Novel Insights into Neuronal CAMK2 Function

Martijn J. Kool

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COLOFON

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NOVEL INSIGHTS INTO NEURONAL CAMK2 FUNCTION

NIEUWE INZICHTEN IN DE FUNCTIE VAN NEURONAAL CAMK2

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PREFACE

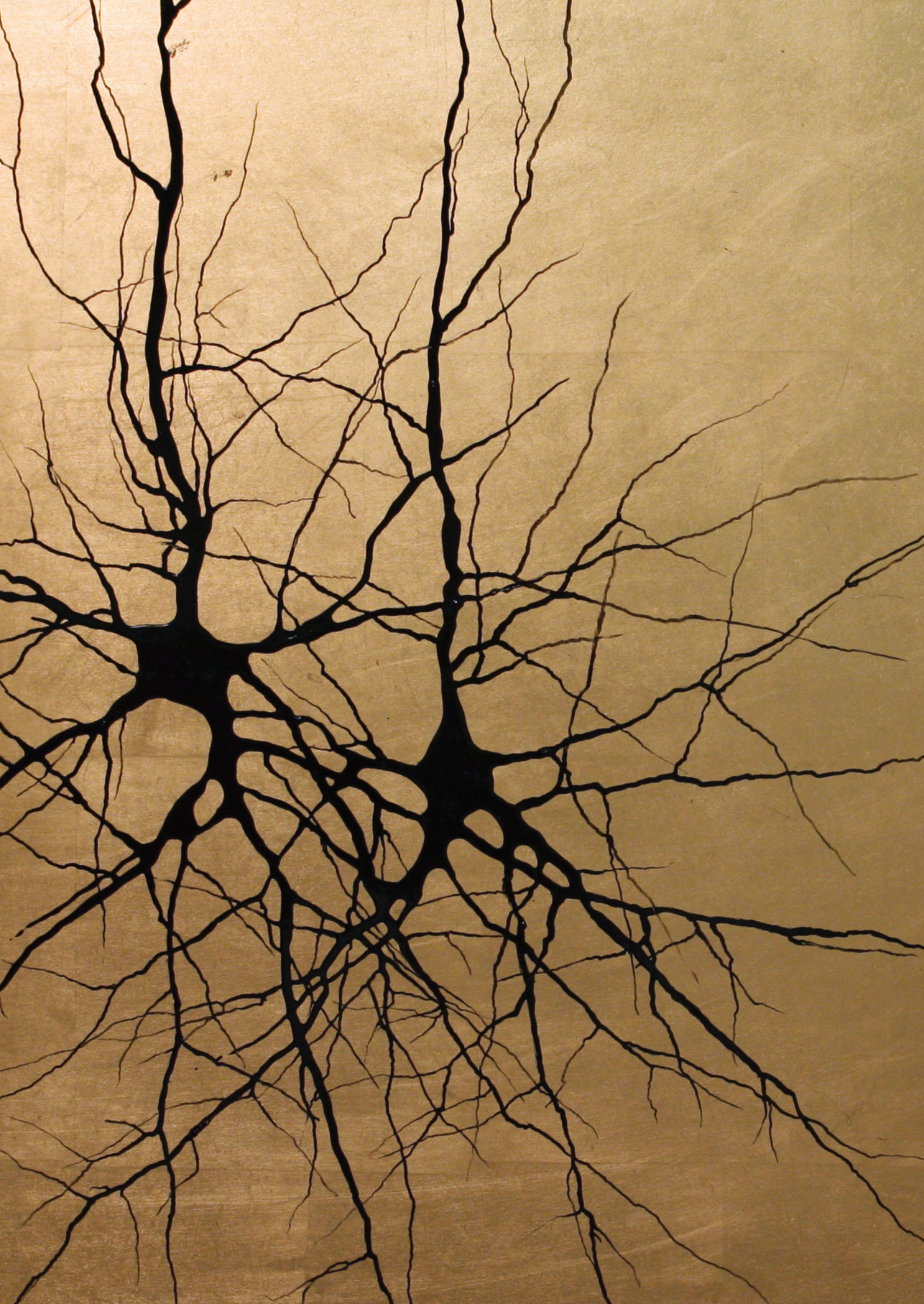
This dissertation will focus on the role of the two majorly abundant protein kinases CAMK2A and CAMK2B present in the brain. I have decided to highlight not all but only certain aspects of the literature on CAMK2A and CAMK2B for three reasons.

First, CAMK2A has already been profoundly studied considering the vast body of literature on this protein subunit. CAMK2B however, has not been given the same level of attention as its highly homologous protein isoform CAMK2A. As such, a substantial part of the introduction of this dissertation will try to summarize all current knowledge on this relatively unknown protein subunit CAMK2B.

Second, this dissertation will try to elucidate the unique and common functions of CAMK2A and CAMK2B, thereby helping in understanding the full spectrum of the CAMK2 protein in neuronal function. Both protein subunits arose from a common ancestral gene, making these two proteins highly homologous. Therefore, for many functions in the brain it is not known which are carried out by CAMK2A, which are carried out by CAMK2B or which are carried out by both.

Third, as a result of this high homology, dissociating between these two proteins using pharmacological inhibitors has so far proved impossible. Regardless, CAMK2 inhibitors are widely used to unravel novel functions of CAMK2. Thorough knowledge of these inhibitors is important to distinguish between direct effects of the drugs versus off-target effects. Therefore, the introduction of this dissertation will try to summarize the knowledge on most current drugs available for CAMK2 and assert their specificity.

On a different note, the use of the words CAMK2A, CAMK2B and CAMK2 can be confusing, both for outsiders as well as among experts in the field. To clarify, this dissertation will refer to CAMK2 as the entire holoenzyme, either containing both CAMK2A and CAMK2B (heteromeric holoenzyme) as well as only CAMK2A (homomeric holoenzyme). If certain traits can be ascribed to single subunits of the holoenzyme, the specific name of that isoform (e.g. CAMK2A, CAMK2B) will be mentioned.



Chapter 1

General Introduction

"Nothing in biology makes sense except in the light of evolution"

Theodosius Dobzhansky

1.1 A BILLION-YEAR-OLD GENE

Ca^{2+}/Calmodulin-dependent protein kinase II (CAMK2) is a protein that can be transcribed from 4 different genes which are estimated to have evolved from a common ancestral gene over 1 billion years ago (Ryan and Grant, 2009). CAMK2 is thought to have arisen at the start of the kingdom of Metazoa (Ryan and Grant, 2009) and has gradually evolved and acquired a variety of functions in a plethora of species.

As a result, the *CAMK2* gene can be found throughout the animal kingdom, with only one genetic copy in (among others): sponges (*A. Queenslandica* (Ryan and Grant, 2009) and *Suberitus domuncula* (Krasko et al., 1999)), nematodes (*C. Elegans*; in which *CAMK2* is referred to as *uncoordinated (unc)-43* (Reiner et al., 1999)) and insects (*D. melanogaster* (Ohsako et al., 1993)), three copies in (among others): frogs (*Xenopus laevis* (Stevens et al., 2001)) and 4 copies in (among others): mice (*Mus Musculus* (Hanley et al., 1989)), chicken (*Gallus Gallus* (Li et al., 1998)), and humans. The multiple gene copies of *CAMK2* found in frogs and higher vertebrates most likely arose from duplication of a common ancestral *CAMK2* gene (Tombes et al., 2003). These duplications in turn are thought to have arisen from two whole genome duplication events (paleopolyploidy) that have occurred in a common ancestor of chordates and echinodermata in the deuterostomes clade (McLysaght et al., 2002). Therefore, the different *CAMK2* genes can be referred to as paralogs.

Today, the CAMK2 protein is mainly known for its neuronal and cardiac functions. Interestingly however, the ancestral CAMK2 protein may have started as a non-neuronal and non-cardiac protein, since species in the phylum porifera (sponges) that have 1 copy have neither nervous nor circulatory system. Therefore, the initial function of CAMK2 in calcium homeostasis might have been much broader than its current specific functions in the heart and the brain. This thesis will specifically focus on CAMK2 functions in the brain.

The four protein isoforms known in higher vertebrates are CAMK2A, CAMK2B, CAMK2G and CAMK2D (A for *alpha*, B for *beta*, G for *gamma* and D for *delta*), all coming from the 4 paralog genes *Camk2a*, *Camk2b*, *Camk2g* and *Camk2d* located on different chromosomes (**Table 1**). *Camk2b* and *Camk2g* are the most closely related of these, and it is likely that *Camk2a* arose later in evolution as it is not present in amphibians (Tombes et al., 2003).

Table 1. Overview of CAMK2 proteins and their chromosomal location in mice and humans

Protein	Gene[a]	Mouse Chromosome	Human Chromosome
CAMK2A	***Camk2a***	18	5
CAMK2B	***Camk2b***	11	7
CAMK2G	***Camk2g***	14	10
CAMK2D	***Camk2d***	3	4

[a] Based on the nomenclature for mice

Taken together, it can safely be assumed that in higher vertebrates the four CAMK2 paralogs have each developed to fulfill unique roles. On the other hand, considering their divergence from a common ancestral gene, an argument can also be made that certain functions present in the ancestral gene have been conserved in two, three or maybe all four paralogs.

1.2 CAMK2 STRUCTURE, DOMAIN ORGANIZATION AND REGULATION

CAMK2 is quite an unusual protein, in the sense that it can auto-assemble into a larger protein, called a holoenzyme. This can be done using only proteins transcribed from one gene (homomers), or with a mixture of proteins coming from different CAMK2 genes (heteromers). In this section, the structure of this holoenzyme will be discussed as well as the domain organization and the regulation of a single CAMK2 protein.

1.2.1 CAMK2 Structure

As mentioned above, CAMK2 can form a holoenzyme. More specifically, it can form a dodecameric holoenzyme, coming from the Greek *dodeca* (twelve) and *meric* (part of), meaning that CAMK2 is a holoenzyme consisting of, on average, twelve protein subunits

(Colbran and Brown, 2004; Chao et al., 2011). The holoenzyme is made of two rings of 6 subunits each, placed on top of each other in a doughnut-like shape (Rosenberg et al., 2005) (**Figure 1**).

CAMK2A and CAMK2B can form heteromeric oligomers (Shen et al., 1998). The same study mentioned but did not show that CAMK2G and CAMK2D can also form heteromeric holoenzymes with CAMK2A and CAMK2B, although later reports suggested that CAMK2G can move to the nucleus without translocation of any of the other isoforms (Ma et al., 2014). One does not necessarily exclude the other, as CAMK2G can detach from CAMK2A or CAMK2B upon translocation to the nucleus. Exchange of subunits between holoenzymes has been observed as well, but so far only for CAMK2A subunits (Stratton et al., 2013). Nevertheless, it is still not definitively clear whether CAMK2G and CAMK2D can form heteromers with CAMK2A and CAMK2B.

1.2.2 Domain organization and regulation

The CAMK2 protein can be divided into multiple functional domains. These domains can be found in all 4 isoforms. Considering yet again that these genes have come from a common ancestral gene, it is perhaps not surprising that all 4 CAMK2 isoforms are highly homologous in their domain organization. In rats for example, it has been found that there is an 89-93% sequence homology between two of these domains (the catalytic and regulatory domain which will be discussed below) (Tobimatsu and Fujisawa, 1989).

Basically, all CAMK2 isoforms contain a catalytic domain, a regulatory domain, a variable domain and an association domain (**Figure 1**). The catalytic domain contains the ATP- and substrate-binding sites and interaction sites for anchoring proteins. This domain can also catalyze the phosphotransferase reaction (Lisman et al., 2002). The regulatory domain consists of an autoinhibitory domain, which contains a segment that closely resembles protein substrates. Under basal conditions, this so-called pseudosubstrate segment binds to the substrate binding region (S-site) of the catalytic domain. This way, activity of the enzyme is inhibited (Smith et al., 1992). Upon binding of Ca^{2+}/Calmodulin, binding of the pseudosubstrate segment to the S-site can be relieved. The variable domain is responsible for most differences between the isoforms. This is where differences in affinity for Ca^{2+}/Calmodulin and inserts for targeting of CAMK2 to subcellular localizations can be found. Finally, the association domain at the C-terminal of the kinase is responsible for the assembly of multiple CAMK2 subunits into a hetero- or homomeric holoenzyme.

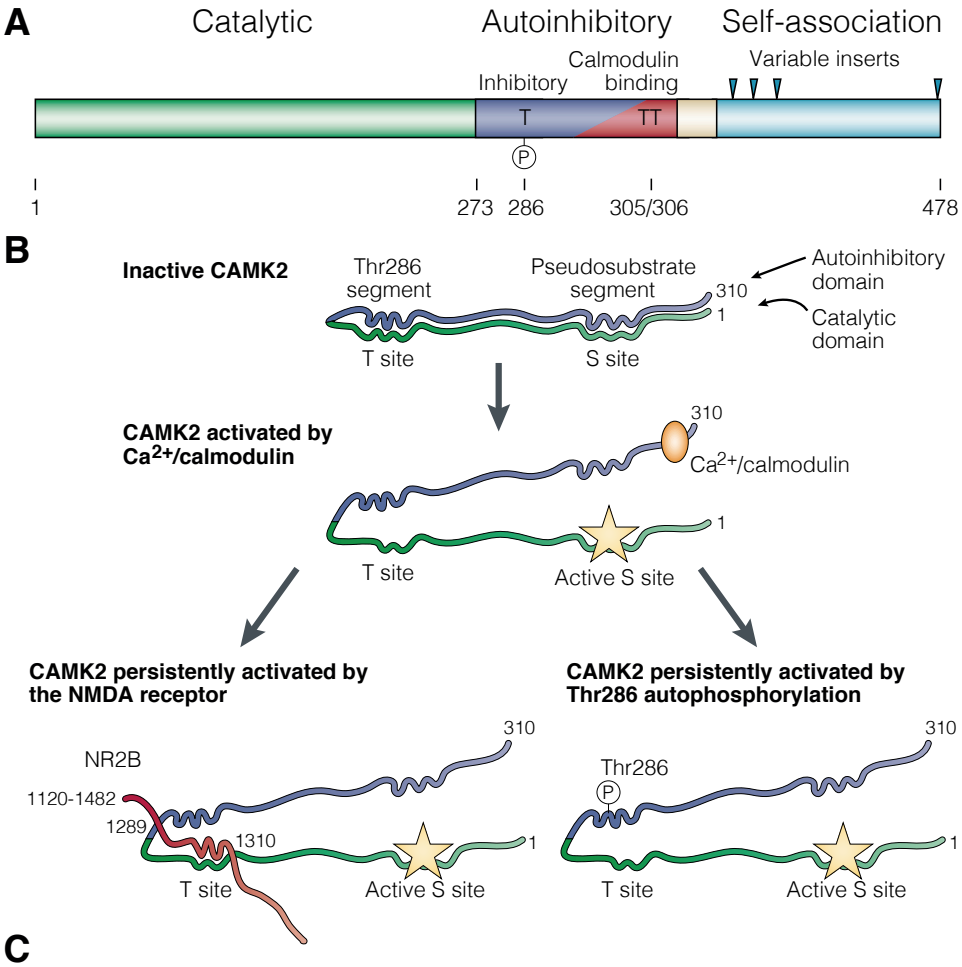

A
Catalytic
Autoinhibitory
Self-association
Calmodulin binding
Inhibitory
Variable inserts
T
TT
P
1
273
286
305/306
478
B
Inactive CAMK2
Thr286 segment
Pseudosubstrate segment
310
1
Autoinhibitory domain
Catalytic domain
T site
S site
CAMK2 activated by Ca2+/calmodulin
310
Ca2+/calmodulin
1
T site
Active S site
CAMK2 persistently activated by the NMDA receptor
CAMK2 persistently activated by Thr286 autophosphorylation
310
NR2B
1120-1482
1289
1310
1
T site
Active S site
310
Thr286
P
1
T site
Active S site
C

Figure 1. CAMK2 domain organization, regulation and structure. (A) Domain organization of CAMK2 including the most important phosphorylation sites (indicated with T). **(B)** The autoinhibitory and catalytic domains form a gate that regulates activity. The enzyme is inhibited when the gate is closed because the autoinhibitory domain binds to the catalytic domain at the S and T sites (top). The binding of Ca^{2+}/calmodulin releaves the autoinhibitory domain and activates CAMK2 (middle). The NMDA (N-methyl-D-aspartate) receptor NR2B subunit can bind to the T site, keeping the gate open and the enzyme active even after the dissociation of calmodulin (bottom left). In the presence of Ca^{2+}/calmodulin, the Thr286 site can be phosphorylated by a neighbouring subunit. This is also sufficient to keep the enzyme autonomously active even after dissociation of calmodulin (bottom right). **(C)** Three-dimensional structure of CAMK2. (Left) This view shows only one of the hexameric rings formed by the catalytic regions of six subunits. (Right) Stereo view of CAMK2 seen from a perspective perpendicular to that shown left. The association domains of the 12 subunits form the gear-like structure. Adapted from Lisman et al., Nature Reviews Neuroscience, 2002 and Kolodziej et al., J. Biol. Chem., 2000.

But what are the roles of these domains in regulating the activity of CAMK2? Under basal conditions, access to the substrate-binding site (S-site, on the catalytic domain) on CAMK2 is blocked by the pseudosubstrate segment of the protein (Braun and Schulman, 1995a; Colbran and Brown, 2004; Coultrap and Bayer, 2012) (**Figure 1**). CAMK2 is activated upon influx of calcium into the cell. Four calcium ions can bind the protein Calmodulin, which in turn will bind to the Ca^{2+}/Calmodulin footprint present on the pseudosubstrate segment of the CAMK2 isoforms (R296 to A309 for CAMK2A, R297 to A310 in the other three isoforms (V310 in CAMK2G)) (Vallano, 1989). Binding of Ca^{2+}/Calmodulin to this footprint releases the pseudosubstrate segment from the catalytic domain, making the catalytic domain available to phosphorylate different substrates. When two neighboring subunits have bound Ca^{2+}/Calmodulin and their catalytic domains are available for phosphorylation, one catalytic domain can phosphorylate the neighboring subunit in the regulatory domain at threonine 286 (T286) (Miller and Kennedy, 1986; Hanson et al., 1994). Phosphorylation of T286 (T287 in CAMK2B, CAMK2G and CAMK2D) greatly enhances the affinity for Ca^{2+}/Calmodulin, a mechanism referred to as CaM-trapping. This trapping of Ca^{2+}/Calmodulin can keep the CAMK2 holoenzyme activated more easily. However, when calcium levels in the cell drop to basal levels Ca^{2+}/Calmodulin can detach from CAMK2. Phosphorylated T286 will render the CAMK2 subunit autonomously active as it will function to keep the pseudosubstrate segment relieved from the catalytic domain. This autonomous activity, also referred to as Ca^{2+}-independent activity, is ~40-80% compared to the maximal activity (when Ca^{2+}/Calmodulin is bound (Patton et al., 1990; Coultrap et al., 2010). When Ca^{2+}/Calmodulin is no longer bound, it allows phosphorylation of yet another site, TT305/6 (TT306/7 in the other three isoforms). This site is located within the footprint for Ca^{2+}/Calmodulin

binding, thus phosphorylation of this site prevents Ca^{2+}/Calmodulin from binding, thereby inhibiting Ca^{2+}/Calmodulin-dependent activation of CAMK2. If further loss of phosphorylation at T286 occurs, the pseudosubstrate segment will attach to the catalytic domain thereby inhibiting the enzyme and only upon dephosphorylation of TT305/6 can CAMK2 be activated again (as reviewed in (Lisman et al., 2002)).

1.3 CAMK2 LOCALIZATION, SYNAPTIC FUNCTIONS AND NEURONAL EXCITABILITY

1.3.1 Localization

Localization of CAMK2 can be described on multiple levels. This can be done on the basis of expression within a cell, within brain regions or even the complete organism. Differences in localization can occur upon activation of CAMK2 and there is even a difference in localization between *Camk2a* and *Camk2b* messenger RNA (mRNA). All these differences in localization are briefly described here.

CAMK2A and CAMK2B are the most abundant CAMK2 isoforms in the brain. CAMK2A is mainly expressed in excitatory neurons of the hippocampus and cortex (Jones et al., 1994; McDonald et al., 2002; Zou et al., 2002), whereas CAMK2B can be found in both excitatory and inhibitory neurons and in oligodendrocytes. Besides the brain, CAMK2B can also be found in skeletal muscle cells and pancreas islet cells (Bayer et al., 1998; Rochlitz et al., 2000; Cahoy et al., 2008; Martinez-Lozada et al., 2014). The other two isoforms, CAMK2G and CAMK2D, can be found throughout the body and have important functions in cardiac tissue. These functions in cardiac tissue are beyond the scope of this thesis and will not be discussed here. Recently, CAMK2G was also found to have an important role in neuronal tissue (Ma et al., 2014).

Differences in localization between CAMK2's most prominent neuronal subunits, CAMK2A and CAMK2B, can also be described in terms of a ratio between the two in different brain regions. For example, CAMK2A and CAMK2B are expressed at a ratio of 3:1 in hippocampus (Bennett et al., 1983; Miller and Kennedy, 1985; Brocke et al., 1999) whereas in the cerebellum the inverse ratio is found (Miller and Kennedy, 1985). In general, CAMK2A levels are much lower in pons and midbrain compared to forebrain and hippocampus (Erondu and Kennedy, 1985). However, these ratios should be interpreted with caution, since ratios on the level of brain region (e.g. hippocampus) do not at all represent the levels of CAMK2A and CAMK2B at the level of single cells. Hippocampal region CA1 for example is known to have varying levels of CAMK2A (Brocke et al., 1999). Moreover, in some excitatory CA1 cells CAMK2A RNA could not

even be detected (Brocke et al., 1995), implying that CAMK2A is not always present in excitatory hippocampal CA1 pyramidal cells. Also for the cerebellum, ratios of 1:4 for CAMK2A and CAMK2B have been found, but upon closer inspection CAMK2A is only expressed in cerebellar Purkinje cells, where ratios of CAMK2A and CAMK2B are 1:1. CAMK2B on the other hand can be found throughout all neuronal cell types of the cerebellum (Conlee et al., 2000; Hansel et al., 2006).

Within their respective cell types, CAMK2A and CAMK2B can be found in dendrites and in spines. Concentrations of CAMK2 are particularly high in a region within spines called the postsynaptic density (PSD). Activation or deactivation of the protein however can change the ratios of expression between dendrite and PSD (Shen and Meyer, 1999). Upon activation, CAMK2 detaches from actin and moves to the PSD, a localization shift that is reversed upon deactivation. Moreover, autophosphorylation can increase the time CAMK2 remains in the PSD (Shen and Meyer, 1999). Therefore, as we will see later, mutations of CAMK2 that interfere with the activation of the protein not only disable activation but also change the localization of the protein (Shen and Meyer, 1999; Elgersma et al., 2002).

Under basal condition, CAMK2 concentration is twice as high in spines as in the shaft (Merrill et al., 2005; Feng et al., 2011) which upon activation can only further increase. In spines, up to 2-6% of total protein is made up of CAMK2. The density of CAMK2 on average is 80 holoenzymes per 0,1μm^2 of PSD (Chen et al., 2005; Cheng et al., 2006) with up to ~240 holoenzymes in large mushroom-shaped spines (Feng et al., 2011).

Interestingly, there are also differences in localization of mRNA between CAMK2A and CAMK2B. *Camk2a* mRNA is present at the spines and can provide a quick local translation mechanism after LTP induction to increase levels of CAMK2A. In contrast to *Camk2a* mRNA, *Camk2b* mRNA is not found in spines (Burgin et al., 1990; Benson et al., 1992; Mayford et al., 1996b).

1.3.2 Presynaptic functions

CAMK2 can be found on both sides of the synapse where it has numerous functions. Most of the functions are beyond the scope of this thesis. Therefore, I will limit the summary to presynaptic and postsynaptic functions in light of presynaptic or postsynaptic plasticity.

