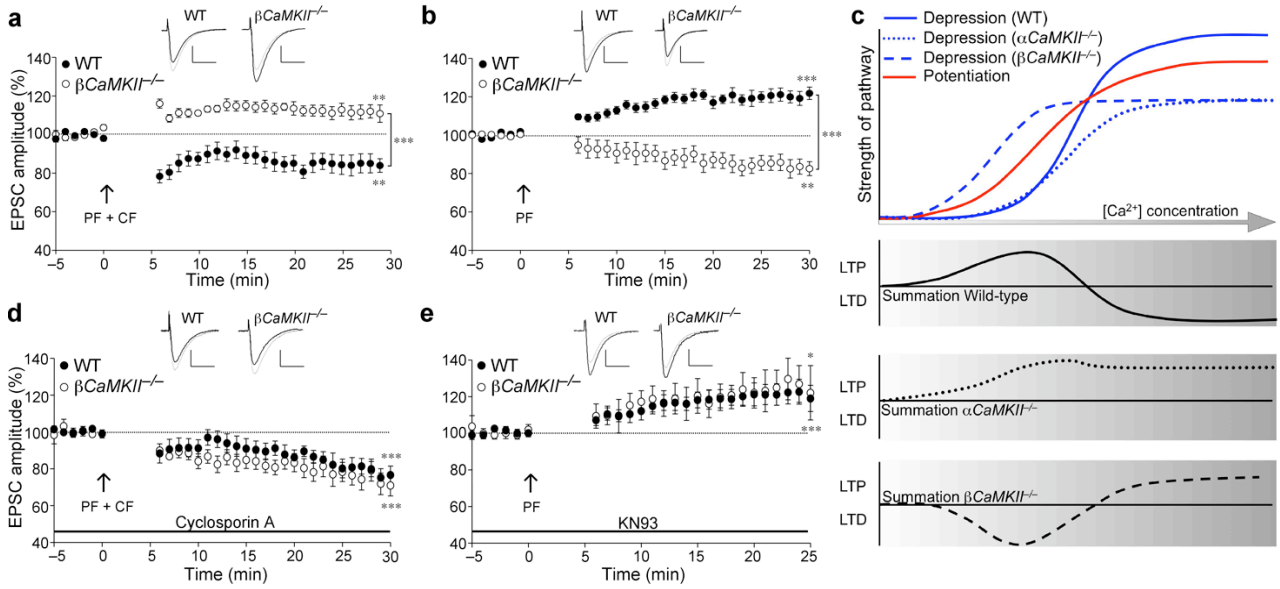


Fig. 2 Bidirectional inversion of plasticity at the parallel fiber–Purkinje cell synapse. ***(a)*** *Paired parallel fiber and climbing fiber stimulation yields LTD in wild–type (n=12) Purkinje cells but LTP in Camk2b$^{-/-}$ (n=11) Purkinje cells.* ***(b)*** *Parallel fiber stimulation only, results in LTP in wild–type (n=11) Purkinje cells but yields LTD in Camk2b$^{-/-}$ (n=13) Purkinje cells.* ***(c)*** *Schematic model, visualizing how changes in the CaMKII driven depression pathway (top) can result in the different plasticity curves as observed in wild–type, Camk2a$^{-/-}$and Camk2b$^{-/-}$ mice (bottom). The model illustrates that a reduced strength of the kinase driven depression pathway ('down–shift'), combined with a reduced threshold for calcium activation ('left–shift') results in an inversion of the plasticity curve in Camk2b$^{-/-}$ mice.* ***(d)*** *LTD is rescued in Camk2b$^{-/-}$slices in the presence of PP2B inhibitor Cyclosporin A (wild–type: n=6; Camk2b$^{-/-}$: n=13).* ***(e)*** *LTP is rescued in Camk2b$^{-/-}$slices using the CaMKII inhibitor KN93 (wild–type: n=12; Camk2b$^{-/-}$: n=7). Insets show representative traces before (grey line) and after (black line) tetanization (scale bars 100pA/10ms). Error bars represent SEM. Asterisks with brackets indicate statistical significance between wild–type and mutant slices (Student's t–test over last 5 min). Asterisks without brackets indicate significant difference from baseline (paired t–test). *P<0.05; **P<0.01; ***P<0.005.*

Even though the plasticity induced by the protocols described above is known to be expressed postsynaptically[9-12], the observed changes in synaptic plasticity could potentially result from additional presynaptic changes. However, *Camk2b$^{-/-}$* mutants showed no difference in basal excitatory synaptic transmission and Purkinje cells were innervated by a single climbing fiber (**Supplementary Fig. 5**, **6**), suggesting that the bidirectional inversion of parallel fiber–Purkinje cell plasticity is indeed caused by changes in postsynaptic plasticity.

To better understand how the loss of βCaMKII can result in a bidirectional inversion of plasticity at the parallel fiber–Purkinje cell synapse, we propose the following plasticity rules that collectively provide a model which fully accounts for the observed data: First, the synaptic depression and potentiation pathways are independent competing processes driven by kinases and phosphatases, respectively[9,10,12], each regulated by a distinct sensitivity for calcium. LTP is generated when phosphatase activity outweighs kinase activity, and LTD is generated when kinase activity outweighs phosphatase activity (schematically depicted in **Fig. 2c**, **Supplementary Fig. 7**). Second, since LTP induction is not affected by deleting the *Camk2a* gene[6], nor by inhibition of CaMKII kinase activity[11], we assume that αCaMKII and βCaMKII activity is contributing to the synaptic depression pathway only (**Fig. 2c**, **Supplementary Fig. 7**). Hence, loss of either αCaMKII or βCaMKII

selectively decreases the depression driving force, and patterns of stimulation that normally produce LTD, will therefore yield LTP in the *Camk2a*$^{-/-}$ mutant[6] or *Camk2b*$^{-/-}$ mutant (**Fig. 2a**). Consequently, we predicted that blocking the opposing potentiation pathway, by inhibiting calcium sensitive phosphatase PP2B (calcineurin) activity[12], should restore LTD induction in the *Camk2b*$^{-/-}$ mutant. Indeed, the presence of cyclosporin A during LTD induction, yielded a significant induction of LTD in the *Camk2b*$^{-/-}$ mutant (**Fig. 2d**). This indicates that the PP2B–driven synaptic potentiation pathway is in direct competition with the CaMKII–driven synaptic depression pathway, and that loss of βCaMKII activity can be compensated by inactivating PP2B activity.

Although the aforementioned plasticity rules readily explain the shift from LTD to LTP as seen in the *Camk2a*$^{-/-}$[6] and *Camk2b*$^{-/-}$ mutants, they are not sufficient to explain why an LTP inducing protocol results in LTD in *Camk2b*$^{-/-}$ mutants. An inversion of the plasticity curve as observed in the *Camk2b*$^{-/-}$ mutant, can be achieved only if the depression driving force in *Camk2b*$^{-/-}$ mutants is shifted to the left (indicated in **Fig. 2c**), reflecting a lower threshold for activation of this pathway. As αCaMKII is a major driver of the depression pathway[6], this model would predict that activation of αCaMKII is facilitated in the *Camk2b*$^{-/-}$ mutant, leading to precocious induction of the LTD pathway under low calcium conditions. If this is true, the presence of a CaMKII inhibitor under LTP inducing conditions should rescue the LTP deficits in the *Camk2b*$^{-/-}$ mutant (**Fig. 2c**, **Supplementary Fig. 7**). This was indeed the case: addition of the CaMKII inhibitor KN93 restored LTP in *Camk2b*$^{-/-}$slices to wild–type levels (**Fig. 2e**). These results confirm that the induction of the LTD pathway under low calcium conditions in the *Camk2b*$^{-/-}$ mutant is indeed caused by the inadvertent activation of αCaMKII.

What could be the cause of the reduced threshold of αCaMKII activation in *Camk2b*$^{-/-}$ mutants? Notably, the reduced threshold for αCaMKII activation is not caused by increased expression of αCaMKII (**Fig. 1**, **Supplementary Fig. 8**). In addition, there is not a strong increase in basal levels of autonomously activated αCaMKII, as the reduction of calcium–dependent activity was not significantly different from the reduction of calcium–independent activity (P=0.5), and no increase in T286P–αCaMKII was observed (**Supplementary Fig. 1**, **8**). Moreover, the competitive CaMKII inhibitor KN93, which rescued the LTP deficit, can not inhibit αCaMKII if it is already in the autonomously active state, arguing strongly against high basal levels of autonomously active αCaMKII in *Camk2b*$^{-/-}$ mutants.

A more likely explanation comes from the biochemical differences between αCaMKII and βCaMKII. βCaMKII contains an additional domain which enables βCaMKII to cluster the entire CaMKII holoenzyme to F–actin[13-15]. Since all cerebellar αCaMKII is associated with βCaMKII in wild–type mice (**Supplementary Fig. 8**), the loss of βCaMKII will cause a loss of αCaMKII clustering to F–actin, potentially increasing the availability of CaMKII holoenzymes[13,15]. In addition, elegant *in vitro* experiments showed that translocation to the PSD is 4 times shorter for αCaMKII homo–oligomers as compared to mixed αCaMKII/βCaMKII hetero–oligomers, and even 24 times shorter as compared to βCaMKII homo–oligomers[15]. Such a substantial reduction in the translocation rate of CaMKII holoenzymes could well explain the reduced threshold for CaMKII activation in the *Camk2b*$^{-/-}$ mutants.

Taken together, we show that *Camk2b* is not an essential gene for overall brain development or Purkinje cell maturation, but rather plays an essential role in motor coordination and cerebellar plasticity. Although we cannot rule out that these defects are potentially caused by developmental changes, the observation that we can rescue both the

LTD and LTP deficits by pharmacology suggests a direct role of βCaMKII in regulating bidirectional synaptic plasticity at the parallel fiber–Purkinje cell synapse. Specifically, we show that parallel fiber–Purkinje cell plasticity is regulated by a balanced and integrated coordination of kinase and phosphatase activity, and that βCaMKII modulates the polarity of synaptic plasticity in two functionally distinct ways: Like αCaMKII[6], βCaMKII activity is required for driving the synaptic depression pathway under high calcium conditions. However under low calcium conditions, βCaMKII prevents activation of this pathway. Overall, these findings provide us with a unique insight in the molecular mechanisms governing the rules for bidirectional synaptic plasticity.

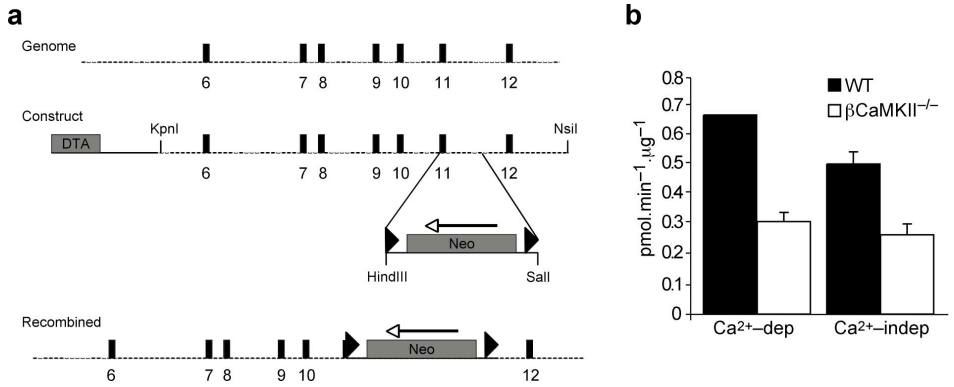
References

1. Bienenstock, E.L., Cooper, L.N. & Munro, P.W. *J Neurosci* **2**, 32-48 (1982).
2. Erondu, N.E. & Kennedy, M.B. *J Neurosci* **5**, 3270-7 (1985).
3. Silva, A.J., Stevens, C.F., Tonegawa, S. & Wang, Y. *Science* **257**, 201-6 (1992).
4. Elgersma, Y. *et al. Neuron* **36**, 493-505 (2002).
5. Giese, K.P., Fedorov, N.B., Filipkowski, R.K. & Silva, A.J. *Science* **279**, 870-3 (1998).
6. Hansel, C. *et al. Neuron* **51**, 835-43 (2006).
7. Thiagarajan, T.C., Piedras-Renteria, E.S. & Tsien, R.W. *Neuron* **36**, 1103-14 (2002).
8. Tanaka, K. *et al. Neuron* **54**, 787-800 (2007).
9. Coesmans, M., Weber, J.T., De Zeeuw, C.I. & Hansel, C. *Neuron* **44**, 691-700 (2004).
10. Lev-Ram, V., Wong, S.T., Storm, D.R. & Tsien, R.Y. *Proc Natl Acad Sci U S A* **99**, 8389-93 (2002).
11. Kakegawa, W. & Yuzaki, M. *Proc Natl Acad Sci U S A* **102**, 17846-51 (2005).
12. Belmeguenai, A. & Hansel, C. *J Neurosci* **25**, 10768-72 (2005).
13. Shen, K., Teruel, M.N., Subramanian, K. & Meyer, T. *Neuron* **21**, 593-606. (1998).
14. Okamoto, K., Narayanan, R., Lee, S.H., Murata, K. & Hayashi, Y. *Proc Natl Acad Sci U S A* **104**, 6418-23 (2007).
15. Shen, K. & Meyer, T. *Science* **284**, 162-6 (1999).

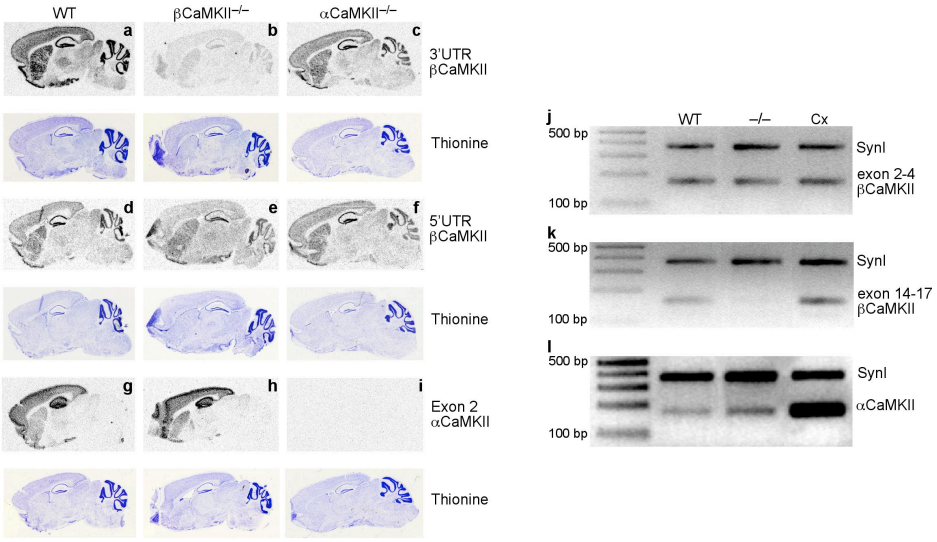
Acknowledgements

We are grateful to D. Andreev, G. Buitendijk, P. Plak, A. Belmeguenai, D. Jaarsma, M. Vandeputte, M. Elgersma, E. Haasdijk, H. Goedknegt, B. Dortland, M. Aghadavoud and J. Kongasan for technical assistance and to G. Borst and R. Colbran for stimulating discussions.

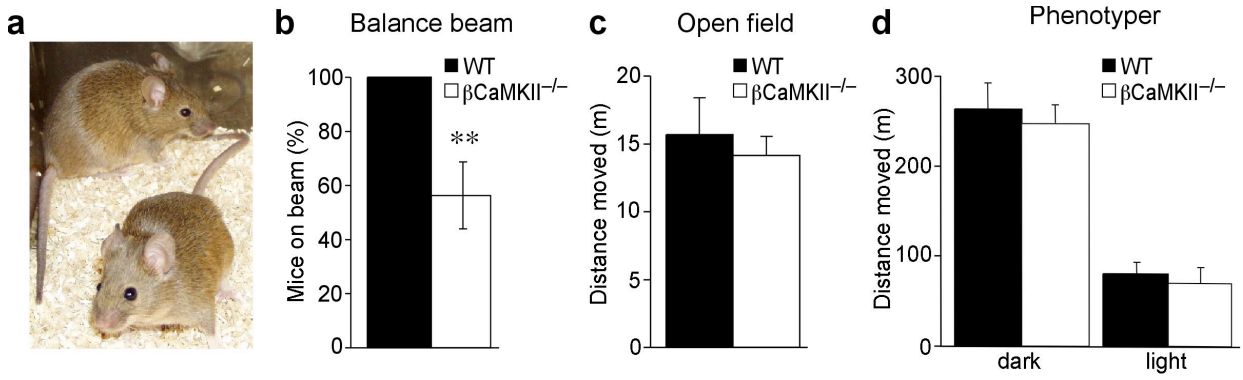
Supplementary figures and legends



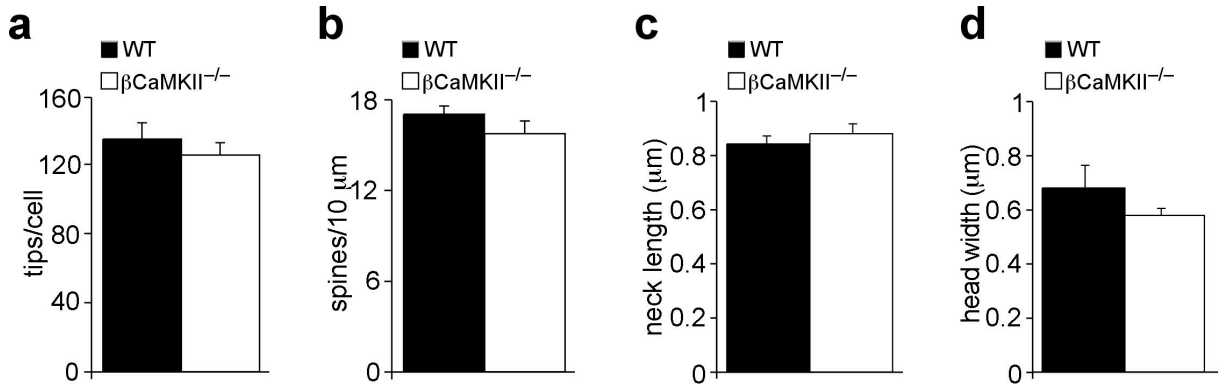
Supplementary Fig 1. Generation of the Camk2b$^{-/-}$ mutant mice. ***(a)*** *Strategy to generate the Camk2b$^{-/-}$ mutant mice. (Top) Wild-type Camk2b locus with the location of exons 6-12 depicted as black boxes. (Middle) Targeting construct used for generation of Camk2b$^{-/-}$ mutant mice with the gene encoding the Diphteria Toxin A chain (DTA) outside the homologous recombination sites and the Neomycin resistance gene (NEO) inserted in exon 11. Shown are the restriction sites used for insertion of the NEO gene and the NsiI site used to linearize the targeting construct. (Bottom) Mutant Camk2b locus after homologous recombination.* ***(b)*** *Ca^{2+}-dependent and Ca^{2+}-independent CaMKII activity are significantly ($P<0.0001$;$P<0.005$ respectively) reduced in the cerebellum of Camk2b$^{-/-}$ mutant mice ($n=5$) as compared to wild-type mice ($n=5$).*



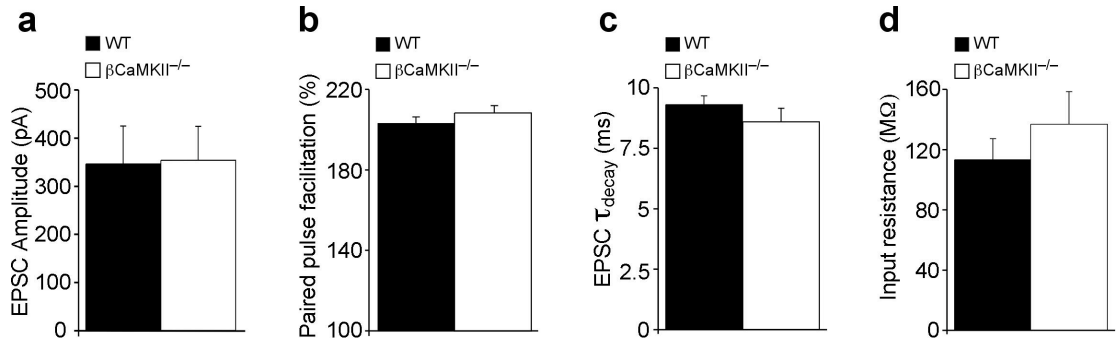
Supplementary Fig. 2. In situ hybridization and RT-PCR analysis of Camk2b and Camk2a mRNA in mutant and wild-type mice. ***(a-i)*** *In situ hybridization of Camk2a and Camk2b mRNA in wild-type (left panels), Camkb$^{-/-}$ (middle panels), and Camk2a$^{-/-}$ mice (right panels). Thionin staining (blue stained slices) was performed after the in situ hybridization to investigate brain morphology.* ***(a-f)*** *In situ hybridization shows that Camk2b mRNA is highly abundant in cerebellum, cortex, hippocampus and striatum of wild-type mice. Camk2b$^{-/-}$ mutant mice show decreased levels of 3' UTR Camk2b mRNA* ***(b)*** *but normal levels of 5' UTR Camk2b mRNA in Camk2b$^{-/-}$ mice* ***(e)****, suggesting a successful truncation of the gene.* ***(g-i)*** *Camk2a mRNA level is not affected in Camk2b$^{-/-}$ mutant mice.* ***(j-l)*** *semi-quantitative RT-PCR analysis shows normal Camk2a mRNA levels but strongly reduced 3' (exon 14-17) Camk2b mRNA levels in the cerebellum of Camk2b$^{-/-}$ mutant mice. PCR primers were directed against exon 2-4* ***(j)*** *and exon 14-17* ***(k)*** *of the Camk2b (which are up- or downstream of the inserted NEO gene, respectively) and directed against exon 2-5 for the Camk2a gene* ***(i)****. As a quantitative control, primers against Synapsin I mRNA were included. The 'Cx' lane is a RT-PCR on the same amount of mRNA (0.3 µg) derived from wild-type cortical tissue. To assure that the PCR conditions were in the linear range, a serial dilution was performed on all samples (not shown).*