CAMK2, even though it was originally discovered as a presynaptic protein and named Synapsin I kinase (DeLorenzo et al., 1979; Kennedy and Greengard, 1981; Kennedy et al., 1983), is mostly known for its postsynaptic role. However, CAMK2 also has important roles presynaptically. Most research on a presynaptic role for CAMK2 has been performed

on *Camk2a* mutant mice in the well-studied CA3-CA1 synapse in the hippocampus, where it is involved in neurotransmitter release and short-term synaptic plasticity, but not in basal synaptic transmission and paired-pulse facilitation (PPF) (Llinás et al., 1985; Lin et al., 1990; Nichols et al., 1990; Chapman et al., 1995; Hinds et al., 2003; Hojjati et al., 2007; Jiang et al., 2008; Pang et al., 2010). The role of presynaptic CAMK2B still remains elusive, as well as the presynaptic role of the holoenzyme CAMK2 in long-term potentiation (LTP). Studies so far have been using pharmacological approaches to investigate the function of presynaptic CAMK2. In one study using organotypic cultures of hippocampal slices blocking presynaptic CAMK2 in the CA3-CA3 synapse, LTP was reduced by 50% (Lu and Hawkins, 2006). Another study showed in dissociated hippocampal neurons that pharmacologically blocking presynaptic CAMK2 prevented the induction of LTP (Ninan and Arancio, 2004). These studies show an important role for presynaptic CAMK2 in the induction of LTP. A drawback of using inhibitors however is that they cannot distinguish between different isoforms like CAMK2A or CAMK2B. Additionally, inhibitors may fail to elucidate the full function of CAMK2 because they only block enzymatic activity, whereas it is known that CAMK2 can also have structural roles (Hojjati et al., 2007; Borgesius et al., 2011). In **Chapter 4** we have used a genetic approach to elucidate the presynaptic role of CAMK2A and CAMK2B in the CA3 area of the hippocampus.

1.3.3 Postsynaptic functions

CAMK2 is both necessary (Silva et al., 1992b) and sufficient (Lledo et al., 1995) to induce LTP postsynaptically. Upon activation by Ca^{2+}/Calmodulin and further autonomous activation by T286 autophosphorylation, CAMK2 can move to the PSD (Shen and Meyer, 1999). There, CAMK2 can bind the NMDA-receptor (NMDAr). More specifically, after T286 phosphorylation, a site opposite to T286, the T-site, can bind GluN2B (Bayer et al., 2001) or the NR1 subunit (Leonard et al., 1999) (**Figure 1**). It can bind specifically near S1303 in NR2B (in a way that does not require T286 phosphorylation) or near amino acids 839 and 1120 which does require autophosphorylation (Bayer et al., 2001). This way, CAMK2 is strategically placed at the primary site of calcium entry into spines to control synaptic strength. CAMK2 bound to the NMDAr via the T-site can persist for more than 30 minutes after removal of calcium (Bayer et al., 2006). Interfering with the binding of CAMK2 to the NMDAr near S1303 greatly reduces the duration (and thereby the maintenance) of LTP (Barria and Malinow, 2005).

But how does CAMK2 strengthen synaptic transmission? Multiple mechanisms are known. First, the AMPA-receptor (AMPAr) subunit GluA1 contains a phosphorylation site, Serine 831 (S831) that can be phosphorylated by CAMK2. When this happens, CAMK2

enhances channel conductance by 50% (Mammen et al., 1997; Barria et al., 1997a; Derkach et al., 1999; Kristensen et al., 2011). Second, autophosphorylated CAMK2 that binds to the NMDAr organizes a structural process along with many AMPAr anchoring proteins that will lead to an increase of AMPA receptors docking in the PSD (Lisman and Zhabotinsky, 2001). Additionally, a member of the transmembrane AMPA-receptor regulatory proteins stargazin (TARP g-2), an auxiliary protein on extrasynaptic AMPA receptors, can be phosphorylated (Tomita et al., 2005; Opazo et al., 2010). As a result, extrasynaptic AMPA receptors are trapped at the synapse by binding of stargazin to PSD95, a protein highly abundant in the PSD (Opazo et al., 2012). In summary, CAMK2 can increase synaptic strength both by increasing AMPAr channel conductance *per* channel and by increasing the total number of AMPA receptors in the spine. In both ways, CAMK2 will greatly enhance postsynaptic sensitivity for presynaptic signals.

CAMK2 has also been implicated in regulating long-term depression (LTD), which involves the weakening of synapses (Stevens et al., 1994). During prolonged weak stimuli, known to induce LTD, CAMK2 phosphorylates GluA1 on S567 (Coultrap et al., 2014). Phosphorylation of GluA1 on this site can reduce synaptic GluA1 localization (Lu et al., 2010), thereby weakening the synapse. This form of LTD is NMDA-receptor dependent and requires autonomous activity of CAMK2 (i.e. T286/7 phosphorylation) (Coultrap et al., 2014). Direct binding of CAMK2 to the NMDA-receptor can be reduced by the induction of LTD (Aow et al., 2015). CAMK2 is also known to phosphorylate GABA-receptors and can thereby change the strength of inhibitory synapses (Wei et al., 2004; Houston et al., 2008). Other forms of LTD, such as group I metabotropic glutamate receptor-mediated long-term depression mGluR-LTD may also depend on CAMK2. The role of CAMK2 in this form of LTD is still unclear since blocking CAMK2 using KN62 can either facilitate (Schnabel et al., 1999) or inhibit group I mGluR-LTD (Mockett et al., 2011).

1.3.4 CAMK2 and intrinsic neuronal excitability

Besides synaptic plasticity, neuronal excitability can also be controlled by CAMK2. For example, CAMK2A-T286A mutant mice show increased intrinsic excitability in CA1 pyramidal neurons (Sametsky et al., 2009). According to this study CAMK2A functions to downregulate CA1 intrinsic neuronal excitability following synaptic stimulation (Sametsky et al., 2009). Another study found a role for CAMK2 in downregulating intrinsic excitability after induction of LTP. This was done by stimulating the Schaffer-collaterals while simultaneously depolarizing postsynaptic CA1 neurons. This downregulation requires CAMK2 activity, postsynaptic Ca^{2+} influx, NMDA-receptors, backpropagating action potentials, protein synthesis and increased I_h (Fan et al., 2005). CAMK2-mediated downregulation of intrinsic excitability has also been observed in vestibular nucleus

neurons (Nelson et al., 2005) and medium spiny neurons in the striatum (Klug et al., 2012). In contrast, one report found that stimulating synaptic inputs correlating with postsynaptic neuronal spikes in CA1 pyramidal cells elicited both LTP and lowered the threshold for action potential generation, thereby increasing intrinsic excitability using a CAMK2-dependent mechanism (Xu et al., 2005). With the exception of the last study, CAMK2 seems to have an inhibiting function on excitability, but the exact mechanisms involved are yet to be elicited.

Most of the abovementioned studies have used pharmacological inhibition to study the role of CAMK2 in excitability. Surprisingly, *Camk2* mutants have only rarely been used to address CAMK2's function in neuronal excitability and unitary synaptic transmission. In **Chapter 5** we address these issues by using an inducible knockout mouse for both CAMK2A and CAMK2B.

1.3.5 Regulation of receptors by CAMK2

Receptors can function as gateways to conduct signals across the biological membrane of a cell. Many receptors in the brain are important for signaling with the extracellular environment including other neurons. CAMK2 is known to bind to and regulate multiple receptors. An overview of these receptors found so far is depicted in **Table 2**. Even though CAMK2 has been found to interact with receptors in cardiac tissue (e.g. see (Maier, 2011) and (Respress et al., 2012) or for review see (Bers and Grandi, 2009)) and in pancreatic cells (Kline et al., 2013), this section will only focus on neuronal receptors.

In long-term potentiation, one of the most important channels known to be phosphorylated by CAMK2 is the AMPA receptor. As mentioned above, CAMK2 can phosphorylate the AMPA receptor subunit GluA1 on S831, which will increase channel conductance (Barria et al., 1997a; 1997b). Loss of autophosphorylation in the T286A knock-in mutant does not reduce phosphorylation at this GluA1 site, probably due to Ca^{2+}-dependent activity of CAMK2 or by PKC, which can competitively phosphorylate GluA1 at S831. Both Serine 845 (S845), a PKA-dependent phosphorylation site on GluA1, and S831 regulate LTP and LTD as has been demonstrated in mutants where the Serines were mutated to Alanines (S831A and S845A), to prevent phosphorylation at these sites (phospho-deficient mutants) (Lee et al., 2003). Interestingly, single mutants (S831A or S845A) have normal LTP and only S845A mutants show impaired LTD whereas S831A mutants do not (Lee et al., 2010), indicating that S831 and S845 act in concert to induce LTP. However, S831 and S845 phosphorylation as one of the crucial steps in the mechanisms of LTP has been called into question by the discovery that mainly in adulthood only 1% of AMPA receptors at synapses is phosphorylated in the basal state which was only marginally increased upon induction of chemical LTP using

Table 2. Overview of receptors regulated by CAMK2

Protein	Type [a]	Site	Function	Reference
GluA1	P	Ser831	Increase channel conductance by 50%	Barria et al., 1997
	P	Ser567	Limits trafficking to synapses	Lu et al., 2010
GluN1	B	–	Recruitment of CAMK2 close to Ca^{2+} source	Leonard et al., 1999
GluN2B	B	near Ser1303	Crucial for LTP expression	Barria et al., 2005
	B	839-1120	–	Bayer et al., 2001
mGluR1	P+B	Thr871	Recruits CAMK2A to the receptor facilitating desensitization	Jin et al., 2013a
mGluR5	B	C-terminus	Recruit and store CAMK2A in synapses	Jin et al., 2013b
$Ca_v\alpha1.2$ [b]	P+B	C-terminus	Ca2+-dependent facilitation	Hudmon et al., 2005
$Ca_v\beta1.2$ [b]	P	Thr498	Ca2+-dependent facilitation	Koval et al., 2010, Grueter 2008
	B	near Leu 493	Ca2+-dependent facilitation	Koval et al., 2010, Grueter 2008
$Ca_v1.3$ [b]	B	–	Augmentation of Ca^{2+} currents (together with densin)	Jenkins et al., 2010
$Ca_v2.1$ [c]	B	1897-1912	Enhance P/Q-VGCC activity	Jiang et al., 2008
$Ca_v2.2$ [d]	P	C-terminus	Enhance interactions with SNARE protein complexes	Yokoyama et al., 2005
$Ca_v3.2$ [e]	P	Ser1198	Regulation of T-VGCC	Welsby et al., 2003
D3R	P+B	Ser229	Inhibiting dopamine signaling	Liu et al., 2009
$Na_v1.5$	P	Ser571	Regulate cell excitability	Hund et al., 2010
$K_v4.2$	P	Ser438 + Ser459	Increase cell-surface expression	Varga et al., 2004
EAG	P	Thr787	Increase current amplitude/slows down inactivation	Wang et al., 2002
$GABA_A$	P	mult. sites	Trafficking/modulation of channel activity	reviewed in Houston et al., 2009

[a] Type of regulation; P = phosphorylation; B = binding
[b] This subunit belongs to the L-type voltage-gated calcium channel family
[c] This subunit belongs to the P/Q-type voltage-gated calcium channel family
[d] This subunit belongs to the N-type voltage-gated calcium channel family
[e] This subunit belongs to the T-type voltage-gated calcium channel family
D3R = dopamine receptor D3; EAG = ether à go-go; – = unknown

glycine (Hosokawa et al., 2015). Further research is warranted to clarify this important discrepancy. Recently S567 was identified as another phosphorylation site on GluA1 which can be phosphorylated by CAMK2 both *in vitro* and *in vivo* (Lu et al., 2010).

Of equal importance in the mechanisms of LTP and also regulated by CAMK2 is the NMDA receptor. As mentioned earlier, CAMK2 can bind two sites on the NMDA receptor subunit GluN2B. First, CAMK2 can bind near S1303 which does not require T286 phosphorylation (Strack and Colbran, 1998; Barria and Malinow, 2005). Second, CAMK2 can bind between amino acids 839 to 1120 in a way that does require T286 autophosphorylation (Bayer et al., 2001). Binding of CAMK2 to the GluN1 subunit has been described as well (Leonard et al., 1999).

CAMK2 can also bind to both members of group I metabotropic glutamate receptors (mGluR1 and 5), which are known to increase NMDA receptor activity (for review, see (Mao et al., 2014)). CAMK2 can phosphorylate mGluR1 at T871 (Jin et al., 2013b) and can bind to the C-terminal of mGluR5, although the exact binding sequence is still unknown and no phosphorylation sites have been found so far (Jin et al., 2013a). Interestingly, Calmodulin can also bind to the same C-terminal region on mGluR5, but it cannot bind to mGluR1 (Choi et al., 2011). On mGluR5, this can create competition for binding between CAMK2 and Calmodulin. Moreover, binding of CAMK2 to mGluR5, in contrast to mGluR1, is reduced upon activation of CAMK2 (Jin et al., 2013a). As mentioned above, the role of CAMK2 in DHPG-induced LTD, which acts through both mGluR1 and mGluR5, is unclear. In **Chapter 4** we investigated the role of CAMK2A and CAMK2B in DHPG-induced LTD.

Additionally, some members of the voltage-gated calcium channel family (VGCC) are also regulated by CAMK2. For example, CAMK2 can bind and phosphorylate both the $Ca_v\alpha1.2$ and the $Ca_v\beta1.2$ subunits (Hudmon et al., 2005; Grueter et al., 2008; Koval et al., 2010). Furthermore, CAMK2 can bind $Ca_v1.3$, belonging to the L-type VGCC mainly present on dendrites and soma, (Jenkins et al., 2010) thereby forming a crucial link in excitation-transcription coupling that is critical for learning and memory (Wang et al., 2017). CAMK2 can presynaptically bind $Ca_v2.1$, a P/Q-type VGCC (Jiang et al., 2008) and phosphorylate $Ca_v2.2$, an N-type VGCC in the C-terminus thereby enhancing interactions with SNARE protein complexes (Hell et al., 1994; Yokoyama et al., 2005) and postsynaptically phosphorylate $Ca_v3.2$ on S1198, a T-type VGCC (Welsby et al., 2003).

CAMK2 can bind and phosphorylate the dopamine D3 receptor (D3R) on S229 in mice, thereby negatively regulating dopamine signaling (Liu et al., 2009). In mice CAMK2 can phosphorylate Nav1.5 channels on S571 (Hund et al., 2010) and phosphorylate $K_v4.2$

channels on S438 and S459 thereby increasing surface expression of these channels and decreasing excitability of the cell (Varga et al., 2004). EAG (ether à go-go) channels are also phosphorylated by CAMK2 in *Drosophila*, enhancing current amplitude and slowing down inactivation of these channels (Wang et al., 2002). Finally, different subunits of the $GABA_A$ receptors can be phosphorylated on multiple sites in mice, mediating different functions such as increasing cell-surface expression and modulating channel activity (as reviewed in (Houston et al., 2009)).

1.4 DIFFERENCES IN CAMK2A AND CAMK2B FUNCTIONING

Despite the similarity between CAMK2A and CAMK2B (for examples, see (Hudmon and Schulman, 2002) and (Tobimatsu and Fujisawa, 1989)), few differences have been described as well. One of these examples is the binding of CAMK2B to actin, which will be discussed further in another section below. Studying differences of the isoforms of the CAMK2 family can be troubling, since pharmacologically there are no blockers that can target only one isoform specifically and knockout models for isoforms other than CAMK2A are still scarce. Despite these limitations some differences in functionality have been described.

The first difference in functionality between CAMK2A and CAMK2B is their binding affinity for Calmodulin. The EC50 for Calmodulin, the effective binding at which 50% of maximum activity is achieved, is ~8 fold lower for CAMK2B homomers (15nM) than for CAMK2A homomers (130nM) (Brocke et al., 1999).

Second, in substrate specificity there are a few differences known between CAMK2A and CAMK2B. For example, the C-terminus of densin-180 selectively binds to CAMK2A (not to CAMK2B) (Robison et al., 2005). Another part more towards the N-terminus of densin binds both CAMK2A and CAMK2B (Jiao et al., 2011). CAMK2B specifically phosphorylates Cdc20-APC (Puram et al., 2011), while CAMK2A doesn't. CAMK2B, but not CAMK2A, can phosphorylate actin under basal conditions, (O'Leary et al., 2006). Finally, CAMK2B binds Arc/Arg3.1 at low levels of Ca^{2+}/Calmodulin (upon Ca^{2+}/Calmodulin binding the affinity for Arc drops), but CAMK2A does not.

Despite the high homology between CAMK2A and CAMK2B, the above-mentioned examples also indicate unique functions. The next to section of this introduction will focus on these differences. However, consideration of their shared involvement in learning and LTP and their overlap in substrates prompted the idea that they can also be redundant. For example, the reduction, but not the complete absence, of LTP in *Camk2a* knockout mice spawned the idea that the residual LTP was caused by CAMK2B. In

Chapter 4 we address this by using an inducible knockout mouse for both CAMK2A and CAMK2B. Furthermore, the simultaneous deletion of both CAMK2A and CAMK2B allows us to unravel new functions that were previously unknown due to this redundancy.

1.5 CAMK2A

The literature on CAMK2A has expanded enormously ever since its discovery almost 40 years ago. In light of the scope of this dissertation, this vast body of literature cannot be summarized completely. This summary of CAMK2A will only briefly address the different knockout and knock-in mutants that have been generated over the years. For a more detailed overview of CAMK2A I recommend excellent reviews such as (Lisman et al., 2002; 2012; Hell, 2014).

CAMK2A is one of the most abundant protein kinases in the brain which is mostly present in the postsynaptic density of spines (Kennedy, 1997). Much of what we know of CAMK2A is due to the generation of knockout or knock-in mice. As early as 1992, a *Camk2a* knockout mouse was generated, the first knockout mouse in the field of learning and memory. These knockout mice showed impaired spatial learning in the Morris watermaze and impaired LTP at the Schaffer-collateral pathway in the hippocampus (Silva et al., 1992a; 1992b). Additional *Camk2a* knockout mice have been generated that confirmed the LTP and hippocampal learning impairments (Hinds et al., 1998; Elgersma et al., 2002). Interestingly, *Camk2a* knockout mice show reduced, but no complete absence of LTP. Further behavioral analysis revealed that *Camk2a* knockout mice also suffer from reduced fear-related responses, decreased aggression, increased pain sensitivity, increased startle response, increased vigilance and decreased mating (hardly any mating in knockouts) (Chen et al., 1994).

More knock-in mouse mutants have been made for CAMK2A. The importance of T286 phosphorylation was shown by mutating Threonine286 into a phospho-deficient alanine residue (T286A), thereby preventing autonomous activity of CAMK2A. These mice showed almost complete loss of NMDA-receptor-dependent LTP in the hippocampus and impairments in the Morris watermaze (Giese et al., 1998). Interestingly, the LTP impairment in the T286A mutant mice seemed greater than the impairment in the full knockout mutant. This could be explained by a dominant-negative effect of the T286A mutation, which can affect other isoforms present in the holoenzyme (e.g. CAMK2B).

Another knock-in mutant created was the CAMK2A-T286D, with an aspartate instead of a threonine to mimic phosphorylation at this site. These mice also showed impairment in LTP and impaired spatial learning (Mayford et al., 1996a). The impairment in LTP and

learning in this knock-in mutant is thought to be caused by increased phosphorylation of TT305/6 which was found *in vitro* using these mutations, overruling the autonomous activity by inhibiting the enzyme (Pi et al., 2010). Perhaps not surprising, knock-in mutants for the inhibitory phosphorylation sites have been generated as well. In CAMK2A-TT305/5VA mice (phospho-deficient), CAMK2A can no longer be inhibited and these mice show more rigid and less fine-tuned learning and a lower threshold for LTP induction. Finally, in CAMK2A-T305D (phospho-mimic) mice CAMK2A can no longer bind Ca^{2+}/Calmodulin and is therefore constantly in the inactive state. These mice show no learning and complete loss of LTP, which again can be explained by a dominant-negative effect of CAMK2A, similar to what is seen in T286A mice (Elgersma et al., 2002).

Not only is CAMK2A involved in LTP at the Schaffer-collateral pathway in the hippocampus, CAMK2A is also essential for experience-dependent plasticity (Glazewski et al., 1996). This type of plasticity can be induced in the barrel cortex by whisker deprivation. The autonomous activity of CAMK2A is especially important, as homozygous CAMK2A-T286A mice have impaired experience-dependent plasticity (Glazewski et al., 2000). Even on the single cell level, using whole-cell electrophysiology, the loss of autonomous activity of CAMK2A results in impaired LTP (Hardingham et al., 2003).

The use of knockout mice is an elegant tool to assess the molecular mechanisms underlying learning and memory. However, caution is warranted in the interpretations when using these mouse models. For example, even though expression of CAMK2A starts at P1 (Bayer et al., 1999), a developmental role for CAMK2A cannot be ruled out. It is possible that CAMK2A has a developmental role in the first postnatal days, which could cause the impairments in hippocampal learning and LTP seen in adult knockout mice. Second, it could be hypothesized that acute deletion has different effects compared to germline deletion. The latter could induce compensatory mechanisms, which might not be at play upon acute deletion. Third, the use of global knockout mice puts a restriction on spatial involvement of the candidate gene. For example, in the case of CAMK2A, it is unknown which brain regions are involved in the phenotypes present in global *Camk2a* mutant mice. In **Chapter 2** we investigate these issues using a floxed *Camk2a* mutant mouse in which we induce genetic knockout of *Camk2a* in adult mice to dissect the temporal and region-specific requirements for CAMK2A.

1.6 CAMK2B

Even though CAMK2B was discovered relatively shortly after CAMK2A (Kennedy et al., 1983), it was neglected for a long time likely due to the lack of a mouse model. However,

CAMK2B has been recently gaining attention in literature. CAMK2B has some very interesting and unique properties such as binding to actin thereby playing an important role in morphology of the cell. This part of the introduction will focus on everything known on CAMK2B: from the actin binding properties of CAMK2B to specific functions in LTP and homeostatic scaling.

1.6.1 Actin Binding

One of the first functional differences found between CAMK2A and CAMK2B was their localization. When overexpressed separately, CAMK2A is mainly found to be cytosolic, whereas overexpression of CAMK2B co-localizes with PSD95 and the actin cytoskeleton (Shen et al., 1998). When overexpressed together, overexpression of both CAMK2A and CAMK2B changes the localization of CAMK2A to a similar distribution found for CAMK2B (Shen et al., 1998; Okamoto et al., 2004). This suggests that CAMK2B can determine the subcellular localization of CAMK2A. Indeed, when CAMK2B is removed, the localization of CAMK2A changes (Shen et al., 1998; Thiagarajan et al., 2002; Borgesius et al., 2011). The mechanism by which CAMK2B can target itself and CAMK2A to spines is by binding the major cytoskeleton protein actin. Actin is present in dendritic spines and it is involved in structural maintenance of spines and plasticity (Capani et al., 2001; Star et al., 2002; Okamoto et al., 2004; Hayashi and Majewska, 2005). CAMK2B can bind both polymeric F-actin (filamentous) (Fink et al., 2003; Okamoto et al., 2004; O'Leary et al., 2006; Okamoto et al., 2007; Lin and Redmond, 2008) and free monomeric G-actin (globular) (Sanabria et al., 2009).

CAMK2B is bound to actin in its basal state. Upon activation however, CAMK2B can detach from actin (Fink et al., 2003; O'Leary et al., 2006; Okamoto et al., 2007; Lin and Redmond, 2008). Various experiments have tried to assess the mechanisms of CAMK2B detachment. CAMK2B detachment from F-actin is regulated by an autophosphorylation event within the actin binding domain on at least two different putative sites: S331 and S371 (Martinez-Lozada et al., 2014). Absence of Ca^{2+}/Calmodulin or mutations that interfere with CAMK2B activity result in a failure of CAMK2B to detach from actin. For example, expression of a CAMK2B protein with a mutation in its Calmodulin footprint (A303R), that prevents binding Ca^{2+}/Calmodulin, or with a mutation in the catalytic domain (K43R), thereby lacking kinase activity, results in failure of CAMK2B to detach from actin (Lin and Redmond, 2008). Another mutant protein mimicking continuous autophosphorylation (T287D; similar to the T286D mutation in CAMK2A) reversely cannot bind actin (Lin and Redmond, 2008) and decreases dendritic branching (Puram et al., 2011). Finally, CAMK2 inhibitor KN93, which competes with Ca^{2+}/Calmodulin binding, detaches CAMK2B from actin (Lin and Redmond, 2008). Interestingly,

monomeric CAMK2B, which lacks an association domain but still has the actin-binding domain intact, does not co-localize with actin. Therefore, the association domain and possibly oligomerization itself is also necessary for targeting CAMK2B to actin (Lin and Redmond, 2008).