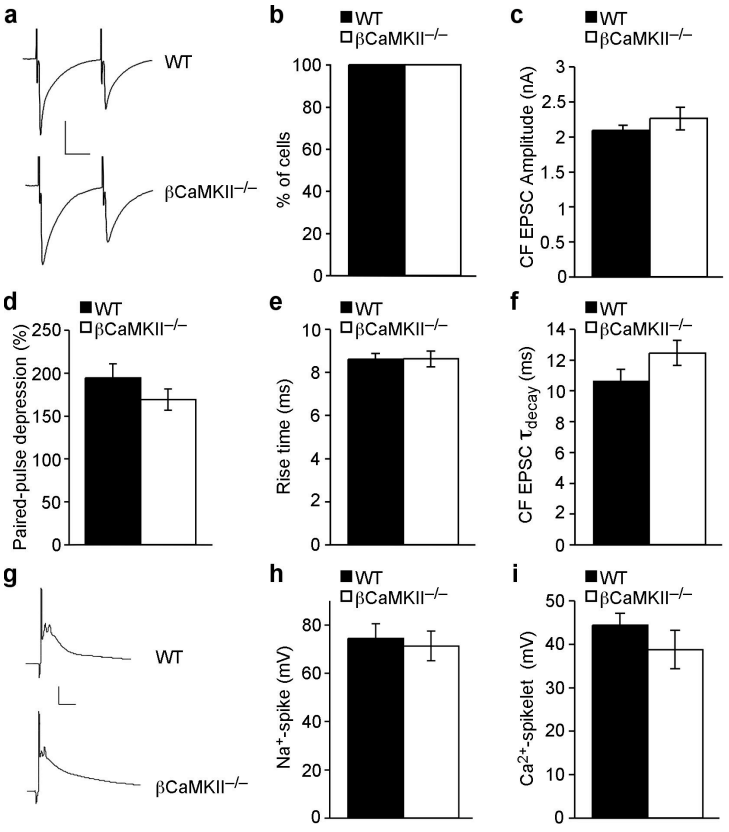
Supplementary Fig. 3. Assessment of general locomotion deficits of Camk2b$^{-/-}$ mutant mice. ***(a)*** *Camk2b$^{-/-}$ mutants (front) are viable and healthy. The mouse in the back is a littermate wild-type mouse (age 4 months). But despite their healthy appearance, Camk2b$^{-/-}$ mutants show signs of cerebellar ataxia.* ***(b)*** *Balance beam test. Indicated is the number of mice that were able to stay on a 1.5 cm thick square beam. All wild-type mice (n=16) were able to stay on the beam for 60 sec, whereas only 9/16 of the Camk2b$^{-/-}$ mutant mice could stay on the beam for one minute (P<0.01, paired t-test). Error bars are standard errors of mean taken from binomial distribution.* ***(c,d)*** *The balance beam and rotarod deficits are not caused by a lack of desire to move, as Camk2b$^{-/-}$ mice move a similar distance as wild-type mice in the open-field* ***(b)*** *(n= 5/5; P=0.6) and in an home cage-like environment (Phenotyper, Noldus Information Technology, The Netherlands)* ***(c)*** *(n=12/12; P=0.7 during dark phase, P=0.6 during light phase). Error bars represent standard error of the mean.*



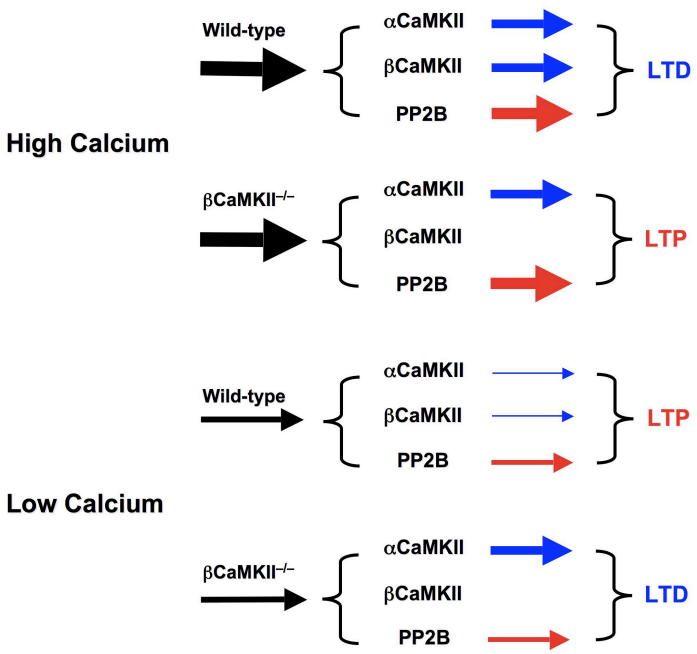
Supplementary Fig. 4. Assessment of cerebellar Purkinje cell morphology in Camk2b$^{-/-}$ mutant mice. *Quantification of the arborization* ***(a)*** *and number of spines* ***(b)*** *of Golgi-Cox stained Purkinje cells showed no significant differences between wild-type mice and mutants (P=0.4 and P=0.2, respectively). Arborization was determined by counting the number of tips per Purkinje cell and the number of spines per 10 mm dendrite. Quantification of the neck length* ***(c)*** *and head width* ***(d)*** *of Purkinje cell spines using diOlistic labeling also showed no significant differences (P=0.5 and P=0.3, respectively). Number of mice used: WT (3); Camk2b$^{-/-}$ (3). Error bars represent standard error of the mean.*



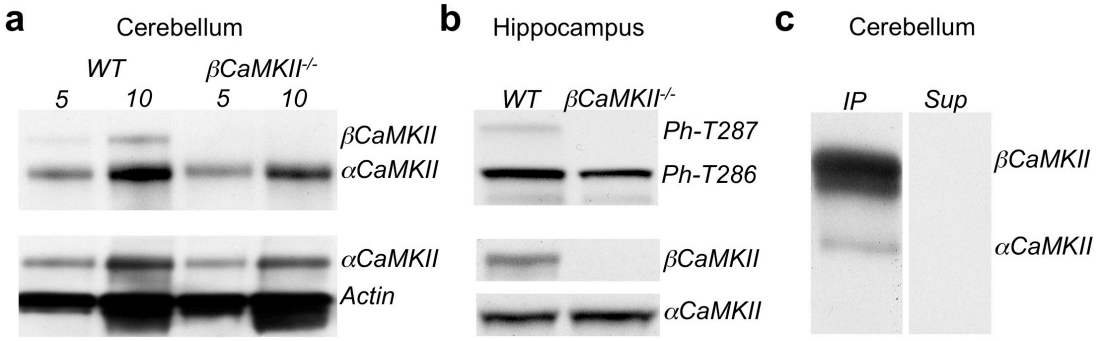
Supplementary Fig. 5. Parallel fiber-Purkinje cell transmission and basal properties of Purkinje cells are not significantly different in Camk2b$^{-/-}$ mutant mice. ***(a)*** *Camk2b$^{-/-}$ Purkinje cells show a normal EPSC amplitude (P=0.9; n=11 WT, n=9 Camk2b$^{-/-}$ mutants).* ***(b)****, Camk2b$^{-/-}$ Purkinje cells show normal paired pulse facilitation (50 ms inter-stimulus interval) (P=0.3; n=13 WT, n=13 Camk2b$^{-/-}$ mutants).* ***(c)*** *Camk2b$^{-/-}$ Purkinje cells show normal t_{decay} (single-exponential fit) of the EPSC (P=0.3; n=13 WT, n=10 Camk2b$^{-/-}$ mutants).* ***(d)*** *Camk2b$^{-/-}$ Purkinje cells show normal average input resistance (calculated by a 10mV hyperpolarizing voltage step; see supplementary methods) (P=0.4; n=9 WT, n=7 Camk2b$^{-/-}$ mutants). Error bars represent standard error of the mean.*



Supplementary Fig. 6. Climbing fiber-Purkinje cell synaptic transmission is normal in Camk2b$^{-/-}$ mutant mice. ***(a)*** *Average trace of six double CF-EPSCs for wild-type (upper) and Camk2b$^{-/-}$ (lower) cells. Horizontal scale bar indicates 20 ms; vertical indicates 1 nA.* ***(b)*** *All recorded Purkinje cells of both wild type (n=10) and Camk2b$^{-/-}$ (n=13) mice showed absence of multiple climbing fiber innervation. Shown is the percentage of Purkinje cells with mono-climbing fiber innervation.* ***(c)*** *Camk2b$^{-/-}$ Purkinje cells show normal CF-EPSC amplitudes (P=0.6; n=10 WT, n=13 Camk2b$^{-/-}$).* ***(d)*** *Camk2b$^{-/-}$ Purkinje cells show normal paired-pulse depression of the CF-EPSCs at an inter-stimulus interval of 50 ms (P=0.2; n=10 WT, n=13 Camk2b$^{-/-}$).* ***(e)*** *Camk2b$^{-/-}$ Purkinje cells show normal 10-90% rise times of the first CF-EPSC (P=1.0; n=10 WT, n=13 Camk2b$^{-/-}$).* ***(f)*** *Camk2b$^{-/-}$ Purkinje cells show normal t_{decay} (single-exponential fit) of the first CF-EPSC (P=0.2; n=10 WT, n=13 Camk2b$^{-/-}$).* ***(g)*** *Average trace of 20 CF-EPSPs for wild type (upper) and Camk2b$^{-/-}$ (lower). Horizontal scale bar indicates 20 ms; vertical indicates 20 mV.* ***(h)*** *Camk2b$^{-/-}$ Purkinje cells show normal amplitudes of Na^+-spikes in CF-EPSPs (P=0.7; n=7/7).* ***(i)*** *Camk2b$^{-/-}$ Purkinje cells show normal amplitudes of the first Ca^{2+}-spikelet in CF-EPSPs (P=0.3; n= 7/7). Error bars represent standard error of the mean.*



Supplementary Fig. 7. Role of CaMKII and PP2B in parallel fiber-Purkinje cell plasticity. *Simplified model depicting the role of αCaMKII, βCaMKII and phosphatase PP2B in LTP and LTD at the parallel fiber-Purkinje cell synapse. In wild-type Purkinje cells high calcium influx (top) activates the phosphatase PP2B driven synaptic potentiation pathway but also results in maximal induction of the CaMKII driven synaptic depression pathway, which effectively out-competes PP2B, leading to a net LTD. In contrast, in the absence of βCaMKII the synaptic depression pathway (which requires αCaMKII and βCaMKII activity) is no longer able to out-compete PP2B, resulting in net LTP. Low calcium influx (bottom) only moderately activates PP2B, but as there is no or insignificant activation of CaMKII, the net result is LTP. In Camk2b$^{-/-}$ Purkinje cells, low calcium influx results in precocious activation of αCaMKII, which is sufficient to out-compete PP2B, resulting in net LTD.*



Supplementary Fig. 8. Neither αCaMKII protein level nor its autophosphoryation is increased in Camk2b$^{-/-}$ mutants. ***(a)*** *Expression of αCaMKII protein is not increased in the cerebellum. If anything, there is a slight reduction at the protein level. Shown are Western blots of cerebellar lysates of 2 wild-type mice and 2 mutants; with 5mg and 10 mg loaded of each sample. Blots were probed with a specific αCaMKII (Chemicon; clone 6G9) or βCaMKII (Zymed; clone CB-b1) antibody.* ***(b)*** *The amount of Ph-T286-αCaMKII is not changed in the hippocampus of Camk2b$^{-/-}$ mutants. Ph-T286-αCaMKII could not be detected in the cerebellum, which is likely due to the 60-fold lower concentration of αCaMKII in cerebellar lysates (not shown).* ***(c)*** *Immunoprecipitation using a βCaMKII-specific antibody, reveals that all αCaMKII is asscoiated with βCaMKII in the cerebellum of wild-type mice. IP: immunoprecipitated fraction; Sup: supernatant fraction after immunoprecipitation.*

Supplementary Materials and Methods

Generation of Camk2b mutant mice

The *Camk2b* knock-out targeting construct was generated as follows. The *Camk2b* genomic sequence (ENSMUSG00000057897) was obtained from a public database (Ensembl) and used to design the primers for the targeting constructs. PCR fragments encompassing exon 6-11 (5.3 Kb; exon denotation according to ENSMUST00000019133) using:
5′ primer: 5′-GGTACCTGAGGAAGGTGCCAGCTCTGTCCC-3′ and
3′ primer: 5′–TTATCGATTCTCCTGTCTGTGCATCATAGAGG-3′
and exon 11-13 (6 Kb) using:
5′ primer: 5′-GCGGCCGCCTGTTAAAGGAATGGTTCTC-3′ and
3′ primer: 5′-ATGCATCTAAAAGGCAGGCAGGATGATCTGC-3′ were amplified using High Fidelity Taq Polymerase (Roche) on ES cell genomic DNA and cloned on either site of a PGK-Neomycin selection cassette. All exons were sequenced to verify that no mutations were introduced accidentally. For counter selection, a gene encoding Diphtheria toxin chain A (DTA) was inserted at the 5′ of the targeting construct. The targeting construct was linearized and electroporated into E14 ES cells (derived from 129P2 mice). Cells were cultured in BRL cell conditioned medium in the presence of Leukemia inhibitory factor (LIF). After selection with G418 (200 μg/ml), targeted clones were identified by PCR (long-range PCR from Neomycin resistance gene to the region flanking the targeted sequence). A clone with normal karyotype was injected into blastocysts of C57Bl/6 mice. Male chimeras were crossed with female C57Bl/6 mice (Harlan). The resulting F1 heterozygous mice (in the 129P2-C57Bl/6 background) were used to generate F2 homozygous mutants and wild-type littermate controls. These mice were used for all the behavioral and electrophysiological experiments. The experimenter was blind for the genotype, but homozygous mice were easily recognizable by the ataxic gait. Therefore, data was also analyzed by a second person blind to the genotype. Mice were housed on a 12 hours light/dark cycle with food and water available *ad libitum* and used between 2 and 6 months of age for all experiments described (including electrophysiology). All animal procedures were approved by a Dutch Ethical Committee (DEC) for animal experiments.

Molecular analysis of mutants

In situ hybridization. Whole brains were quickly isolated and frozen on dry ice. Sagittal slices (14μm) were cut using Cryostat (Jung CM 300, Leica. Instruments, Nussloch, Germany) and thaw-mounted on slides (Superfrost®Plus, Menzel-Glaser, Germany). In situ hybridization was performed as described previously [1]. Probes used were antisense to the 5′ UTR of *Camk2b*:
5′-GGCCATGGCGGTGGCGATCGGGCTCGGCGTGCGCTCGGC-3′,
antisense to the 3′UTR of *Camk2b*:
5′-GGTACCTACAGACTAACACGGTCGGCATGGGCACATTCACG-3′ and antisense to exon 2 of *Camk2a*:
5′-GCTGCATACTCATGGCCGGTACAGAGCTTGACACAGCGTCGGAC-3′. Afterwards slices were stained with thionin.

RT-PCR. RNA was isolated from cerebellum and cortex, by homogenization in TRIzol® Reagent (Invitrogen). A semi-quantitative RT-PCR (verified by serial dilutions) was performed using Superscript™III Reverse transcriptase (Invitrogen) and RNAsin (Promega). Expression of the *Camk2b* mRNA in the heterozygous and homozygous

Camk2b mutant mice, was analyzed separately up- and downstream of the Neomycin cassette by detection of exon 2-4 of *Camk2b*, using 5′ primer 5′-CGACGCTGTGTCAAGCTCTGTAC-3′ and 3′ primer: 5′-GAAGCCCTCTTCAGAGATGC-3′ and detection of exon 14-17 of *Camk2b*, using 5′ primer: 5′-GAAAGCAGATGGAGTCAAG-3′ and 3′ primer: 5′-GTTGTGTTGGTGCTGTCG-3′. The expression of *Camk2a* mRNA was analyzed by detection of exon 2-5 of *Camk2a*, using 5′ primer: 5′-GCGCAGGTGTGTGAAGGTGC-3′ and 3′ primer: 5′-CACAATGTCTTCAAACAGTTCC-3′. A control PCR was performed with pimers against exon 5-8 of Synapsin I using 5′ primer: 5′-TGCCCAGATGGTTCGACTAC-3′ and 3′ primer: 5′-TGTCAGACATGGCAATCTGC-3′.

Western blot and immunoprecipitations. Lysates for westernblot were prepared by quick dissection of the brain and by homogenization of the brain tissue in lysis buffer (10mM TRIS-HCl 6.8, 2.5% SDS, 2mM EDTA) and protease and phosphatase inhibitor cocktails (Sigma). The concentration of the lysates was adjusted to 1 mg/ml. 10 µg was used for Western blot analysis, unless otherwise specified. Western blots in Fig. 1 were probed with antibodies directed against the N-terminal CaMKII (H-300, 1:500; Santa-Cruz). Westernblots shown in Supplementary Fig. 8 were probed with αCaMKII (clone 6G9; 1:1000; Chemicon), βCaMKII (clone CB-b1; 1:10000; Zymed), Ph-T286/T87 CaMKII antibody (1:5000; #06-881, Upstate Cell Signaling Solutions) or actin (clone C4; 1:10.000; Chemicon). Bands were visualized using Enhanced Chemo Luminescence (Pierce). For immunoprecipitations P2 synaptosomes were solubilized in a final buffer containing 25mM Hepes, 150mM NaCl, 1%. Triton and diluted to a final concentration to 2.5 mg/ml protein. For the immunoprecipitation we used 1 ml of homogenate (2.5 mg) and added 10 mg βCaMKII antibody (Zymed, clone CB-b1).

Kinase activity. Ca^{2+}-dependent and Ca^{2+}-independent activity were determined in fresh cerebellar lysates, using Autocamtide 2 as a substrate (CaMKII assay kit from Upstate cell signaling solutions, Boiling Springs, USA). Each assay was tested for linearity (serial dilution of lysates), and data was normalized to a wild-type (Ca^{2+}-dependent) control, which was present at each run.

Immunocytochemistry. Brains from adult mice were fixed with 4% paraformaldehyde by transcardial perfusion. Immunocytochemistry was performed on free-floating 40µm thick frozen sections employing a standard avidin-biotin-immunoperoxidase complex method (ABC, Vector Laboratories, USA) with βCaMKII (Zymed), αCaMKII (Chemicon) or Calbindin (Sigma) as the primary antibody and diaminobenzidine (0.05%) as the chromogen [2].

Morphological analysis: For dendritic arborization measurements we used Golgi-Cox staining on unfixed cerebelli using the FD Rapid GolgiStain Kit (FD NeuroTechnologies Inc., USA), according to the manufacturers' instructions. Sagittal sections, 100 µm thick, were cut on a microtome with cryostat adaptations. Purkinje cell counting and selection for further detailed analysis was done by an observer who was blind to the genotype. The tips of the outer branches were manually counted using a 40X objective and were used as a measure for dendritic branching. A calibration grid was used to count the number of spines per 10 µm, using a 100X objective. For the spine analysis, brains from adult mice were fixed with 4% paraformaldehyde by transcardial perfusion. To examine dendritic spine number and morphology, 200 micrometer cerebellar sections were subjected to 'diOlistic' labeling [3]. Tungsten particles (1.7 micrometer; Bio-Rad, Hercules, CA) coated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate crystals (DiI;

Invitrogen, Eugene, OR) were propelled into fixed tissue with biolistic Helios gene gun (Bio-Rad) through a membrane filter with a 3 μm pore size (Falcon 3092; BD Biosciences, Franklin Lakes, NJ). Sections were postfixed in 4% paraformaldehyde overnight and then mounted for confocal microscopy. A Zeis LSM510 confocal microscope, with a Zeiss 63x objective was used to obtain an image series of sections at 0.5 μm intervals. All imaging and analysis was performed in blinded manner. For quantification n=6 cells per genotype were identified (number of mice: WT (3); *Camk2b*$^{-/-}$ (3)), and protrusions were measured as described previously [4]. Morphometric analysis and quantification were performed using MetaMorph software (Universal Imaging Corporation, West Chester, PA) by investigators who were blind to genotype and experimental manipulation.

Behavioral analysis

Balance beam. Mice were placed on a 1.5 cm wide square beam, which was elevated 30 cm, and were allowed to stay on the beam for a maximum of 60 seconds.

Accelerating Rotarod. Motor coordination on the accelerating (4–40 rpm, in 5 minutes) rotarod (model 7650, Ugo Basile Biological Research Apparatus, Varese, Italy) was measured in 5 trials with 1 hour inter trial interval. The indicated time is the time spent on the rotarod, or time until the mouse made 3 consecutive rotations on the rotarod.

Open field. Mice we placed in a dimly–lit open field (120 cm diameter), and the total distance moved was recorded for 5 minutes (SMART software, Panlab, Barcelona, Spain).

Phenotyper. Mice were placed in the Phenotyper (Noldus Information Technology, Wageningen, The Netherlands) for 24 hours, and the total distance moved was determined. The phenotyper is a home-cage like environment, with a camera in the top-lid.

Electrophysiology

Sagittal slices of the cerebellar vermis (200-250 μm) of 16-22 week old mice, were made using a Vibratome (Leica VT1000S, Leica, Nussloch, Germany) and afterwards kept for >2 hours in ACSF (containing (in mM): 124 NaCl, 5 KCl, 1.25 Na_2HPO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 D-glucose aerated with 95% O_2 and 5% CO_2) before the recordings started. All drugs were purchased from Sigma. Experiments were performed at room temperature and in the presence of bath-applied 100 mM picrotoxin (Sigma) to block $GABA_A$-receptors. KN-93 (1 μM) and Cyclosporin A (5 μM dissolved in 0.5% EtOH) were added where indicated to block kinase and phosphatase activity respectively[5]. Purkinje cells were visualized using an upright microscope (Axioskop 2 FS *plus*, Carl Zeiss, Jena, Germany) equipped with a 40X water immersed objective. Whole-cell patch-clamp recordings were performed using an EPC-10 amplifier (HEKA Electronics, Lambrecht, Germany). For long-term plasticity recordings, electrodes were filled with a solution containing (in mM): 9 KCl, 10 KOH, 120 K-gluconate, 3.48 $MgCl_2$, 10 HEPES, 4 NaCl, 4 Na_2ATP, 0.4 Na_3GTP and 17.5 sucrose (pH 7.25, osmolarity ~290 osm). Currents were filtered at 3 kHz and digitized at 8 kHz. For extracellular stimulation, glass pipettes were filled with external saline. Long-term plasticity at the parallel fiber – Purkinje cell synapse was assessed by either pairing parallel fiber and climbing fiber stimulation at 1 Hz for 5 minutes (PF-LTD protocol) or by parallel fiber stimulation alone at 1 Hz for 5 minutes (PF-LTP protocol). Test responses were evoked at a frequency of 0.05 Hz (2 stimuli; 50 ms inter stimulus interval) with ~0.5-6 μA pulses that were applied for 500 (LTP) or 700 μs (LTD). Holding potentials in the range of -60 to -75 mV were chosen to prevent spontaneous spike activity. In all experiments, cells were switched to current-clamp mode for tetanization.

Recordings were excluded if R_S or R_I varied by >15% over the course of the experiment. Paired-pulse facilitation was tested using double test pulses with 50 ms inter stimulus interval. EPSC kinetics were recorded using Cs^{2+}-based intracellular recordings (containing (in mM) 115 $CsMeSO_4$, 20 TEA-Cl, 10 HEPES, 2.5 $MgCl_2$, 4 Na_2ATP, 0.4 Na_3GTP, 10 Na_2-Phosphocreatine, 0.6 EGTA, pH 7.25-7.30, osmolarity ~290 osm) while 50-80% online R_S-compensation (residual R_S < 10 MΩ) was applied. For climbing fiber-mediated EPSC recordings Purkinje cells were held at -10mV and 5 mM QX-314 were added to the internal medium to prevent spiking activity. During these recordings we checked for multiple climbing fiber innervation by gradually increasing the stimulus intensity[7].

Statistical analysis
Statistical analysis was performed using StatView (SAS institute, USA). For behavioral analysis (more than one measurement per group) we used a repeated measurements ANOVA, followed by a *post hoc* Fisher's PLSD analysis whenever it was appropriate. For comparison of two groups (one measurement each) we used a Student's *t*-test. Differences in LTD/LTP between wild-type mice and mutants were assessed by running a two-sample Student's *t*-test on the average values of the 5 last minutes of the recording (as indicated in the graphs). To assess whether significant LTP/LTD was obtained within a group, we used a paired *t*-test, in which we compared for each animal the average baseline value with the average value of the last 5 minutes of the recording (as indicated in the graphs). For climbing fiber-mediated EPSC analysis we averaged 6 responses per cell and used a single-exponential fit to calculate the decay time constant, 10-90% rise time and amplitude. Twenty climbing fiber-mediated EPSCs were averaged to calculate the absolute amplitude of the Na^+-spike and the first Ca^{2+}-spikelet.

Supplementary References

1. Wisden, W., Morris, B.J., Darlison, M.G., Hunt, S.P. & Barnard, E.A. *Neuron* **1**, 937-47 (1988).
2. Hansel, C. *et al. Neuron* **51**, 835-43 (2006).
3. Gan, W.B., Grutzendler, J., Wong, W.T., Wong, R.O. & Lichtman, J.W. *Neuron* **27**, 219-25 (2000).
4. Hoogenraad, C.C. *et al. Dev Cell* **12**, 587-602 (2007).
5. Belmeguenai, A. & Hansel, C. *J Neurosci* **25**, 10768-72 (2005).

Chapter IV

A non-enzymatic role of βCaMKII in hippocampal synaptic plasticity and learning

Geeske M. Van Woerden*, Nils Zuiderveen Borgesius*, Gabrielle H.S. Buitendijk, Ype Elgersma

Manuscript in preparation

*These authors contributed equally

CaMKII plays an essential role in hippocampal synaptic plasticity, learning and memory. However, most research has focused on αCaMKII and little is known about the role of βCaMKII. Using two different *Camk2b* mouse mutants we assessed the role of βCaMKII in learning and hippocampal plasticity. We found that a *Camk2b*^{A303R} mutant, in which the Ca^{2+}/calmodulin binding domain of βCaMKII was mutated to prevent its activation, showed no deficits in hippocampal long-term potentiation (LTP) or contextual fear conditioning. In contrast, the absence of βCaMKII caused marked impairments in both hippocampal plasticity and learning. Taken together these results strongly suggest that βCaMKII plays an essential role in hippocampal learning and LTP, but that this role is independent of its ability to be activated. Hence, our data provides compelling support for a model in which βCaMKII plays a non-enzymatic, structural role in hippocampal function.

Introduction

Calcium/calmodulin-dependent kinase type II (CaMKII) is one of the most abundant proteins of the hippocampus, and its role in hippocampal plasticity and learning has been thoroughly investigated by pharmacological and genetic approaches. The hippocampus expresses two major isoforms of CaMKII: (alpha (α) and beta (β) CaMKII) which are encoded by two distinct genes (*CaMK2a* and *CaMK2b*, respectively)[1]. Because αCaMKII and βCaMKII are highly homologous they cannot be distinguished using a pharmacological approach. Since almost all genetic approaches have focused on αCaMKII (see reviews[2-5]), little is known about the role of βCaMKII. However, recent studies showed that βCaMKII is essential for cerebellar plasticity and learning, and inducible up-regulation of βCaMKII activity in the dentate gyrus interferes with memory consolidation[6, 7].

Interestingly, there are several indications that the role of βCaMKII may to some extent be independent of its activity. For instance, whereas βCaMKII activity is required for parallel fiber-Purkinje cell LTD induction after high-calcium influx, βCaMKII plays a non-enzymatic role under low-calcium conditions, where it functions to prevent precocious activation of αCaMKII[6].

It is likely that such a non-enzymatic function relies on the extra domain that is present in the variable region of βCaMKII only[8-11]. *In vitro* studies showed that this domain enables the βCaMKII subunits to cluster the entire CaMKII holoenzyme to F-actin in an activity-controlled manner[10, 11 12, 13]. In addition, these studies showed that βCaMKII requires this F-actin binding domain to control dendritic arborization *in vitro*, and that a βCaMKII-A303R mutant which cannot be activated by Ca^{2+}/Calmodulin and therefore can not be released from the F-actin can still exert this effect. Mainly due to these different actin-binding properties, αCaMKII and βCaMKII have opposing effects on synaptic strength in cultured neurons[14]. Additional evidence that the actin binding domain in the variable region of βCaMKII probably plays a critical role in synaptic function comes from the observation that exons in this domain are controlled at the transcriptional level by alternative splicing[1, 15]. Indeed, the presence of just one single exon in the variable region determines the interaction with F-actin[15].

To investigate the role of βCaMKII in hippocampal plasticity and learning, and to test to what extend its function relies on its enzymatic or non-enzymatic (F-actin binding) properties, we generated a *Camk2b*$^{-/-}$ (knockout) mouse and a *Camk2b*^{A303R} mutant in which the Ca^{2+}/calmodulin binding site (Ala303) is mutated. This mutated βCaMKII protein is still able to bind to F-actin, but it cannot be activated by Ca^{2+}/calmodulin[12, 16].

We show that these *Camk2b*^{A303R} mice show normal hippocampal plasticity and learning. In contrast, *Camk2b*$^{-/-}$ mice show impaired hippocampal plasticity and learning. Together these results indeed show that βCaMKII plays a non-enzymatic role in hippocampal plasticity and learning.

Results

Generation and molecular characterization of the *Camk2b*^{A303R} and *Camk2b*$^{-/-}$ mice

To dissect the precise mechanism by which βCaMKII regulates hippocampal synaptic plasticity, we used the *Camk2b*$^{-/-}$ mutant[6] and we created a knock-in mutant of the *Camk2b* gene, in which we substituted Alanine^{303} for an Arginine (A303R) (**Fig. 1**). This amino acid is located in the Ca^{2+}/calmodulin binding domain and mutating it into an Arginine has been shown to block binding of Ca^{2+}/calmodulin, thereby preventing kinase activation of βCaMKII[11, 12, 15]. Importantly, this mutation does not affect its binding to F-actin[11, 12, 15].

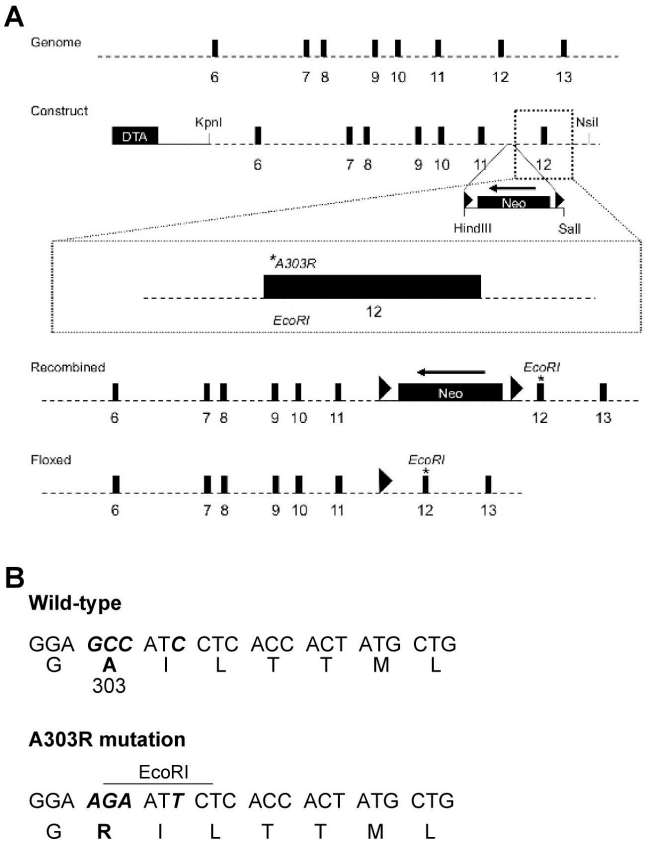


Figure 1. Generation of Camk2b^{A303R} mice. ***(a)*** *Schematic diagram for the generation of the Camk2b^{A303R} mutants. (Genome) wild-type Camk2b locus with the exons around Ala303 depicted as black boxes. (Construct) Targeting construct used for introducing the mutation in Ala303. The asterisk in exon 12 indicates the mutation at Ala303. The LoxP sites flanking the neomycin gene are depicted as triangles. The Diphtheria Toxin cassette (DTA) was cloned in the construct for positive selection. (Recombined) Mutant Camk2b^{A303R} locus after homologous recombination in ES cells. (Floxed) Mutant Camk2b^{A303R} locus after Cre recombination.* ***(b)*** *Camk2b sequence of the exon encoding Ala303. (Upper) Wild-type DNA indicating the location of Ala303 in bold. (Bottom) DNA of the A303R mutant. The basepares and aminoacids which are mutated are shown in bold-italics. Note the presence of the EcoRI restriction site after introducing the mutation.*

Immunostaining and Western blot analysis of brains of *Camk2b*$^{-/-}$ mutants confirmed the absence of βCaMKII in the hippocampus of the *Camk2b*$^{-/-}$ mouse (**Fig. 2a,b**). Since *in vitro* experiments showed that up-regulation of βCaMKII causes down-regulation of αCaMKII[14], we tested whether the absence of βCaMKII caused up-regulation of αCaMKII *in vivo*. However we did not observe up-regulation of αCaMKII protein levels (**Fig. 2b**) although there is a slight reduction in Thr^{286} phosphorylation of αCaMKII (**Fig. 2b**).

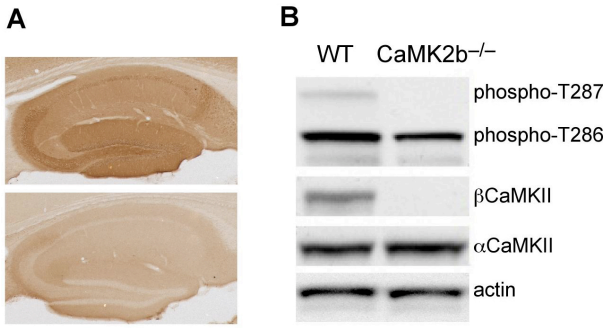


Figure 2. Molecular analysis of the Camk2b$^{-/-}$ mice. ***(a)*** *Immunocytochemistry using an antibody specific for βCaMKII showed complete absence of βCaMKII in the hippocampus.* ***(b)*** *Western blot analysis using antibodies specific for αCaMKII and βCaMKII showed complete absence of βCaMKII in the hippocampus, without any change in the levels of αCaMKII. Furthermore, western blot analysis using an antibody specific for detecting the phosphorylation levels of αCaMKII-T286 and βCaMKII-T287 revealed a slight reduction of T286 phosphorylation, whereas the T287 phosphorylation is completely absent.*

Immunostaining and Western blot analysis of brains of homozygous pointmutants (designated *Camk2b^{A303R}* mice) revealed no change in expression of βCaMKII and αCaMKII (**Fig. 3a,b**) in the *Camk2b^{A303R}* mouse indicating that the mutation does not affect expression levels of βCaMKII and αCaMKII. Since binding of Ca^{2+}/calmodulin is a prerequisite for auto-phosphorylation of CaMKII at Thr286/287 as reviewed by[5, 17, 18] we used the Phospho-Thr286/287 antibody to confirm that this mutation renders the βCaMKII^{A303R} protein insensitive to Ca^{2+}/calmodulin activation. Indeed Western blot analysis showed that βCaMKII- Thr^{287} was entirely absent in the *Camk2b^{A303R}* mouse (**Fig. 3b**), confirming *in vitro* studies that the A303R mutation blocks activation of βCaMKII[12, 15]. We also observed a slight reduction of αCaMKII Thr^{286} autophosphorylation in this mutant (**Fig. 3b**), which is not unexpected given that most of the αCaMKII subunits are associated with βCaMKII subunits, and that it takes two adjacent activated CaMKII subunits to get intersubunit autophosphorylation at Thr286/287[5, 17, 18]. These data show that in *Camk2b$^{-/-}$* mice βCaMKII is absent and protein expression of αCaMKII is unaltered, whereas in *Camk2b^{A303R}* mice, we show that the A303R mutation was successful, preventing activation of βCaMKII without affecting the levels of expression of both βCaMKII and αCaMKII.

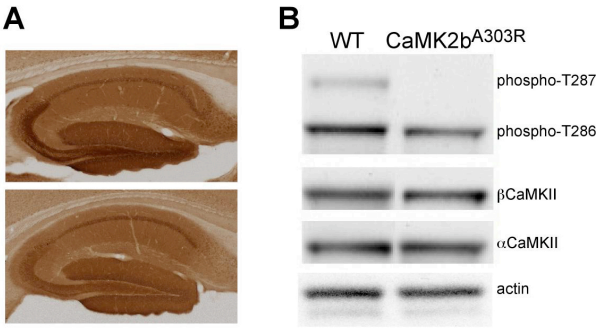


Figure 3. Molecular analysis of the Camk2b^{A303R} mice. ***(a)*** *Immunocytochemistry staining using an antibody specific for βCaMKII showed no difference in βCaMKII staining in the hippocampus of Camk2b^{A303R} mice compared to wild-type mice.* ***(b)*** *Western blot analysis using antibodies specific for αCaMKII and βCaMKII showed no difference in the levels of αCaMKII and βCaMKII in the hippocampus of the Camk2b^{A303R} mice. Antibodies specific for detecting the phosphorylation levels of αCaMKII-Thr286 and βCaMKII-Thr287 revealed a slight reduction of Thr286 phosphorylation, whereas the Thr287 phosphorylation is completely absent in the hippocampus of the Camk2b^{A303R} mice.*

Previous studies showed that down- or upregulation of βCaMKII *in vitro,* caused respectively decreased or increased dendritic arborization[12]. Since this phenomenon was only observed in transfected premature cultures, and not in mature cultures, βCaMKII appears to be critical for normal dendritic development. However, we did not find any gross changes in overall brain development nor in the development of the Purkinje cells of the *Camk2b$^{-/-}$* mouse in our earlier study[6]. Thionin staining of brain sections of the *Camk2b^{A303R}* mice also did not reveal gross changes in overall brain morphology (**Fig. 4a**). In addition, more detailed examination of the hippocampus using the thionin staining did

not reveal any changes in hippocampal structure at the light microscope level (**Fig. 4b**) of *Camk2b*$^{-/-}$ mice. Furthermore we investigated the morphology of the CA1 pyramidal cells of *Camk2b*$^{-/-}$ using Golgi-Cox staining. We found no significant change in the density of spines (6.35 ± 0.36 n=15 cells from 3 mice; 7.07 ± 0.61 n=15 from 3 mice for wild-type and *Camk2b*$^{-/-}$ mice respectively; one-way ANOVA $F_{1,28}$=1.34; p>0.28; **Fig. 4c,d**). Together these data show that neuronal development in *Camk2b*$^{-/-}$ mice is normal.

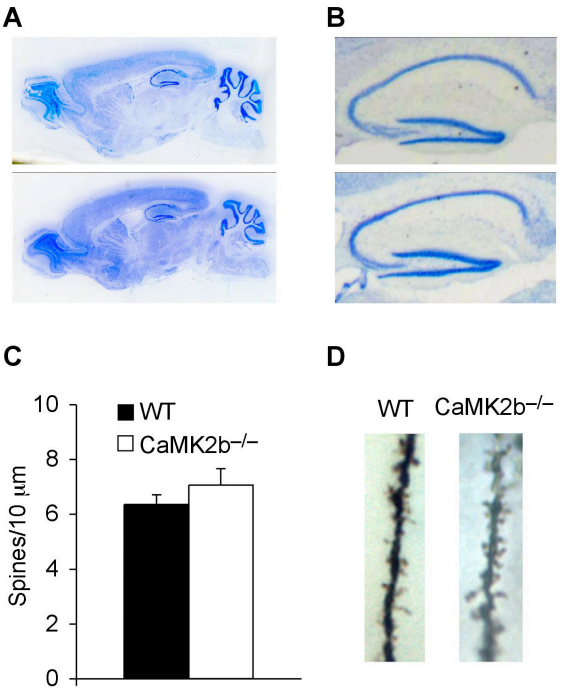


Figure 4. Morphological analysis of Camk2b^{A303R} and Camk2b$^{-/-}$ mice. ***(a)*** *Thionin staining showed no gross morphological difference in the brain of Camk2b^{A303R} mice.* ***(b)*** *Thionin staining showed no apparent morphological changes in the hippocampus of Camk2b$^{-/-}$ mice compared to wild-type mice.* ***(c,d)*** *Golgi analysis of the hippocampal pyramidal cells did not reveal any difference in spine density (p=0.3; wild-type, n=5; Camk2b$^{-/-}$, n=5). Error bars indicate SEM*

Behavioral characterization of the *Camk2b*^{A303R} and *Camk2b*$^{-/-}$ mice

Like the *Camk2b*$^{-/-}$ mice[6], *Camk2b*^{A303R} mice showed clear ataxia. In addition, when tested on the accelerating rotarod, a motor performance test, the *Camk2b*^{A303R} mice had significantly shorter latency times than their wild-type littermates (repeated measures ANOVA $F_{1,18}$=39.05; p<0.001; **Fig. 5a**). These results are consistent with our previous findings that suggested that cerebellar function relies critically on both the enzymatic and non-enzymatic mechanisms of βCaMKII.

To investigate the requirement of βCaMKII activation in hippocampus-dependent learning, we tested the *Camk2b*^{A303R} mice in the contextual fear conditioning task. In this test mice are conditioned to associate a certain context with a mild aversive foot shock. Learning is assessed by measuring freezing behavior, *i.e.* the cessation of all movement except respiration, which is a natural expression of fear in mice. *Camk2b*^{A303R} mice showed indistinguishable freezing behavior from their wild-type littermates when tested on the contextual fear conditioning task (one-way ANOVA $F_{1,18}$=0.06; p>0.80; **Fig. 5b**) without any change in baseline freezing behavior (one-way ANOVA $F_{1,18}$=0.86; p>0.36). These results suggest that normal context conditioning does not require βCaMKII activation by Ca^{2+}/calmodulin nor its autonomous activity by Thr^{287} phosphorylation.

To see if the presence of βCaMKII protein is necessary for fear learning, we also tested the *Camk2b*$^{-/-}$ mice. In contrast to the intact fear conditioning of *Camk2b*^{A303R} mice, we found significantly less freezing in the *Camk2b*$^{-/-}$ mutants compared to their wild-type littermates (one-way ANOVA $F_{1,39}$=22.9; p<0.001; **Fig. 5c**) without any change in baseline freezing behavior (one-way ANOVA $F_{1,19}$=0.91; p>0.35; **Fig. 5c**) indicating an impairment of fear learning. Importantly, the motor coordination deficits of *Camk2b*$^{-/-}$ mice cannot explain the deficits in fear conditioning, since *Camk2b*$^{-/-}$ mice have similar motor dysfunction as *Camk2b*^{A303R} mice[6], which showed normal fear conditioning. These data show that presence of βCaMKII protein, independent of its kinase activation, is necessary for contextual fear learning.

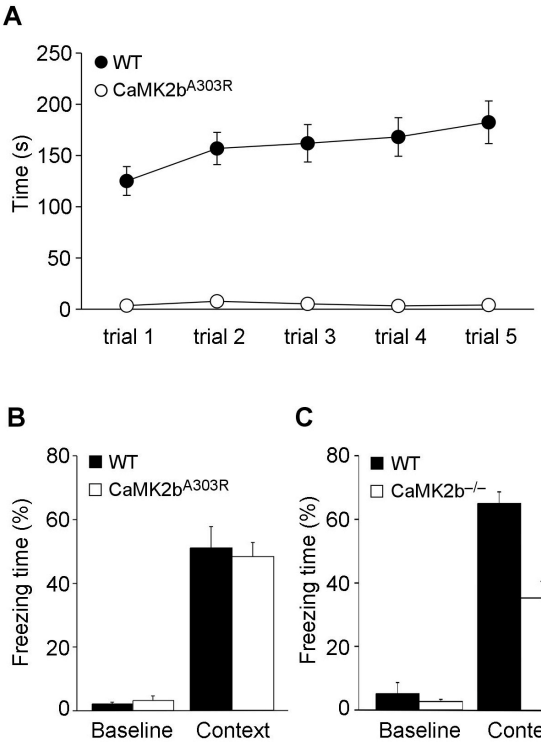


Figure 5. Behavioral analysis of Camk2b^{A303R} and Camk2b$^{-/-}$ mice. ***(a)*** *Motor performance assessed by the accelerating rotarod. Camk2b^{A303R} mice (n=6) stayed on the rotarod for a significantly shorter period compared to wild-type mice (n=14) (p<0.001). The latency until the mice fell off the rod is shown. Error bars indicate SEM.* ***(b)*** *Camk2b^{A303R} mice (n=6) show normal fear conditioning learning, and normal baseline freezing compared to wild-type mice (n=14) (p=0.8 and p=0.4 respectively).* ***(c)*** *Camk2b$^{-/-}$ mice (n=16) show reduced fear conditioning learning (p<0.001), whereas baseline freezing is normal compared to wild-type mice (n=25) (p=0.4). Error bars indicate SEM.*

Hippocampal synaptic plasticity in the *Camk2b^{A303R}* and *Camk2b$^{-/-}$* mice

The behavioral data presented above strongly suggests that hippocampal dependent learning requires the presence of βCaMKII, but not its enzymatic activity. To investigate if the same applies to hippocampal plasticity, we investigated the properties of the Schaffer-collateral pathway synapses of the *Camk2b^{A303R}* mouse in acute hippocampal slices using fEPSP measurements. No significant differences were found in basal synaptic transmission or PPF ((Basal synaptic transmission: repeated measures ANOVA fiber volley $F_{1,124}$=0.96; p>0.30 (**Suppl. Fig. 1a**); and fEPSP $F_{1,124}$=0.10; p>0.70 (**Suppl. Fig. 1b**); n=68 and 58 for wild-type and *Camk2b^{A303R}* mice respectively; **Fig. 6a**), and (PPF repeated measures ANOVA: $F_{1,151}$=0.004; p>0.95; n=82 and 71 for wild-type and *Camk2b^{A303R}* mice respectively; **Fig. 6b**)). We then investigated the ability to induce long-term potentiation (LTP) at the Schaffer-collateral pathway. We tested LTP using the commonly used 100 Hz for one second protocol and found no difference between *Camk2b^{A303R}* and wildtypes (132.9 ± 5.5 n=28; 132.9 ± 7.8 n=19 for wild-type and *Camk2b^{A303R}* mice respectively; one-way ANOVA $F_{1,45}$=0.28; p<0.60; **Fig. 6c**). Additionally, when applying the 10 Hz for 10 sec protocol, which is closer to the LTP induction threshold and is within the natural occurring Theta rhythm frequency, no difference was found between the *Camk2b^{A303R}* and wildtypes (148.2 ± 7.3 n=12; 145.3 ± 5.9 n=9 for wild-type and *Camk2b^{A303R}* mice respectively; one-way ANOVA $F_{1,19}$=0.77; p<0.77; **Fig. 6d**). These results show that for these forms of hippocampal synaptic plasticity, the activation of βCaMKII is not required.