Why is only CAMK2B able to bind actin, and not CAMK2A (but see (Khan et al., 2016))? The answer can be found in the variable domain of the protein, which in CAMK2B contains regions necessary for actin binding that are not present in CAMK2A. In general, CAMK2B contains three exons in the variable region (V) that are not present in the *Camk2a* gene (V1, V3 and V4). Two other exons in the variable region, V2 and V5, are not unique to CAMK2B as they are present in both CAMK2A and CAMK2B. V1 is most important for actin binding (O'Leary et al., 2006), but presence of V4 can further strengthen this binding. These exons in the variable region also give rise to alternative splice variants known to CAMK2B. Most prominently expressed in the adult brain *in vivo* is the full-length b splice variant (Brocke et al., 1995). This full-length splice variant is capable of binding actin (O'Leary et al., 2006). Other described splice variants are b_M, b_E, b' and b'_E. The $_E$ stands for *embryonic*, since these splice variants are expressed mainly during embryonic development (Brocke et al., 1995). The $_M$ in b_M stands for *muscular*, because this splice variant is mainly found in skeletal muscles (Bayer et al., 1998). An overview of these splice variants along with the exons from the variable region that are translated into protein can be found in **Table 3**. In short, V1 is present in the full-length CAMK2B splice variant, but not in the b_E and b'_E. Therefore, these splice variants cannot bind actin (Brocke et al., 1995; O'Leary et al., 2006). V1 is also present in b', but compared to full-length b it misses V4 and therefore binding of b' to actin is decreased (Shen et al., 1998; Zheng et al., 2014).

Table 3. Overview of CAMK2B splice variants

Splice Variant	Characteristic	Expression[a]	Actin Binding	Exons[b]
β	full length	throughout the brain	strong	V1-V5
βE	embryonic	throughout the brain	no binding	V2-V5
β'	-	pancreatic islet cells, hippocampus, cortex	moderate/strong	V1-V3, V5
β'E	embryonic	pancreatic islet cells, hippocampus, cortex	no binding	V2, V3, V5
βM	muscular	skeletal muscles, pancreatic islet cells, neurons	weak	V1-V6

[a]As has so far been described in literature
[b]As expressed in the translated splice variant

1.6.2 CAMK2B and neuronal morphology

1.6.2.1 CAMK2B and dendritic arborization and spine morphology

Since actin is a major constituent of the cytoskeleton of eukaryotic cells and the cytoskeleton is responsible for the morphology of these cells, actin-binding proteins like CAMK2B influencing the cytoskeleton can also regulate morphology, like dendritic arborization and spine density. Many studies have been conducted to elucidate the role of CAMK2B in morphology of neurons. The effects of changing expression of CAMK2B by either knockdown or overexpression on dendritic arborization vary among reports, as will become clear below. Not only the use of different techniques but also temporal differences in the manipulation performed can change the effect on morphology. For example, one report showed that overexpression of CAMK2B increases dendritic arborization and filopodia motility and knockdown of CAMK2B decreases arborization (Fink et al., 2003). According to another report knockdown of CAMK2B actually increases dendritic arborization (Puram et al., 2011). Since both studies use the same technique (primary neuronal cultures) the contrast in findings might lie in the difference in the time point the neurons were harvested from the rat brain to obtain cultured hippocampal neurons, E18.5 (Puram et al., 2011) versus postnatal day 6 or 10 (Fink et al., 2003). This effect of CAMK2B was only found in young cultures and not in adult cultures, implying that CAMK2B only has an effect on dendritic arborization within a critical window of development (Fink et al., 2003).

The effect of overexpression or knockdown of CAMK2B remains inconclusive with respect to spine density. Overexpressing CAMK2B in cultured hippocampal neurons increases spine density whereas knockdown decreases it (Fink et al., 2003). Overexpressing CAMK2B can increase mEPSC frequency, an effect likely to be mediated by an increase in dendritic branching or spine density (Thiagarajan et al., 2002). However, the knockdown effect on spine density is less consistent. Another study found that upon knockdown of CAMK2B in organotypic slices of the hippocampus spine density does not change but interestingly spine volume decreases and neck length increases, conversing mature spines into immature spines (Okamoto et al., 2007).

To further complicate the issue, mice that are knockout for CAMK2B (*Camk2b*$^{-/-}$) have normal gross morphology (Bachstetter et al., 2014) and show no changes in dendritic arborization, spine density, neck length and head width in cerebellar Purkinje cells (Van Woerden et al., 2009). Similarly in the hippocampus, spine density is unchanged in *Camk2b*$^{-/-}$ mice (Borgesius et al., 2011). It is possible that *Camk2b*$^{-/-}$ mice have no phenotype in arborization and spine density because the loss of CAMK2B is compensated for during development. In contrast, the acute effects of overexpression

or knockdown within the neuronal cultures *in vitro* are perhaps not compensated due to their rapid onset. Another possibility is that morphology itself is regulated differently when neurons are in culture compared to *in vivo*. For example, there are clear differences between the extracellular environment in these two conditions and extracellular matrix proteins are known to influence morphogenesis (Rozario and DeSimone, 2010; Gordon et al., 2013).

Overall, it seems clear that under certain *in vitro* conditions, CAMK2B can influence the morphology of neurons. In the knockout mouse models for CAMK2B, however, these observations have not been made. This could mean that in *Camk2b* knockout mice, compensatory mechanisms are at play.

1.6.2.2 Gating mechanism for structural plasticity

What is the function of CAMK2B attachment to actin and detachment upon entry of calcium mentioned before and what does it mean for the morphology, organization and functioning of the spine? The detachment of CAMK2B allows actin regulatory proteins like cofilin and the Arp2/3 complex to act on the polymerization of actin. The activation of CAMK2B leads to a brief time window in which the actin cytoskeleton within a spine can be remodeled. Re-attachment of CAMK2B closes this time window and stabilizes the new F-actin structure for extended periods of time. In this model, CAMK2B acts as a negative regulator of actin remodeling in spines and as a molecular-temporal gate for synaptic plasticity (Kim et al., 2015). However, actin detachment alone is not sufficient to induce structural changes to the cytoskeleton. Glutamate receptor signaling has to accompany this detachment in order for structural changes to occur. This way, coincidence detection underlies the structural plasticity changes, which can work as a double-verification step (Kim et al., 2015).

1.6.2.3 Other pathways in neuronal morphology regulated by CAMK2B

Next to providing a gating mechanism in spines, other mechanisms are known by which CAMK2B regulates morphology. Although much is still unclear, a small summary of all known pathways in which CAMK2B is involved will be discussed here. In granule neurons CAMK2B can phosphorylate NeuroD at Ser336 which regulates dendritic growth and maintenance (Gaudillière et al., 2004). CAMK2B can also bind to PCM1 using a unique centrosomal binding sequence (CTS) that is not present in CAMK2A. Using this CTS to bind to PCM1, CAMK2B can be targeted to the centrosome where it can phosphorylate Cdc20 on Ser51. Upon phosphorylation, Cdc20 will leave the centrosome, which will lead to dendrite retraction and pruning (Puram et al., 2011). Activation of group 1 mGluR and subsequent activation of PKC has been shown to phosphorylate CAMK2B in cerebellar Purkinje cells promoting spine formation and elongation (Sugawara et al.,

2017). In addition, Rem2 is also a substrate of CAMK2B. Phosphorylation of Rem2 by CAMK2B reduces dendritic branching (Ghiretti et al., 2013). CAMK2B can phosphorylate LIM-kinase1 (LIMK1) on Thr508 and thereby activate the cofilin-phosphorylating activity of LIMK1, which is part of a pathway involved in BDNF-induced enhancement of primary neurite formation (Saito et al., 2013). Taken together, CAMK2B can interact with multiple proteins to regulate neuronal morphology. However, it is still unclear whether these interactions are present throughout the brain or whether pathways through which CAMK2B can regulate morphology differ between cell types or brain regions.

1.6.3 CAMK2B in different cell types

1.6.3.1 CAMK2B in interneurons

CAMK2B and CAMK2A are widely expressed throughout pyramidal cells in the cortex and the hippocampus, but in interneurons in these brain regions only CAMK2B is abundantly expressed and CAMK2A is not expressed at all (Liu and Jones, 1996; Sík et al., 1998; Thiagarajan et al., 2002). Considering the important role for CAMK2A in mediating plasticity in both cortical and CA1 hippocampal pyramidal cells, the absence of CAMK2A in interneurons poses the question whether CAMK2B might be responsible for plasticity in interneurons. Especially interneurons in the stratum radiatum of the hippocampus are a likely candidate for CAMK2B to regulate plasticity since LTP expressed there from glutamatergic synapses is mostly NMDA receptor-dependent (Lamsa et al., 2005). Indeed, CAMK2 inhibitors KN62, KN93 and an autoinhibitory peptide blocks LTP in these interneurons (Wang and Kelly, 2001; Lamsa et al., 2007a).

However, interneurons within the hippocampus can be quite diverse (Klausberger and Somogyi, 2008). Interneurons in the stratum radiatum as mentioned above are mostly expressing NMDA receptor-dependent LTP. However, most interneurons in the stratum oriens of the hippocampus are expressing NMDA receptor-independent LTP (Perez et al., 2001; Kullmann and Lamsa, 2007; Lamsa et al., 2007b). If CAMK2B is also important for plasticity in the latter group of interneurons, it poses the interesting question where the source of Ca^{2+} is coming from in these interneurons if not from the NMDA-receptor. Interestingly, these neurons have a high number of GluA2-lacking AMPA receptors, which are, next to sodium-permeable, also calcium-permeable (Matta et al., 2013). If indeed CAMK2B plays an important role in these interneurons, then the source of calcium needed to activate CAMK2B could well be the GluA2-lacking AMPA receptors. Importantly, there are LTP pathways known in pyramidal cells that involve calcium-permeable AMPA receptors that do not depend on CAMK2. This could also be

the case for interneurons that express calcium-permeable AMPA receptors (Asrar et al., 2009). Therefore, whether there is a role for CAMK2B in these cells in LTP remains to be uncovered.

1.6.3.2 CAMK2B in oligodendrocytes

In addition to its abundant expression in excitatory and inhibitory cells, CAMK2B is also present in oligodendrocytes (Cahoy et al., 2008; Martinez-Lozada et al., 2014). There it regulates translation of proteins (Flores-Méndez et al., 2013), maturation of oligodendrocytes and myelination of axons (Waggener et al., 2013). Knockdown of CAMK2B mRNA restrains the establishment of an expanded process network that could be compared to the dendritic arborization in neurons (Waggener et al., 2013). Upon stimulation of oligodendrocytes with glutamate, CAMK2B becomes active and an autophosphorylation event occurs at S371 to further promote oligodendrocyte maturation (Martinez-Lozada et al., 2014). This phosphorylation event is also observed in neurons (Kim et al., 2015). Also, *Camk2b*$^{-/-}$ mice show decreased thickness of myelin sheath whereas *Camk2b*$^{A303R/A303R}$ mice have normal myelination, implying that CAMK2B, in contrast to oligodendrocyte maturation, has a structural role in myelination of axons (Waggener et al., 2013).

1.6.4 CAMK2B in LTP and homeostatic scaling

1.6.4.1 CAMK2B in hippocampal LTP

Does CAMK2B have a similar role in LTP as CAMK2A in pyramidal neurons? CAMK2B seems to have a structural role in hippocampal CA1 neurons. *Camk2b*$^{-/-}$ mice have impaired hippocampus-dependent learning and LTP at the CA3-CA1 Schaffer-collateral pathway. In contrast, *Camk2b*$^{A303R/A303R}$ mice expressing mutant CAMK2B that can no longer bind Ca^{2+}/Calmodulin show normal learning and LTP. One striking difference between these two mutants is that the *Camk2b*$^{A303R/A303R}$ mutant can still target CAMK2A to spines, which is most likely the explanation for the lack of a phenotype in these mice (Borgesius et al., 2011).

It is interesting that *Camk2b*$^{A303R/A303R}$ mice do not show impaired hippocampus-dependent learning even though the mutation in these mice prevents CAMK2B from detaching from actin (Borgesius et al., 2011). The question then arises whether structural LTP is still present in *Camk2b*$^{A303R/A303R}$ mice. According to (Kim et al., 2016), detachment from actin is essential for both electrophysiologically measured functional and structural LTP. It could therefore be hypothesized that CAMK2B-A303R mice have normal functional LTP but do not have the structural changes in spine size that are accompanied with it.

Perhaps CAMK2B-A303R mice are able to keep a gate at the spine neck by binding to actin. For example, CAMK2B with the length of its own homomere can bind two filaments of actin (Sanabria et al., 2009), which keeps these filaments relatively close, preventing diffusion of other proteins out of the spine (see also (Allison et al., 2000)). It could be hypothesized that the CAMK2B-A303R mice do not show a phenotype because of keeping this gate closed, whereas the CAMK2B knockout mice have a problem keeping this gate closed and as a result, CAMK2A can diffuse out of the spine.

1.6.4.2 CAMK2B in cerebellar LTP

Considering the high expression of CAMK2B in the cerebellum, it is perhaps not surprising that it has an important role in LTP there as well. Many studies have focused more specifically on the cerebellar Purkinje cells, the only cell type in the cerebellum that also expresses CAMK2A (Walaas et al., 1988).

The plasticity rules are different in the cerebellum compared to the hippocampus (Bienenstock et al., 1982; Lev-Ram et al., 2002; Coesmans et al., 2004). At the parallel fiber-Purkinje cell synapse, phosphatases are responsible for LTP induction, whereas kinases are responsible for LTD induction (Coesmans et al., 2004; Belmeguenai and Hansel, 2005; Kakegawa and Yuzaki, 2005). Stimulation of the parallel fibers results in a low influx of calcium postsynaptically in Purkinje cells, which will activate mainly phosphatases. This low influx will allow phosphatases to outcompete kinases allowing phosphatases to induce LTP at the parallel fiber-Purkinje cell synapse. Upon stimulation of parallel fibers in combination with the climbing fiber, which will result in a high influx of calcium, kinases will outcompete phosphatases resulting in LTD at the parallel fiber-Purkinje cell synapse. Note that for the cortex and hippocampus these rules are inversed, meaning that at low calcium levels phosphatases still outcompete kinases, but in these brain regions this results in LTD. High influx of calcium results in kinases outcompeting phosphatases leading to the induction of LTP.

Interestingly, deleting CAMK2A in the cerebellum using *Camk2a*$^{-/-}$ mutant mice impairs the induction of LTD. Upon low influx of calcium phosphatases will outcompete kinases leading to LTP. Upon high influx of calcium, phosphatases will still outcompete kinases due to the lack of the abundant kinase CAMK2A, and hence LTP will also be induced at higher calcium influx (Hansel et al., 2006).

However, in *Camk2b*$^{-/-}$ mice, the loss of CAMK2B results in a complete reversal of the plasticity rules governed at the parallel fiber-Purkinje cell synapse (Van Woerden et al., 2009). First, low calcium influx caused by stimulating parallel fibers results in LTD. Second, high calcium influx caused by stimulating both parallel fibers and the climbing

fiber results in LTP (Van Woerden et al., 2009). The latter observation can be explained by an overall lack of an abundant kinase, resulting in phosphatases outcompeting kinases at high levels of calcium influx, comparable to high calcium influx in *Camk2a*$^{-/-}$ mice. But that does not explain the former observation, where low influx of calcium results in LTD. The authors explain this observation by addressing an important function of CAMK2B: binding to actin (Okamoto et al., 2007). The lack of CAMK2B can result in more available CAMK2A not bound to actin in heteromeric holoenzymes that can mediate precocious kinase activity. Furthermore, previous experiments have shown that homomeric CAMK2A holoenzymes can move to the PSD 4 times faster than mixed CAMK2A/CAMK2B holoenzymes and 24-fold faster than homomeric CAMK2B holoenzymes (Shen et al., 1998), which can explain the ready availability of CAMK2A in the absence of CAMK2B. This way, CAMK2B orchestrates the direction of plasticity at the parallel fiber-Purkinje cell synapse.

1.6.4.3 CAMK2B in homeostatic scaling

CAMK2B and CAMK2A can also play an important role in homeostatic scaling. Blocking AMPA receptors for up to 24 hours increases the CAMK2B/CAMK2A ratio in neurons and increases expression of surface GluA1, mediated by enzymatic activity of CAMK2B, which in turn increases in mEPSC amplitude and frequency if the AMPA block is alleviated (Thiagarajan et al., 2002; Groth et al., 2011). Blocking all inhibition decreases the CAMK2B/CAMK2A ratio, and decreases the frequency and increases the amplitude of mEPSCs. Interestingly, the up and down regulation are reciprocal, the total levels of CAMK2 do not change. Because of this, excitatory neurons can regulate homeostatic scaling upon strong input by increasing levels of CAMK2A, which has an 8-fold weaker binding affinity for Ca^{2+}/Calmodulin, and when input is low, increasing levels of CAMK2B, which has a stronger binding affinity for Ca^{2+}/Calmodulin (Brocke et al., 1999; Thiagarajan et al., 2002). This provides a neuronal tuning mechanism to different levels of activity in the cell. This tuning mechanism has been suggested to work through the scaffolding protein GKAP (Shin et al., 2012). The question remains, how these tuning mechanisms play a role *in vivo* where the window of activity changes is narrower than in the conditions used in these papers, and the activity can vary more between different synapses within the same neuron.

1.6.5 CAMK2B and the synaptic tag hypothesis

In 1997 Uwe Frey and Richard Morris postulated the idea of a 'synaptic tag' in long-term potentiation (Frey and Morris, 1997). Although the nature of this tag is still unclear, the authors stated that this tag will enable synapses that have undergone LTP to capture newly synthesized plasticity-related proteins to maintain increased synaptic strength

over the course of hours to days or even years. Interestingly, disruption of the actin cytoskeleton can disrupt this synaptic tag (Ramachandran and Frey, 2009). In addition, KN62 can prevent the establishment of this tag (Sajikumar et al., 2007). This would imply that CAMK2B could be involved in the formation of this tag since it binds actin and can be inhibited by KN62. Indeed, CAMK2B has been proposed to be at least part of this synaptic tag (Okuno et al., 2012; 2017), but not as a synaptic tag but rather by acting oppositely as an inversed synaptic tag by binding Arc (also called Arg3.1) at inactive synapses. Arc is an immediate early gene whose induction highly correlates with augmented neuronal activity (Link et al., 1995; Lyford et al., 1995; Guzowski et al., 1999; Ramírez-Amaya et al., 2005). Arc can bind inactive CAMK2B predominantly at synapses that have not been active for a while. Because of this, the binding of Arc with inactive CAMK2B can act as an inversed synaptic tag, creating a tag for inactive synapses to stop newly synthesized plasticity-related proteins from entering the synapse, keeping them only available for synapses that do not have this inverse synaptic tag (Okuno et al., 2012; 2017) and have undergone LTP.

1.6.6 CAMK2B in locomotion

As discussed before, on a behavioral level, *Camk2b*$^{-/-}$ mice have hippocampus-dependent learning problems in contextual fear conditioning (Borgesius et al., 2011). Since CAMK2B has an important role in actin binding and plasticity, even inversing the plasticity rules in the cerebellum, a brain region important for motor control, the question rises whether knockout animals for *Camk2b* show outspoken motor phenotypes. Indeed, ever since the generation of the first *Camk2b* knockout mouse (*Camk2b*$^{-/-}$) (Van Woerden et al., 2009), it has become clear that CAMK2B has an important function in locomotion. *Camk2b*$^{-/-}$ mice show a reduced latency on the accelerating rotarod and an impairment to stay on the balance beam (Van Woerden et al., 2009). These phenotypes were later independently verified in another CAMK2B knockout mouse (Bachstetter et al., 2014) together with new phenotypes including lower weight in the first 3-9 weeks of age (but normal birth weight), increased fat mass at 12 weeks of age, decreased strength in the forelimbs in the grip strength test, decreased spontaneous activity in the running wheel, reduced anxiety related behavior and decreased nest building (Bachstetter et al., 2014). Most of these phenotypes can be ascribed to the impaired locomotion in these mice. Since CAMK2B is expressed in abundance in major brain regions implicated in locomotion from early development, it is unclear whether these motor problems (i) have a developmental origin, i.e. an important role for CAMK2B during early development, (ii) are due to an important post-developmental role for CAMK2B in one or multiple brain regions involved in locomotion or (iii) a combination of these options. In **Chapter 3** we

try to dissect the spatiotemporal requirements of CAMK2B in locomotion by crossing the *Camk2b*$^{f/f}$ mouse with specific *Cre*-lines deleting the *Camk2b* gene in brain regions known to be important for locomotion.

1.7 CAMK2 INHIBITORS

Because knockout mice for *Camk2a* and *Camk2b* were not always available, much research has used pharmacological inhibitors to elucidate the kinetic role of CAMK2 in the brain. However, it is of utmost importance that: (i) these inhibitors are applied at the right concentration (ii) these inhibitors are CAMK2-specific and (iii) off-target effects are known and considered. This section will focus on which CAMK2 inhibitors are mainly used in literature and which of these above issues have been addressed.

In recent years, multiple inhibitors for CAMK2 have been developed. Some are specific for CAMK2, whereas others inhibit a broader range of Ca^{2+}/Calmodulin-dependent protein kinases. Potency can also vary significantly between drugs. An overview of the most used inhibitors for CAMK2 can be found in **Table 4**, divided into three different classes, along with their specificity and known off target effects. In short, the first class are the artificially developed drugs KN62 and KN93, the second class are peptides based on the autoinhibitory domain of CAMK2 like AIP and AC3-I and the third class consists of the group of endogenous proteins like CAMKIIN and newly developed peptides based on CAMKIIN.

One of the first inhibitors developed was KN62 in 1990 (Tokumitsu et al., 1990). However, KN62 is hydrophobic which limits the application of this drug *in vitro* or *in vivo*, as it must be dissolved in DMSO, which can be harmful for the tissues or cultured cells. Therefore, in 1991, a new inhibitor, KN93, was developed which was dissolvable in water but still able to cross the cell membrane (Sumi et al., 1991). KN62 and KN93 both compete with Calmodulin in binding to CAMK2 (Tokumitsu et al., 1990; Sumi et al., 1991). Because of their competitive nature with Calmodulin, it could be expected that KN62 and KN93 have off-target effects with other CaM binding proteins. Indeed, KN62 was found to block CAMK5 (Mochizuki et al., 1993) and CAMK4 *in vitro* (Enslen et al., 1994).

Next to having these broader effects on closely related protein kinases, additional off-target effects of KN93 and KN62 are known. For example, KN62 and KN93 have an effect on basal activity levels of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis (Ishii et al., 1991; Sumi et al., 1991). These effects were also found by their respective inactive analogues KN04 and KN92 (Tombes et al., 1995) in a similar dose-dependent manner in a later study (Marley and Thomson, 1996), indicating

Table 4. Overview of CAMK2 inhibitors and their side-effects

Inhibitor	Year	Specificity	IC50	Binding on	Off-target effects	Reference
CaM-binding competitive inhibitors						
KN62	1990	CAMK1, CAMK2 CAMK4, CAMK5	370nM	Competes with CaM-binding [a]	TH, $P2X_7$	Tokumitsu et al., 1990
KN04	1990	–	–	Inact. analogue KN62	TH, $P2X_7$	Ishikawa et al., 1990
KN93	1991	CAMK1, CAMK2 CAMK4, CAMK5	370nM	Competes with CaM-binding [a]	TH, $Ca_v1.2$, $Ca_v1.3$, I_{KR}	Sumi et al., 1991
KN92	1995	–	–	Inact. analogue KN93	TH, $Ca_v1.2$, $Ca_v1.3$, I_{KR}	Tombes et al., 1995
Substrate-based inhibitors						
AC3-I	1995	CAMK2	3μM	Peptide-substrate competitive	PKD1	Braun et al., 1995
AIP	1995	CAMK2	100nM	Peptide-substrate competitive	–	Ishida et al., 1995
Endogenous inhibitors - CaMKIIN						
CaMKIINα	1998	CAMK2	50nM	Peptide-substrate competitive	-	Chang et al., 1998
CaMKIINβ	1998	CAMK2	50nM	Peptide-substrate competitive	-	Chang et al., 1998
CN21a	2007	CAMK2	0.1μM	Peptide-substrate competitive	-	Vest et al., 2007
CN19o	2011	CAMK2	0.4nM	Peptide-substrate competitive	-	Coultrap et al., 2011
CN17β	2013	CAMK2	40nM	Peptide-substrate competitive	-	Gomez-Monterrey et al., 2013

nM = nanoMolair; μM = microMolair; CaM = calmodulin; inact. = inactive; TH = Tyrosine hydroxylase; $P2X_7$ = P2X purinoceptor 7; PKD1 = protein kinase D1; AC3-I = Autocamtide-3 Inhibitor; AIP = Autocamtide-2 Inhibitor

[a] Autophosphorylated CAMK2 (T286/T287) is not blocked by these inhibitors

that these effects were not mediated through CAMK2 but were indeed off-target. Furthermore, KN93 and its inactive analogue KN92 are both known to decrease $Ca_V1.2$ and $Ca_V1.3$ currents, an effect not seen when using a more specific CAMK2 inhibitor, autocamtide-2-related inhibitory protein (AIP; will be further discussed below) (Gao et al., 2006). Moreover, KN62 is known to block P2X7 receptors, an effect also accomplished by the inactive analogue KN04 (Gargett and Wiley, 1997). Finally, KN93 and KN92 are known to block inward rectifying potassium channels (I_{KR}) in mammalian ventricular myocytes (Hegyi et al., 2015).