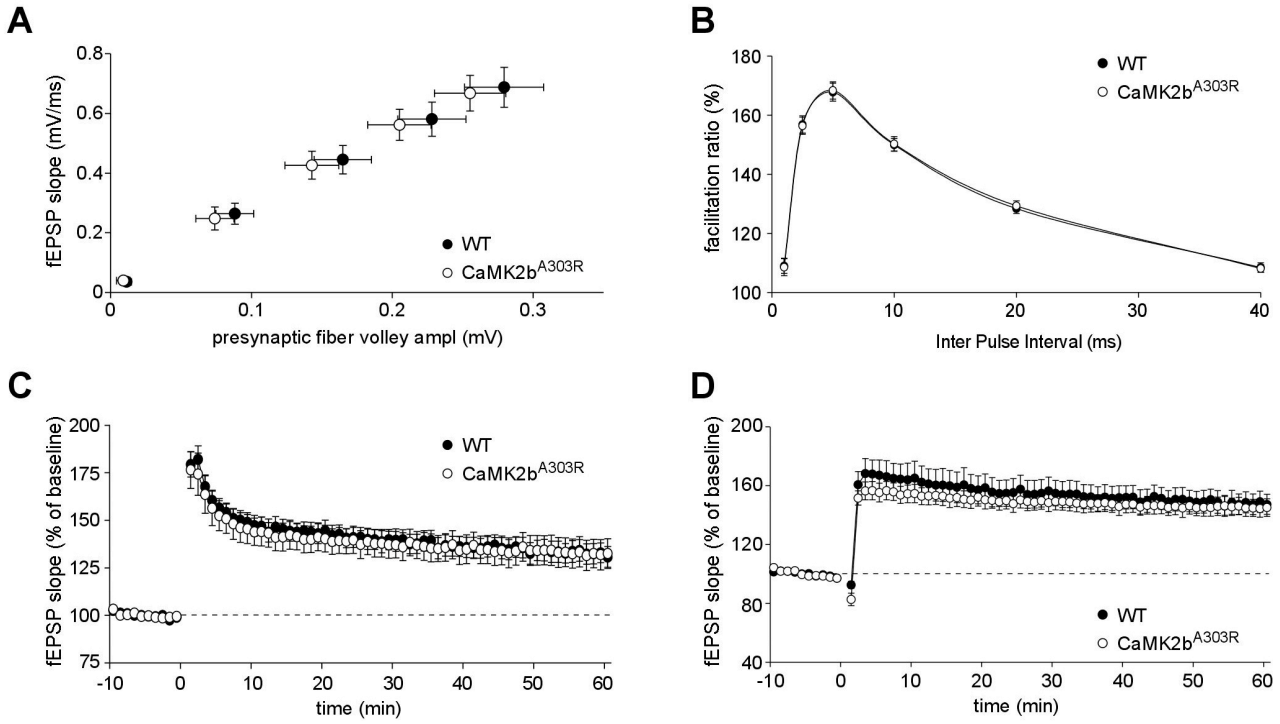


Figure 6. Normal hippocampal synaptic plasticity in Camk2b^{A303R} mice. ***(a)*** *Camk2b^{A303R} mice show normal synaptic transmission (fiber volley, p=0.3; fEPSP slope, p=0.7; wild-type, n=68; camk2b^{A303R}, n=58).* ***(b)*** *Camk2b^{A303R} mice show normal PPF (p=0.9; wild-type, n=82; camk2b^{A303R}, n=71).* ***(c)*** *Camk2b^{A303R} mice show normal 100 Hz LTP (p=0.6; wild-type, n=28; camk2b^{A303R}, n=19).* ***(d)*** *Camk2b^{A303R} mice show normal 10 Hz LTP (p=0.8; wild-type, n=12; camk2b^{A303R}, n=9).*

To test if hippocampal plasticity requires βCaMKII protein at all, we applied the same electrophysiological analysis for *Camk2b$^{-/-}$* mice. No significant impairment in basal synaptic transmission was observed in the *Camk2b$^{-/-}$* mice (**Fig. 7a**), since they do not show a significant change in fiber volley (repeated measures ANOVA $F_{1,135}$=0.22; p>0.50; **Suppl. Fig. 2a**) nor in fEPSP slope (repeated measures ANOVA $F_{1,135}$=1.4; p>0.23; **Suppl. Fig. 2b**). However, Paired Pulse Facilitation (PPF), a form of short-term pre-synaptic plasticity, was slightly but significantly enhanced across a broad range of intervals in *Camk2b$^{-/-}$* mice (repeated measures ANOVA $F_{1,168}$=10.66; p<0.002; **Fig. 7b**), which could be due to a small reduction in vesicle release probability. When we tested LTP, we found a significant impairment in *Camk2b$^{-/-}$* mutants compared to wild-type littermates for both 100 Hz as well as 10 Hz (100 Hz, 152.6 ± 7.7 n=27; 119.3 ± 4.4 n=21 for wild-type and *Camk2b$^{-/-}$* mice respectively; one-way ANOVA $F_{1,46}$=13.14; p<0.001; **Fig. 7c**; 10 Hz, 135.7 ± 4.6 n=12; 119.8 ± 6.0 n=9 for wild-type and *Camk2b$^{-/-}$* mice respectively; one-way ANOVA $F_{1,19}$=6.89; p<0.05; **Fig. 7d**). Taken together, these experiments show that βCaMKII plays an essential role in hippocampal plasticity and learning, but that the activation of βCaMKII is not required for this function.

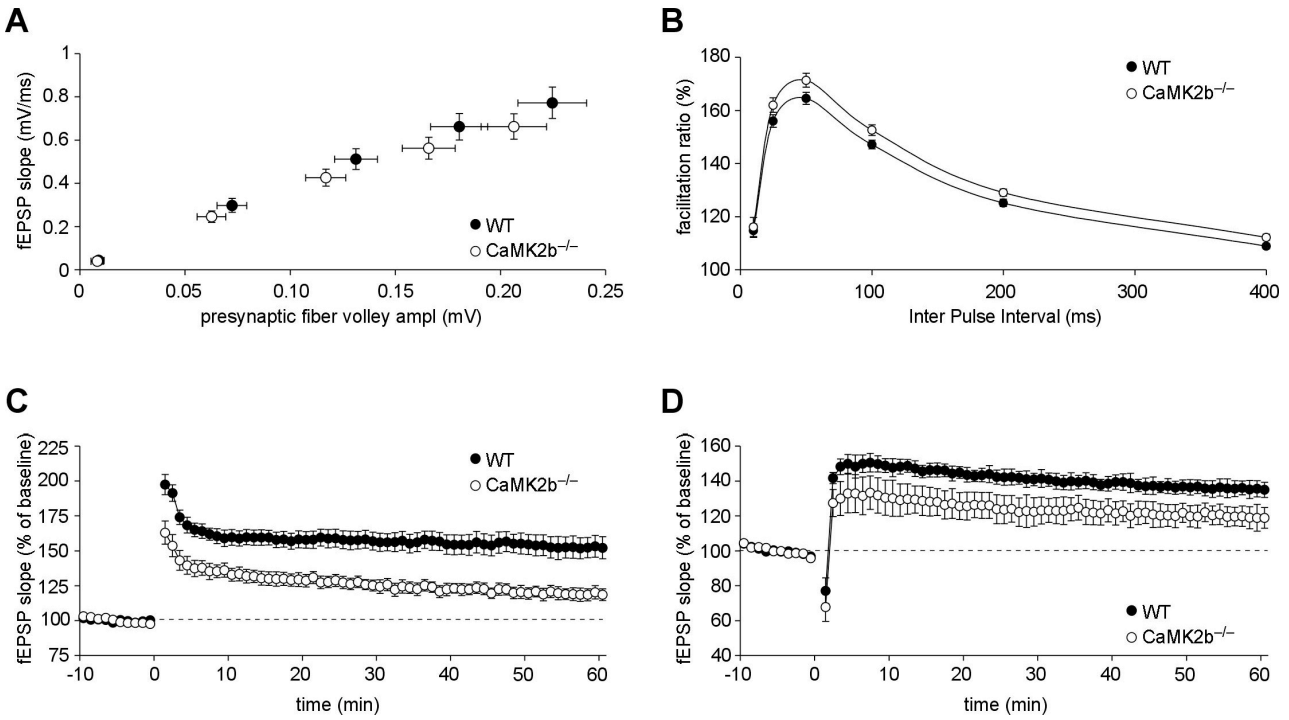


Figure 7. Enhanced PPF and impaired 10 and 100 Hz LTP in Camk2b$^{-/-}$ mice. ***(a)*** *Camk2b$^{-/-}$ mice show normal synaptic transmission (fiber volley, p=0.5; fEPSP slope, p=0.2; wild-type, n=74; camk2b$^{-/-}$, n=63).* ***(b)*** *Camk2b$^{-/-}$ mice show enhanced PPF (p<0.01; wild-type, n=82; camk2b$^{-/-}$, n=88).* ***(c)*** *Camk2b$^{-/-}$ mice show impaired 100 Hz LTP (p<0.001; wild-type, n=27; camk2b$^{-/-}$, n=21).* ***(d)*** *Camk2b$^{-/-}$ mice show impaired 10 Hz LTP (p<0.05; wild-type, n=12; camk2b$^{-/-}$, n=9).*

Discussion

Here we describe the role of βCaMKII in hippocampal synaptic plasticity and fear learning, using two different βCaMKII mutants; a null-mutant (*Camk2b$^{-/-}$*) and an inactive mutant (*Camk2b^{A303R}*). We have shown that neither the absence nor the inactivation of βCaMKII affects brain development at the light microscopic level, contrary to what would be expected based on *in vitro* studies from literature. Furthermore, we have shown that the absence of βCaMKII impairs fear learning and hippocampal synaptic plasticity while inactivating βCaMKII does not. Thereby strongly suggesting that βCaMKII has a non-enzymatic, structural role in fear learning and hippocampal synaptic plasticity.

Development and homeostasis

Considering the earlier expression of βCaMKII compared to αCaMKII[19, 20] and the effects of βCaMKII overexpression on cultured hippocampal neurons[12] or neuroblastoma cells[21], an important role in neuronal development could be expected. In contrast, we have found no perturbations of neuronal development in neither the *Camk2b$^{-/-}$* nor the *Camk2b^{A303R}* mice. An explanation for this discrepancy could lie in the different experimental systems used, which results in two important differences. First, our mouse mutants have changed βCaMKII throughout development. In contrast, neurons used for cultures, developed in the presence of βCaMKII protein but are suddenly confronted with decreased βCaMKII expression and assessed only 24 hours later. This may not allow them sufficient time to compensate for this change. Second, given the low neuronal density, neurons in culture are mostly dependent on intrinsic signaling mechanisms to develop, whereas neurons in a brain also rely on cues from neighboring cells and synaptic inputs. It would be interesting

to see how neurons derived from *Camk2b*$^{-/-}$ mice develop in culture, to clarify the differences between the *in vivo* and *in vitro* experiments.

Synaptic plasticity

Our data clearly show that NMDA receptor dependent LTP (10 and 100 Hz) is impaired when βCaMKII is absent (*Camk2b*$^{-/-}$) but not when βCaMKII is inactive (*Camk2b*^{A303R}), suggesting an exclusive non-enzymatic role of βCaMKII in hipocampal plasticity. This finding is at odds with the notion that the increased affinity for Ca^{2+}/calmodulin is also important for the role of βCaMKII in neuronal function. For instance, it has been shown that in cultured neurons the α/β ratio is regulated by neuronal activity[14] which was suggested to be an homeostasis mechanism, where reduced activity would increase Ca^{2+} sensitivity of the CaMKII holoenzymes by decreasing the α/β ratio and vice versa because βCaMKII has higher affinity for Ca^{2+}/calmodulin. Although we do find an increased LTP threshold in the *Camk2b*$^{-/-}$ mice ((LTP deficit with 10 and 100 Hz but not with a stronger 200 Hz tetanus (data not shown)), this does not appear to be the result of a reduced Ca^{2+}/calmodulin sensitivity caused by the lack of the more sensitive βCaMKII sub-unit for two reasons. First, the *Camk2b*^{A303R} mutant shows normal LTP, despite the loss of Ca^{2+}/calmodulin binding. Second, loss of the αCaMKII protein did not result in a lower threshold for LTP induction in the heterozygous or homozygous *Camk2a* knockout mouse, in which the subunit compensation has been shifted towards βCaMKII.

Given that Ca^{2+}/calmodulin binding is required to release the α- and βCaMKII containing holoenzyme from the actin skeleton and to allow subsequent translocation to the PSD[11], one would expect an LTP and learning impairment in the *Camk2b*^{A303R} mouse. So why does the *Camk2b*^{A303R} mouse not show a deficit in hippocampal plasticity? One possible explanation is that binding to F-actin is an absolute requirement for normal LTP, and that the remaining unbound αCaMKII in the *Camk2b*^{A303R} synapses are sufficient to target to the PSD and exert their (enzymatic) function. Hence, this would imply that synaptic plasticity requires binding of CaMKII to F-actin, but not necessarily its activity induced release from F-actin. This notion is in agreement with *in vitro* studies that showed that the βCaMKII^{A303R} protein is still able to regulate dendritic arborization. Importantly, a similar, but not identical, mutation in the Ca^{2+}/calmodulin binding domain of αCaMKII (T305D) abolishes learning and LTP in homozygous mice, and even has a dominant negative effect in LTP[22], in sharp contrast to lack of effect of the A303R mutation. The T305D mutation in αCaMKII mimics natural inhibitory autophosphorylation of αCaMKII and not only prevents Ca^{2+}/calmodulin binding and Thr^{286} autophosphorylation, it also strongly reduces the levels of post-synaptic density (PSD) associated CaMKII[22]. Taken together these findings strongly suggest that CaMKII binding to F-actin and CaMKII targeting to the PSD are critical steps in synaptic plasticity. However, these steps do not have to occur successively, but can operate independent of each other.

References

1. Hudmon, A. & Schulman, H. Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. *Biochem J* **364**, 593-611 (2002).
2. Elgersma, Y., Sweatt, J.D. & Giese, K.P. Mouse genetic approaches to investigating calcium/calmodulin-dependent protein kinase II function in plasticity and cognition. *J Neurosci* **24**, 8410-8415 (2004).
3. Lisman, J.E. & McIntyre, C.C. Synaptic plasticity: a molecular memory switch. *Curr Biol* **11**, R788-791 (2001).
4. Griffith, L.C. Calcium/calmodulin-dependent protein kinase II: an unforgettable kinase. *J Neurosci* **24**, 8391-8393 (2004).
5. Lisman, J., Schulman, H. & Cline, H. The molecular basis of CaMKII function in synaptic and behavioural memory. *Nat Rev Neurosci* **3**, 175-190 (2002).
6. van Woerden, G.M., *et al*. betaCaMKII controls the direction of plasticity at parallel fiber-Purkinje cell synapses. *Nat Neurosci* **12**, 823-825 (2009).
7. Cho, M.H., Cao, X., Wang, D. & Tsien, J.Z. Dentate gyrus-specific manipulation of beta-Ca2+/calmodulin-dependent kinase II disrupts memory consolidation. *Proc Natl Acad Sci U S A* **104**, 16317-16322 (2007).
8. Miller, S.G., Patton, B.L. & Kennedy, M.B. Sequences of autophosphorylation sites in neuronal type II CaM kinase that control Ca2(+)-independent activity. *Neuron* **1**, 593-604 (1988).
9. Bulleit, R.F., Bennett, M.K., Molloy, S.S., Hurley, J.B. & Kennedy, M.B. Conserved and variable regions in the subunits of brain type II Ca2+/calmodulin-dependent protein kinase. *Neuron* **1**, 63-72 (1988).
10. Shen, K., Teruel, M.N., Subramanian, K. & Meyer, T. CaMKIIbeta functions as an F-actin targeting module that localizes CaMKIIalpha/beta heterooligomers to dendritic spines. *Neuron* **21**, 593-606 (1998).
11. Shen, K. & Meyer, T. Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. *Science* **284**, 162-166 (1999).
12. Fink, C.C., *et al*. Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CaMKII. *Neuron* **39**, 283-297 (2003).
13. Lin, Y.C. & Redmond, L. Neuronal CaMKII acts as a structural kinase. *Commun Integr Biol* **2**, 40-41 (2009).
14. Thiagarajan, T.C., Piedras-Renteria, E.S. & Tsien, R.W. alpha- and betaCaMKII. Inverse regulation by neuronal activity and opposing effects on synaptic strength. *Neuron* **36**, 1103-1114 (2002).
15. O'Leary, H., Lasda, E. & Bayer, K.U. CaMKIIbeta association with the actin cytoskeleton is regulated by alternative splicing. *Mol Biol Cell* **17**, 4656-4665 (2006).
16. Lin, Y.C. & Redmond, L. CaMKIIbeta binding to stable F-actin in vivo regulates F-actin filament stability. *Proc Natl Acad Sci U S A* **105**, 15791-15796 (2008).
17. Hudmon, A. & Schulman, H. Neuronal CA2+/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. *Annu Rev Biochem* **71**, 473-510 (2002).
18. Colbran, R.J. Targeting of calcium/calmodulin-dependent protein kinase II. *Biochem J* **378**, 1-16 (2004).
19. Bayer, K.U., Lohler, J., Schulman, H. & Harbers, K. Developmental expression of the CaM kinase II isoforms: ubiquitous gamma- and delta-CaM kinase II are the early isoforms and most abundant in the developing nervous system. *Brain Res Mol Brain Res* **70**, 147-154 (1999).
20. Sahyoun, N., *et al*. Early postnatal development of calmodulin-dependent protein kinase II in rat brain. *Biochem Biophys Res Commun* **132**, 878-884 (1985).
21. Nomura, T., Kumatoriya, K., Yoshimura, Y. & Yamauchi, T. Overexpression of alpha and beta isoforms of Ca2+/calmodulin-dependent protein kinase II in neuroblastoma cells -- H-7 promotes neurite outgrowth. *Brain Res* **766**, 129-141 (1997).

22. Elgersma, Y., *et al*. Inhibitory autophosphorylation of CaMKII controls PSD association, plasticity, and learning. *Neuron* **36**, 493-505 (2002).
23. Roche, K.W., O'Brien, R.J., Mammen, A.L., Bernhardt, J. & Huganir, R.L. Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. *Neuron* **16**, 1179-1188 (1996).
24. Hansel, C., *et al*. alphaCaMKII Is essential for cerebellar LTD and motor learning. *Neuron* **51**, 835-843 (2006).

Material and Methods

Generation of the Camk2b^{A303R} mutants.
The *Camk2b^{A303R}* targeting construct was generated as follows. The *Camk2b* genomic sequence (ENSMUSG00000057897) was obtained from a public database (Ensembl) and used to design the primers for the targeting constructs. PCR fragments encompassing exon 6-11 using
5′ primer: 5′-GGTACCTGAGGAAGGTGCCAGCTCTGTCCC-3′ and
3′ primer: 5′–GTCGACCAGGGTAGTCACGGTTGTCC-3′ (5.3 Kb; exon denotation according to ENSMUST00000019133) and exon 11-12 using
5′ primer: 5′-GCGGCCGCCTGTTAAAGGAATGGTTCTC-3′ and
3′ primer: 5′-ATGCATCTAAAAGGCAGGCAGGATGATCTGC-3′ (6 Kb)
were amplified using High Fidelity Taq Polymerase[23] on ES cell genomic DNA and cloned on either site of a PGK-Neomycin selection cassette. All exons were sequenced to verify that no mutations were introduced accidentally. Site directed mutagenesis was used to introduce the pointmutation Ala303Arg. For counter selection, a gene encoding Diphteria toxin chain A (DTA) was inserted at the 5′ of the targeting construct. The targeting construct was linearized and electroporated into E14 ES cells (derived from 129P2 mice). Cells were cultured in BRL cell conditioned medium in the presence of Leukemia inhibitory factor (LIF). After selection with G418 (200 μg/ml), targeted clones were identified by PCR (long-range PCR from Neomycin resistance gene to the region flanking the targeted sequence). A clone with normal karyotype was injected into blastocysts of C57Bl/6 mice. Male chimeras were crossed with female C57Bl/6 mice (Harlan). The resulting F1 heterozygous mice (in the 129P2-C57Bl/6 background) were used to generate F2 homozygous mutants and wild-type littermate controls. These mice were used for all the behavioral and electrophysiological experiments. The experimenter was blind for the genotype, but homozygous mice were easily recognizable by the ataxic gait. Therefore, a second person blind to the genotype also analyzed data. Mice were housed on a 12 hours light/dark cycle with food and water available *ad libitum* and used between 2 and 6 months of age for all experiments described (including electrophysiology). All animal procedures were approved by a Dutch Ethical Committee (DEC) for animal experiments.

Molecular analysis
Western blot. Lysates were prepared by quick dissection of the brain and by homogenization of the brain tissue in lysis buffer (10mM TRIS-HCl 6.8, 2.5% SDS, 2mM EDTA and protease and phosphatase inhibitor cocktails (Sigma). The concentration of the lysates was adjusted to 1 mg/ml. 10 μg was used for Western blot analysis. Western blots were probed with antibodies directed against αCaMKII (MAB3119, 1:10,000; Chemicon), βCaMKII (CB-β1, 1:10,000; Zymed), Ph-T286/T87 CaMKII antibody (1:5000; #06-881, Upstate Cell Signaling Solutions) and Actin (MAB1501R, 1:2000; Chemicon). Blots were

stained using Enhanced ChemoLuminescence (#32106, Pierce) Western blot quantification was performed using NIH-Image.
Immunocytochemistry. Immunocytochemistry was performed on free-floating 40μm thick frozen sections employing a standard avidin-biotin-immunoperoxidase complex method (ABC, Vector Laboratories, USA) with, βCaMKII (CB-β1, 1:2000; Zymed) as the primary antibody and diaminobenzidine (0.05%) as the chromogen[24]. For gross brain morphology sections were stained with thionin.
Dendritic arborization. Golgi-Cox staining on unfixed hippocampi of 3 *Camk2b*$^{-/-}$ mutants and 3 wild-type mice was performed using the FD Rapid GolgiStain Kit (FD NeuroTechnologies Inc., USA), according to the manufacturers' instructions. Saggital sections, 100μm thick, were cut on a microtome with cryostat adaptations. Pyramidal cell counting and selection for further detailed analysis was done by two independent observers who were both blind for genotype. A calibration grid was used to count the number of spines per 10μm, using a 40X objective.

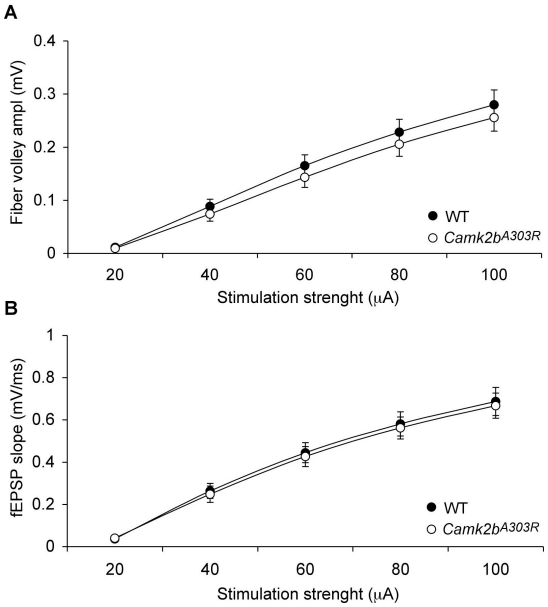
Behavioral analysis
Accelerating Rotarod. Motor coordination on the accelerating (4–40 rpm, in 5 minutes) rotarod (model 7650, Ugo Basile Biological Research Apparatus, Varese, Italy) was measured in 5 trials with 1 hour inter trial interval. The indicated time is the time spent on the rotarod, or time until the mouse made 3 consecutive rotations on the rotarod.
Fear conditioning was performed in a conditioning chamber (Med. Associates) equipped with a grid floor via which the foot shock could be administered. Each mouse was placed inside the conditioning chamber for 180 seconds. A foot shock (2 s, 0.4 mA) was delivered 148 s after placement in the chamber. Twenty-four hours later, context-dependent freezing was measured during 3 minutes.

Electrophysiology
After the animals had been sacrificed sagittal slices (400 μm) were made submerged in ice-cold artificial CSF (ACSF) using a vibratome and hippocampi were dissected out. These sagittal hippocampal slices were maintained at room temperature for at least 1.5 hours to recover before experiments were initiated. Then they were placed in a submerged recording chamber and perfused continuously at a rate of 2 ml/min with ACSF equilibrated with 95% O_2, 5% CO_2 at 31°C. ACSF contained the following (in mM): 120 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose. Extracellular recording of field EPSP (fEPSPs) were made in CA1 stratum radiatum with platinum/iridium electrodes (Frederick Haer Companu, Bowdoinham, ME). A bipolar Pt/Ir was used to stimulate Schaffer-collateral/commissural afferents with a stimulus duration of 100 μs. Stimulus response curves were obtained at the beginning of each experiment, 20 minutes after placing the electrodes. LTP was evoked using two different tetani: (i) 10 Hz (1 train of 10 seconds at 10 Hz) and (ii) 100 Hz (1 train of 1 second 100 Hz). The 10 Hz protocols were performed at two-thirds and the 100 Hz protocol at one-third of the maximum fEPSP. fEPSP measurements were done once per minute. Potentiation was measured as the normalized increase of the mean fEPSP slope for the duration of the baseline. Only stable recordings were included and this judgment was made blind to genotype. Average LTP was defined as the mean last 10 minutes of each protocol.

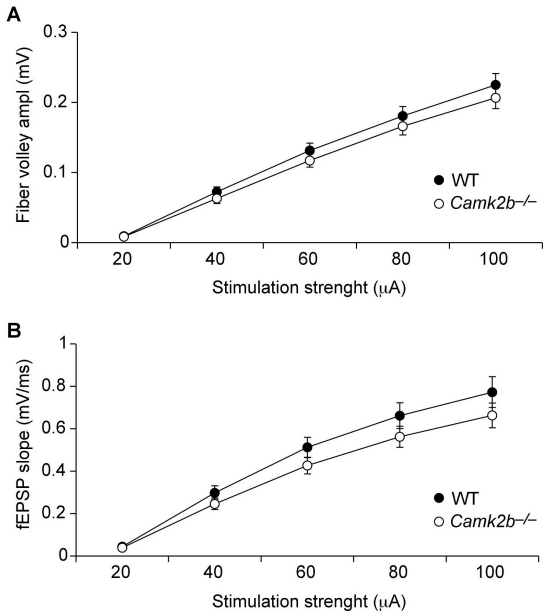
Statistical analysis

All results are presented as means ± SEM and all statistical analysis were done using SPSS 15 software. A one-way ANOVA was used for was used to analyze differences between genotypes for spine density, freezing time and LTP induction (based on the average of the last 10 min.). A repeated-measures ANOVA was used to analyze differences between genotypes for rotarod, fiber volley, fEPSP slope, and PPF.

Supplementary figures



Supplementary Figure 1. Normal basal synaptic transmission in Camk2b^{A303R} mice. ***(a,b)*** *Camk2b^{A303R} mice show normal synaptic transmission* ***((a)*** *fiber volley, p=0.3;* ***(b)*** *fEPSP slope, p=0.7; wild-type, n=68; camk2b^{A303R}, n=58).*



Supplementary Figure 2. Normal basal synaptic transmission in Camk2b$^{-/-}$ mice. ***(a,b)*** *Camk2b$^{-/-}$ mice show normal basal synaptic transmission* ***((a)*** *fiber volley, p=0.5;* ***(b)*** *fEPSP slope, p=0.2; wild-type, n=74; camk2b$^{-/-}$, n=63)*

Chapter V

Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of aCaMKII inhibitory phosphorylation

Geeske M. van Woerden, Karin D. Harris, Mohammed Reza Hojjati, Richard M. Gustin, Shenfeng Qiu, Rogerio de Avila Freire, Yong-hui Jiang, Ype Elgersma and Edwin J. Weeber

Published in Nat Neurosci. 2007 March;10(3):280-2

Angelman Syndrome (AS) is a severe neurological disorder characterized by mental retardation, motor dysfunction and epilepsy. We now show that the molecular and cellular deficits of an AS mouse model can be rescued by introducing an additional mutation at the inhibitory phosphorylation site of αCaMKII. Moreover, these double mutants do no longer show the behavioral deficits seen in AS mice, suggesting that these deficits are the direct result of increased αCaMKII inhibitory phosphorylation.

Angelman Syndrome (AS) is caused by loss of function of imprinted genes on human chromosome 15q11-13 or by mutations in the *UBE3A* gene which resides in this region[1-4]. Imprinting of this gene results in exclusive maternal expression in hippocampal neurons and cerebellar Purkinje cells[5]. The *UBE3A* gene encodes E6-AP, a ubiquitin protein ligase, but its role remains elusive. Heterozygous mice with a maternally inherited *Ube3a* mutation (from here on designated as AS mice) exhibit seizures, motor and cognitive abnormalities similar to the symptoms seen in AS patients[6]. Biochemical analysis of these mice indicates a reduction of calcium/calmodulin-dependent kinase type 2 (CaMKII) activity, and increased phosphorylation of the inhibitory Thr305 and Thr306 site[7]. Because increased CaMKII inhibitory phosphorylation has a severe impact on neuronal function[8-10], we now investigated whether the increased inhibitory CaMKII phosphorylation is directly responsible for the major deficits seen in AS. Female AS mice were crossed with heterozygous males that carry the targeted *αCaMKII-T305V/T306A* mutation, which prevents αCaMKII inhibitory phosphorylation (designated as CaMKII-305/6$^{+/-}$ mice) (**Supplementary methods**). This resulted in offspring with four different genotypes: wild-type (WT) mice, mutants carrying the AS mutation or αCaMKII-305/6$^{+/-}$ mutation, and mice carrying the double AS/CaMKII-305/6$^{+/-}$ mutation. As shown previously[7], Western blot analysis of hippocampal lysates, showed a significant increase of Thr305 phosphorylation in AS mice (130%; P<0.05 Fisher's PLSD). In contrast, inhibitory phosphorylation was significantly reduced in CaMKII-305/6$^{+/-}$ mutants (40%, P<0.001), and remained at this low level in AS/CaMKII-305/6$^{+/-}$ double mutants (**Fig. 1a and Supplementary Fig.1**). Moreover, the decreased kinase activity of the AS mutants was restored to near wild-type levels in the AS/CaMKII-305/6$^{+/-}$ double mutants (**Fig. 1b)**. Thus, these mutants are suitable to test to what extend increased CaMKII inhibitory phosphorylation is underlying the neurological phenotype of AS mice.

All mutants appeared healthy, but adult (> 2 months) AS mice showed a small (20%) but significant increase in bodyweight (P<0.005 Fisher's PLSD; **Supplementary Fig. 2**). Although obesity is not a characteristic feature of the disease, it has been reported in 15% of all AS children[11] and in the majority of AS children carrying the *Ube3a* mutation[12]. Moreover, obesity is common feature of children with a previously unrecognized form of AS[13]. Notably, increased bodyweight was absent in AS/CaMKII-305/6$^{+/-}$ double mutants (P>0.3). Since αCaMKII expression is restricted to neurons, these results suggest that the increased bodyweight of AS mice has a neurological basis, and is caused by increased αCaMKII Thr$^{305/306}$ phosphorylation.

Epilepsy is a common feature of AS[11, 14]. However, since epilepsy is absent in some AS patients and background-dependent in AS mice[8], several genes seem to be involved in the development of this phenotype. Nevertheless, since reduced αCaMKII activity can result in epilepsy[8], it could potentially contribute to the increased seizure propensity of AS mice. Using audiogenic stimulation, we observed seizures in 50% of the AS mice, whereas no seizures were observed in WT mice or CaMKII-305/6$^{+/-}$ mutants. Notably, there was a 3

fold reduction of seizure propensity in AS/CaMKII-305/6$^{+/-}$ double mutants as compared to the AS mutants (P<0.05 Pearson's test, **Fig. 1c**). This suggests that the increased CaMKII inhibitory phosphorylation strongly contributes to the increased seizure susceptibility of the AS mutants.

Motor coordination deficits are common in all AS patients and AS mice[6, 14]. The *UBE3A* gene is imprinted in cerebellar Purkinje cells[5] and we have previously shown that αCaMKII is essential for cerebellar motor learning and Purkinje cell plasticity[15]. Therefore, these deficits could well be caused by deregulation of cerebellar CaMKII. Motor performance of the mutants was assessed using an accelerating rotarod. Indeed there was a significant effect of genotype ($F_{3,31}$=6.9, P<0.005 repeated measures ANOVA): AS mice stayed significantly shorter on the rotarod compared to their wild-type littermates (P<0.0001 Fisher's PLSD; **Fig.1d**). In contrast, CaMKII-305/6$^{+/-}$ mutants performed better than wild-type mice (P<0.005) whereas AS/CaMKII-305/6$^{+/-}$ mice were normal (WT versus AS/CaMKII-305/6$^{+/-}$: P=0.5). These results suggest that regulation of CaMKII inhibitory phosphorylation plays a critical role in motor coordination performance and that the increased CaMKII inhibitory phosphorylation directly underlies the motor performance deficits of AS mice.

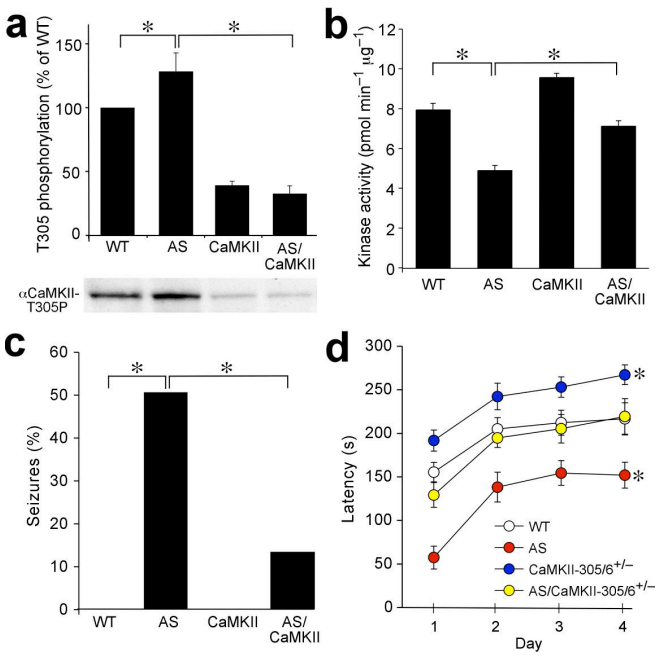


Figure 1. *Reduction of αCaMKII inhibitory phosphorylation rescues CaMKII activity, seizure propensity and motor performance deficits associated with AS.* ***(a)*** *CaMKII Thr305 phosphorylation is reduced in hippocampal lysates of AS/CaMKII-305/6$^{+/-}$ double mutants* ***(b)*** *Restoration of CaMKII activity in hippocampal synaptosomes of AS/CaMKII-305/6$^{+/-}$ double mutants.* ***(c)*** *Propensity to audiogenic seizures of AS mutants is reduced in AS /CaMKII-305/6$^{+/-}$ double mutants.* ***(d)*** *Opposing effects of the AS mutation and CaMKII-305/6$^{+/-}$ mutation on performance on the accelerating rotarod. Asterisks indicate significant differences from wild-type mice. Error bars represent s.e.m. All animal experiments were approved by the Dutch Ethical Committee, or in accordance with Institutional Animal Care and Use Committee guidelines. See text and* ***Supplementary methods*** *for statistics, experimental details and number of subjects used.*

The *UBE3A* gene is also imprinted in the hippocampus[5]. The critical role of αCaMKII in hippocampus-dependent learning[8] makes it likely that the increased inhibitory phosphorylation of αCaMKII underlies the cognitive deficits in AS patients and AS mice[6]. Hippocampal-dependent learning was first assessed using the Morris watermaze task. A probe trial given after 6 days of training revealed a significant effect of genotype when the number of target platform crossings was compared between mutants ($F_{3,66}$=3.7, P<0.05 ANOVA). Indeed, whereas WT and CaMKII-305/6$^{+/-}$ mutants showed significant more crossings of the target platform position as compared to the other positions, AS mice did not (WT P<0.0001; CaMKII-305/6$^{+/-}$ P<0.0001; AS P=0.16, Paired t-test between target and average of other positions)(**Fig 2a,b**). The learning deficit of AS mutants was not due to impaired motor performance or decreased motivation to escape, since all mutants showed similar escape latencies in the hidden platform task ($F_{3,31}$=0.8, P=0.5 repeated measures ANOVA, **Fig 2c**)

as well as in the visible platform task (**Supplementary Fig. 3**). Moreover, swim speed of AS mice was similar as wild-type mice, and the spatial learning deficits were overcome by additional training (**Supplementary Fig. 3**). Importantly, the spatial learning deficits of AS mice at day 6, were again completely rescued by the CaMKII-305/6$^{+/-}$ mutation, since the AS/CaMKII-305/6$^{+/-}$ double mutants showed a highly selective search strategy (target crossing versus average of other positions P<0.0001, Paired t-test).

Hippocampal-dependent learning was also assessed using contextual fear conditioning. Context-dependent memory was tested 24 hours or 7 days following training (**Fig 2d**). A significant effect of genotype was observed at both time points with AS mice freezing significantly less than any other group (24 hours: $F_{3,25}$= 9, P<0.0005 ANOVA all P<0.01 Fisher's PLSD; 7 days: $F_{3,25}$= 26, P<0.0001 ANOVA; all P<0.0001 Fisher's PLSD). In contrast, CaMKII-305/6$^{+/-}$ mutants showed significantly more freezing in the 7 days memory test (all P<0.05 Fisher's PLSD) but not in the 24 hours memory test. Once more, the phenotype was rescued by the AS/CaMKII-305/6$^{+/-}$ double mutation (WT versus AS/CaMKII-305/6$^{+/-}$ 24 hours: P=0.6; 7 days: P=0.4 Fisher's PLSD). Since cued conditioning was normal in the AS mice (**Supplementary Fig. 4**), we conclude that AS mice have a hippocampal learning deficit, and that this deficit can be rescued by decreasing αCaMKII inhibitory phosphorylation.

αCaMKII inhibitory phosphorylation plays an important role in setting the threshold for the induction of long-term potentiation (LTP): homozygous CaMKII-305/6 mice show increased LTP at sub-threshold stimulation, and normal LTP when a strong stimulation protocol is applied[9]. Conversely, AS mice show a striking LTP deficit, which is rescued by a strong stimulation protocol[6, 7]. To directly test whether the LTP deficit of AS mice is due to excessive Thr$^{305/306}$ phosphorylation, we measured LTP in hippocampal slices of the mutants. Consistent with previous findings, AS mice showed a severe LTP deficit compared to wild-type mice ($F_{3,87}$=0.3, P<0.0001 ANOVA; all P<0.001 Fishers PLSD)(**Fig. 2e**). However, LTP was normal in the CaMKII-305/6$^{+/-}$ mice (P=0.5 Fishers PLSD) as well as in the AS/CaMKII-305/6$^{+/-}$ double mutants (P=0.4 Fishers PLSD), suggesting that the plasticity deficits of AS mice are the result of increased CaMKII inhibitory phosphorylation.

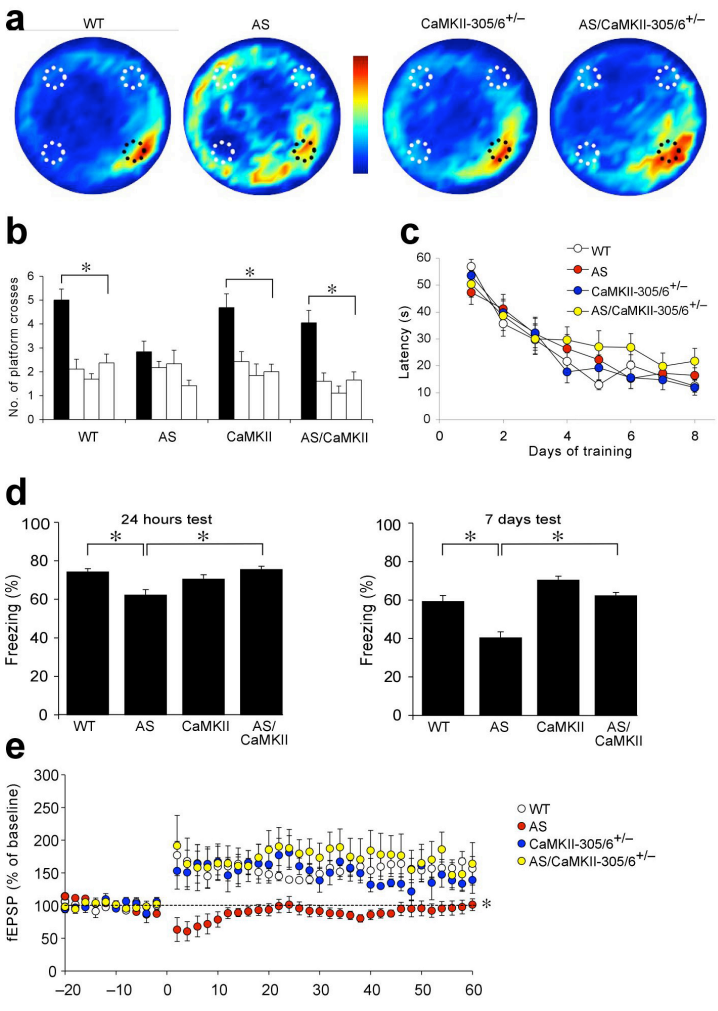


Figure 2. *Hippocampal learning and plasticity of AS mice is rescued by the CaMKII-305/6$^{+/-}$ mutation.* ***(a-c)*** *Learning in the Morris watermaze.* ***(a)*** *A probe trial shows that AS mice search less focussed for the platform as compared to WT and CaMKII-305/6$^{+/-}$ mice. The plots are a visual representation of all search tracks combined, in which the colour indicates the mean time spent at a certain position. The black circle indicates the target platform position used during training. The other (virtual) positions are indicated in white.* ***(b)*** *Quantification of the platform positions as indicated in* ***(a)****. Bars represent target position, adjacent right, opposite, adjacent left position respectively. Asterisks indicate that target position crossing is significantly more than crossing of the other positions.* ***(c)*** *Escape latency to reach the platform shows no difference between the mutants.* ***(d)*** *AS mutants show impaired contextual conditioning. Freezing was assessed either 24 hours, or 7 days after conditioning.* ***(e)*** *LTP deficits of AS mice are rescued by the CaMKII-305/6$^{+/-}$ mutation. Error bars represent s.e.m. See text and* ***Supplementary methods*** *for statistics, experimental details and number of subjects used.*

Although further research is needed to link E6-AP to CaMKII inhibitory phosphorylation, this is the first study to report a functional connection between CaMKII and a human learning and memory disorder. It also represents a fundamental breakthrough in our basic understanding of the molecular basis of the motor, seizure and cognitive facets observed in Angelman syndrome.