In 1995 two more potent inhibitory drugs, autocamtide-2-related inhibitory protein (AIP) and autocamtide-3 inhibitor (AC3-I), were developed (Braun and Schulman, 1995b; Ishida et al., 1995). These drugs are >100 times more selective for CAMK2 than they are for PKC, PKA or CAMK4. Both AIP and AC3-I mimic the autoinhibitory domain of CAMK2A but have their phosphorylatable Thr286/287 substituted for an Alanine. They block CAMK2 by competing with substrates for binding to the catalytic domain. Off-target effects have been described. For example, AC3-I can inhibit cellular actions of protein kinase D1 (PKD1) (Backs et al., 2009). A control peptide for AC3-I is available (AC3-C), but it is not as often used as KN92 or KN04 (Patel et al., 1999; Wu et al., 2009). AIP or AC3-I are sometimes modified by lipids to enhance cell membrane permeation, but some effects unrelated to CAMK2 inhibition were shown for these peptides as well (Wu et al., 2009).

A third class of CAMK2 inhibitors is a group of endogenous proteins that inhibit CAMK2 with high affinity. Two small proteins have been identified, CaM-KIINa and CaM-KIINb, sometimes collectively called CAMKIIN, and both proteins can be found throughout the brain (Chang et al., 2001; Pellicena and Schulman, 2014). The a and b in these proteins do not relate to the CAMK2 isoforms as these proteins block all CAMK2 isoforms equally with an IC50 of 50nM (Chang et al., 2001). Further analysis of the core inhibitory domain of CAMKIIN led to the generation of CAMKIINtide, a 28 amino acid long peptide inhibitor (Chang et al., 1998). CAMKIIN and CAMKIINtide both block CAMK2 in the kinase domain, meaning that they can only block CAMK2 after it has been activated by Ca^{2+}/Calmodulin or when it is autophosphorylated. Further adjustments of CAMKIINtide led to even more potent, more specific and shorter peptides, such as CN21a (Vest et al., 2007), CN19o with an IC50 < 0.4nM (Coultrap and Bayer, 2011) and CN17b (Gomez-Monterrey et al., 2013).

1.8 CAMK2 IN BRAIN DISEASE

By the end of this introduction it should become clear that CAMK2 is involved in a plentitude of pathways. Therefore, it should not be surprising that CAMK2 is implicated in many brain diseases. Examples of diseases are epilepsy ((Churn et al., 2000; McNamara et al., 2006; Yamagata et al., 2006), reviewed in (Liu and Murray, 2012)), Alzheimer's disease (Kummer et al., 2014) (reviewed in (Ghosh and Giese, 2015)), Parkinson's disease (Picconi et al., 2004), depression (Li et al., 2013), bipolar disorder (Ament et al., 2015), alcohol addiction (Easton et al., 2013; Schöpf et al., 2015) and Angelman syndrome (Weeber et al., 2003; Van Woerden et al., 2007).

Most studies did not differentiate between different isoforms. Therefore, it is not clear whether a single isoform or all isoforms of CAMK2 are involved in the pathophysiology of the disease. One exception is a study that found a role for CAMK2B in depression (Li et al., 2013). In congenitally learned helpless rats, an animal model for depression, CAMK2B protein is upregulated in the lateral habenula, a brain region implicated in depression (Li et al., 2013). Overexpression of CAMK2B in wildtype mice and rats results in depression-like behavior. Conversely, reducing CAMK2B levels in these rats rescues depression-like behavior, indicating that CAMK2B is an important mediator of depression-like behavior in the lateral habenula. Interestingly, CAMK2G is even more upregulated in this animal model, but was not further tested, so the role for CAMK2G in depression remains elusive (Li et al., 2013). Even though the mechanism for depression in this study is limited only to CAMK2B and not to CAMK2A, a potential role for CAMK2A in depression cannot be ruled out since it has been found to be down-regulated in the hippocampus and prefrontal cortex of humans with depression who committed suicide (Fuchsova et al., 2015).

However, papers describing isolated deletions or mutations in one of the *Camk2* genes in humans have been scarce. One paper described heterozygous deletion of human *CAMK2A* as part of a large deletion encompassing several genes in two persons with Treacher Collins syndrome with unexpected intellectual disability, where the deletion of *CAMK2A* could explain the intellectual disability (Vincent et al., 2014).

Another missense mutation in CAMK2A, CAMK2A-E183V, was found and associated with autism-spectrum disorder (Iossifov et al., 2014). This CAMK2A-E183V mutation was later shown to decrease CAMK2A kinase activity and interactions with CAMK2 associated proteins and to increase CAMK2A turnover in cells (Stephenson et al., 2017). Moreover, CAMK2A-E183V increases dendritic arborization and decreases spine density and basal

synaptic transmission. CAMK2A-E183V knock-in mice also show aberrant behavioral phenotypes including hyperactivity, decreased social interaction and increased repetitive behaviors (Stephenson et al., 2017).

In an evaluation of 100 patients with intellectual disability and an IQ <50 with unaffected parents, a missense mutation in *CAMK2G* (Arg292Pro) was found in one of these patients (de Ligt et al., 2012). *CAMK2G* being the first paralog found in intellectual disability in humans was somewhat surprising considering the strong abundance and specific neuronal expression of CAMK2A and CAMK2B compared to the lower expression of CAMK2G as well as its non-specificity in neurons. Recently however, new important roles for CAMK2G in the coupling of neuronal excitation to transcription are being unraveled, increasing our understanding of CAMK2G and possible pathophysiology if indeed the CAMK2G mutation turns out to be cause of the intellectual disability (Ma et al., 2014; Cohen et al., 2015; 2016).

Very recently, 19 rare *de novo* mutations were found in individuals with intellectual disability in both *CAMK2A* and *CAMK2B* and screened for their effect on CAMK2 function and neuronal migration. Some mutations increased CAMK2 autophosphorylation and others decreased it, but interestingly, all mutations that interfered with autophosphorylation also affected neuronal migration, implicating a tight regulation between autophosphorylation and development (Küry et al., 2017). With new genomic screening techniques becoming more affordable than ever, it will be very interesting to see what the future holds in store for us when it comes to *CAMK2A, CAMK2B, CAMK2G* or *CAMK2D* mutations in humans.

1.9 SCOPE OF THIS DISSERTATION

This dissertation will focus on both CAMK2A and CAMK2B, on both their independent role in neuronal and cognitive functioning as well as their collective role. In **Chapter 2** we focus on the CAMK2A's function in adulthood. Using an inducible *Camk2a* knockout mutant, we investigate the spatial and temporal requirements of CAMK2A in hippocampal-dependent learning and LTP. In **Chapter 3** we use a similar approach with an inducible *Camk2b* knockout mutant, now investigating the molecular, spatial and temporal requirements of CAMK2B in locomotion. Finally, in **Chapter 4 and 5** we use a similar approach with mice where both CAMK2A and CAMK2B are deleted throughout the brain, uncovering previously unknown functions of CAMK2 in survival (**Chapter 4**) and neuronal excitability (**Chapter 5**).

REFERENCES

Allison DW, Chervin AS, Gelfand VI, Craig AM (2000) 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.

Ament SA et al. (2015) Rare variants in neuronal excitability genes influence risk for bipolar disorder. Proceedings of the National Academy of Sciences of the United States of America 112:3576–3581.

Aow J, Doré K, Malinow R (2015) Conformational signaling required for synaptic plasticity by the NMDA receptor complex. Proceedings of the National Academy of Sciences 112:14711–14716.

Asrar S, Zhou Z, Ren W, Jia Z (2009) Ca(2+) permeable AMPA receptor induced long-term potentiation requires PI3/MAP kinases but not Ca/CaM-dependent kinase II. PLoS ONE 4:e4339.

Bachstetter AD, Webster SJ, Tu T, Goulding DS, Haiech J, Watterson DM, Van Eldik LJ (2014) Generation and Behavior Characterization of CaMKIIβ Knockout Mice Skoulakis EMC, ed. PLoS ONE 9:e105191.

Backs J, Backs T, Neef S, Kreusser MM, Lehmann LH, Patrick DM, Grueter CE, Qi X, Richardson JA, Hill JA, Katus HA, Bassel-Duby R, Maier LS, Olson EN (2009) The delta isoform of CaM kinase II is required for pathological cardiac hypertrophy and remodeling after pressure overload. Proceedings of the National Academy of Sciences of the United States of America 106:2342–2347.

Barria A, Derkach V, Soderling T (1997a) 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.

Barria A, Malinow R (2005) NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. Neuron 48:289–301.

Barria A, Muller D, Derkach V, Griffith LC, Soderling TR (1997b) Regulatory phosphorylation of AMPA-type glutamate receptors by CaM-KII during long-term potentiation. Science 276:2042–2045.

Bayer KU, De Koninck P, Leonard AS, Hell JW, Schulman H (2001) Interaction with the NMDA receptor locks CaMKII in an active conformation. Nature 411:801–805.

Bayer KU, Harbers K, Schulman H (1998) alphaKAP is an anchoring protein for a novel CaM kinase II isoform in skeletal muscle. EMBO J 17:5598–5605.

Bayer KU, LeBel E, McDonald GL, O'Leary H, Schulman H, De Koninck P (2006) Transition from reversible to persistent binding of CaMKII to postsynaptic sites and NR2B. Journal of Neuroscience 26:1164–1174.

Bayer KU, Löhler J, Schulman H, Harbers K (1999) 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.

Belmeguenai A, Hansel C (2005) A role for protein phosphatases 1, 2A, and 2B in cerebellar long-term potentiation. Journal of Neuroscience 25:10768–10772.

Bennett MK, Erondu NE, Kennedy MB (1983) Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. J Biol Chem 258:12735–12744.

Benson DL, Gall CM, Isackson PJ (1992) Dendritic localization of type II calcium calmodulin-dependent protein kinase mRNA in normal and reinnervated rat hippocampus. Neuroscience 46:851–857.

Bers DM, Grandi E (2009) Calcium/calmodulin-dependent kinase II regulation of cardiac ion channels. J Cardiovasc Pharmacol 54:180–187.

Bienenstock EL, Cooper LN, Munro PW (1982) Theory for the development of neuron selectivity: orientation specificity and binocular interaction in visual cortex. J Neurosci 2:32–48.

Borgesius NZ, Van Woerden GM, Buitendijk GHS, Keijzer N, Jaarsma D, Hoogenraad CC, Elgersma Y (2011) βCaMKII plays a nonenzymatic role in hippocampal synaptic plasticity and learning by targeting αCaMKII to synapses. Journal of Neuroscience 31:10141–10148.

Braun AP, Schulman H (1995a) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. Annu Rev Physiol 57:417–445.

Braun AP, Schulman H (1995b) A non-selective cation current activated via the multifunctional Ca(2+)-calmodulin-dependent protein kinase in human epithelial cells. The Journal of Physiology 488 (Pt 1):37–55.

Brocke L, Chiang LW, Wagner PD, Schulman H (1999) Functional implications of the subunit composition of neuronal CaM kinase II. J Biol Chem 274:22713–22722.

Brocke L, Srinivasan M, Schulman H (1995) Developmental and regional expression of multifunctional Ca2+/calmodulin-dependent protein kinase isoforms in rat brain. J Neurosci 15:6797–6808.

Burgin KE, Waxham MN, Rickling S, Westgate SA, Mobley WC, Kelly PT (1990) In situ hybridization histochemistry of Ca2+/calmodulin-dependent protein kinase in developing rat brain. J Neurosci 10:1788–1798.

Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, Lubischer JL, Krieg PA, Krupenko SA, Thompson WJ, Barres BA (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. Journal of Neuroscience 28:264–278.

Capani F, Martone ME, Deerinck TJ, Ellisman MH (2001) Selective localization of high concentrations of F-actin in subpopulations of dendritic spines in rat central nervous system: a three-dimensional electron microscopic study. J Comp Neurol 435:156–170.

Chang BH, Mukherji S, Soderling TR (1998) Characterization of a calmodulin kinase II inhibitor protein in brain. Proceedings of the National Academy of Sciences of the United States of America 95:10890–10895.

Chang BH, Mukherji S, Soderling TR (2001) Calcium/calmodulin-dependent protein kinase II inhibitor protein: localization of isoforms in rat brain. Neuroscience 102:767–777.

Chao LH, Stratton MM, Lee I-H, Rosenberg OS, Levitz J, Mandell DJ, Kortemme T, Groves JT, Schulman H, Kuriyan J (2011) A mechanism for tunable autoinhibition in the structure of a human Ca2+/calmodulin- dependent kinase II holoenzyme. Cell 146:732–745.

Chapman PF, Frenguelli BG, Smith A, Chen CM, Silva AJ (1995) The alpha-Ca2+/calmodulin kinase II: a bidirectional modulator of presynaptic plasticity. Neuron 14:591–597.

Chen C, Rainnie DG, Greene RW, Tonegawa S (1994) Abnormal fear response and aggressive behavior in mutant mice deficient for alpha-calcium-calmodulin kinase II. Science 266:291–294.

Chen X, Vinade L, Leapman RD, Petersen JD, Nakagawa T, Phillips TM, Sheng M, Reese TS (2005) Mass of the postsynaptic density and enumeration of three key molecules. Proceedings of the National Academy of Sciences of the United States of America 102:11551–11556.

Cheng D, Hoogenraad CC, Rush J, Ramm E, Schlager MA, Duong DM, Xu P, Wijayawardana SR, Hanfelt J, Nakagawa T, Sheng M, Peng J (2006) Relative and absolute quantification of postsynaptic density proteome isolated from rat forebrain and cerebellum. Mol Cell Proteomics 5:1158–1170.

Choi KY, Chung S, Roche KW (2011) Differential binding of calmodulin to group I metabotropic glutamate receptors regulates receptor trafficking and signaling. Journal of Neuroscience 31:5921–5930.

Churn SB, Kochan LD, DeLorenzo RJ (2000) Chronic inhibition of Ca(2+)/calmodulin kinase II activity in the pilocarpine model of epilepsy. Brain Res 875:66–77.

Coesmans M, Weber JT, De Zeeuw CI, Hansel C (2004) Bidirectional parallel fiber plasticity in the cerebellum under climbing fiber control. Neuron 44:691–700.

Cohen SM, Li B, Tsien RW, Ma H (2015) Evolutionary and functional perspectives on signaling from neuronal surface to nucleus. Biochem Biophys Res Commun 460:88–99.

Cohen SM, Ma H, Kuchibhotla KV, Watson BO, Buzsáki G, Froemke RC, Tsien RW (2016) Excitation-Transcription Coupling in Parvalbumin-Positive Interneurons Employs a Novel CaM Kinase-Dependent Pathway Distinct from Excitatory Neurons. Neuron:1–17.

Colbran RJ, Brown AM (2004) Calcium/calmodulin-dependent protein kinase II and synaptic plasticity. Curr Opin Neurobiol 14:318–327.

Conlee JW, Shapiro SM, Churn SB (2000) 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.

Coultrap SJ, Bayer KU (2011) Improving a natural CaMKII inhibitor by random and rational design. PLoS ONE 6:e25245.

Coultrap SJ, Bayer KU (2012) CaMKII regulation in information processing and storage. Trends in Neurosciences 35:607–618.

Coultrap SJ, Buard I, Kulbe JR, Dell'Acqua ML, Bayer KU (2010) CaMKII Autonomy Is Substrate-dependent and Further Stimulated by Ca2+/Calmodulin. Journal of Biological Chemistry 285:17930–17937.

Coultrap SJ, Freund RK, O'Leary H, Sanderson JL, Roche KW, Dell'acqua ML, Bayer KU (2014) Autonomous CaMKII Mediates Both LTP and LTD Using a Mechanism for Differential Substrate Site Selection. Cell Reports 6:431–437.

de Ligt J, Willemsen MH, van Bon BWM, Kleefstra T, Yntema HG, Kroes T, Vulto-van Silfhout AT, Koolen DA, de Vries P, Gilissen C, del Rosario M, Hoischen A, Scheffer H, de Vries BBA, Brunner HG, Veltman JA, Vissers LELM (2012) Diagnostic exome sequencing in persons with severe intellectual disability. N Engl J Med 367:1921–1929.

DeLorenzo RJ, Freedman SD, Yohe WB, Maurer SC (1979) Stimulation of Ca2+-dependent neurotransmitter release and presynaptic nerve terminal protein phosphorylation by calmodulin and a calmodulin-like protein isolated from synaptic vesicles. Proceedings of the National Academy of Sciences of the United States of America 76:1838–1842.

Derkach V, Barria A, Soderling TR (1999) Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. Proceedings of the National Academy of Sciences of the United States of America 96:3269–3274.

Easton AC et al. (2013) αCaMKII Autophosphorylation Controls the Establishment of Alcohol Drinking Behavior. Neuropsychopharmacology 38:1636–1647.

Elgersma Y, Fedorov NB, Ikonen S, Choi ES, Elgersma M, Carvalho OM, Giese KP, Silva AJ (2002) Inhibitory autophosphorylation of CaMKII controls PSD association, plasticity, and learning. Neuron 36:493–505.

Enslen H, Sun P, Brickey D, Soderling SH, Klamo E, Soderling TR (1994) Characterization of Ca2+/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. J Biol Chem 269:15520–15527.

Erondu NE, Kennedy MB (1985) Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain. J Neurosci 5:3270–3277.

Fan Y, Fricker D, Brager DH, Chen X, Lu H-C, Chitwood RA, Johnston D (2005) Activity-dependent decrease of excitability in rat hippocampal neurons through increases in I(h). Nat Neurosci 8:1542–1551.

Feng B, Raghavachari S, Lisman J (2011) Quantitative estimates of the cytoplasmic, PSD,and NMDAR-bound pools of CaMKII in dendritic spines. Brain Res 1419:46–52.

Fink CC, Bayer K-U, Myers JW, Ferrell JE, Schulman H, Meyer T (2003) Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CaMKII. Neuron 39:283–297.

Flores-Méndez MA, Martinez-Lozada Z, Monroy HC, Hernández-Kelly LC, Barrera I, Ortega A (2013) Glutamate-dependent translational control in cultured Bergmann glia cells: eIF2α phosphorylation. Neurochem Res 38:1324–1332.

Frey U, Morris RG (1997) Synaptic tagging and long-term potentiation. Nature 385:533–536.

Fuchsova B, Alvarez Juliá A, Rizavi HS, Frasch AC, Pandey GN (2015) Altered expression of neuroplasticity-related genes in the brain of depressed suicides. Neuroscience 299:1–17.

Gao L, Blair LAC, Marshall J (2006) CaMKII-independent effects of KN93 and its inactive analog KN92: reversible inhibition of L-type calcium channels. Biochem Biophys Res Commun 345:1606–1610.

Gargett CE, Wiley JS (1997) The isoquinoline derivative KN-62 a potent antagonist of the P2Z-receptor of human lymphocytes. Br J Pharmacol 120:1483–1490 Available at: http://onlinelibrary.wiley.com/doi/10.1038/sj.bjp.0701081/epdf.

Gaudillière B, Konishi Y, la Iglesia de N, Yao GL, Bonni A (2004) A CaMKII-NeuroD signaling pathway specifies dendritic morphogenesis. Neuron 41:229–241.

Ghiretti AE, Kenny K, Marr MT, Paradis S (2013) CaMKII-Dependent Phosphorylation of the GTPase Rem2 Is Required to Restrict Dendritic Complexity. Journal of Neuroscience 33:6504–6515.

Ghosh A, Giese KP (2015) Calcium/calmodulin-dependent kinase II and Alzheimer's disease. Mol Brain 8:78.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ (1998) Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. Science 279:870–873.

Glazewski S, Chen CM, Silva A, Fox K (1996) Requirement for alpha-CaMKII in experience-dependent plasticity of the barrel cortex. Science 272:421–423.

Glazewski S, Giese KP, Silva A, Fox K (2000) The role of alpha-CaMKII autophosphorylation in neocortical experience-dependent plasticity. Nat Neurosci 3:911–918.

Gomez-Monterrey I, Sala M, Rusciano MR, Monaco S, Maione AS, Iaccarino G, Tortorella P, D'Ursi AM, Scrima M, Carotenuto A, De Rosa G, Bertamino A, Vernieri E, Grieco P, Novellino E, Illario M, Campiglia P (2013) Characterization of a selective CaMKII peptide inhibitor. Eur J Med Chem 62:425–434.

Gordon J, Amini S, White MK (2013) General Overview of Neuronal Cell Culture. In: Methods in Molecular Biology, pp 1–8 Methods in Molecular Biology. Totowa, NJ: Humana Press.

Groth RD, Lindskog M, Thiagarajan TC, Li L, Tsien RW (2011) Beta Ca2+/CaM-dependent kinase type II triggers upregulation of GluA1 to coordinate adaptation to synaptic inactivity in hippocampal neurons. Proceedings of the National Academy of Sciences of the United States of America 108:828–833.

Grueter CE, Abiria SA, Wu Y, Anderson ME, Colbran RJ (2008) Differential regulated interactions of calcium/calmodulin-dependent protein kinase II with isoforms of voltage-gated calcium channel beta subunits. Biochemistry 47:1760–1767.

Guzowski JF, McNaughton BL, Barnes CA, Worley PF (1999) Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles. Nat Neurosci 2:1120–1124.

Hanley RM, Payne ME, Cruzalegui F, Christenson MA, Means AR (1989) Sequence of the cDNA for the alpha subunit of calmodulin kinase II from mouse brain. Nucleic Acids Res 17:3992.

Hansel C, de Jeu M, Belmeguenai A, Houtman SH, Buitendijk GHS, Andreev D, De Zeeuw CI, Elgersma Y (2006) alphaCaMKII Is essential for cerebellar LTD and motor learning. Neuron 51:835–843.

Hanson PI, Meyer T, Stryer L, Schulman H (1994) Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. Neuron 12:943–956.

Hardingham N, Glazewski S, Pakhotin P, Mizuno K, Chapman PF, Giese KP, Fox K (2003) Neocortical long-term potentiation and experience-dependent synaptic plasticity require alpha-calcium/calmodulin-dependent protein kinase II autophosphorylation. Journal of Neuroscience 23:4428–4436.

Hayashi Y, Majewska AK (2005) Dendritic spine geometry: functional implication and regulation. Neuron 46:529–532.

Hegyi B, Chen-Izu Y, Jian Z, Shimkunas R, Izu LT, Banyasz T (2015) KN-93 inhibits IKr in mammalian cardiomyocytes. Journal of Molecular and Cellular Cardiology 89:173–176.

Hell JW (2014) CaMKII: Claiming Center Stage in Postsynaptic Function and Organization. Neuron 81:249–265.

Hell JW, Appleyard SM, Yokoyama CT, Warner C, Catterall WA (1994) Differential phosphorylation of two size forms of the N-type calcium channel alpha 1 subunit which have different COOH termini. J Biol Chem 269:7390–7396.

Hinds HL, Goussakov I, Nakazawa K, Tonegawa S, Bolshakov VY (2003) Essential function of alpha-calcium/calmodulin-dependent protein kinase II in neurotransmitter release at a glutamatergic central synapse. Proceedings of the National Academy of Sciences of the United States of America 100:4275–4280.

Hinds HL, Tonegawa S, Malinow R (1998) CA1 long-term potentiation is diminished but present in hippocampal slices from alpha-CaMKII mutant mice. Learn Mem 5:344–354.

Hojjati MR, Van Woerden GM, Tyler WJ, Giese KP, Silva AJ, Pozzo-Miller L, Elgersma Y (2007) Kinase activity is not required for alphaCaMKII-dependent presynaptic plasticity at CA3-CA1 synapses. Nat Neurosci 10:1125–1127.

Hosokawa T, Mitsushima D, Kaneko R, Hayashi Y (2015) Stoichiometry and phosphoisotypes of hippocampal AMPA-type glutamate receptor phosphorylation. Neuron 85:60–67.

Houston CM, He Q, Smart TG (2009) CaMKII phosphorylation of the GABA(A) receptor: receptor subtype- and synapse-specific modulation. The Journal of Physiology 587:2115–2125.

Houston CM, Hosie AM, Smart TG (2008) Distinct regulation of beta2 and beta3 subunit-containing cerebellar synaptic GABAA receptors by calcium/calmodulin-dependent protein kinase II. Journal of Neuroscience 28:7574–7584.

Hudmon A, Schulman H (2002) Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. Biochem J 364:593–611.

Hudmon A, Schulman H, Kim J, Maltez JM, Tsien RW, Pitt GS (2005) CaMKII tethers to L-type Ca2+ channels, establishing a local and dedicated integrator of Ca2+ signals for facilitation. The Journal of Cell Biology 171:537–547.

Hund TJ, Koval OM, Li J, Wright PJ, Qian L, Snyder JS, Gudmundsson H, Kline CF, Davidson NP, Cardona N, Rasband MN, Anderson ME, Mohler PJ (2010) A βIV-spectrin/CaMKII signaling complex is essential for membrane excitability in mice. J Clin Invest 120:3508–3519.

Iossifov I et al. (2014) The contribution of de novo coding mutations to autism spectrum disorder. Nature:1–17.

Ishida A, Kameshita I, Okuno S, Kitani T, Fujisawa H (1995) A novel highly specific and potent inhibitor of calmodulin-dependent protein kinase II. Biochem Biophys Res Commun 212:806–812.

Ishii A, Kiuchi K, Kobayashi R, Sumi M, Hidaka H, Nagatsu T (1991) A selective Ca2+/calmodulin-dependent protein kinase II inhibitor, KN-62, inhibits the enhanced phosphorylation and the activation of tyrosine hydroxylase by 56 mM K+ in rat pheochromocytoma PC12h cells. Biochem Biophys Res Commun 176:1051–1056.

Jenkins MA, Christel CJ, Jiao Y, Abiria S, Kim KY, Usachev YM, Obermair GJ, Colbran RJ, Lee A (2010) Ca2+-dependent facilitation of Cav1.3 Ca2+ channels by densin and Ca2+/calmodulin-dependent protein kinase II. Journal of Neuroscience 30:5125–5135.

Jiang X, Lautermilch NJ, Watari H, Westenbroek RE, Scheuer T, Catterall WA (2008) Modulation of CaV2.1 channels by Ca2+/calmodulin-dependent protein kinase II bound to the C-terminal domain. Proceedings of the National Academy of Sciences of the United States of America 105:341–346.

Jiao Y, Jalan-Sakrikar N, Robison AJ, Baucum AJ, Bass MA, Colbran RJ (2011) Characterization of a central Ca2+/calmodulin-dependent protein kinase IIalpha/beta binding domain in densin that selectively modulates glutamate receptor subunit phosphorylation. J Biol Chem 286:24806–24818.

Jin D-Z, Guo M-L, Xue B, Mao L-M, Wang JQ (2013a) Differential regulation of CaMKIIα interactions with mGluR5 and NMDA receptors by Ca(2+) in neurons. J Neurochem 127:620–631.

Jin DZ, Guo ML, Xue B, Fibuch EE, Choe ES, Mao LM, Wang JQ (2013b) Phosphorylation and Feedback Regulation of Metabotropic Glutamate Receptor 1 by Calcium/Calmodulin-Dependent Protein Kinase II. Journal of Neuroscience 33:3402–3412.

Jones EG, Huntley GW, Benson DL (1994) Alpha calcium/calmodulin-dependent protein kinase II selectively expressed in a subpopulation of excitatory neurons in monkey sensory-motor cortex: comparison with GAD-67 expression. J Neurosci 14:611–629.

Kakegawa W, Yuzaki M (2005) A mechanism underlying AMPA receptor trafficking during cerebellar long-term potentiation. Proceedings of the National Academy of Sciences of the United States of America 102:17846–17851.

Kennedy MB (1997) The postsynaptic density at glutamatergic synapses. Trends in Neurosciences 20:264–268.

Kennedy MB, Bennett MK, Erondu NE (1983) Biochemical and immunochemical evidence that the "major postsynaptic density protein" is a subunit of a calmodulin-dependent protein kinase. Proceedings of the National Academy of Sciences of the United States of America 80:7357–7361.

Kennedy MB, Greengard P (1981) Two calcium/calmodulin-dependent protein kinases, which are highly concentrated in brain, phosphorylate protein I at distinct sites. Proceedings of the National Academy of Sciences of the United States of America 78:1293–1297.

Khan S, Conte I, Carter T, Bayer KU, Molloy JE (2016) Multiple CaMKII Binding Modes to the Actin Cytoskeleton Revealed by Single-Molecule Imaging. Biophys J 111:395–408.

Kim K, Lakhanpal G, Lu HE, Khan M, Suzuki A, Kato-Hayashi M, Narayanan R, Luyben TT, Matsuda T, Nagai T, Blanpied TA, Hayashi Y, Okamoto K (2015) A Temporary Gating of Actin Remodeling during Synaptic Plasticity Consists of the Interplay between the Kinase and Structural Functions of CaMKII. Neuron 87:813–826.

Kim K, Saneyoshi T, Hosokawa T, Okamoto K, Hayashi Y (2016) Interplay of enzymatic and structural functions of CaMKII in long-term potentiation. J Neurochem 139:959–972.

Klausberger T, Somogyi P (2008) Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science 321:53–57.

Kline CF, Wright PJ, Koval OM, Zmuda EJ, Johnson BL, Anderson ME, Hai T, Hund TJ, Mohler PJ (2013) βIV-Spectrin and CaMKII facilitate Kir6.2 regulation in pancreatic beta cells. Proceedings of the National Academy of Sciences of the United States of America 110:17576–17581.

Klug JR, Mathur BN, Kash TL, Wang H-D, Matthews RT, Robison AJ, Anderson ME, Deutch AY, Lovinger DM, Colbran RJ, Winder DG (2012) Genetic inhibition of CaMKII in dorsal striatal medium spiny neurons reduces functional excitatory synapses and enhances intrinsic excitability. PLoS ONE 7:e45323.

Koval OM, Guan X, Wu Y, Joiner M-L, Gao Z, Chen B, Grumbach IM, Luczak ED, Colbran RJ, Song L-S, Hund TJ, Mohler PJ, Anderson ME (2010) CaV1.2 beta-subunit coordinates CaMKII-triggered cardiomyocyte death and afterdepolarizations. Proceedings of the National Academy of Sciences of the United States of America 107:4996–5000.

Krasko A, Schröder HC, Perovic S, Steffen R, Kruse M, Reichert W, Müller IM, Müller WE (1999) Ethylene modulates gene expression in cells of the marine sponge Suberites domuncula and reduces the degree of apoptosis. J Biol Chem 274:31524–31530.

Kristensen AS, Jenkins MA, Banke TG, Schousboe A, Makino Y, Johnson RC, Huganir R, Traynelis SF (2011) Mechanism of Ca2+/calmodulin-dependent kinase II regulation of AMPA receptor gating. Nature Publishing Group 14:727–735.

Kullmann DM, Lamsa KP (2007) Long-term synaptic plasticity in hippocampal interneurons. Nat Rev Neurosci 8:687–699.

Kummer MP, Hammerschmidt T, Martinez A, Terwel D, Eichele G, Witten A, Figura S, Stoll M, Schwartz S, Pape H-C, Schultze JL, Weinshenker D, Heneka MT, Urban I (2014) Ear2 deletion causes early memory and learning deficits in APP/PS1 mice. Journal of Neuroscience 34:8845–8854.

Küry S et al. (2017) De Novo Mutations in Protein Kinase Genes CAMK2A and CAMK2B Cause Intellectual Disability. Am J Hum Genet 101:768–788.

Lamsa K, Heeroma JH, Kullmann DM (2005) Hebbian LTP in feed-forward inhibitory interneurons and the temporal fidelity of input discrimination. Nat Neurosci 8:916–924.

Lamsa K, Irvine EE, Giese KP, Kullmann DM (2007a) NMDA receptor-dependent long-term potentiation in mouse hippocampal interneurons shows a unique dependence on Ca2+/calmodulin-dependent kinases. The Journal of Physiology 584:885–894.

Lamsa KP, Heeroma JH, Somogyi P, Rusakov DA, Kullmann DM (2007b) Anti-Hebbian long-term potentiation in the hippocampal feedback inhibitory circuit. Science 315:1262–1266.

Lee H-K, Takamiya K, Han J-S, Man H, Kim C-H, Rumbaugh G, Yu S, Ding L, He C, Petralia RS, Wenthold RJ, Gallagher M, Huganir RL (2003) Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. Cell 112:631–643.

Lee H-K, Takamiya K, He K, Song L, Huganir RL (2010) Specific Roles of AMPA Receptor Subunit GluR1 (GluA1) Phosphorylation Sites in Regulating Synaptic Plasticity in the CA1 Region of Hippocampus. J Neurophysiol 103:479–489.

Leonard AS, Lim IA, Hemsworth DE, Horne MC, Hell JW (1999) Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor. Proceedings of the National Academy of Sciences of the United States of America 96:3239–3244.

Lev-Ram V, Wong ST, Storm DR, Tsien RY (2002) A new form of cerebellar long-term potentiation is postsynaptic and depends on nitric oxide but not cAMP. Proceedings of the National Academy of Sciences of the United States of America 99:8389–8393.

Li G, Liu N, Liu OL, Mower GD, Gregg RG, Cooper NG (1998) Molecular cloning and analysis of Ca2+/calmodulin-dependent protein kinase II from the chicken brain. J Mol Neurosci 11:135–139.

Li K, Zhou T, Liao L, Yang Z, Wong C, Henn F, Malinow R, Yates JR, Hu H (2013) βCaMKII in Lateral Habenula Mediates Core Symptoms of Depression. Science 341:1016–1020.

Lin JW, Sugimori M, Llinás RR, McGuinness TL, Greengard P (1990) Effects of synapsin I and calcium/calmodulin-dependent protein kinase II on spontaneous neurotransmitter release in the squid giant synapse. Proceedings of the National Academy of Sciences of the United States of America 87:8257–8261.

Lin Y-C, Redmond L (2008) CaMKIIbeta binding to stable F-actin in vivo regulates F-actin filament stability. Proceedings of the National Academy of Sciences of the United States of America 105:15791–15796.

Link W, Konietzko U, Kauselmann G, Krug M, Schwanke B, Frey U, Kuhl D (1995) Somatodendritic expression of an immediate early gene is regulated by synaptic activity. Proceedings of the National Academy of Sciences of the United States of America 92:5734–5738.

Lisman J, Schulman H, Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. Nat Rev Neurosci 3:175–190.

Lisman J, Yasuda R, Raghavachari S (2012) Mechanisms of CaMKII action in long-term potentiation. Nat Rev Neurosci 13:169–182.

Lisman JE, Zhabotinsky AM (2001) A model of synaptic memory: a CaMKII/PP1 switch that potentiates transmission by organizing an AMPA receptor anchoring assembly. Neuron 31:191–201.

Liu X-B, Murray KD (2012) Neuronal excitability and calcium/calmodulin-dependent protein kinase type II: location, location, location. Epilepsia 53 Suppl 1:45–52.

Liu X-Y, Mao L-M, Zhang G-C, Papasian CJ, Fibuch EE, Lan H-X, Zhou H-F, Xu M, Wang JQ (2009) Activity-dependent modulation of limbic dopamine D3 receptors by CaMKII. Neuron 61:425–438.

Liu XB, Jones EG (1996) Localization of alpha type II calcium calmodulin-dependent protein kinase at glutamatergic but not gamma-aminobutyric acid (GABAergic) synapses in thalamus and cerebral cortex. Proceedings of the National Academy of Sciences of the United States of America 93:7332–7336.

Lledo PM, Hjelmstad GO, Mukherji S, Soderling TR, Malenka RC, Nicoll RA (1995) Calcium/calmodulin-dependent kinase II and long-term potentiation enhance synaptic transmission by the same mechanism. Proceedings of the National Academy of Sciences of the United States of America 92:11175–11179.

Llinás R, McGuinness TL, Leonard CS, Sugimori M, Greengard P (1985) Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. Proceedings of the National Academy of Sciences of the United States of America 82:3035–3039.

Lu F-M, Hawkins RD (2006) Presynaptic and postsynaptic Ca(2+) and CamKII contribute to long-term potentiation at synapses between individual CA3 neurons. Proceedings of the National Academy of Sciences of the United States of America 103:4264–4269.

Lu W, Isozaki K, Roche KW, Nicoll RA (2010) Synaptic targeting of AMPA receptors is regulated by a CaMKII site in the first intracellular loop of GluA1. Proceedings of the National Academy of Sciences of the United States of America 107:22266–22271.

Lyford GL, Yamagata K, Kaufmann WE, Barnes CA, Sanders LK, Copeland NG, Gilbert DJ, Jenkins NA, Lanahan AA, Worley PF (1995) Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. Neuron 14:433–445.

Ma H, Groth RD, Cohen SM, Emery JF, Li B, Hoedt E, Zhang G, Neubert TA, Tsien RW (2014) γCaMKII Shuttles Ca(2+)/CaM to the Nucleus to Trigger CREB Phosphorylation and Gene Expression. Cell 159:281–294.

Maier LS (2011) CaMKII regulation of voltage-gated sodium channels and cell excitability. Heart Rhythm 8:474–477.

Mammen AL, Kameyama K, Roche KW, Huganir RL (1997) Phosphorylation of the alpha-amino-3-hydroxy-5-methylisoxazole4-propionic acid receptor GluR1 subunit by calcium/calmodulin-dependent kinase II. J Biol Chem 272:32528–32533.

Mao L-M, Jin D-Z, Xue B, Chu X-P, Wang JQ (2014) Phosphorylation and regulation of glutamate receptors by CaMKII. Sheng Li Xue Bao 66:365–372.

Marley PD, Thomson KA (1996) The Ca++/calmodulin-dependent protein kinase II inhibitors KN62 and KN93, and their inactive analogues KN04 and KN92, inhibit nicotinic activation of tyrosine hydroxylase in bovine chromaffin cells. Biochem Biophys Res Commun 221:15–18.

Martinez-Lozada Z, Waggener CT, Kim K, Zou S, Knapp PE, Hayashi Y, Ortega A, Fuss B (2014) Activation of sodium-dependent glutamate transporters regulates the morphological aspects of oligodendrocyte maturation via signaling through calcium/calmodulin-dependent kinase IIβ's actin-binding/-stabilizing domain. Glia 62:1543–1558.

Matta JA, Pelkey KA, Craig MT, Chittajallu R, Jeffries BW, McBain CJ (2013) Developmental origin dictates interneuron AMPA and NMDA receptor subunit composition and plasticity. Nature Publishing Group 16:1032–1041.

Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, Kandel ER (1996a) Control of memory formation through regulated expression of a CaMKII transgene. Science 274:1678–1683.

Mayford M, Baranes D, Podsypanina K, Kandel ER (1996b) The 3'-untranslated region of CaMKII alpha is a cis-acting signal for the localization and translation of mRNA in dendrites. Proceedings of the National Academy of Sciences of the United States of America 93:13250–13255.

McDonald AJ, Muller JF, Mascagni F (2002) GABAergic innervation of alpha type II calcium/calmodulin-dependent protein kinase immunoreactive pyramidal neurons in the rat basolateral amygdala. J Comp Neurol 446:199–218.

McLysaght A, Hokamp K, Wolfe KH (2002) Extensive genomic duplication during early chordate evolution. Nat Genet 31:200–204.

McNamara JO, Huang YZ, Leonard AS (2006) Molecular signaling mechanisms underlying epileptogenesis. Sci STKE 2006:re12.

Merrill MA, Chen Y, Strack S, Hell JW (2005) Activity-driven postsynaptic translocation of CaMKII. Trends Pharmacol Sci 26:645–653.

Miller SG, Kennedy MB (1985) 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.

Miller SG, Kennedy MB (1986) Regulation of brain type II Ca2+/calmodulin-dependent protein kinase by autophosphorylation: a Ca2+-triggered molecular switch. Cell 44:861–870.

Mochizuki H, Ito T, Hidaka H (1993) Purification and characterization of Ca2+/calmodulin-dependent protein kinase V from rat cerebrum. J Biol Chem 268:9143–9147.

Mockett BG, Guevremont D, Wutte M, Hulme SR, Williams JM, Abraham WC (2011) Calcium/Calmodulin-Dependent Protein Kinase II Mediates Group I Metabotropic Glutamate Receptor-Dependent Protein Synthesis and Long-Term Depression in Rat Hippocampus. Journal of Neuroscience 31:7380–7391.

Nelson AB, Gittis AH, Lac du S (2005) Decreases in CaMKII Activity Trigger Persistent Potentiation of Intrinsic Excitability in Spontaneously Firing Vestibular Nucleus Neurons. Neuron 46:623–631.

Nichols RA, Sihra TS, Czernik AJ, Nairn AC, Greengard P (1990) Calcium/calmodulin-dependent protein kinase II increases glutamate and noradrenaline release from synaptosomes. Nature 343:647–651.

Ninan I, Arancio O (2004) Presynaptic CaMKII is necessary for synaptic plasticity in cultured hippocampal neurons. Neuron 42:129–141.

O'Leary H, Lasda E, Bayer KU (2006) CaMKIIbeta association with the actin cytoskeleton is regulated by alternative splicing. Mol Biol Cell 17:4656–4665.

Ohsako S, Nishida Y, Ryo H, Yamauchi T (1993) 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.

Okamoto K-I, Nagai T, Miyawaki A, Hayashi Y (2004) Rapid and persistent modulation of actin dynamics regulates postsynaptic reorganization underlying bidirectional plasticity. Nat Neurosci 7:1104–1112.

Okamoto K-I, Narayanan R, Lee SH, Murata K, Hayashi Y (2007) The role of CaMKII as an F-actin-bundling protein crucial for maintenance of dendritic spine structure. Proceedings of the National Academy of Sciences of the United States of America 104:6418–6423.

Okuno H, Akashi K, Ishii Y, Yagishita-Kyo N, Suzuki K, Nonaka M, Kawashima T, Fujii H, Takemoto-Kimura S, Abe M, Natsume R, Chowdhury S, Sakimura K, Worley PF, Bito H (2012) Inverse Synaptic Tagging of Inactive Synapses via Dynamic Interaction of Arc/Arg3.1 with CaMKII&beta. Cell 149:886–898.

Okuno H, Minatohara K, Bito H (2017) ARTICLE IN PRESS. Seminars in Cell and Developmental Biology:1–8.

Opazo P, Labrecque S, Tigaret CM, Frouin A, Wiseman PW, De Koninck P, Choquet D (2010) CaMKII triggers the diffusional trapping of surface AMPARs through phosphorylation of stargazin. Neuron 67:239–252.

Opazo P, Sainlos M, Choquet D (2012) Regulation of AMPA receptor surface diffusion by PSD-95 slots. Curr Opin Neurobiol 22:453–460.

Pang ZP, Cao P, Xu W, Sudhof TC (2010) Calmodulin Controls Synaptic Strength via Presynaptic Activation of Calmodulin Kinase II. Journal of Neuroscience 30:4132–4142.

Patel R, Holt M, Philipova R, Moss S, Schulman H, Hidaka H, Whitaker M (1999) Calcium/calmodulin-dependent phosphorylation and activation of human Cdc25-C at the G2/M phase transition in HeLa cells. J Biol Chem 274:7958–7968.

Patton BL, Miller SG, Kennedy MB (1990) 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.

Pellicena P, Schulman H (2014) CaMKII inhibitors: from research tools to therapeutic agents. Front Pharmacol 5:21.

Perez Y, Morin F, Lacaille JC (2001) A hebbian form of long-term potentiation dependent on mGluR1a in hippocampal inhibitory interneurons. Proceedings of the National Academy of Sciences of the United States of America 98:9401–9406.

Pi HJ, Otmakhov N, Lemelin D, De Koninck P, Lisman J (2010) Autonomous CaMKII can promote either long-term potentiation or long-term depression, depending on the state of T305/T306 phosphorylation. Journal of Neuroscience 30:8704–8709.

Picconi B, Gardoni F, Centonze D, Mauceri D, Cenci MA, Bernardi G, Calabresi P, Di Luca M (2004) Abnormal Ca2+-calmodulin-dependent protein kinase II function mediates synaptic and motor deficits in experimental parkinsonism. Journal of Neuroscience 24:5283–5291.

Puram SV, Kim AH, Ikeuchi Y, Wilson-Grady JT, Merdes A, Gygi SP, Bonni A (2011) A CaMKIIb signaling pathway at the centrosome regulates dendrite patterning in the brain. Nature Publishing Group 14:973–983.

Ramachandran B, Frey JU (2009) Interfering with the actin network and its effect on long-term potentiation and synaptic tagging in hippocampal CA1 neurons in slices in vitro. Journal of Neuroscience 29:12167–12173.

Ramírez-Amaya V, Vazdarjanova A, Mikhael D, Rosi S, Worley PF, Barnes CA (2005) Spatial exploration-induced Arc mRNA and protein expression: evidence for selective, network-specific reactivation. Journal of Neuroscience 25:1761–1768.

Reiner DJ, Newton EM, Tian H, Thomas JH (1999) Diverse behavioural defects caused by mutations in Caenorhabditis elegans unc-43 CaM kinase II. Nature 402:199–203.

Respress JL, van Oort RJ, Li N, Rolim N, Dixit SS, deAlmeida A, Voigt N, Lawrence WS, Skapura DG, Skårdal K, Wisløff U, Wieland T, Ai X, Pogwizd SM, Dobrev D, Wehrens XHT (2012) Role of RyR2 phosphorylation at S2814 during heart failure progression. Circulation Research 110:1474–1483.

Robison AJ, Bartlett RK, Bass MA, Colbran RJ (2005) Differential modulation of Ca2+/calmodulin-dependent protein kinase II activity by regulated interactions with N-methyl-D-aspartate receptor NR2B subunits and alpha-actinin. J Biol Chem 280:39316–39323.

Rochlitz H, Voigt A, Lankat-Buttgereit B, Göke B, Heimberg H, Nauck MA, Schiemann U, Schatz H, Pfeiffer AF (2000) Cloning and quantitative determination of the human Ca2+/calmodulin-dependent protein kinase II (CaMK II) isoforms in human beta cells. Diabetologia 43:465–473.

Rosenberg OS, Deindl S, Sung R-J, Nairn AC, Kuriyan J (2005) Structure of the autoinhibited kinase domain of CaMKII and SAXS analysis of the holoenzyme. Cell 123:849–860.

Rozario T, DeSimone DW (2010) The extracellular matrix in development and morphogenesis: A dynamic view. Dev Biol 341:126–140.

Ryan TJ, Grant SGN (2009) The origin and evolution of synapses. Nat Rev Neurosci 10:701–712.

Saito A, Miyajima K, Akatsuka J, Kondo H, Mashiko T, Kiuchi T, Ohashi K, Mizuno K (2013) CaMKIIβ-mediated LIM-kinase activation plays a crucial role in BDNF-induced neuritogenesis. Genes Cells:n/a–n/a.

Sajikumar S, Navakkode S, Frey JU (2007) Identification of compartment- and process-specific molecules required for "synaptic tagging" during long-term potentiation and long-term depression in hippocampal CA1. Journal of Neuroscience 27:5068–5080.

Sametsky EA, Disterhoft JF, Ohno M (2009) Autophosphorylation of αCaMKII downregulates excitability of CA1 pyramidal neurons following synaptic stimulation. Neurobiology of Learning and Memory 92:120–123.

Sanabria H, Swulius MT, Kolodziej SJ, Liu J, Waxham MN (2009) {beta}CaMKII regulates actin assembly and structure. J Biol Chem 284:9770–9780.

Schnabel R, Palmer MJ, Kilpatrick IC, Collingridge GL (1999) A CaMKII inhibitor, KN-62, facilitates DHPG-induced LTD in the CA1 region of the hippocampus. Neuropharmacology 38:605–608.