Acknowledgements

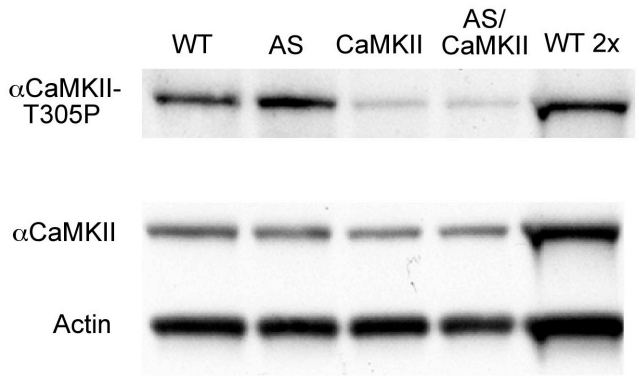
The authors wish to thank M. Elgersma and M. Aghadavoud Jolfaei for technical support. This work was supported by the Netherlands Organisation for Scientific Research (NWO-ZonMW), Hersenstichting Nederland (HsN), Netherlands Epilepsy Fund and the Nina Foundation/ Angelman Vereniging.

References

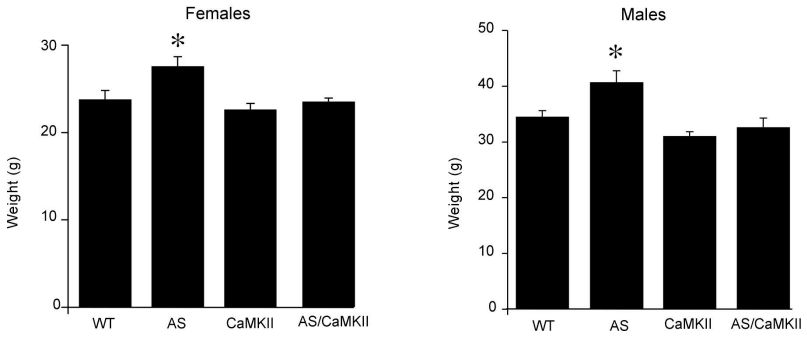
1. Knoll, J.H., *et al.* Angelman and Prader-Willi syndromes share a common chromosome 15 deletion but differ in parental origin of the deletion. *Am J Med Genet* **32**, 285-290 (1989).
2. Sutcliffe, J.S., *et al.* The E6-Ap ubiquitin-protein ligase (UBE3A) gene is localized within a narrowed Angelman syndrome critical region. *Genome Res* **7**, 368-377 (1997).
3. Matsuura, T., *et al.* De novo truncating mutations in E6-AP ubiquitin-protein ligase gene (UBE3A) in Angelman syndrome. *Nat Genet* **15**, 74-77 (1997).
4. Kishino, T., Lalande, M. & Wagstaff, J. UBE3A/E6-AP mutations cause Angelman syndrome. *Nat Genet* **15**, 70-73 (1997).

5. Albrecht, U., *et al.* Imprinted expression of the murine Angelman syndrome gene, Ube3a, in hippocampal and Purkinje neurons. *Nat Genet* **17**, 75-78 (1997).
6. Jiang, Y.H., *et al.* Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron* **21**, 799-811 (1998).
7. Weeber, E.J., *et al.* Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *J Neurosci* **23**, 2634-2644 (2003).
8. Elgersma, Y., Sweatt, J.D. & Giese, K.P. Mouse genetic approaches to investigating calcium/calmodulin-dependent protein kinase II function in plasticity and cognition. *J Neurosci* **24**, 8410-8415 (2004).
9. Elgersma, Y., *et al.* Inhibitory autophosphorylation of CaMKII controls PSD association, plasticity, and learning. *Neuron* **36**, 493-505 (2002).
10. Zhang, L., *et al.* Hippocampal synaptic metaplasticity requires inhibitory autophosphorylation of Ca2+/calmodulin-dependent kinase II. *J Neurosci* **25**, 7697-7707 (2005).
11. Smith, A., *et al.* Clinical features in 27 patients with Angelman syndrome resulting from DNA deletion. *J Med Genet* **33**, 107-112 (1996).
12. Moncla, A., *et al.* Angelman syndrome resulting from UBE3A mutations in 14 patients from eight families: clinical manifestations and genetic counselling. *J Med Genet* **36**, 554-560 (1999).
13. Gillessen-Kaesbach, G., *et al.* A previously unrecognised phenotype characterised by obesity, muscular hypotonia, and ability to speak in patients with Angelman syndrome caused by an imprinting defect. *Eur J Hum Genet* **7**, 638-644 (1999).
14. Clayton-Smith, J. Clinical research on Angelman syndrome in the United Kingdom: observations on 82 affected individuals. *Am J Med Genet* **46**, 12-15 (1993).
15. Hansel, C., *et al.* alphaCaMKII Is essential for cerebellar LTD and motor learning. *Neuron* **51**, 835-843 (2006).

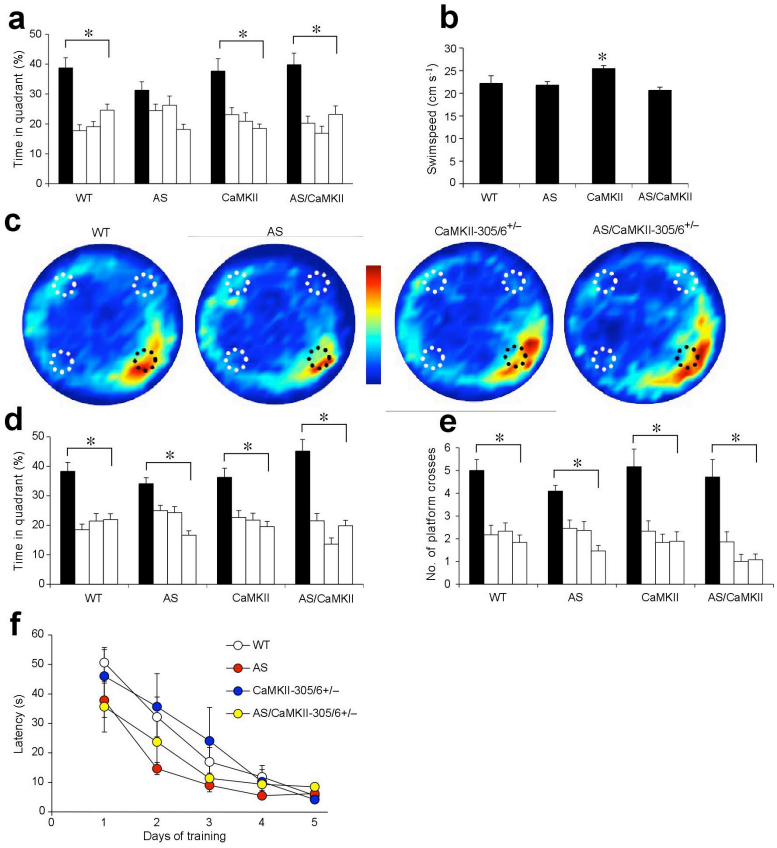
Supplementary figures



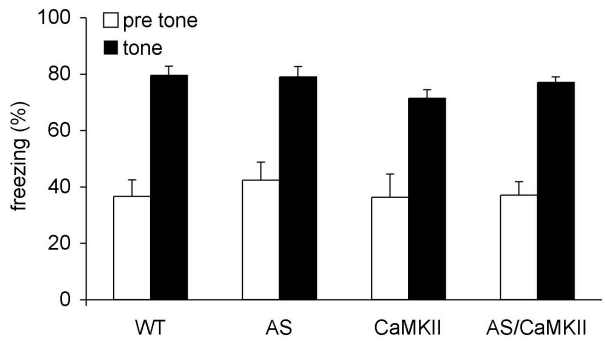
Supplementary Figure 1. *Decreased inhibitory phosphorylation in hippocampal lysates of AS/CaMKII-305/6$^{+/-}$ double mutants. AS mice show a significantly increased CaMKII-T305 phosphorylation, as compared to their WT littermates, which is decreased when crossed with CaMKII-305/6$^{+/-}$ mice. Western blots were first probed with antibodies directed against Thr305-αCaMKII and after stripping re-probed with αCaMKII and Actin. To ensure that the staining was within the linear range, the wild-type samples were also loaded at a two fold higher concentration.*



Supplementary Figure 2. *The increased bodyweight in AS mice returns to WT level in AS/CaMKII-305/6$^{+/-}$ mice. AS mice show a small but significant (P<0.005) increase in bodyweight (females (2.5 months old): $F_{3,48}$=5.6, P<0.005, males (4 months old): $F_{3,13}$ =9.6, P<0.005 ANOVA; Post hoc analysis: all P< 0.005 Fisher's PLSD), which was absent in AS/CaMKII-305/6$^{+/-}$ double mutants (P>0.3 for males and females). Error bars represent SEM.*



Supplementary Figure 3. *Learning of the hidden platform (a-e) and visible (f) platform watermaze. AS mice show impaired hippocampal learning at the probe trial given at day 6 compared to their wild type littermates, which is improved after two additional training days. (**a**) During the probe trial at day 6 AS mice do not spent significantly more time in the target quadrant as compared to all the other quadrants, whereas the AS/CaMKII-305/6$^{+/-}$ mice show high preference for the target quadrant. (**b**) The learning impairment of the AS mice cannot be ascribed to motor impairments or motivational problems, as they swim as fast as their wild type littermates. Only the CaMKII-305/6$^{+/-}$ mice tend to swim faster. (**c,d,e**) At the probe trial given at day 8, the AS mice caught up with their littermate controls and showed selective searching as judged from the time spend in target quadrant (**d**) and from the number of platform crosses (**e**). To exclude that AS mice have visual or motivational problems that could account for the impaired learning, a visible watermaze was performed and the escape latency was determined. This task shows no effect of genotype ($F_{3,14}$=1.4, P=0.3 repeated measures ANOVA). Error bars represent SEM.*



Supplementary Figure 4. *AS mice show no deficits in cued conditioning. Cued conditioning test was used to assess whether the contextual conditioning phenotype of the AS mice was hippocampus-dependent. Cued conditioning did not reveal any difference between the different mutants, indicating that the contextual phenotype found in AS mice is indeed hippocampus-dependent ($F_{3,34}$ =0.6, P=0.6 repeated measures ANOVA). Error bars represent SEM.*

Material and Methods

Breeding of mutants.

Mutants with the *Ube3a* null mutation (designated as AS mice) and mutants carrying the targeted *αCaMKII-T305V/T306A* mutation which prevents phosphorylation at these residues (designated as CaMKII-305/6$^{+/-}$ mice) were developed as described previously[6, 9]. All experiments described in this paper were carried out using hybrid mice in the mixed 129/Sv-C57BL/6 background. To that end, male αCaMKII-305/6$^{+/-}$ mutants in the C57BL/6 background were crossed with female AS mutants in the 129/Sv background (weight, rotarod, watermaze) or with female AS mutants in the 129/Sv-C57BL/6 background (epilepsy, fear conditioning, LTP).

Mice were genotyped when they were 7-10 days, but the experimenter remained blind for the genotype during data collection and the initial analysis. Genotypes were again established after all experiments were done and the code was then broken to perform the final statistical analysis. All experiments were done with 2-4 month old littermates that were housed in groups of 2-3 per homecage. Genotype groups were approximately sex and age matched. Single-housed mice were excluded for the behavioral studies. The mice were kept on a 12h light/dark cycle, with food and water available ad libitum. The behavioral experiments were performed during the light period of the cycle. All animal experiments were approved by the Dutch Ethical Committee, or in accordance with guidelines of Institutional Animal Care and Use Committee of Vanderbilt University.

Western blot analysis of the phosphorylation state of CaMKII.

Lysates were prepared by quickly dissecting the hippocampus followed by homogenization in lysis buffer (10mM TRIS-HCl 6.8, 2.5% SDS, 2mM EDTA and protease and phosphatase inhibitor cocktails (Sigma)). Lysates were then adjusted to 1 mg/ml, and 10 µg was used for SDS-PAGE analysis and Western blotting. Western blots were first probed with antibodies directed against Thr305-αCaMKII (pB60[9], 1:5000) and after stripping re-probed with αCaMKII (MAB3119, 1:10000; Chemicon), and Actin (MAB1501R, 1:2000; Chemicon). Blots were quantified using NIH-Image using the αCaMKII signal as loading control, and normalized against wild-type. The number of mice used: WT (4); AS (4); CaMKII-306/5$^{+/-}$ (4); AS/CaMKII-305/6$^{+/-}$ (4).

CaMKII Activity Measurement

Mice were sacrificed by cervical dislocation and hippocampi were quickly isolated in ice-cold cutting solution and sonicated in cold homogenization buffer containing 4 mM HEPES, 320 mM sucrose and protease inhibitor cocktail (Sigma, Saint Louis, MO). The homogenates were centrifuged at 1500 g for 10 min. The supernatant was then centrifuged at 16000 g for 20 min to yield a pellet containing the crude synaptosome fraction. This fraction was resuspended in 200µl homogenization buffer to measure the Ca^{2+}/calmodulin dependent CaMKII activity using the assay kit from Millipore Inc. (14-217). The number of mice used: WT (6); AS (6); CaMKII-306/5$^{+/-}$ (6); AS/CaMKII-305/6$^{+/-}$ (6).

Weight and seizure susceptibility

Female AS mice started to show increased bodyweight after 2 months, whereas male AS mice started to become significantly heavier after 4 months. Number of mice used: females (8-9 weeks): WT (13); AS (7); CaMKII-305/6$^{+/-}$ (10); AS/CAMKII-305/6$^{+/-}$, (13). Males (16-18 weeks): WT (4); AS (3); CaMKII-305/6$^{+/-}$ (6); AS/CaMKII-305/6$^{+/-}$ (4). To assess seizure susceptibility we induced audiogenic seizures by vigorously scratching scissors across the metal grating of the mouse cage lid for 20 seconds, or shorter if a seizure developed before that time. The number of mice used in this paradigm: WT (11); AS (10); CaMKII-305/6$^{+/-}$ (12); AS/CaMKII-305/6$^{+/-}$ (15).

Motor performance.

We tested motor function in F1 mice using the accelerating rotarod (4-40 rpm, in 5 minutes; model 7650, Ugo Basile Biological Research Apparatus, Varese, Italy). Mice were given two trials per day with a 45-60 min inter trial interval for 4 consecutive days. For each day we calculated the average of the time spent on the rotarod, or of the time until the mouse made 3 consecutive rotations on the rotarod. Maximum duration of a trial was 5 min. The number of mice used: WT (10); AS (7); CaMKII-305/6$^{+/-}$ (10); AS/CaMKII-305/6$^{+/-}$ (8).

Water Maze.

To test spatial memory we used the Water Maze. Prior to the test mice were handled extensively (2 min/day; 5 days). Our pool is 1.2 m in diameter and has an 11 cm diameter platform submerged 1 cm below the water surface. The water is painted milk-white with non-toxic paint and water temperature is kept constant at 26°C. We use dimmed lighting, and mouse-tracking is performed using SMART version 2.0 (Panlab, Barcelona, Spain). Mice were given 2 trials per day, with 30 sec inter trial interval for 8 consecutive days. At a training session, the mouse was first placed on the platform for 30 sec. Then it was placed in the water at a pseudo-random start position and it was given a maximum of 60 seconds to find the platform. If the mouse did not find the platform within 60 seconds, it was placed back on the platform. After 30 seconds on the platform, this training procedure was repeated once more. The platform position remained at the same position during all trials.

One hour after the training on day 6 and day 8, a probe trial was given to test spatial learning. Mice were placed on the platform for 30 seconds, after which the platform was removed from the pool and the mice were placed in the pool at the opposite side of the previous platform position. The mice were then allowed to search for the platform for 60 seconds. The number of mice used for this paradigm: WT (18); AS (11); CaMKII-306/5$^{+/-}$ (18); AS/CaMKII-305/6$^{+/-}$ (14).

The visible water maze was performed done in the same way as the hidden platform water maze, except that this time the platform was flagged with a small cue on top the platform. The number of (naïve) mice used for this paradigm: WT (6); AS (5); CaMKII-306/5$^{+/-}$ (4); AS/CaMKII-305/6$^{+/-}$ (3).

Fear Conditioning.

Fear conditioning was performed in a testing chamber (26 × 22 × 18 cm; San Diego Instruments, San Diego, CA) made of Plexiglas equipped with a grid floor via which the foot shock could be administered and a CCD camera to monitor activity. The conditioning chamber was placed inside a soundproof isolation cubicle. Training and testing occurred in the presence of white light and white noise. Each mouse was placed inside the conditioning chamber for 150 seconds before the onset of a 2 s foot shock (0.5 mA). After 150 seconds, a second foot shock was delivered, and the mouse was returned to its home cage after another 2.5 min. Testing of context-dependent fear was performed either 24 hours or 1 week after the conditioning in the same context.

For cued conditioning, each mouse was placed inside the conditioning chamber for 2 min before the onset of a conditioned stimulus (CS; an 85 dB tone), which lasted for 30 s. training occurred in the presence of white light and white noise. A 2 s US foot shock (0.5 mA) was delivered immediately after the termination of the CS. Each mouse remained in the chamber for an additional 120 s, followed by another CS–US pairing. Each mouse was returned to its home cage after another 2.5 min. Cued fear conditioning was tested in the presence of a masked context consisting of a small Plexiglas cube attached to an opaque

Plexiglas floor insert, different lighting conditions, and a vanilla odor. Each mouse was placed in this novel context for 3 min at approximately 24 h after training, and they were exposed to the CS for another 3 min.

Freezing behavior was recorded and processed by SDI Photobeam Activity System software throughout each testing session. The mouse was considered to be freezing after a lack of movement for 2 s. Number of animals used: 24 hours context: WT (6); AS (6); CaMKII-305/$6^{+/-}$ (8); AS/CaMKII-305/$6^{+/-}$ (9) ; 1 week context: (WT (7); AS (8); CaMKII-305/$6^{+/-}$ (7); AS/CaMKII-305/$6^{+/-}$ (8). 24 hours cued: WT (11); AS (11); CaMKII-305/$6^{+/-}$ (7); AS/CaMKII-305/$6^{+/-}$ (9) ;

Shock threshold was assessed by placing the animal in the conditioning chamber and by delivering foot shocks starting at 0.075 mA and increasing by 0.05 mA every 30 s. The experiment was terminated at the shock intensity sufficient to induce vocalization. There was no difference in shock sensitivity between the groups ($F_{3,75}$=0.53, P=0.9 ANOVA).

Hippocampal slice preparation and electrophysiology

The brain was quickly removed and placed in ice-cold high sucrose cutting solution containing (in mM): 110 sucrose, 60 NaCl, 3 KCl, 28 $NaHCO_3$, 1.25 NaH_2PO_4, 7 $MgCl_2$, 0.5 $CaCl_2$, 0.6 sodium ascorbate, and 5 D- glucose, pH 7.3–7.4. Horizontal 400 μm sections were cut in sucrose cutting solution using a Vibratome. The slices were stored in 95% O_2/5% CO_2-equilibrated artificial CSF containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 28 $NaHCO_3$, 1.0 $MgCl_2$, 2.0 $CaCl_2$, and 10 D- glucose, (pH 7.3-7.4) and kept at room temperature for at least 1 hour before switching to the interface chamber supported by a nylon mesh and allowed to recover for a minimum of 1 hour prior to recording. The chamber was kept at 32 ± 0.5 °C with a laminar ACSF flow rate of 2–3 ml/min. Extracellular field recordings were obtained from area CA1 of the stratum radiatum. Stimulations were supplied with a bipolar Teflon-coated platinum electrode and recordings were obtained with the use of a glass microelectrode field with ACSF with tip diameter about 1 μm (1–4 MΩ electrical resistance). Tetani used to evoke CA1 LTP consisted of two trains of 100 Hz stimulation for 1s with each train separated by a 20s interval. Stimulus intensities were adjusted to give pEPSPs (population excitatory postsynaptic potentials) with slopes that were <50% of the maximum determined from an input/output curve. Potentiation was measured as the normalized increase of the mean pEPSP for the duration of the baseline recording. Experimental results were obtained from those slices that exhibited stable baseline synaptic transmission for a minimum of 30 min prior to the delivery of the LTP- inducing stimulus. For recording we used an Axon 1320 Digidata data acquisition hardware operated by Axon pClamp 9.2 software. We used maximally two slices per mouse. The number of slices used for the experiment are: WT (11); AS (6); CaMKII-305/$6^{+/-}$ (6); AS/CaMKII-305/$6^{+/-}$ (6).

Statistical analysis.

StatView data analysis software was used for statistical analysis. An ANOVA or repeated measures ANOVA was used for multiple group data, to test for the effect of genotype. If this was significant, the data was further analyzed using the Fisher PLSD Post Hoc test. All figure data represents mean ±SEM. An analysis with a value of p<0.05 was considered to be statistically significant (indicated by an asterisk in the figures).

Chapter VI

General Discussion:
What can still be learned about CaMKII

The overall aim of the studies described in this thesis was to further elucidate the role of CaMKII in plasticity, learning and disease. To obtain more knowledge on CaMKII we made use of different α- and βCaMKII mutants.

To clarify the role of αCaMKII in presynaptic plasticity we analyzed different αCaMKII mutants (chapter II)[1]. By looking at Synapsin I phosphorylation in the different mutants, it was first determined which mutants still exert enzymatic functions. Only in the αCaMKII knockout and the dominant negative αCaMKII^{T305D} mutant showed no phosphorylation of Synapsin I. However, when analyzing presynaptic plasticity, the αCaMKII^{T305D} showed no difference compared to wildtypes, whereas the αCaMKII knockout showed enhanced synaptic augmentation and decreased synaptic fatigue. Together with the data obtained using competitive inhibitors blocking Ca^{2+}/CaM binding, we showed unambiguously that kinase activity of αCaMKII, and phosphorylation of Synapsin I by αCaMKII, is not required for presynaptic plasticity. The decreased synaptic fatigue could be the result of an increase in the number of docked vesicles in the αCaMKII knockout mice, which indeed appeared to be the case. Hence, it can be concluded that αCaMKII plays a structural role at the presynaptic site, limiting the number of docked vesicles, and thereby modulating short-term presynaptic plasticity. Recently, a kinase dead αCaMKII mutant (αCaMKII^{K42R}) was generated and further supporting our findings, this mutant showed no difference in short-term presynaptic plasticity[2]. There is however still some discrepancy on the role of CaMKII in presynaptic plasticity. αCaMKII has been shown to either enhance or inhibit presynaptic plasticity, depending on the experimental setup or physiological circumstances. Therefore, it is hypothesized that presynaptic CaMKII is a bidirectional modulator of neurotransmitter release, depending on the sort of presynaptic activity[3]. It would be very interesting to see what the binding partners for αCaMKII at the presynaptic site are, and how they play a role in regulating the direction of plasticity.

Can we conclude now that presynaptic αCaMKII activity is not important at all? Presynaptic CaMKII activity has been shown to be required for CA3-CA3 LTP in organotypic slices[4], indicating that presynaptic αCaMKII activity is required for other presynaptic measures, for example those that involve a longer time scale. The best way to test this hypothesis would be to generate CA3-specific αCaMKII knockouts and measure the effect on long-term plasticity and learning.

In chapter III and IV we started to unravel the role of βCaMKII in plasticity and learning. Since βCaMKII is the predominant CaMKII isozyme in the cerebellum[5], and αCaMKII was already shown to play an important role in cerebellar motor learning and plasticity[6], it is very likely that this isozyme is of even greater importance for cerebellar functioning. In chapter III of this thesis we started with assessing the role of βCaMKII in cerebellar plasticity using βCaMKII$^{-/-}$ mouse[7]. Surprisingly, even though most *in vitro* studies revealed an important role for βCaMKII in neuronal development and dendritic arborization[8, 9], the βCaMKII$^{-/-}$ mice did not show deficits in overall brain development or Purkinje cell maturation[7]. However, the mice did show severe problems at the parallel fiber-Purkinje cell synapse, in that plasticity at this synapse appeared to be completely reversed: LTP inducing stimulus protocols induces LTD in βCaMKII$^{-/-}$ and vice versa, indicating that this isozyme is of crucial importance for the direction of plasticity at this synapse.

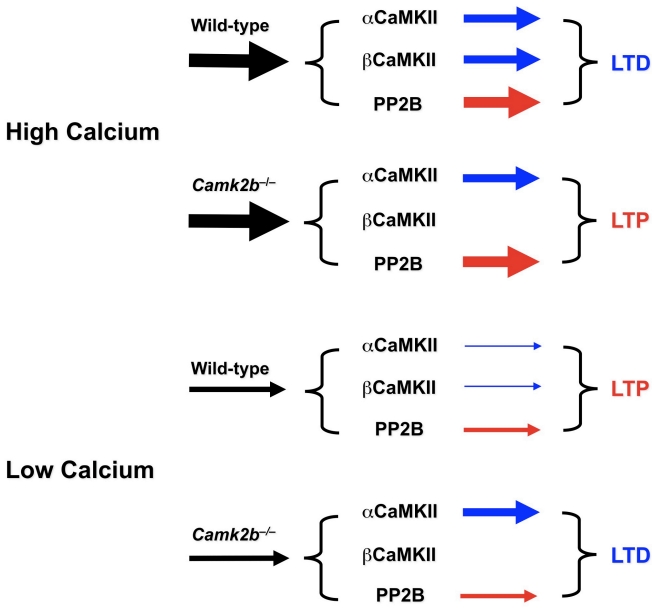


Figure 1. Role of CaMKII and PP2B in parallel fiber-Purkinje cell plasticity. *Simplified model depicting the role of αCaMKII, βCaMKII and phosphatase PP2B in LTP and LTD at the parallel fiber-Purkinje cell synapse. In wild-type Purkinje cells high calcium influx (top) activates the phosphatase PP2B driven synaptic potentiation pathway but also results in maximal induction of the CaMKII driven synaptic depression pathway, which effectively out-competes PP2B, leading to a net LTD. In contrast, in the absence of βCaMKII the synaptic depression pathway (which requires αCaMKII and βCaMKII activity) is no longer able to out-compete PP2B, resulting in net LTP.*

Low calcium influx (bottom) only moderately activates PP2B, but as there is no or insignificant activation of CaMKII, the net result is LTP. In βCaMKII$^{-/-}$ Purkinje cells low calcium influx results in precocious activation of αCaMKII, which is sufficient to out-compete PP2B, resulting in net LTD.

What could explain the inversed plasticity in the βCaMKII$^{-/-}$ mutants? It is widely accepted that in the Purkinje cell there is a balance between phosphatase and kinase activity, with the kinases being more activated during LTD induction (high Ca^{2+} influx) and the phosphatases being more activated during LTP induction (low Ca^{2+} influx) (as reviewed by[10]). According to the impact of αCaMKII deletion on cerebellar function in adult mice[6], it is reasonable to assume that the CaMKIIs are one of the most important kinases in the Purkinje cells of adult mice. Therefore, we propose the following model (fig 1): When one of CaMKII isozymes (αCaMKII or βCaMKII) is absent, the balance during high calcium influx is shifted towards the phosphatase PP2B and LTP instead of LTD will be induced. Using phosphatase blockers we showed that this is indeed the case. However, this phosphatase/kinase balance does not explain the shift from LTP to LTD, during low calcium levels. βCaMKII tethers αCaMKII to the actin cytoskeleton limiting the number of αCaMKII subunits that can get activated during low calcium stimulation. This makes it reasonable to assume, that when βCaMKII is absent, more αCaMKII homomers are available to become activated under low calcium conditions, thereby outweighing PP2B, resulting in LTD instead of LTP, which was confirmed using the CaMKII inhibitor KN93. Hence, we hypothesized that βCaMKII activity is required for LTD expression, whereas under LTP inducing conditions, βCaMKII prevents activation of the LTD pathway. However, we cannot exclude a possible role for other neurons, since βCaMKII$^{-/-}$ is expressed in most of the cell types in the cerebellum[6, 11]. It would be very interesting to

assess the role of βCaMKII in the other cell types as well. Cell-specific βCaMKII knockouts could be a useful tool to study the specific roles of βCaMKII in all these different cell types in the cerebellum.

In chapter IV of this thesis we describe the role of βCaMKII in the hippocampus, using the βCaMKII$^{-/-}$ and the βCaMKII^{A303R} mice. Interestingly, we found that absence of βCaMKII results in impaired LTP and impaired fear conditioning memory in mice, whereas the presence of βCaMKII, even though it cannot be activated (βCaMKII^{A303R}) results in normal LTP and fear conditioning memory in mice. These results indicate that in the hippocampus, the presence of βCaMKII is sufficient for plasticity and learning, hence that the role of βCaMKII in the hippocampus is more likely to be structural instead of enzymatic.

It is still puzzling that the βCaMKII^{A303R} mice do not show a phenotype, even though theoretically, the αCaMKII containing holoenzymes cannot be released from actin and therefore cannot be translocated. It can be hypothesized that the βCaMKII containing holoenzymes attached to actin are close enough to the PSD, so that αCaMKII, even though it cannot be released from the actin cytoskeleton, is still able to phosphorylate some of its substrates, enough to induce LTP. It has been shown that in hippocampal cultures, actin-bound βCaMKII is located mostly in the dendritic spines, and therefore that αCaMKII is already close to the PSD[12]. It can also be hypothesized that in the βCaMKII^{A303R} mice there are more αCaMKII homomers present as to compensate for the αCaMKII subunits that cannot be released from the actin cytoskeleton, and that the actin cytoskeleton now functions as a 'gate', keeping the spine closed to prevent αCaMKII to disperse out of the synapse. Pulldown assays using the βCaMKII antibody, and investigating how much αCaMKII comes down with βCaMKII can answer the question which of these hypotheses is more likely to be true.

What mechanism can explain the phenotype in the βCaMKII$^{-/-}$ mice? When βCaMKII is absent, αCaMKII cannot bind to actin anymore and could therefore be dispersed throughout the neuron, leaving not enough αCaMKII molecules located at the proper position to be able to induce LTP upon stimulation. Indeed, *in vitro* studies showed that reducing the amount of βCaMKII[12], or treating cells with actin depolymerisation drugs[13], results in dispersion of αCaMKII away from the PSD. If it is true that there is not enough αCaMKII at the proper location, it is likely that when the intracellular [Ca^{2+}] increases not only in the spine, but also in the dendrite, more αCaMKII is activated, which then could induce LTP. This could be tested using a 200 Hz protocol, which also activates the voltage-gated calcium channels (VGCCs)[14], inducing a large Ca^{2+} influx at different locations. This could then rescue the LTP deficit in the βCaMKII$^{-/-}$ mice. However, the only way to directly prove the dislocalization of αCaMKII is by using fluorescent microscopy or immunogold labeling and look directly at the localization of αCaMKII in neurons of βCaMKII$^{-/-}$ mice.

Taken together, βCaMKII plays a non-enzymatic role in the hippocampus, and it is hypothesized that it regulates the subcellular localization of αCaMKII. A simplified model of αCaMKII localization in the different βCaMKII mutants is shown in figure 2.

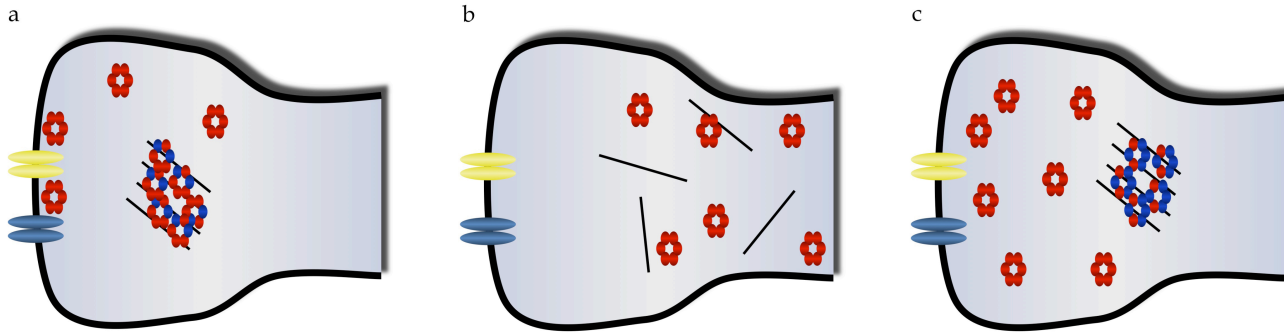


Figure 2: Model for αCaMKII localisation in βCaMKII mutants. *a) WT synapse. The actin cytoskeleton is highly organized and most of the αCaMKII subunits are tethered to the actin cytoskeleton by βCaMKII, b) βCaMKII$^{-/-}$ synapse. The actin cytoskeleton is not organized anymore and the αCaMKII homomers are dispersed throughout the neuron, c) βCaMKII^{A303R} synapse. The actin cytoskeleton is highly organized and prevents αCaMKII homomers to disperse from the spine. Depicted are postsynaptic CA1 synapses at rest stage. Red dots: αCaMKII; blue dots: βCaMKII; black stripes: actin; Yellow ovals: NMDA receptor; Blue ovals: AMPA receptor.*

When considering the results in chapter III and IV it becomes clear that the function of βCaMKII depends on the brain region. Deletion of βCaMKII in the cerebellum has more impact than deletion of βCaMKII in the hippocampus. What causes this difference? It is known that there is a difference in the α:β ratio in the two brain regions. In the hippocampus, the α:β ratio is 3:1[15], whereas in the cerebellum the α:β ratio is 1:4[16]. However, these ratios are an average for the complete hippocampus and cerebellum, when looking at specific cell types, these ratios change. In the hippocampus, αCaMKII is solely expressed in excitatory neurons[17, 18], whereas βCaMKII is expressed also in inhibitory interneurons. In the cerebellum, again αCaMKII is limited to one cell type, namely Purkinje cells[19], whereas βCaMKII is expressed throughout the cerebellum in most of the cell types[11]. Thus, when looking at the cell types where both isoforms are present, *i.e.* excitatory neurons in the hippcampus and Purkinje cells in the cerebellum, the ratio between αCaMKII and βCaMKII changes to approximately 4:1 and 1:1 respectively. It can be assumed that this has direct implications for the importance of βCaMKII in these cell types. Absence of βCaMKII in Purkinje cells is likely to result in a more severe phenotype than absence of βCaMKII in excitatory neurons in the hippocampus, which is exactly what we see. Apart from that, we have shown that βCaMKII fulfills different functions in the different cell types. In Purkinje cells, βCaMKII has both an enzymatic and a structural role, whereas in the hippocampus, βCaMKII is more likely to play only a structural role. Therefore, it is of great interest to look at the different targets of βCaMKII in both cell types to see what the differences are to finally get an idea of the mechanisms underlying plasticity in both the cerebellum and the hippocampus.

It has previously been shown that AS mice have increased inhibitory autophosphorylation at the TT305/6 residues of αCaMKII[20]. In Chapter V we assessed whether the phenotype seen in AS mice is the result of this increased inhibition of αCaMKII. To test this hypothesis, we crossed CaMKII$^{TT305/6VA}$ mice, in which the inhibitory phosphorylation sites are blocked, with AS mice[21]. Surprisingly, introducing this counter mutation in AS mice completely rescued the motor deficits, the learning problems and the impaired plasticity. The epilepsy was largely, although not completely, rescued, indicating that other genes are involved in this phenotype. Taken together, these

results prove that CaMKII plays a major role in the pathophysiology of Angelman Syndrome.

What could be the link between the loss of E6AP and the increase in inhibitory phosphorylation of CaMKII? Three models can be proposed (fig 3).

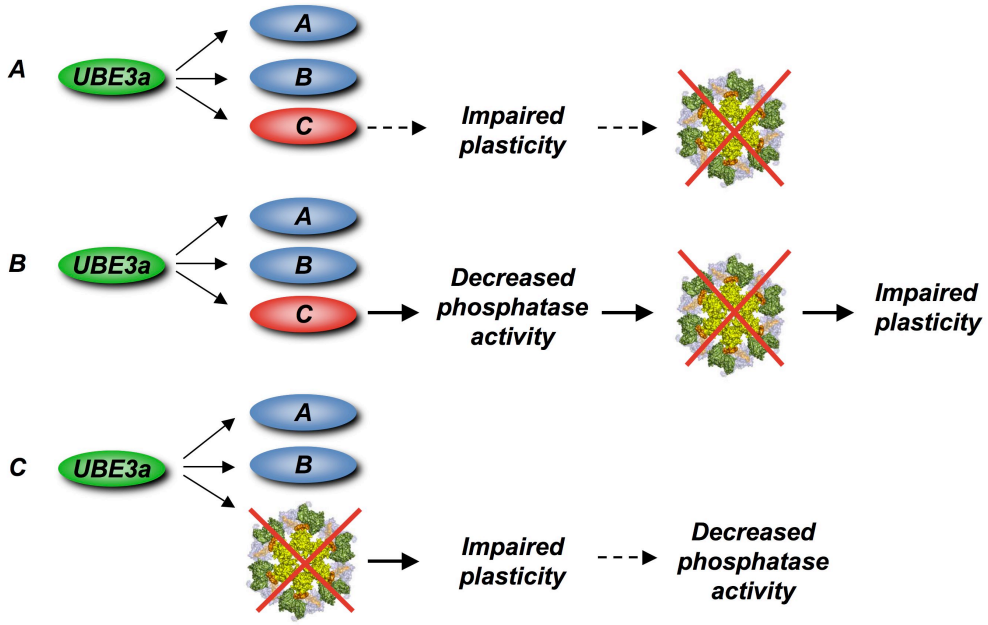


Figure 3. *Three models showing how the absence of UBE3A can cause an increase in self-inhibited CaMKII. See text for a detailed description of these models.*

In the first model (fig 3a) ('indirect model'), it is assumed that UBE3A has many different targets, and that one (or several) of these targets is essential for normal neuronal function and plasticity. The misregulation of this critical target (designated 'C') could cause plasticity defects, which in turn results in self-inhibition of CaMKII. This so called 'basal' self-inhibition occurs in the prolonged absence of calcium, and has been shown to occur *in vitro*[22]. However, the finding that we can rescue the neurological and plasticity deficits seen in AS mice by decreasing the amount of CaMKII that can undergo self-inhibition[21], cannot be explained by this model, making this the least attractive model to pursue.

In the second model (fig 3b) ('phosphatase model') a critical protein controlling phosphatase function is misregulated in the absence of UBE3A. This results in reduced phosphatase function, which leaves CaMKII in the inhibited (T305/T306 phosphorylated) form. This increased self-inhibition leads to plasticity deficits. This model takes into account the finding that PP1/2a activity is reduced in AS mice[20]. Very recently a new target for UBE3A was discovered, the promyelocytic leukemia tumor suppressor (PML)[23]. Intriguingly, another study showed that PML regulates cell fate in the developing neocortex, indicating it plays a role in neuronal function[24]. The exact role of PML in neuronal function is still unknown, but it is shown that PML regulates PP1 and PP2a localization in the cell[24]. This could result in reduced dephosphorylation of CaMKII: overexpression of PML due to absence of UBE3A, results in delocalization of PP1 and PP2a, away from CaMKII, hence less dephosphorylation of CaMKII. This model can

explain how the neurological and plasticity deficits seen in AS mice are rescued by reducing the amount of self-inhibited CaMKII[21].

In the third model (fig 3c) ('direct model') it is hypothesized that CaMKII itself is a direct target for UBE3A. It could be that the self-inhibited form of CaMKII undergoes a conformational shift, which makes it a target for UBE3A. In the absence of UBE3A this self-inhibited form starts to accumulate, which affects the dendritic local synthesis of new CaMKII. The net effect would be that the total amount of CaMKII is unaffected, however most of it is in the self-inhibited form. The accumulation of the self-inhibited form reduces neuronal plasticity. The neuron attempts to counteract the reduced plasticity by decreasing phosphate activity which under normal conditions would favour induction of long-term plasticity (LTP)[25]. This model is compatible with all our current knowledge of the molecular mechanisms underlying synaptic plasticity, and it also explains why all the neurological and plasticity deficits of AS mice can be rescued so well by decreasing the amount of self-inhibited CaMKII[21].

Even though we propose three different models, it could also be that the function of PP1 and PP2a are indeed the cause of the decreased dephosphorylation of CaMKII and that CaMKII is a target for UBE3A, thus a combination of model 2 and 3. To test model 2 it would be interesting to cross PML$^{-/-}$ mice with AS mice and test whether we can rescue the phenotypes again. To test if CaMKII is a target for UBE3A, pull down assays and yeast two hybrid screens could be done.

But knowing the exact pathway or mechanisms underlying Angelman Syndrome, does not necessarily mean that we can go and find a cure for these patients. One question that needs to be addressed first is whether synaptic function becomes normal again upon for example re-expression of the UBE3A protein. This is important, because there is a chance that absence of the protein changes synaptic networks already during development, which cannot be reversed at a later age. If that is the case, then there is less chance of finding a cure for the patients. Therefore it is of high importance to first test in AS mice whether the phenotype can be rescued in adulthood. One of the methods to do that is to use inducible knockouts.

Hopefully, by knowing the exact pathway or mechanisms underlying Angelman Syndrome, we will be able to find a treatment for these patients in the future.

References

1. Hojjati, M.R., *et al*. Kinase activity is not required for alphaCaMKII-dependent presynaptic plasticity at CA3-CA1 synapses. *Nat Neurosci* **10**, 1125-1127 (2007).
2. Yamagata, Y., *et al*. Kinase-dead knock-in mouse reveals an essential role of kinase activity of Ca2+/calmodulin-dependent protein kinase IIalpha in dendritic spine enlargement, long-term potentiation, and learning. *J Neurosci* **29**, 7607-7618 (2009).
3. Wang, Z.W. Regulation of synaptic transmission by presynaptic CaMKII and BK channels. *Mol Neurobiol* **38**, 153-166 (2008).
4. Lu, F.M. & Hawkins, R.D. Presynaptic and postsynaptic Ca(2+) and CamKII contribute to long-term potentiation at synapses between individual CA3 neurons. *Proc Natl Acad Sci U S A* **103**, 4264-4269 (2006).
5. Erondu, N.E. & Kennedy, M.B. Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain. *J Neurosci* **5**, 3270-3277 (1985).
6. Hansel, C., *et al*. alphaCaMKII Is essential for cerebellar LTD and motor learning. *Neuron* **51**, 835-843 (2006).

7. van Woerden, G.M., *et al*. betaCaMKII controls the direction of plasticity at parallel fiber-Purkinje cell synapses. *Nat Neurosci* **12**, 823-825 (2009).
8. Nomura, T., Kumatoriya, K., Yoshimura, Y. & Yamauchi, T. Overexpression of alpha and beta isoforms of Ca2+/calmodulin-dependent protein kinase II in neuroblastoma cells -- H-7 promotes neurite outgrowth. *Brain Res* **766**, 129-141 (1997).
9. Fink, C.C., *et al*. Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CaMKII. *Neuron* **39**, 283-297 (2003).
10. Ito, M. Cerebellar long-term depression: characterization, signal transduction, and functional roles. *Physiol Rev* **81**, 1143-1195 (2001).
11. Conlee, J.W., Shapiro, S.M. & Churn, S.B. Expression of the alpha and beta subunits of Ca2+/calmodulin kinase II in the cerebellum of jaundiced Gunn rats during development: a quantitative light microscopic analysis. *Acta Neuropathol* **99**, 393-401 (2000).
12. Shen, K., Teruel, M.N., Subramanian, K. & Meyer, T. CaMKIIbeta functions as an F-actin targeting module that localizes CaMKIIalpha/beta heterooligomers to dendritic spines. *Neuron* **21**, 593-606 (1998).
13. Allison, D.W., Chervin, A.S., Gelfand, V.I. & Craig, A.M. Postsynaptic scaffolds of excitatory and inhibitory synapses in hippocampal neurons: maintenance of core components independent of actin filaments and microtubules. *J Neurosci* **20**, 4545-4554 (2000).
14. Grover, L.M. & Teyler, T.J. Two components of long-term potentiation induced by different patterns of afferent activation. *Nature* **347**, 477-479 (1990).
15. Brocke, L., Chiang, L.W., Wagner, P.D. & Schulman, H. Functional implications of the subunit composition of neuronal CaM kinase II. *J Biol Chem* **274**, 22713-22722 (1999).
16. Miller, S.G. & Kennedy, M.B. Distinct forebrain and cerebellar isozymes of type II Ca2+/calmodulin-dependent protein kinase associate differently with the postsynaptic density fraction. *J Biol Chem* **260**, 9039-9046 (1985).
17. Benson, D.L., Isackson, P.J., Hendry, S.H. & Jones, E.G. Differential gene expression for glutamic acid decarboxylase and type II calcium-calmodulin-dependent protein kinase in basal ganglia, thalamus, and hypothalamus of the monkey. *J Neurosci* **11**, 1540-1564 (1991).
18. Benson, D.L., Isackson, P.J., Gall, C.M. & Jones, E.G. Contrasting patterns in the localization of glutamic acid decarboxylase and Ca2+/calmodulin protein kinase gene expression in the rat central nervous system. *Neuroscience* **46**, 825-849 (1992).
19. Walaas, S.I., *et al*. Cell-specific localization of the alpha-subunit of calcium/calmodulin-dependent protein kinase II in Purkinje cells in rodent cerebellum. *Brain Res* **464**, 233-242 (1988).
20. Weeber, E.J., *et al*. Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *J Neurosci* **23**, 2634-2644 (2003).
21. van Woerden, G.M., *et al*. Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of alphaCaMKII inhibitory phosphorylation. *Nat Neurosci* **10**, 280-282 (2007).
22. Colbran, R.J. Inactivation of Ca2+/calmodulin-dependent protein kinase II by basal autophosphorylation. *J Biol Chem* **268**, 7163-7170 (1993).
23. Louria-Hayon, I., *et al*. E6AP promotes the degradation of the PML tumor suppressor. *Cell Death Differ* **16**, 1156-1166 (2009).
24. Regad, T., Bellodi, C., Nicotera, P. & Salomoni, P. The tumor suppressor Pml regulates cell fate in the developing neocortex. *Nat Neurosci* **12**, 132-140 (2009).
25. Genoux, D., *et al*. Protein phosphatase 1 is a molecular constraint on learning and memory. *Nature* **418**, 970-975 (2002).