Schöpf I, Easton AC, Solati J, Golub Y, Kornhuber J, Giese KP, Müller CP (2015) αCaMKII autophosphorylation mediates neuronal activation in the hippocampal dentate gyrus after alcohol and cocaine in mice. Neurosci Lett 591:65–68.

Shen K, Meyer T (1999) Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. Science 284:162–166.

Shen K, Teruel MN, Subramanian K, Meyer T (1998) CaMKIIbeta functions as an F-actin targeting module that localizes CaMKIIalpha/beta heterooligomers to dendritic spines. Neuron 21:593–606.

Shin SM, Zhang N, Hansen J, Gerges NZ, Pak DTS, Sheng M, Lee SH (2012) GKAP orchestrates activity-dependent postsynaptic protein remodeling and homeostatic scaling. Nature Publishing Group:1–13.

Silva AJ, Paylor R, Wehner JM, Tonegawa S (1992a) Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science 257:206–211.

Silva AJ, Stevens CF, Tonegawa S, Wang Y (1992b) Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science 257:201–206.

Sík A, Hájos N, Gulácsi A, Mody I, Freund TF (1998) The absence of a major Ca2+ signaling pathway in GABAergic neurons of the hippocampus. Proceedings of the National Academy of Sciences of the United States of America 95:3245–3250.

Smith MK, Colbran RJ, Brickey DA, Soderling TR (1992) Functional determinants in the autoinhibitory domain of calcium/calmodulin-dependent protein kinase II. Role of His282 and multiple basic residues. J Biol Chem 267:1761–1768.

Star EN, Kwiatkowski DJ, Murthy VN (2002) Rapid turnover of actin in dendritic spines and its regulation by activity. Nat Neurosci 5:239–246.

Stephenson JR, Wang X, Perfitt TL, Parrish WP, Shonesy BC, Marks CR, Mortlock DP, Nakagawa T, Sutcliffe JS, Colbran RJ (2017) A Novel Human CAMK2A Mutation Disrupts Dendritic Morphology and Synaptic Transmission, and Causes ASD-Related Behaviors. Journal of Neuroscience 37:2216–2233.

Stevens CF, Tonegawa S, Wang Y (1994) The role of calcium-calmodulin kinase II in three forms of synaptic plasticity. Curr Biol 4:687–693.

Stevens I, Rondelez E, Merlevede W, Goris J (2001) Cloning and differential expression of new calcium, calmodulin-dependent protein kinase II isoforms in Xenopus laevis oocytes and several adult tissues. J Biochem 129:551–560.

Strack S, Colbran RJ (1998) 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.

Stratton M, Lee I-H, Bhattacharyya M, Christensen SM, Chao LH, Schulman H, Groves JT, Kuriyan J (2013) Activation-triggered subunit exchange between CaMKII holoenzymes facilitates the spread of kinase activity. eLife 3:e01610.

Sugawara T, Hisatsune C, Miyamoto H, Ogawa N, Mikoshiba K (2017) Regulation of spinogenesis in mature Purkinje cells via mGluR/PKC-mediated phosphorylation of CaMKIIβ. Proceedings of the National Academy of Sciences of the United States of America 29:201617270.

Sumi M, Kiuchi K, Ishikawa T, Ishii A, Hagiwara M, Nagatsu T, Hidaka H (1991) 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.

Thiagarajan TC, Piedras-Renteria ES, Tsien RW (2002) alpha- and betaCaMKII. Inverse regulation by neuronal activity and opposing effects on synaptic strength. Neuron 36:1103–1114.

Tobimatsu T, Fujisawa H (1989) Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. J Biol Chem 264:17907–17912.

Tokumitsu H, Chijiwa T, Hagiwara M, Mizutani A, Terasawa M, Hidaka H (1990) KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazi ne, a specific inhibitor of Ca2+/calmodulin-dependent protein kinase II. J Biol Chem 265:4315–4320.

Tombes RM, Faison MO, Turbeville JM (2003) Organization and evolution of multifunctional Ca2+/CaM-dependent protein kinase genes. Gene 322:17–31.

Tombes RM, Grant S, Westin EH, Krystal G (1995) G1 cell cycle arrest and apoptosis are induced in NIH 3T3 cells by KN-93, an inhibitor of CaMK-II (the multifunctional Ca2+/CaM kinase). Cell Growth & Differentiation 6:1063.

Tomita S, Stein V, Stocker TJ, Nicoll RA, Bredt DS (2005) Bidirectional Synaptic Plasticity Regulated by Phosphorylation of Stargazin-like TARPs. Neuron 45:269–277.

Vallano ML (1989) Separation of isozymic forms of type II calcium/calmodulin-dependent protein kinase using cation-exchange chromatography. J Neurosci Methods 30:1–9.

Van Woerden GM, Harris KD, Hojjati MR, Gustin RM, Qiu S, De Avila Freire R, Jiang Y-H, Elgersma Y, Weeber EJ (2007) Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of alphaCaMKII inhibitory phosphorylation. Nat Neurosci 10:280–282.

Van Woerden GM, Hoebeek FE, Gao Z, Nagaraja RY, Hoogenraad CC, Kushner SA, Hansel C, De Zeeuw CI, Elgersma Y (2009) betaCaMKII controls the direction of plasticity at parallel fiber-Purkinje cell synapses. Nature Publishing Group 12:823–825.

Varga AW, Yuan L-L, Anderson AE, Schrader LA, Wu G-Y, Gatchel JR, Johnston D, Sweatt JD (2004) Calcium-calmodulin-dependent kinase II modulates Kv4.2 channel expression and upregulates neuronal A-type potassium currents. Journal of Neuroscience 24:3643–3654.

Vest RS, Davies KD, O'Leary H, Port JD, Bayer KU (2007) Dual mechanism of a natural CaMKII inhibitor. Mol Biol Cell 18:5024–5033.

Vincent M, Collet C, Verloes A, Lambert L, Herlin C, Blanchet C, Sanchez E, Drunat S, Vigneron J, Laplanche J-L, Puechberty J, Sarda P, Geneviève D (2014) Large deletions encompassing the TCOF1 and CAMK2A genes are responsible for Treacher Collins syndrome with intellectual disability. Eur J Hum Genet 22:52–56.

Waggener CT, Dupree JL, Elgersma Y, Fuss B (2013) CaMKIIβ Regulates Oligodendrocyte Maturation and CNS Myelination. Journal of Neuroscience 33:10453–10458.

Walaas SI, Lai Y, Gorelick FS, DeCamilli P, Moretti M, Greengard P (1988) 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.

Wang JH, Kelly P (2001) Calcium-calmodulin signalling pathway up-regulates glutamatergic synaptic function in non-pyramidal, fast spiking rat hippocampal CA1 neurons. The Journal of Physiology 533:407–422.

Wang X, Marks CR, Perfitt TL, Nakagawa T, Lee A, Jacobson DA, Colbran RJ (2017) A novel mechanism for Ca 2+/calmodulin-dependent protein kinase II targeting to L-type Ca 2+channels that initiates long-range signaling to the nucleus. Journal of Biological Chemistry:jbc.M117.788331.

Wang Z, Wilson GF, Griffith LC (2002) Calcium/calmodulin-dependent protein kinase II phosphorylates and regulates the Drosophila eag potassium channel. J Biol Chem 277:24022–24029.

Weeber EJ, Jiang Y-H, Elgersma Y, Varga AW, Carrasquillo Y, Brown SE, Christian JM, Mirnikjoo B, Silva A, Beaudet AL, Sweatt JD (2003) Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. Journal of Neuroscience 23:2634–2644.

Wei J, Zhang M, Zhu Y, Wang JH (2004) Ca2+–calmodulin signalling pathway up-regulates GABA synaptic transmission through cytoskeleton-mediated mechanisms. Neuroscience 127:637–647.

Welsby PJ, Wang H, Wolfe JT, Colbran RJ, Johnson ML, Barrett PQ (2003) A mechanism for the direct regulation of T-type calcium channels by Ca2+/calmodulin-dependent kinase II. Journal of Neuroscience 23:10116–10121.

Wu Y, Gao Z, Chen B, Koval OM, Singh MV, Guan X, Hund TJ, Kutschke W, Sarma S, Grumbach IM, Wehrens XHT, Mohler PJ, Song L-S, Anderson ME (2009) Calmodulin kinase II is required for fight or flight sinoatrial node physiology. Proceedings of the National Academy of Sciences of the United States of America 106:5972–5977.

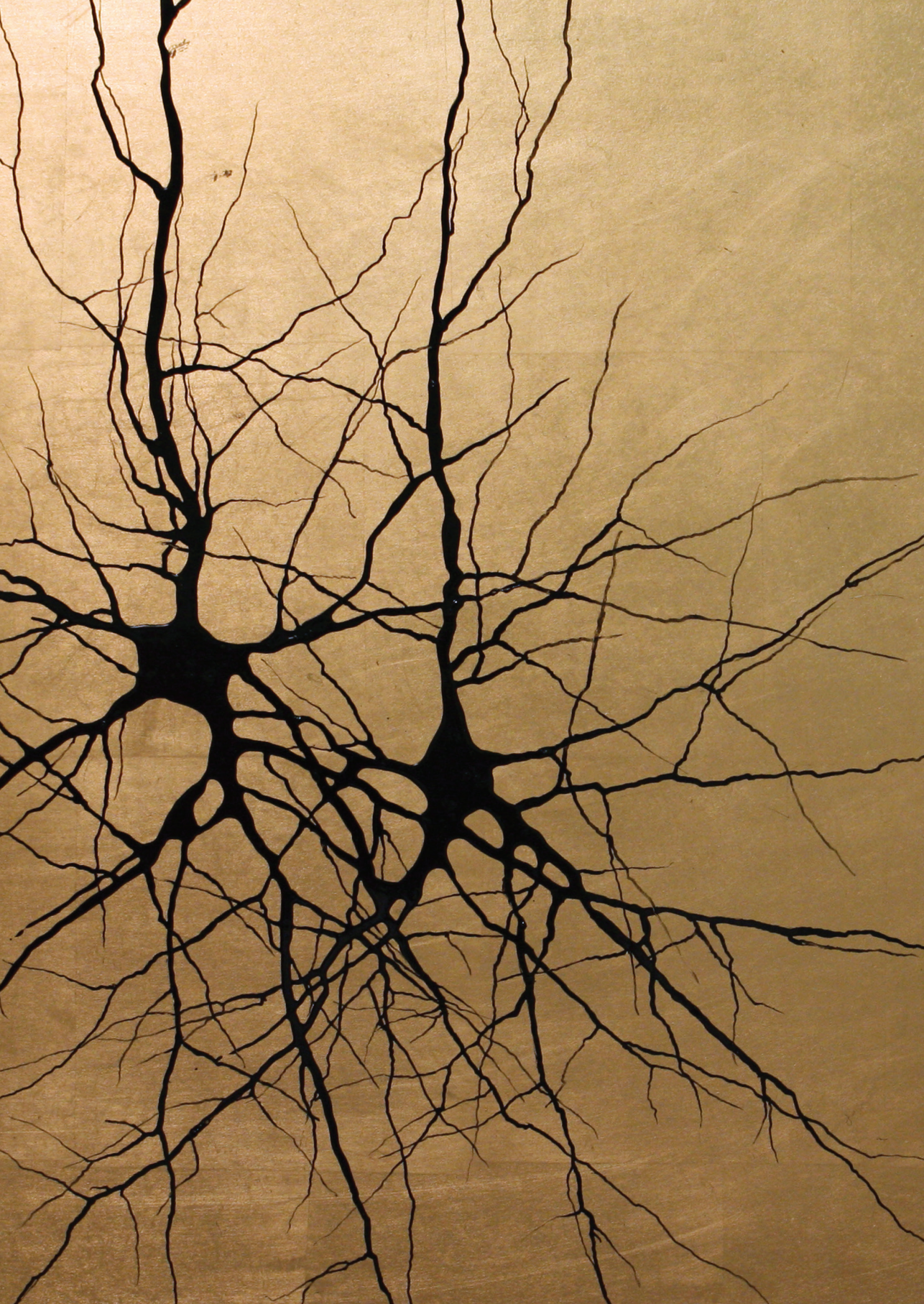
Xu J, Kang N, Jiang L, Nedergaard M, Kang J (2005) Activity-dependent long-term potentiation of intrinsic excitability in hippocampal CA1 pyramidal neurons. Journal of Neuroscience 25:1750–1760.

Yamagata Y, Imoto K, Obata K (2006) 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.

Yokoyama CT, Myers SJ, Fu J, Mockus SM, Scheuer T, Catterall WA (2005) Mechanism of SNARE protein binding and regulation of Cav2 channels by phosphorylation of the synaptic protein interaction site. Molecular and Cellular Neuroscience 28:1–17.

Zheng J, Redmond L, Xu C, Kuang J, Liao W (2014) Alternative splicing in the variable domain of CaMKIIβ affects the level of F-actin association in developing neurons. Int J Clin Exp Pathol 7:2963–2975.

Zou D-J, Greer CA, Firestein S (2002) Expression pattern of alpha CaMKII in the mouse main olfactory bulb. J Comp Neurol 443:226–236.



Chapter 2

Temporal and region-specific requirements of αCaMKII in spatial and contextual learning

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ABSTRACT

The alpha isoform of the calcium/calmodulin-dependent protein kinase II (αCaMKII) has been extensively implicated in molecular and cellular mechanisms underlying spatial and contextual learning in a wide variety of species. Germline deletion of *Camk2a* leads to severe deficits in spatial and contextual learning in mice. However, the temporal and region-specific requirements for αCaMKII have remained largely unexplored. Here, we generated conditional *Camk2a* mutants to examine the influence of spatially-restricted and temporally-controlled expression of αCaMKII. Forebrain-specific deletion of the *Camk2a* gene resulted in severe deficits in water maze and contextual fear learning, while mice with deletion restricted to the cerebellum learned normally. Furthermore, we found that temporally-controlled deletion of the *Camk2a* gene in adult mice is as detrimental as germline deletion for learning and synaptic plasticity. Taken together, we confirm the requirement for αCaMKII in the forebrain, but not the cerebellum, in spatial and contextual learning. Moreover, we highlight the absolute requirement for intact αCaMKII expression at the time of learning.

INTRODUCTION

Mouse genetic studies with targeted mutations of the *Camk2a* gene have greatly contributed to establishing the causal link between synaptic plasticity and learning (Silva et al., 1992a; Silva et al., 1992b; Giese et al., 1998; Elgersma et al., 2002). However, the interpretation of results using germline knockout mice requires consideration of possible neurodevelopmental influences on the phenotype of adult mice, given the absence of gene expression not only at the time of learning but also throughout neural development. Perhaps most importantly for interpreting the extensive literature utilizing germline *Camk2a* knockout mice, it remains unclear how the absence of αCaMKII during the early postnatal period might adversely influence brain development, thereby leading to the observed deficits in spatial and contextual learning. In contrast, most current models of αCaMKII function in synaptic plasticity and learning posit its requirement at the time of learning, independent of any developmental influences (for review see (Elgersma et al., 2004)). Therefore, distinguishing between the potential developmental influences of αCaMKII versus its necessity at the time of learning remains a highly important question.

Many distinct brain regions have robust αCaMKII expression, for which germline *Camk2a* knockout mice have not provided a definitive experimental model to dissociate region-specific influences. In the cortex and hippocampus, αCaMKII is exclusively found in glutamatergic pyramidal neurons (Benson et al., 1992; Jones et al., 1994; Sik et al., 1998), whereas in the striatum it is localized to GABAergic medium-spiny neurons (Benson et al., 1992; Takeuchi et al., 2002). Additionally, αCaMKII is expressed in cerebellar Purkinje cells, where it is required for intact parallel fiber-Purkinje cell long-term depression (LTD) (Hansel et al., 2006), a form of plasticity suggested to contribute not only to motor learning, but also to spatial and contextual learning (Lalonde and Strazielle, 2003; Burguiere et al., 2005; Goddyn et al., 2006; Burguiere et al., 2010; Galliano et al., 2013). Therefore, examining the region-specific role of αCaMKII on learning offers unique insights into the functioning of this highly abundant synaptic protein, and more generally on the systems-level neurobiology of spatial memory.

In the current study, we engineered a conditional *Camk2a* allele in order to fully parameterize the contribution of αCaMKII to learning and memory, thereby permitting spatiotemporal control over deletion. We found that loss of αCaMKII in adulthood leads to similar deficits as observed in the global knockout, which lacks αCaMKII expression from conception. Moreover, we confirm the critical importance of αCaMKII in the cortex and hippocampus for intact spatial and contextual learning, while its expression in the cerebellum is dispensable. Taken together, the present study shows that in contrast to

cerebellar αCaMKII, the loss of forebrain αCaMKII has severe adverse effects on spatial learning in mice. Furthermore, deletion of *Camk2a* in adulthood is sufficient to fully recapitulate the learning impairments of global knockout mice. This finding confirms the critical necessity of forebrain αCaMKII expression at the time of learning.

MATERIALS AND METHODS

Animals

The following mice were used in this study: *Camk2a*$^{+/+}$ (WT), *Camk2a*^{flox} (homozygously floxed *Camk2a* mice), *Camk2a*$^{flox/L7\text{-}cre-}$ and *Camk2a*$^{flox/L7\text{-}cre+}$ (Purkinje cell-specific αCaMKII knockout), *Camk2a*$^{flox/Emx\text{-}cre-}$ and *Camk2a*$^{flox/Emx\text{-}cre+}$ (telencephalon-restricted αCaMKII knockout), *Camk2a*$^{flox/CreER-}$ *and Camk2a*$^{flox/CreER+}$ (global αCaMKII knockout with temporal control over gene deletion), and germline αCaMKII knockout mice (*Camk2a*$^{-/-}$). All mice were backcrossed >10 times into the C57BL/6JOlaHsd background.

Mice were group housed and maintained under a 12h light/dark cycle (lights on at 07:00 A.M.), with *ad libitum* access to food and water. Behavioural testing was performed during the light phase. The experimenter remained blind to the genotype throughout the experiments and data analysis. All behavioural experiments were performed using littermates. Mice were between 2-5 months of age. Experimental group assignments were made in consideration of sex- and age-matching. All experiments were performed in accordance with the Dutch Animal Ethical Committee (DEC).

Generation of floxed Camk2a mice

Camk2a floxed mutant ES cells were generated as follows: a genomic clone of approximately 8 Kb encoding the *Camk2a* exon 2 (amino acids 22–53) was isolated by screening a mouse library. The targeting construct was made by inserting a PGK neomycin cassette flanked by LoxP sites into the EcoRI site approximately 1.2 Kbp downstream of exon 2. Another LoxP site was inserted into the SmaI site 60 bp upstream of exon 2. Targeted ES clones (61 out of 130) were identified by Southern blot analysis. PCR analysis revealed that 20 out of these 61 clones contained the additional 5′ LoxP site. The PGK neo cassette was removed by transient expression of Cre recombinase (pBS185, Gibco). Consequently, a mutant allele was created in which *Camk2a* exon 2 was flanked by LoxP sites (Fig. 1*A*).

L7-cre

Heterozygous floxed αCaMKII mice were crossed with L7/pcp-2 Cre transgenic mice (RRID:IMSR_JAX:004146) (Oberdick et al., 1990; Barski et al., 2000). Male *Camk2a*$^{flox/L7\text{-}}$

$^{cre+}$ mice from this F1 offspring were crossed with female *Camk2a*$^{flox/L7\text{-}cre-}$ mice to obtain the 2 experimental genotypes investigated: *Camk2a*$^{flox/L7\text{-}cre-}$ and *Camk2a*$^{flox/L7\text{-}cre+}$. L7 is expressed in cerebellar Purkinje cells *in utero*. Therefore, in *Camk2a*$^{flox/L7\text{-}cre+}$ mice, deletion of *Camk2a* is Purkinje-cell specific and occurs prior to the normal postnatal expression of αCaMKII (Smeyne et al., 1991).

Emx1-cre

Camk2a^{flox} mice were crossed with Emx1-Cre transgenic mice (RRID:IMSR_RBRC01345) (Iwasato et al., 2004). Male *Camk2a*$^{flox/Emx\text{-}cre+}$ mice from this F1 offspring were crossed with female *Camk2a*^{flox} mice. Male *Camk2a*$^{flox/Emx\text{-}cre+}$ mice from this F2 offspring were crossed again with female *Camk2a*^{flox} to obtain the 2 experimental genotypes: *Camk2a*$^{flox/Emx\text{-}cre-}$ and *Camk2a*$^{flox/Emx\text{-}cre+}$. Emx1 is expressed in glutamatergic telencephalic neurons *in utero* (Simeone et al., 1992). Therefore, in *Camk2a*$^{flox/Emx\text{-}cre+}$ mice, deletion of *Camk2a* is restricted to the telencephalon and occurs prior to the normal postnatal expression of αCaMKII.

CreER

Cre^{ER} mice were generated by engineering a fusion protein between cre recombinase and the ligand binding domain of a tamoxifen-responsive mutant human estrogen receptor, driven by the widespread CMV early enhancer/chicken beta actin (CAG) promoter (RRID:IMSR_JAX:004682) (Hayashi and McMahon, 2002). Only upon tamoxifen binding will the Cre^{ER} protein translocate into the nucleus and catalyse loxP recombination (Van Duyne, 2001). Therefore, tamoxifen administration in adulthood allows the normal endogenous expression of αCaMKII protein throughout development. For breeding of the experimental genotypes, male *Cre*$^{ER+}$ mice were crossed with female *Camk2a*^{flox} mice. The resulting F2 male *Camk2a*$^{flox/CreER+}$ mice were crossed with female *Camk2a*^{flox} mice to obtain the *Camk2a*$^{flox/CreER+}$ and *Camk2a*$^{flox/CreER-}$ genotypes.

Tamoxifen injections for CreER experiments

Adult deletion of *Camk2a* was achieved using 4 injections of tamoxifen. Tamoxifen (Sigma-Aldrich) was dissolved in sunflower oil in a dilution of 20 mg/ml. Injections were performed once daily for 4 consecutive days at a dose of 0.10 mg/g of bodyweight. Behavioural testing was initiated four weeks after the first day of tamoxifen injection (Hayashi and McMahon, 2002). Importantly, in order to control for any potential influence of tamoxifen itself, all mice (both *Camk2a*$^{flox/CreER+}$ and *Camk2a*$^{flox/CreER-}$) received tamoxifen injections. Notably however, previous studies have demonstrated that using this dosing regimen, the administration of tamoxifen has no discernible influence on emotional reactivity, neurological functioning, or learning (Vogt et al., 2008).

Morris water maze

All mice were handled for one minute daily beginning a week prior to the experiment. The water maze is a circular pool with a diameter of 1.2 meters, filled with an opaque mixture of water and white paint. Water temperature was maintained at 25-26 degrees Celsius. The escape platform had a diameter of 11 cm and was submerged 1 cm beneath the water surface. The swimming paths of the animals were captured by a closed-circuit video camera mounted directly above the centre of the pool and analysed using Smart version 2.0 (Panlab rib_00052). The room was illuminated by ceiling lamps set to a low level of light intensity. Visually-salient and readily-distinguishable distal cues were displayed on each wall of the room.

Mice were trained using two 60 s trials per day, with a 30 s inter-trial interval, for five consecutive days. During training, mice were placed on the platform for 30 s and subsequently placed into the pool at pseudo-random starting positions. After reaching the platform, the mice were allowed to remain for 30 s, before returning to their homecage. If a mouse was unable to locate the platform within 60 s, the trial was concluded and the mouse was gently placed on the platform by the experimenter, and remained there for 30 s. The platform location was in a fixed position throughout all trials. For each trial, latency, distance covered, mean swim speed and path were measured.

On day 5, a probe trial was performed one hour after the training, in which the platform was removed to assess spatial learning. During the probe trial, mice were placed in the pool at the opposite side of the platform position and were allowed to search for 60 s. The amount of time spent in each quadrant and platform location crossings were measured for each probe trial. For statistical analysis, the time in the target quadrant was compared to the average of the other three quadrants.