Summary

CaMKII is one of the most abundant proteins and probably also one of the most extensively studied kinases of the brain. To date, already more than 2000 articles have been published on CaMKII, however, there is still no full understanding on the exact functions of the protein in the different parts of the brain. The two most abundant CaMKII isozymes present in the brain are αCaMKII and βCaMKII. In this thesis we attempt to further elucidate the role of these two isozymes in learning, synaptic plasticity and disease.

Chapter II focuses on the role of αCaMKII in presynaptic plasticity. Using targeted mouse mutants and pharmacologic inhibition of αCaMKII, we demonstrate that the presence of αCaMKII protein is required for normal short-term presynaptic plasticity, but not its activation, autophosphorylation nor its ability to phosphorylate Synapsin I. Furthermore we show that αCaMKII regulates the number of docked vesicles independent of its ability to be activated. These results indicate that αCaMKII plays a non-enzymatic role in short-term presynaptic plasticity at hippocampal CA3-CA1 synapses.

βCaMKII is the second most abundant CaMKII in the brain, however, so far not much is known about the role of βCaMKII in plasticity and learning. One of the reasons for the lack of knowledge is the fact that for a long time no mouse model was available. Our lab is the first lab to generate a βCaMKII null-mutant. Since βCaMKII is the predominant CaMKII isoform of the cerebellum, we first assessed the function of βCaMKII in the cerebellum using this mouse model (chapter III). We showed that βCaMKII plays an essential role in controlling the direction of plasticity at the parallel fiber–Purkinje cell synapse: a protocol that induces synaptic depression in wild–type mice, results in synaptic potentiation in βCaMKII knock–out mice, and *vice versa.* We propose a model in which βCaMKII plays a structural role at the parallel fiber-Purkinje cell synapse during the induction of LTP, and in which the enzymatic function of βCaMKII is necessary for the induction of LTD. These findings provide us with unique experimental insight into the mechanisms that transduce graded calcium signals into either synaptic depression or potentiation.

In chapter IV we looked at the role of βCaMKII in hippocampal plasticity and learning, using two different βCaMKII mutants. Here we show that the absence of βCaMKII severely impairs fear conditioning and hippocampal long-term potentiation (LTP). Remarkably, preventing βCaMKII activation by mutating the Ca^{2+}/Calmodulin binding domain (A303R) has effect on neither plasticity nor learning. Taken together, these results strongly suggest a non-enzymatic, structural role in hippocampal learning and LTP for βCaMKII.

CaMKII is known to be involved in several neurological and psychiatrical disorders, one of which is Angelman Syndrome. In this neurological disorder, CaMKII shows increased phosphorylation at the inhibitory phosphorylation sites (TT305/6). In chapter V we show that the molecular and cellular deficits of an AS mouse model can be rescued by introducing a mutation at the inhibitory phosphorylation site of αCaMKII. Moreover, these double mutants do no longer show the behavioral deficits seen in AS mice, suggesting that these deficits are the direct result of increased αCaMKII inhibitory phosphorylation.

Taken together, our research has helped to further elucidate the role of CaMKII in learning, plasticity and disease.

Samenvatting

CaMKII is een van de meest voorkomende eiwitten en waarschijnlijk een van de meest bestudeerde kinases in het brein. Tot op heden zijn er al meer dan 2000 artikelen gepubliceerd over CaMKII, maar desondanks is er nog steeds geen volledige duidelijkheid over de exacte functies van het eiwit in de verschillende gebieden in het brein. De twee meest voorkomende CaMKII isozymen aanwezig in het brein zijn αCaMKII en βCaMKII. In dit proefschrift doen we een poging om de rol van deze twee isozymen in de processen van leren, synaptische plasticiteit en ziekten verder te ontrafelen.

Hoofdstuk II concentreert zich op de rol van αCaMKII in presynaptische plasticiteit. Door gebruik te maken van muismutanten en farmacologische inhibitie van αCaMKII, laten we zien dat de aanwezigheid van het αCaMKII eiwit nodig is voor normale presynaptische plasticiteit, maar dat activatie, autofosforylatie, noch zijn mogelijkheid om Synapsin I te fosforyleren daarvoor nodig zijn. Verder laten we zien dat αCaMKII het aantal membraan gebonden neurotransmitter blaasjes reguleert zonder dat het daarvoor geactiveerd hoeft te worden. Deze resultaten impliceren dat αCaMKII een niet-enzymatische rol speelt in korte-termijn presynaptische plasticiteit in hippocampale CA3-CA1 synapsen.

βCaMKII is de tweede meest voorkomende CaMKII in het brein, maar desondanks is er tot op heden weinig bekend over de rol van βCaMKII in plasticiteit en leren. Een van de redenen daarvoor is het feit dat er lange tijd geen enkel muismodel voorhanden was. Ons lab is het eerste lab dat een βCaMKII knock-out muis heeft gegenereerd. Omdat βCaMKII de overheersende CaMKII isoform is in het cerebellum, hebben we eerst gekeken naar de functie van βCaMKII in het cerebellum (Hoofdstuk III). We laten zien dat βCaMKII een essentiële rol speelt in het controleren van de richting van plasticiteit in de parallel vezel-Purkinje cel synaps: een protocol om synaptische depressie te induceren in wild–type muizen, resulteert in synaptische potentiatie in βCaMKII knock-out muizen, en *vice versa.* We stellen een model voor waar βCaMKII in de parallel vezel-Purkinje cel synaps een structurele rol speelt gedurende de inductie van LTP en waar enzymatische rol van βCaMKII noodzakelijk is om LTD te krijgen. Door deze bevindingen hebben we nu een uniek experimenteel inzicht in de mechanismen die graduele calcium signalen omzet in synaptische depressie of juist potentiatie.

In hoofdstuk IV hebben we gekeken naar de rol van βCaMKII in hippocampale plasticiteit en leren, door gebruik te maken van twee verschillende βCaMKII mutanten. We laten hier zien dat afwezigheid van βCaMKII de context conditionering en hippocampale lange-termijn potentiatie ernstig verstoort. Opmerkelijk is dat het verhinderen van activatie van βCaMKII door het muteren van het Ca^{2+}/Calmodulin bindend domein (A303R), geen effect heeft op plasticiteit of leren. Samengenomen suggereren deze resultaten een niet-enzymatische, structurele rol voor βCaMKII in hippocampaal leren en LTP.

Het is bekend dat CaMKII betrokken is bij verschillende neurologische en psychiatrische stoornissen, waaronder Angelman Syndroom (AS). In deze neurologische ziekte is meer CaMKII gefosforyleerd op de inhibitoire fosforylatie plaatsen (TT305/6). In hoofdstuk V laten we zien dat de moleculaire en cellulaire deficiënties in een AS muismodel verholpen kunnen worden door een mutatie in de inhibitoire fosforylatie plaatsen van αCaMKII. Bovendien laten deze dubbelmutanten geen gedragsproblemen

meer zien, die wel te zien waren in AS muizen, wat suggereert dat deze problemen het directe resultaat zijn van de verhoogde inhibitoire fosforylatie van αCaMKII.

Samen genomen hebben onze studies een bijdrage geleverd aan het verder ontrafelen van de rol van CaMKII in leren, plasticiteit en ziekten.

Dankwoord

En eindelijk is het dan zover: we zijn beland bij het meest gelezen onderdeel van de thesis. Ik heb een aardig lijstje van mensen die ik graag wil bedanken, maar ongetwijfeld zal ik her en der wat mensen vergeten. Alvast mijn oprechte excuses hiervoor en mocht ik u/je inderdaad zijn vergeten, dan geldt deze voor u/jou: Hartelijk dank!!

De eerste die ik wil bedanken is mijn promotor en directe begeleider: Prof.dr. Ype Elgersma. Ik ben je ontzettend dankbaar dat je me de kans gaf om in jouw net beginnende groep kennis te maken met de wetenschap en uiteindelijk een promotietraject in te gaan. Ik heb veel van je geleerd, vooral praktisch, maar zeker ook intellectueel gezien. Je was er altijd als ik vragen had, of het nu door de weeks was gewoon op het werk, of in het weekend als ik precies wist te bellen op het moment dat je in de tuin bezig was. Ik weet dat ik zeer vaak geen AIO wilde zijn en vaak veel liever analist was geworden, maar toch met jouw geduld, steun en motivatie ligt hier nu toch een boekje. Ook in de persoonlijke sfeer heb je me vaak gesteund, als ik weer eens tegen mezelf aanliep of bijvoorbeeld problemen had met het kopen of verkopen van een huis. We hebben best veel meegemaakt in de afgelopen 6 jaar. Ik heb voor mijn gevoel dan ook enorm geluk gehad om een baas te treffen als jij. Dank je voor alles!!

Uiteraard wil ik ook graag Prof.dr. Chris de Zeeuw bedanken, voor de steun en hulp op de achtergrond en de interessante discussies over wetenschap, of over minder wetenschappelijke onderwerpen.

Members of the Elgersma lab: First of all, thank you all for the wonderful atmosphere you create, for the 'gezelligheid' but also for the interesting and/or completely useless conversations we've sometimes had. Minetta, Ik heb veel van je geleerd en nog steeds als ik iets niet weet, dan kan ik meestal wel bij jou terecht. Je bent, denk ik, de belangrijkste persoon in het lab, door je organiseren en superviseren van alle muizenzaken. Ik ken niemand zo georganiseerd en gestructureerd als jij. Ook het samen muziek maken op de piano, of zingend op het lab heb ik altijd erg leuk gevonden. Dank je voor het doorlezen van het proefschrift en je commentaren erop. Ik ben trots dat je mijn paranimf wil zijn, dank je voor alles! Susan, dank je voor alle discussies die we hadden, of het nou werkgerelateerd is of niet. Ook wil ik je bedanken voor al je input op mijn proefschrift. Ik denk dat een samenwerking tussen ons echt een goed idee zou zijn! Nils, je bent de volgende die aan de beurt is voor de verdediging. Ik ben ervan overtuigd dat dat goed zal gaan. Ik vind het prettig om met je samen te werken aan, en te discussiëren over oa de CaMKII muizen. Dank je voor je geduld en voor je input op het proefschrift. Caroline, de voorkant van het boekje is erg mooi geworden dankzij jou. Dank je voor je support in de afgelopen periode en voor al je input op het proefschrift. Ik kijk ernaar uit om samen met je te gaan werken aan Angelman Syndrome. Stathis, handsome… finally somebody in the lab with equal interests (piano of course….). Always chocolate for everybody, good in understanding what a mouse thinks and does, and always willing to help with anything (statistics, behavior or when a strong man is needed). What can I say, you're the best. Miki, your Italian influence brings something special in the lab. Thank you for teaching me a little bit about patching and letting me see that it is actually not so boring at all. And thank you for always being a "pain in the ass"… Azar, thank you for the discussions we had and your patience with me when I didn't understand something. Mehrnoush, I really

admire your patience!! All the genotyping! I don't know what we would do without you. Thank you! Laura, ik ben blij dat je bij ons op het lab bent gekomen. Ik vind het leuk om met je samen te werken en over motoren te praten. Wanneer gaan we een ritje maken?? Thijs, dank je voor de interessante discussies en je interesses voor alles wat er gebeurt op het lab. Lianne, ondanks dat je al weer even bezig bent met je coschappen, zal je een belangrijk persoon in het lab blijven, vnl vanwege de patienten studies. Dank je voor je hulp. And finally, Mohammad and Mitja. Mohammad, it was a pleasure for me to work together with you on several different projects. I hope you are doing well in Iran. Mitja, dank je voor een leuke tijd.

Dan een paar mensen die mij ook veel hebben geleerd: Phebe en Simone. Dank jullie voor het geduld dat jullie met mij hadden, wat nodig was aangezien ik helemaal geen moleculaire achtergrond heb. Phebe, dank je dat je me de blastocyst injectie wilde leren. Je bent een goede docent geweest. Ook wil ik hier John Kongasan bedanken voor alle hulp.
Erika en Elize. Waar moet ik beginnen. Elize, ik hou het kort: Je bent geweldig, een kanjer, ik ben je erg dankbaar voor al je hulp en uitleg. Je weet het, ik hou van je! Erika, ik zal nooit de vele koffie-uren vergeten in de vroege ochtend (die goede oude tijd) en de gesprekken die we hadden. Ik waardeer je eerlijkheid en je openheid. Dank je ook voor al je hulp met vele immuno's of breintjes die weer eens gesneden moesten worden en voor je geduld als ik het niet helemaal deed op de manier waarop jij het graag gedaan zag.
Hans, lieverd. De keren dat jij weer eens iets in elkaar gezet hebt of hebt geholpen met alle opstellingen voor onze gedragsexperimenten zijn ontelbaar. Een van de belangrijkste mannen van de afdeling. Dank je! Kenneth en Annette, dank jullie voor het altijd goed organiseren en coördineren van de vele bestellingen die gedaan worden. Loes en Edith, dank jullie voor het regelen van de vele administratieve rompslomp en uiteraard voor het lekkers in de snoeppot. Loes, daarnaast wil ik je graag bedanken voor alle gesprekken die we hebben gehad, je steun, maar ook vooral het muzikale plezier dat we vaak hebben gehad. Je bent een beetje mijn tweede moeder geworden.

I would like to thank Prof.dr. Steven Kushner, the youngest professor in medicine in the Netherlands. Steven, I'm very happy that I've met you and Amy. I would really like to thank you for the interesting, and (for me) helpful discussions that we've had, but also for the nice conversations I had with you every time you were in the lab. I hope many more will follow. En daarbij natuurlijk ook Denise en Katie, hartelijk dank!

Thank you members of the Hoogenraad lab. Samantha thank you for help with the confocal microscope and the help with printing the thesis. Dank je Bjorn voor al je hulp met de gene gun. Nanda hartelijk dank voor de samenwerking met de neuronenculturen van de muizen. Casper dank voor alle hulp en de discussies.

Het Freek lab. Freek, ik vond het een hele waardvolle ervaring om met je samen te mogen werken. Dank je voor alles wat ik van je geleerd heb! Ik hoop vaker met je te kunnen samenwerken. Petra, mijn eerste HLO student. Ik heb ook zeker heel veel van jou geleerd!! Ik vond het leuk je te begeleiden. Mandy, dank je voor de pcr's en hulp op het histolab in het verleden en natuurlijk voor alle gezelligheid. Master Gao, wonderful to discuss with you about CaMKII, specially about the localization of the different isozyms in the different cell types. I'm looking forward to your thesis. Elisa, when I thank you, I want to thank all the members of the sclerati house: Mari, Bazzi, Alessio, and Mini mouse.

Grazie per la vostra amicizia. Mini mouse, solo una parola: supercalifragilestichespiralitoso. And Bazzi, thank you so much! You really made me think that maybe kissing a guy actually is not that bad…

Rogerio, mijn allereerste student. Ik vond het erg leuk om met je samen te werken en heb je vriendschap altijd erg gewaardeerd (de geluiden die je kan produceren soms iets minder, al ben je wel minder luidruchtig dan voorheen). Dank je voor een hele leuke tijd en voor de keren dat je er voor me was. Martijn, en Alexandra dank jullie voor de gesprekken over wetenschap en andere zaken. Tiziana, thank you, especially when I started to write my thesis you were a big support for me. Eleonora, thank you, I definitely learned a lot from you too!

Yvonne en het studententeam van de snijzaal, dank jullie voor het regelen en organiseren van de preparaten voor de verschillende practica. Gert-Jan, al tijdens mijn studie geneeskunde heb ik veel van je geleerd, maar nog steeds op het gebied van functionele anatomie. Dank je dat ik mag helpen met het onderwijs geven. Ik vind het erg leuk om te doen (ook al komt het niet altijd even goed uit…). Dick, Tom en Joan, ook jullie hebben me tijdens mijn studie veel bijgebracht over anatomie, en ik vind het nu erg leuk om naast jullie te mogen staan om de nieuwe lichting artsen iets bij te brengen over de wonderen van het menselijk lichaam.

Iedereen van het EDC, dank jullie voor het verzorgen van de muizen!

Everybody that I didn't mention here, I'm sorry, but specially for you: thank you!!!!

Dan wil ik graag naast de collega's van de afdeling ook nog een aantal andere mensen bedanken. Te beginnen bij Dhr Van Der Weijden. Ik wil u hier toch even bedanken omdat ik daar eerder niet goed de kans voor heb gehad. U heeft een belangrijke rol gespeeld in een belangrijke fase in mijn leven. Verder wil ik graag Mw Vertriest bedanken. In de afgelopen 6 jaar bracht u toch wel een stukje regelmaat en rust. Ik ben geen makkelijk persoon, maar mede dankzij u sta ik nu op het punt om te promoveren. Hartelijk dank! Dan zijn er natuurlijk nog een paar belangrijke vrienden om te noemen. Het contact is niet erg intensief, maar daardoor niet minder belangrijk, dus Claudia, Lianne, Anneke, Karien, Nicole en Jacinta, dank jullie wel voor jullie vriendschap en steun! Petra en Erik-Jan, ook jullie wil ik graag even noemen hier. Ik heb het erg naar mijn zin gehad bij jullie en ik zal ook zeker nog af en toe aan komen waaien om even bij te praten. Dank jullie!

De laatste die ik nog wil noemen zijn Sjoerd, Janneke, Martha en Huub. Sjoerd, je bent en blijft mijn grote broer. Nu eindelijk heb ik meer tijd om aan de motor te gaan sleutelen, ik leer graag van je! Janneke, dankzij jou heb ik tenminste nog iets gezien van de wereld. Al vind ik Zwitserland toch nog steeds je beste keuze. Ik prijs mezelf erg gelukkig met een zus als jij. Dank je voor al je steun en toeverlaat en vooral dat je mijn paranimf wil zijn. Nu is het nog wachten op de eerste Van Woerden & Van Woerden paper! Moeders, als jij niet steeds zo geduldig iedere keer weer gezegd had: stap voor stap, niet ineens naar het eind willen, dan was ik denk ik niet zo ver gekomen. We hebben al met al best veel meegemaakt, maar ik denk dat we het uiteindelijk helemaal nog niet zo slecht hebben gedaan. Ik ben je zeer dankbaar voor alles wat je me geleerd en meegegeven hebt! Huub, ik heb ook veel van jou geleerd waar ik zeker ook op het lab veel aan heb, dank je.

Curriculum Vitae

Geeske M. van Woerden werd geboren op 3 april 1980 in Utrecht. Na het behalen van het VWO diploma aan het St. Gregorius college te Utrecht in 1998, is ze begonnen aan de Geneeskunde opleiding aan het Erasmus MC in Rotterdam. Het wetenschappelijk onderzoek aan het eind van haar doctoraal opleiding werd gedaan op de afdeling Neurowetenschappen. Na het behalen van haar doctoraal examen is ze door gegaan met promotie onderzoek op de afdeling neurowetenschappen in de groep van Prof. Dr. Y. Elgersma.

Publication list

betaCaMKII controls the direction of plasticity at parallel fiber-Purkinje cell synapses.
Van Woerden GM, Hoebeek FE, Gao Z, Nagaraja RY, Hoogenraad CC, Kushner SA, Hansel C, De Zeeuw CI, Elgersma Y.
Nat Neurosci. 2009 Jul;12(7):823-5. Epub 2009 Jun 7.

Spred1 is required for synaptic plasticity and hippocampus-dependent learning.
Denayer E, Ahmed T, Brems H, Van Woerden G, Borgesius NZ, Callaerts-Vegh Z, Yoshimura A, Hartmann D, Elgersma Y, D'Hooge R, Legius E, Balschun D.
J Neurosci. 2008 Dec 31;28(53):14443-9.

Rapid changes in hippocampal CA1 pyramidal cell function via pre- as well as postsynaptic membrane mineralocorticoid receptors.
Olijslagers JE, de Kloet ER, Elgersma Y, van Woerden GM, Joëls M, Karst H.
Eur J Neurosci. 2008 May;27(10):2542-50.

Cognitive deficits in Tsc1+/- mice in the absence of cerebral lesions and seizures.
Goorden SM, van Woerden GM, van der Weerd L, Cheadle JP, Elgersma Y.
Ann Neurol. 2007 Dec;62(6):648-55.

Kinase activity is not required for alphaCaMKII-dependent presynaptic plasticity at CA3-CA1 synapses.
Hojjati MR, van Woerden GM, Tyler WJ, Giese KP, Silva AJ, Pozzo-Miller L, Elgersma Y.
Nat Neurosci. 2007 Sep;10(9):1125-7. Epub 2007 Jul 29.

Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of alphaCaMKII inhibitory phosphorylation.
van Woerden GM, Harris KD, Hojjati MR, Gustin RM, Qiu S, de Avila Freire R, Jiang YH, Elgersma Y, Weeber EJ.
Nat Neurosci. 2007 Mar;10(3):280-2. Epub 2007 Jan 28.

Modulation of presynaptic plasticity and learning by the H-ras/extracellular signal-regulated kinase/synapsin I signaling pathway.
Kushner SA, Elgersma Y, Murphy GG, Jaarsma D, van Woerden GM, Hojjati MR, Cui Y, LeBoutillier JC, Marrone DF, Choi ES, De Zeeuw CI, Petit TL, Pozzo-Miller L, Silva AJ.
J Neurosci. 2005 Oct 19;25(42):9721-34.