Pavlovian fear conditioning

Mice were placed in a steel chamber (30 x 20 cm) with a grid floor, and clear plastic door and ceiling (Med Associates). On day 1, each mouse was placed inside the conditioning chamber for 180 s. A single foot-shock (2 s, 0.4 mA) was delivered following a placement-to-shock interval of 148 s. Twenty-four hours later, mice were returned to the same context for 180 s. Behaviour during training and testing was recorded using a closed-circuit video camera mounted directly in front of the chambers. Freezing behaviour, defined as the cessation of all movement except respiration, was quantified using an automated video-based algorithm with detection parameters calibrated by independent manual scoring (Anagnostaras et al., 2010).

Western Blots

Mice were anaesthetised with isoflurane and decapitated. Cortex, hippocampus and cerebellum were rapidly dissected and frozen in liquid nitrogen. Tissue samples were homogenized using a dounce homogenizer in lysis buffer (0.1 M Tris-HCL, pH 6.8; 4% SDS) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein concentrations were determined using the BCA method (Pierce), adjusted to 1 mg/ml. 10 µg of protein was loaded per lane. The following antibodies were used: αCaMKII (1:10.000, Millipore Cat# MAB8699 RRID:AB_2067919), bCaMKII (1:10.000, Abcam Cat# ab34703 RRID:AB_2275072) and Actin (1:20000; EMD Millipore Cat# MAB1501R RRID:AB_94235). Samples were electrophoresed using 10% Bis-Tris gels (Bio-Rad) and blotted on nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% non-fat dry milk in Tris Buffered Saline (TBS) with 0.1% Tween-20 (TBST). Blots were incubated in primary antibody, diluted in TBST with 2% dry milk, followed by incubation in secondary antibody (Affinipure goat anti-mouse, 1:3.000, AB_2307348). The blots were incubated in chemiluminescence reagent (ECL, Pierce) exposed to film and analysed with ImageJ-64 software (NIH).

Immunohistochemistry

Mice were deeply anaesthetized with pentobarbital and perfused transcardially with a series of Phosphate Buffered Saline (PBS) followed by freshly prepared 4% paraformaldehyde (PFA, Sigma). Brains were carefully removed and fixed for two hours in 4% PFA, followed by 24 h incubation in 0.1 M phosphate buffer (PB) and 10% sucrose. Brains were then embedded in gelatine blocks (10% gelatine, 10% sucrose), post-fixed in 10% formaldehyde and 30% sucrose for 3 h, and kept in 30% sucrose overnight at 4°C. The embedded brains were sectioned using a freezing microtome with a section thickness of 40 µm. Sections were processed free floating, with diaminobenzidine (0.05%) as the chromogen.

Slices were rinsed, blocked in 3% H_2O_2 and PBS, rinsed again and kept at 80°C in 10 mM sodium citrate for 20 min. After rinsing, slices were preincubated for one hour at room temperature in 10% normal horse serum (NHS), 0.5% Triton and PBS and kept overnight at 4°C in 10% NHS, 0.5% Triton, PBS and AffiniPure Fab Fragment (donkey anti mouse, 1:200, Jackson, rid_000053). Subsequently, sections were kept in 10% NHS, 0.5% Triton, PBS and primary antibody (αCaMKII, 1:9000, Millipore Cat# MAB8699 RRID:AB_2067919) for 48 h at 4°C. After rinsing sections were kept for 2 h at room temperature in the secondary antibody (RAM-HRP, 1:200, AB_2307349), in 10% NHS, 0.5% Triton and PBS. Finally, slices were stained in 0.05 M PB, 0.05% diaminotenzidinetetrachloride (DAB) and

3% H202 for 2 min. Slices were then mounted on slides with chrome(III)potassiumsulfate-dodecahydrate, dehydrated in alcohol (70%, 90%, 100%), cleared with xylene and cover slipped with Permount (Fisher Scientific).

Electrophysiology

Adult mice (15-20 weeks old) were anesthetized with isoflurane and decapitated after which the brain was taken out quickly and submerged in ice- cold oxygenated (95%) and carbonated (5%) artificial cerebrospinal fluid (ACSF) (<4.0°C). ACSF contained the following substances (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. Using a vibratome 400 µm thick sagittal slices were made and hippocampi were dissected out afterwards. Hippocampal slices were allowed to recover in a bath of oxygenated and carbonated ACSF at room temperature for 1.5 hours. At onset of the experiment slices were placed submerged in a recording chamber that was continuously perfused at a rate of 2 ml/min with oxygenated and carbonated 30°C ACSF. All slices were recorded from with platinum (Pt)/iridium (Ir) electrodes. Bipolar Pt/Ir stimulating electrodes were used to stimulate the slice with a stimulus duration of 100 µs. Stimulating and recording electrodes were placed on the CA3-CA1 Schaffer-collateral afferents and the dendrites of CA1 pyramidal cells in the stratum radiatum (150-200 µm from s. pyramidale), respectively. Upon placement of electrodes, slices were given 30 minutes to rest before onset of measurements. All PPF experiments were done at one-third of slice maximum. Long-term potentiation (LTP) induced using 100 Hz tetanization (1 train of 1 second at 100 Hz) was also evoked at one-third of slice maximum. Theta burst LTP (4 trains of 4 stimuli at 100 Hz, 200 ms apart) were evoked at two-thirds of slice maximum. Unstable recordings were excluded and determinations were made blind to genotype. The magnitude of LTP was defined as the average of the last 10 measurements of the normalized fEPSP slope.

Data analysis and statistics

Hypothesis testing was performed using SPSS version 17 (SPSS Inc) as indicated in the results section. Outcomes are expressed as mean ± standard error (SE). Pairwise comparisons were performed using either ANOVA with Tukey's post-hoc test, or two-tailed t-tests, depending upon the experimental design and as specified in the text. Group sizes for each experiment are provided in the figure legends.

RESULTS

Spatiotemporal deletion of αCaMKII

To investigate the temporal and region-specific contribution of αCaMKII to the learning deficits observed in global *Camk2a*$^{-/-}$ mice, we first generated a floxed allele of *Camk2a* in order to enable experiments using spatiotemporally-controlled deletion of αCaMKII (Fig. 1*A*). For each of the cre lines used: *L7-cre* (cerebellar Purkinje neurons), *Emx1-cre* (glutamatergic pyramidal neurons of the cortex and hippocampus), *CAG-CreER* (tamoxifen-inducible global deletion), we first confirmed that the protein levels and immunohistochemical distribution of αCaMKII was consistent with the expected pattern of deletion as previously reported using other floxed lines. The conditional mutants were further compared to WT and global KO mice. As expected, *Camk2a*$^{flox/L7\text{-}cre+}$ mice showed a selective loss of αCaMKII expression in cerebellar Purkinje neurons, the only cell type in the cerebellum that normally expresses αCaMKII (Walaas et al., 1988) (Fig. 1*B*,*C*). In *Camk2a*$^{flox/Emx\text{-}cre+}$ mice, αCaMKII was deleted from cortex and hippocampus, but without any changes in the cerebellum. In line with previous reports (Iwasato et al., 2004), recombination was variable across distinct amygdala nuclei. Specifically, high levels of recombination were observed in the basolateral amygdala (BLA) relative to the lateral amygdala (LA) and posterodorsal medial amygdala nucleus (MePD). Also consistent with the Iwasato (2004) findings, we observe a very low residual staining of αCaMKII throughout the telencephalon, with a randomly scattered and sparse distribution of labeled neurons. In contrast, we observed complete tamoxifen-inducible deletion of αCaMKII in adult *Camk2a*$^{flox/CreER+}$ mice. Importantly however, *Camk2a*^{flox} mice lacking cre recombinase had no detectable alteration in their αCaMKII protein levels, confirming that the integrated loxP sites are not interfering with *Camk2a* gene expression (Fig. 1*B*, *C*).

Telencephalic, but not cerebellar, αCaMKII is required for spatial learning

Previous studies have demonstrated that *Camk2a*$^{-/-}$ mice are severely impaired in spatial learning using the hidden version of the Morris water maze (Silva et al., 1992a; Elgersma et al., 2002). However, the temporal and region-specific requirements for endogenous αCaMKII in spatial learning have not previously been determined. Therefore, we examined the impact of temporal and region-specific deletion of αCaMKII on water maze learning, using a 5 day (2 trials/day) training protocol previously shown to result in severe impairments in *Camk2a*$^{-/-}$ global knockout mice in which the same exon was already deleted in germline (Elgersma et al., 2002). In both groups latency times decreased equivalently (Fig. 2*A*, first panel; effect of genotype: $F_{1,15} = 0.46$, $p = 0.51$, repeated measures ANOVA). But, consistent with previous studies, we confirmed

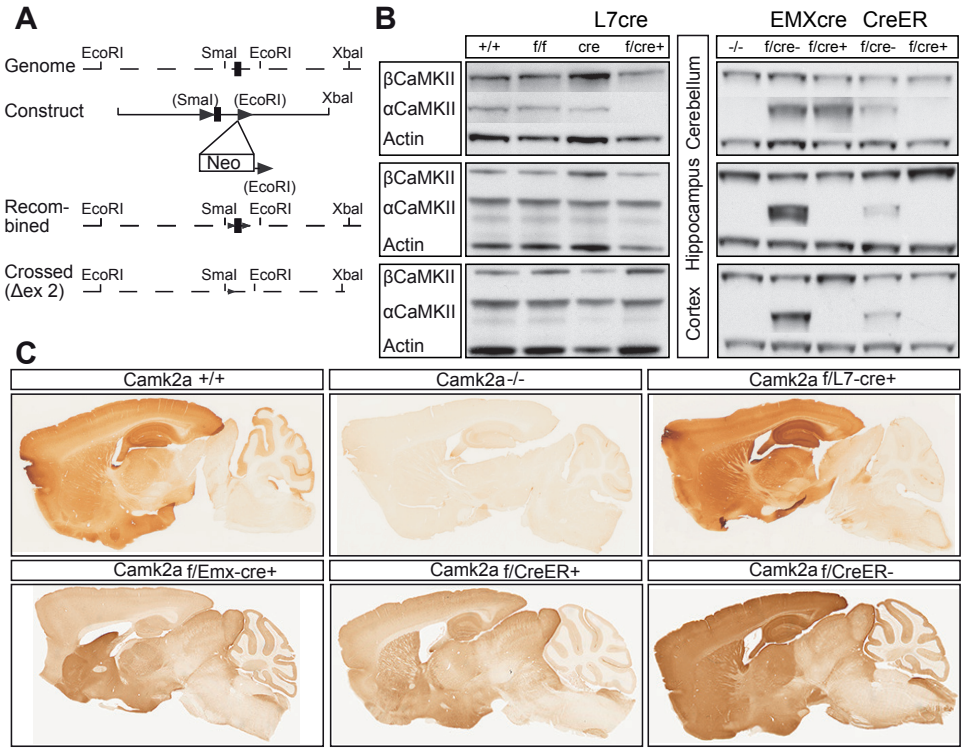


Figure 1: Generation of spatial and temporal specific Camk2a knockout mice. (A) Schematic diagram for the generation of floxed *Camk2a* knockout mice (Genome) Wild-type *Camk2a* locus with exon 2 depicted as a black box. (Construct) Targeting construct used for introducing the LoxP sites and neomycin resistance (Neo) gene in the *Camk2a* locus. The LoxP sites flanking exon 2 and the Neo gene are depicted as triangles. (Recombined) Mutant *Camk2a* flox locus after homologous recombination and Cre recombination in ES cells and consequential deletion of the Neo gene. (Crossed) The mutant *Camk2a* knockout locus after crossing with Cre-positive mice and subsequent deletion of exon 2 in targeted cells. **(B)** Western blots using antibodies for αCaMKII, bCaMKII and actin demonstrate that: (1) αCaMKII level is specifically decreased in the cerebellum of $Camk2a^{flox/L7\text{-}cre+}$ mice, but not in cortex or hippocampus (left panel). (2) αCaMKII level is specifically decreased in the cortex and hippocampus of $Camk2a^{flox/Emx\text{-}cre+}$ mice, but not in the cerebellum (middle panel). (3) αCaMKII level is decreased in all structures of $Camk2a^{flox/CreER+}$ mice (right panel). For all genotypes, Cre-negative mice are unaffected in their αCaMKII and bCaMKII expression levels. **(C)** αCaMKII stainings show: (1) normal staining in $Camk2a^{+/+}$ mice (top left panel). (2) No staining in $Camk2a^{-/-}$ mice (top middle panel). (3) Specific αCaMKII deletion in Purkinje cells in $Camk2a^{flox/L7\text{-}cre+}$ mice (top right panel). (4) Specific αCaMKII deletion in forebrain, but not cerebellar cells in $Camk2a^{flox/Emx\text{-}cre+}$ mice (bottom left panel). (5) Specific αCaMKII deletion throughout the brain in $Camk2a^{flox/CreER+}$ mice (bottom middle panel). (6) Normal staining in $Camk2a^{flox/CreER-}$ mice (bottom right panel).

that *Camk2a*$^{-/-}$ mice have a severe spatial learning deficit, reflected in no significant preference for the target quadrant compared to the other quadrants (t_7 = 2.62, p = 0.80 dependent samples t-test, Fig. 2*B*, first panel), whereas *Camk2a*$^{+/+}$ mice clearly showed a significant preference for the target quadrant ($_{t8}$ = 4.411, p = 0.002, dependent samples t-test; Fig. 2B, first panel).

Previous studies have suggested that the cerebellum may contribute significantly to the acquisition of spatial learning (Lalonde and Strazielle, 2003; Burguiere et al., 2005; Goddyn et al., 2006) (but see also (Galliano et al., 2013)). Therefore, in order to examine the specific contribution of cerebellar αCaMKII on spatial learning in our water maze learning paradigm, we tested *Camk2a*$^{flox/L7\text{-}cre+}$ mice and their *Camk2a*$^{flox/L7\text{-}cre-}$ control littermates on the hidden version of the Morris water maze. During training, latency times decreased equivalently in both genotypes (Fig. 2*A*, second panel; effect of genotype: $F_{1,27}$ = 0.408, p = 0.53, repeated measures ANOVA). Moreover, *Camk2a*$^{flox/L7\text{-}cre+}$ mice are indistinguishable from their littermates in their capacity to locate the platform during the probe trial (average time in platform quadrant, *Camk2a*$^{flox/L7\text{-}cre+}$: t_{13} = 2.868, p = 0.01, paired t-test; *Camk2a*$^{flox/L7\text{-}cre-}$: t_{14} = 3.567, p = 0.003, paired t-test; Fig. 2*B,* second panel). Therefore, cerebellar expression of αCaMKII does not appear to be required for spatial learning given that *Camk2a*$^{flox/L7\text{-}cre+}$ mice have complete deletion of αCaMKII in the cerebellum, yet acquire spatial learning normally under conditions in which global *Camk2a*$^{-/-}$ mice are severely impaired.

We next examined the necessity for telencephalic αCaMKII during spatial learning, using *Camk2a*$^{flox/Emx\text{-}cre+}$ mice and their *Camk2a*$^{flox/Emx\text{-}cre-}$ control littermates. Both groups of mice showed an overall similar reduction in their latency times across the training days (Fig. 2*A*, third panel). Although a repeated measures ANOVA showed a significant difference between the groups (effect of genotype: $F_{1,26}$ = 6.023, p = 0.02), post-hoc Tukey's tests showed that a significant difference between the two groups was restricted to day 3 (t_{26} = 3.171, p = 0.004), and no differences were observed in the initial or final training days. Moreover, consistent with the requirement of telencephalic αCaMKII for spatial learning, *Camk2a*$^{flox/Emx\text{-}cre+}$ mice showed no significant preference for the target quadrant during the probe trial (t_{12} = 1.816, p = 0.09, paired t-test; Fig. 2*B*, third panel). In contrast, the *Camk2a*$^{flox/Emx\text{-}cre-}$ littermates demonstrated robust spatial learning of the hidden platform location (t_{14} = 4.656, p = 0.0004, paired t-test; Fig. 2*B*, third panel). There was no significant difference in thigmotaxis ($_{t26}$ = 0.107, p = 0.79, unpaired t-test), swimspeed ($_{t26}$ = 1.882, p = 0.25, unpaired t-test), and pathlength ($_{t26}$ = 1.904, p = 0.26, unpaired t-test) between the two groups. Together, our results suggest that under these commonly used experimental conditions, spatial learning is strongly dependent on αCaMKII expression in the telencephalon, but not the cerebellum.

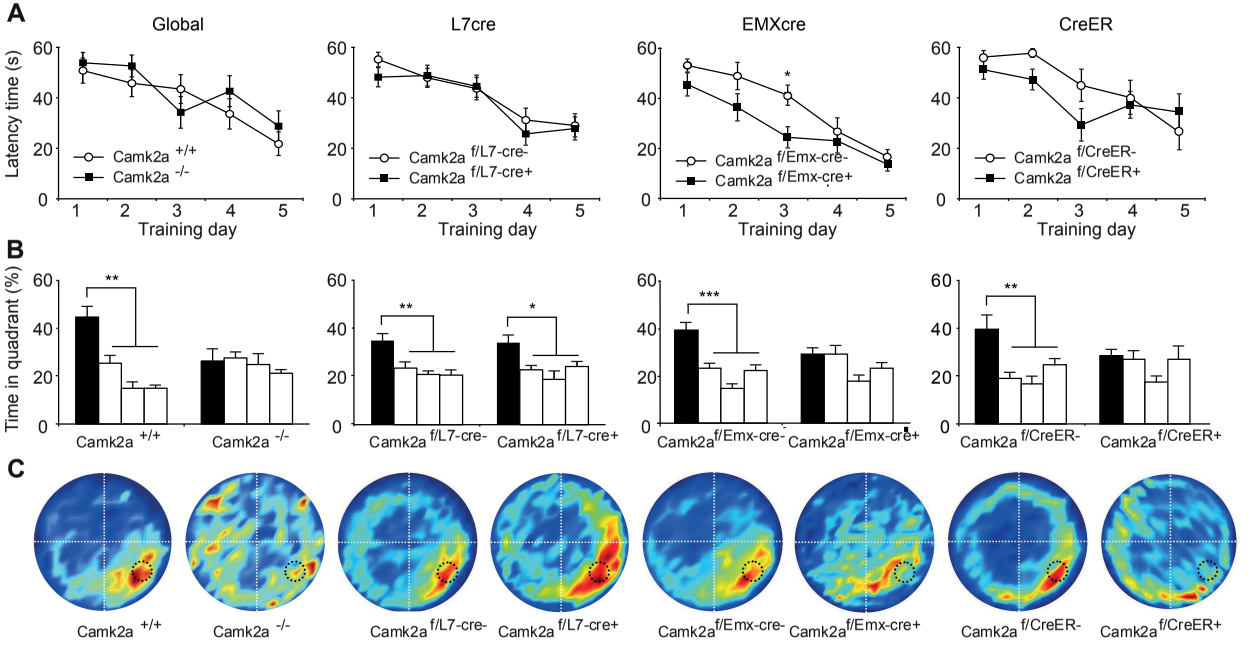


Figure 2: Water maze learning is normal in the absence of cerebellar αCaMKII. (A) Latency times to reach the hidden platform during training are not different between the *Camk2a* mutants and their control littermates. **(B)** Quantification of the time spent in each quadrant at a probe trial given after 5 days of training. Black bar indicates the target quadrant; white bars indicate adjacent right, opposite, and adjacent left quadrants, respectively. Error bars represent standard error of the mean. *Camk2a*$^{+/+}$ mice show a clear preference for the platform quadrant, but not *Camk2a*$^{-/-}$ mice (left panel). *Camk2a*$^{flox/L7\text{-}cre+}$ mice show a clear preference for the platform quadrant (second panel), while *Camk2a*$^{flox/Emx\text{-}cre+}$ mice (third panel) and *Camk2a*$^{flox/CreER+}$ mice (last panel) search equally in all 4 quadrants. All cre-negative control littermates show a clear preference for the platform quadrant. **(C)** Visual representation of all swimming tracks of each group of mice combined from the probe trial given at day 5. The color indicates the time spent at a certain location (red is high, blue is low). Note that *Camk2a*$^{-/-}$, *Camk2a*$^{flox/Emx\text{-}cre+}$ and *Camk2a*$^{flox/CreER+}$ mice mice search randomly while the other groups search preferably around the platform location. Number of mice tested: *Camk2a*$^{+/+}$: 9, *Camk2a*$^{-/-}$: 8, *Camk2a*$^{flox/L7\text{-}cre-}$: 15, *Camk2a*$^{flox/L7\text{-}cre+}$: 14; *Camk2a* $^{flox/Emx\text{-}cre-}$: 15, *Camk2a* $^{flox/Emx\text{-}cre+}$: 13, *Camk2a* $^{flox/CreER-}$: 9, *Camk2a* $^{flox/CreER+}$: 10.

In addition to its region-specific requirements, we also considered how developmentally-regulated expression of αCaMKII influences spatial learning. αCaMKII has been well demonstrated to function critically during NMDA-dependent LTP in the telencephalon, which is widely believed to be an indispensable form of plasticity underlying spatial learning. However, given that αCaMKII expression begins in the early postnatal period, the learning impairments observed in global *Camk2a*$^{-/-}$ mice might result from the absence of αCaMKII during early postnatal development, rather than being required specifically at the time of learning. Therefore, we utilized the tamoxifen-inducible *Camk2a*$^{flox/CreER+}$ mice and their *Camk2a*$^{flox/CreER-}$ littermates to achieve temporal control over the timing of αCaMKII deletion. In particular, we reasoned that by inducing deletion only in adulthood, we could more directly examine the necessity for αCaMKII specifically at the time of learning, while controlling for any potentially confounding abnormalities that might arise from the absence of αCaMKII during early development. Therefore, adult *Camk2a*$^{flox/CreER+}$ mice and their *Camk2a*$^{flox/CreER-}$ control littermates were given daily injections of tamoxifen (0.1 mg/g, i.p.) for four days to induce gene deletion. As shown in Figures 1*B* and 1*C*, tamoxifen-inducible deletion of αCaMKII in adulthood of *Camk2a*$^{flox/CreER+}$ mice was highly efficient, and without any observed deletion in the absence of tamoxifen, thereby maintaining the full integrity of postnatal development.

During the training phase, *Camk2a*$^{flox/CreER+}$ mice and their *Camk2a*$^{flox/CreER-}$ littermates demonstrated a similar reduction in latency times (effect of genotype: $F_{1,17} = 1.982$, $p = 0.18$, repeated measures ANOVA; Fig. 2*A,* last panel). However during the probe trial, *Camk2a*$^{flox/CreER+}$ mice showed no significant preference for the target quadrant ($t_9 = 1.593$, $p = 0.15$, paired t-test; Fig. 2*B*, last panel), whereas their *Camk2a*$^{flox/CreER-}$ littermates demonstrated a search pattern that was highly localized to the location of the hidden platform ($t_8 = 2.511$, $p = 0.04$, paired t-test; Fig. 2*B*, last panel). There was no significant difference in thigmotaxis ($_{t17} = -0.538$, $p = 0.09$, unpaired t-test), swimspeed ($_{t17} = 0.409$, $p = 0.59$, unpaired t-test), and pathlength ($_{t17} = -0.140$, $p = 0.95$, unpaired t-test) between the two groups. Therefore, αCaMKII is specifically required in adulthood at the time of learning for intact acquisition of spatial information, independent of its function during early postnatal development.

Regional and temporally-controlled deletion of αCaMKII on contextual fear learning

Contextual fear conditioning is a highly robust form of associative learning in which unsignaled aversive stimuli (e.g., footshock) are administered within a previously neutral context, in which a range of defensive behaviours (e.g., freezing) are subsequently elicited (Phillips and Ledoux, 1992, 1994). In addition to impaired spatial learning, *Camk2*$^{-/-}$ mice

also have been shown to have deficits in contextual conditioning (Silva et al., 1992a). Therefore, we sought to examine the temporal and region-specific requirements for αCaMKII expression during contextual fear learning.

Consistent with previous studies, we found that *Camk2a*$^{-/-}$ mice with a germline deletion of αCaMKII were severely impaired in contextual fear conditioning (Fig 3*A*; t_{16} = 4.454, p = 0.0004, unpaired t-test) when compared to their *Camk2a*$^{+/+}$ littermates. In contrast, *Camk2a*$^{flox/L7\text{-}cre+}$ mice with cerebellum-specific deletion of αCaMKII showed similar contextual fear learning as their *Camk2a*$^{flox/L7\text{-}cre-}$ control littermates (Fig 3*B*; t_{30} = 0.621, p = 0.57, unpaired t-test), suggesting that Purkinje cell expression of αCaMKII is not required for normal contextual fear conditioning.

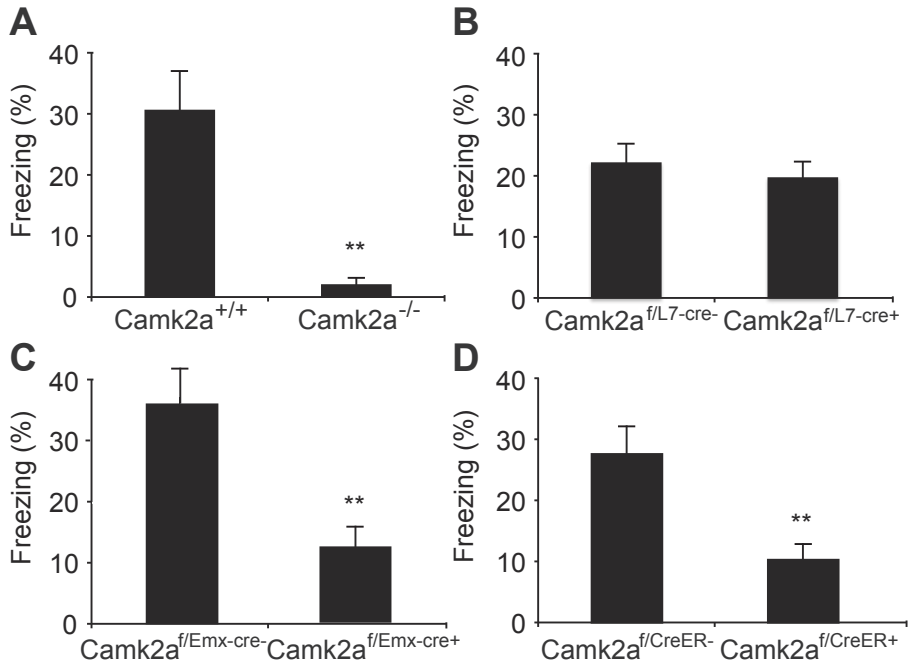


Figure 3: Contextual fear learning is unaffected in the absence of cerebellar αCaMKII. (A) Compared to *Camk2a*$^{+/+}$ mice, *Camk2a*$^{-/-}$ mice show no contextual fear learning. **(B)** *Camk2a*$^{flox/L7\text{-}cre+}$ mice show intact contextual fear learning, when compared to their *Camk2a*$^{flox/L7\text{-}cre-}$ control littermates. In contrast, *Camk2a*$^{flox/Emx\text{-}cre+}$ mice **(C)**, as well as *Camk2a*$^{flox/CreER+}$ **(D)** mice show severe contextual fear learning deficits, when compared to their Cre-negative control littermates. Number of mice tested: *Camk2a*$^{+/+}$: 9, *Camk2a*$^{-/-}$: 9, *Camk2a*$^{flox/L7\text{-}cre-}$: 15, *Camk2a*$^{flox/L7\text{-}cre+}$: 17, *Camk2a*$^{flox/Emx\text{-}cre-}$: 17, *Camk2a*$^{flox/Emx\text{-}cre+}$: 21, *Camk2a*$^{flox/CreER-}$: 10, *Camk2a*$^{flox/CreER+}$: 11. Bars represent the amount of freezing behavior, which is used as an index for recollection of the context. Error bars represent standard error of the mean.

Many previous studies have suggested that contextual fear learning requires NMDA-dependent LTP in the cortex and hippocampus (Levenson et al., 2002) (Kiyama et al., 1998). However, no previous studies have examined the telencephalic region-specific requirement for αCaMKII, despite being among the most widely hypothesized proteins underlying LTP and learning (as reviewed in (Coultrap and Bayer, 2012)). Therefore using *Camk2a*$^{flox/Emx\text{-}cre+}$ mice, we examined whether expression of αCaMKII specifically in the telencephalon was required for contextual conditioning. Indeed, as predicted by the predominant models of synaptic plasticity and learning, *Camk2a*$^{flox/Emx\text{-}cre+}$ mice showed a significant impairment in contextual fear learning, compared with their littermate controls (Fig. 3*C*; $t_{36} = 3.773$, $p = 0.001$; unpaired t-test).

In addition to the region-specific requirements for αCaMKII, we also examined the temporal requirement during contextual fear learning. In particular, we aimed to examine whether the conditioning deficit in the global *Camk2a*$^{-/-}$ mice arises from a developmental abnormality or due to the absence of αCaMKII in adulthood at the time of learning. Therefore, we again utilized *Camk2a*$^{flox/CreER+}$ mice to induce αCaMKII deletion selectively in adulthood, while sparing the potential impact of deletion during early development. Consistent with a requirement for αCaMKII expression at the time of learning, *Camk2a*$^{flox/CreER+}$ mice with temporally-restricted adult deletion showed a severe impairment of contextual learning (Fig. 3*D*; $t_{19} = 4.950$, $p = 0.0001$, unpaired t-test).

Long-term potentiation (LTP) is impaired in mice with adult deletion of αCaMKII

In addition to deficits in the Morris Water maze and contextual fear conditioning, *Camk2a*$^{-/-}$ mice also show an impairment in long-term potentiation (LTP) thought to underlie these hippocampal-dependent forms of learning (Silva et al., 1992b; Elgersma et al., 2002). Therefore, given the spatial and contextual learning deficits in *Camk2a*$^{flox/CreER+}$ mice, we also performed field recordings in acute hippocampal slices to examine whether adult-restricted deletion also impaired synaptic plasticity. Efficacy of synaptic transmission was unaffected in the absence of αCaMKII (Fig. 4*A*). Fiber volley amplitude was not significantly different in *Camk2a*$^{flox/CreER+}$ mice compared to their control littermates (effect of genotype: $F_{1,74} = 2.37$, $p = 0.13$, repeated measures ANOVA; Fig. 4*A*). Postsynaptic fEPSP slope was also independent of αCaMKII, consistent with previous studies using *Camk2a*$^{-/-}$ mice (effect of genotype: $F_{1,108} = 2.97$, $p = 0.09$, repeated measures ANOVA; Fig. 4*A*). Moreover, paired-pulse facilitation (PPF) was also unchanged in *Camk2a*$^{flox/CreER+}$ mice (effect of genotype: $F_{1,59} = 0.26$, $p = 0.61$, repeated measures ANOVA; Fig. 4*B*).

To examine whether the LTP impairment observed in *Camk2a*$^{-/-}$ mice is due to a critical role for αCaMKII during development and/or whether αCaMKII is necessary for the induction of LTP in adulthood, we used two independent protocols to induce LTP (100Hz and theta-burst protocols), given their distinct cellular mechanisms yet shared impairment in *Camk2a*$^{-/-}$ mice. Both stimulation protocols induced robust LTP in control littermates (*Camk2a*$^{flox/CreER-}$; Fig. 4*C,D*). However, *Camk2a*$^{flox/CreER+}$ mice with adult-specific deletion of αCaMKII showed impaired LTP in both the 100Hz (effect of genotype: $F_{1,29}$ = 16.88, p = 0.0003, repeated measures ANOVA; Fig. 4*C*) and theta-burst protocol (effect of genotype: $F_{1,52}$ = 7.85, p = 0.007, repeated measures ANOVA; Fig. 4*D*). Taken together, these findings confirm that αCaMKII is specifically required in adulthood for both learning and synaptic plasticity.

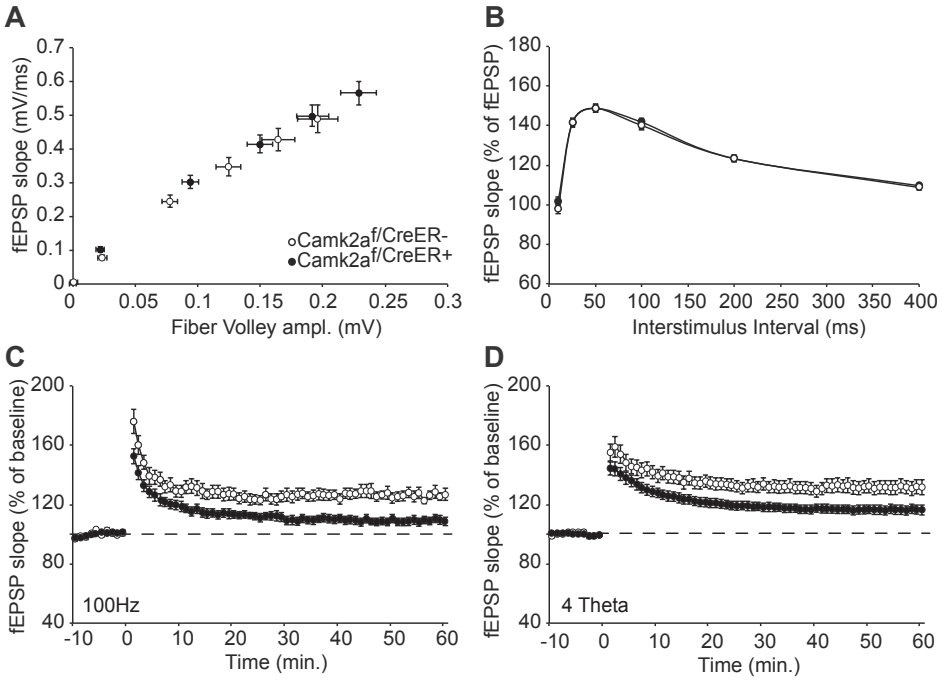


Figure 4: CA3-CA1 Long-term potentiation is impaired upon deletion of αCaMKII in adulthood. (A) *Camk2a*$^{flox/CreER+}$ mice show normal basal synaptic transmission (*Camk2a*$^{flox/CreER+}$ n=37, n=55; *Camk2a*$^{flox/CreER-}$ n= 39, n=55 for number of slices showing fiber volley and fEPSP respectively). **(B)** *Camk2a*$^{flox/CreER+}$ mice show normal paired-pulse facilitation (n=30 and n=31 for *Camk2a*$^{flox/CreER+}$ and *Camk2a*$^{flox/CreER-}$ mice respectively). **(C)** *Camk2a*$^{flox/CreER+}$ mice show impaired 100Hz LTP at the CA3-CA1 synapse in the hippocampus (n=15 and n=16 for *Camk2a*$^{flox/CreER+}$ and *Camk2a*$^{flox/CreER-}$ mice respectively). **(D)** *Camk2a*$^{flox/CreER+}$ mice show impaired 4 Theta LTP at the CA3-CA1 synapse in the hippocampus (n=31 and n=23 for *Camk2a*$^{flox/CreER+}$ and *Camk2a*$^{flox/CreER-}$ mice respectively). Error bars indicate S.E.M.

DISCUSSION

Here we used conditional *Camk2a* mutants to examine the influence of spatially-restricted and temporally-controlled deletion of αCaMKII. The present series of experiments demonstrate that αCaMKII is required during adulthood in the telencephalon, but not the cerebellum, for both spatial and contextual learning as well as hippocampal LTP. Importantly, these results are entirely consistent with previous research showing that global germline deletion of αCaMKII leads to deficits in hippocampal-dependent learning and LTP (Silva et al., 1992a; Silva et al., 1992b; Elgersma et al., 2004). However, no previous experiments had ever been able to control for the possibility that germline deletion of αCaMKII might have led to abnormalities in early brain development, rather than its requirement in adulthood at the time of learning. Moreover, given evidence in the literature regarding the influence of cerebellar Purkinje cell plasticity in spatial and contextual learning (Lalonde and Strazielle, 2003; Burguiere et al., 2005; Goddyn et al., 2006), we also examined region-specific *Camk2a* deletion.

Previous studies using inducible systems, such as (Mayford et al., 1996) and (Wang et al., 2003), investigated the necessity of αCaMKII during plasticity and learning by utilizing overexpression of a mutated temporally controlled and region specific *Camk2a* transgene. Although these studies show that enhanced levels of αCaMKII in forebrain, independent of its activity status, can interfere with learning and memory consolidation, they do not prove the requirement of endogenous αCaMKII for plasticity and learning. Temporal overexpression of the αCaMKII-T286D transgene, which is thought to have a dominant negative effect due to additional phosphorylation on the TT305/6 sites (Pi et al., 2010), caused changes in the frequency threshold for LTP induction, and disruption of several learning paradigms, which is reversed when turning off the transgene (Mayford et al., 1996). These experiments indicate a critical role for CaMKII signaling during learning. However since αCaMKII forms holoenzymes with bCaMKII, these studies do not rule out that the phenotype is caused by deregulation of the entire holoenzyme (rather than just αCaMKII) due to the dominant negative effect of the αCaMKII-T286D protein (Pi et al., 2010). Indeed, the dominant negative mutation αCaMKII-T305D results in a much stronger deficit than *Camk2a*$^{-/-}$ mice (Elgersma et al., 2002).

Temporal overexpression of αCaMKII-F89G (of which the activity can be regulated by specific inhibitors) in adult mice showed that increased CaMKII expression during training and the next 4 weeks had no effects on long-term contextual and cued-fear memories, as long as this level was not changed during consolidation or upon recall (Wang et al., 2003). Hence, these studies indicated that a constant level of CaMKII during the phases of acquisition, consolidation, or recall was critical for normal

learning, but did not address the requirement for endogenous αCaMKII for learning. In contrast, administering tatCN21 (a fusion peptide which inhibits stimulated and autonomous CaMKII activity) did not impair contextual fear memory expression when given one hour before testing (Buard et al., 2010). Importantly however, when tatCN21 was administered one hour prior to training, acquisition was significantly impaired, confirming the necessity of CaMKII for intact learning and in full agreement with our findings.

Notably, we observed that mice with deletion restricted to the cerebellum demonstrated normal spatial and contextual learning, consistent with a recent study examining multiple lines of mice, each with distinct impairments in cerebellar transmission and plasticity, which found no evidence of cognitive impairments, including spatial and contextual learning (Galliano et al., 2013). Future studies should be aimed at further dissecting the regional and cell-type specific contributions of αCaMKII during learning, including the hippocampal subfields and amygdala nuclei. Moreover, it will also be important to explore the differential contributions of αCaMKII during the various phases of memory, including acquisition, consolidation, reconsolidation, maintenance, and retrieval.

We have confirmed that the well-characterized learning deficits in *Camk2a*$^{-/-}$mice are not merely due to absence of the protein during the developmental period. In the present study, mice with temporally-restricted deletion of αCaMKII in adulthood showed equivalent spatial and contextual learning deficits as observed in the global *Camk2a*$^{-/-}$ mutants who lack αCaMKII expression throughout postnatal development. Therefore, we conclude that temporally-controlled deletion of *Camk2a* in adult mice is as detrimental as germline deletion for spatial and contextual learning.

REFERENCES

Anagnostaras SG, Wood SC, Shuman T, Cai DJ, LeDuc AD, Zurn KR, Zurn JB, Sage JR, Herrera GM (2010) Automated assessment of Pavlovian conditioned freezing and shock reactivity in mice using the VideoFreeze system. Front Behav Neurosci 4.

Barski JJ, Dethleffsen K, Meyer M (2000) Cre recombinase expression in cerebellar Purkinje cells. Genesis 28:93-98.

Benson DL, Isackson PJ, Gall CM, Jones EG (1992) 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.

Buard I, Coultrap SJ, Freund RK, Lee YS, Dell'Acqua ML, Silva AJ, Bayer KU (2010) CaMKII "Autonomy" Is Required for Initiating But Not for Maintaining Neuronal Long-Term Information Storage. J Neurosci 30:8214-8220.

Burguiere E, Arabo A, Jarlier F, De Zeeuw CI, Rondi-Reig L (2010) Role of the Cerebellar Cortex in Conditioned Goal-Directed Behavior. Journal of Neuroscience 30:13265-13271.

Burguiere E, Arleo A, Hojjati MR, Elgersma Y, De Zeeuw CI, Berthoz A, Rondi-Reig L (2005) Spatial navigation impairment in mice lacking cerebellar LTD: a motor adaptation deficit? Nature Neuroscience 8:1292-1294.

Coultrap SJ, Bayer KU (2012) CaMKII regulation in information processing and storage. Trends in Neurosciences 35:607-618.

Elgersma Y, Sweatt JD, Giese KP (2004) Mouse genetic approaches to investigating calcium/calmodulin-dependent protein kinase II function in plasticity and cognition. J Neurosci 24:8410-8415.

Elgersma Y, Fedorov NB, Ikonen S, Choi ES, Elgersma M, Carvalho OM, Giese KP, Silva AJ (2002) Inhibitory autophosphorylation of CaMKII controls PSD association, plasticity, and learning. Neuron 36:493-505.

Galliano E, Potters JW, Elgersma Y, Wisden W, Kushner SA, De Zeeuw CI, Hoebeek FE (2013) Synaptic Transmission and Plasticity at Inputs to Murine Cerebellar Purkinje Cells Are Largely Dispensable for Standard Nonmotor Tasks. Journal of Neuroscience 33:12599-12618.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ (1998) Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. Science 279:870-873.

Goddyn H, Leo S, Meert T, D'Hooge R (2006) Differences in behavioural test battery performance between mice with hippocampal and cerebellar lesions. Behavioural Brain Research 173:138-147.

Hansel C, de Jeu M, Belmeguenai A, Houtman SH, Buitendijk GH, Andreev D, De Zeeuw CI, Elgersma Y (2006) alphaCaMKII Is essential for cerebellar LTD and motor learning. Neuron 51:835-843.

Hayashi S, McMahon AP (2002) Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev Biol 244:305-318.

Iwasato T, Nomura R, Ando R, Ikeda T, Tanaka M, Itohara S (2004) Dorsal telencephalon-specific expression of Cre recombinase in PAC transgenic mice. Genesis 38:130-138.

Jones EG, Huntley GW, Benson DL (1994) Alpha Calcium/Calmodulin-Dependent Protein-Kinase-Ii Selectively Expressed in a Subpopulation of Excitatory Neurons in Monkey Sensory-Motor Cortex - Comparison with Gad-67 Expression. Journal of Neuroscience 14:611-629.

Kiyama Y, Manabe T, Sakimura K, Kawakami F, Mori H, Mishina M (1998) Increased thresholds for long-term potentiation and contextual learning in mice lacking the NMDA-type glutamate receptor epsilon 1 subunit. Journal of Neuroscience 18:6704-6712.

Lalonde R, Strazielle C (2003) The effects of cerebellar damage on maze learning in animals. Cerebellum 2:300-309.

Levenson J, Weeber E, Selcher JC, Kategaya LS, Sweatt JD, Eskin A (2002) Long-term potentiation and contextual fear conditioning increase neuronal glutamate uptake. Nature Neuroscience 5:155-161.

Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, Kandel ER (1996) Control of memory formation through regulated expression of a CaMKII transgene. Science 274:1678-1683.

Oberdick J, Smeyne RJ, Mann JR, Zackson S, Morgan JI (1990) A Promoter That Drives Transgene Expression in Cerebellar Purkinje and Retinal Bipolar Neurons. Science 248:223-226.

Phillips RG, Ledoux JE (1992) Differential Contribution of Amygdala and Hippocampus to Cued and Contextual Fear Conditioning. Behavioral Neuroscience 106:274-285.

Phillips RG, Ledoux JE (1994) Lesions of the Dorsal Hippocampal-Formation Interfere with Background but Not Foreground Contextual Fear Conditioning. Learn Memory 1:34-44.

Pi HJ, Otmakhov N, Lemelin D, De Koninck P, Lisman J (2010) Autonomous CaMKII Can Promote either Long-Term Potentiation or Long-Term Depression, Depending on the State of T305/T306 Phosphorylation. J Neurosci 30:8704-8709.

Sik A, Hajos N, Gulacsi A, Mody I, Freund TF (1998) The absence of a major Ca2+ signaling pathway in GABAergic neurons of the hippocampus. P Natl Acad Sci USA 95:3245-3250.

Silva AJ, Paylor R, Wehner JM, Tonegawa S (1992a) Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science 257:206-211.

Silva AJ, Stevens CF, Tonegawa S, Wang Y (1992b) Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science 257:201-206.

Simeone A, Gulisano M, Acampora D, Stornaiuolo A, Rambaldi M, Boncinelli E (1992) 2 Vertebrate Homeobox Genes Related to the Drosophila-Empty Spiracles Gene Are Expressed in the Embryonic Cerebral-Cortex. Embo Journal 11:2541-2550.

Smeyne RJ, Oberdick J, Schilling K, Berrebi AS, Mugnaini E, Morgan JI (1991) Dynamic Organization of Developing Purkinje-Cells Revealed by Transgene Expression. Science 254:719-721.

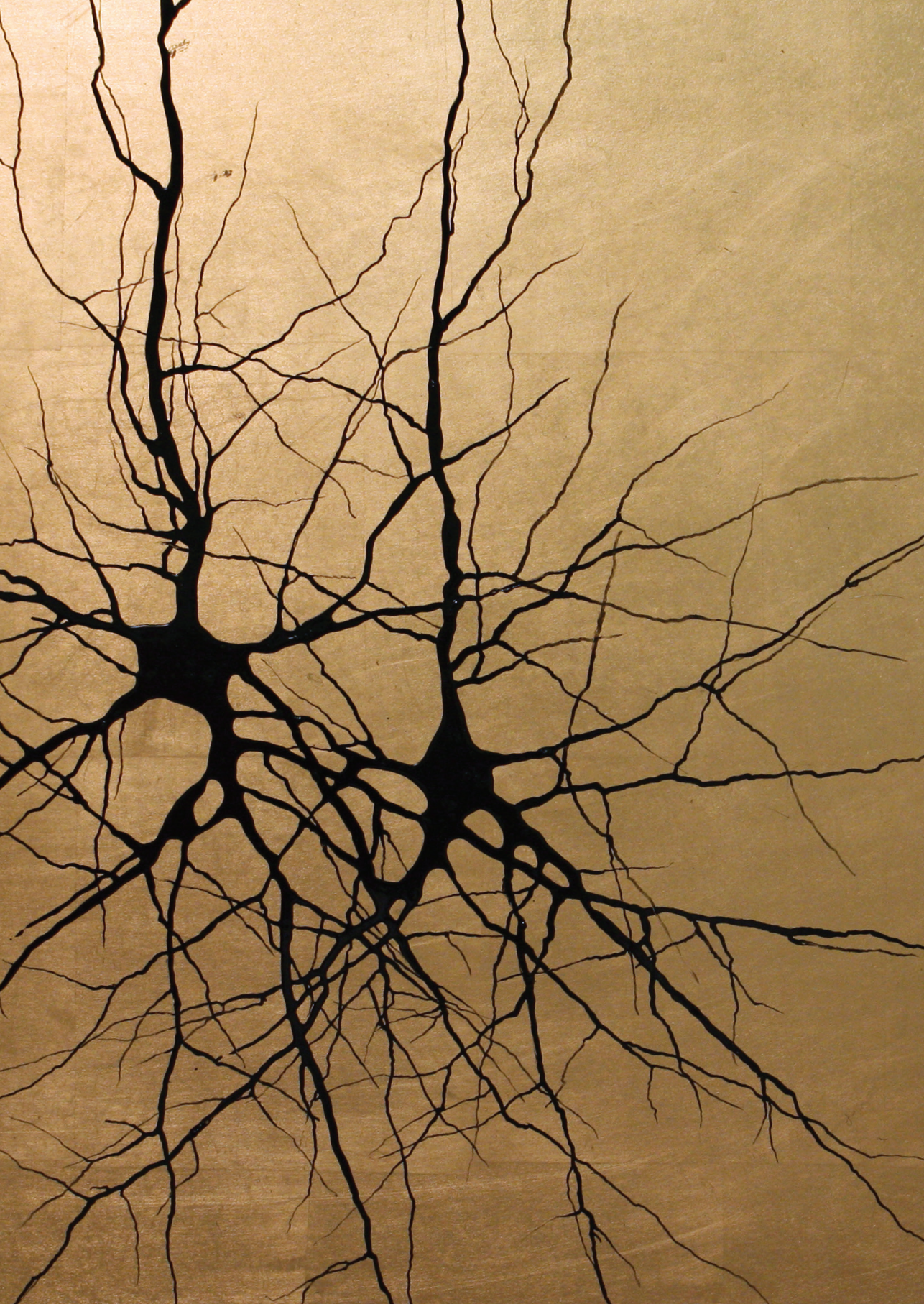
Takeuchi Y, Fukunaga K, Miyamoto E (2002) Activation of nuclear Ca(2+)/calmodulin-dependent protein kinase II and brain-derived neurotrophic factor gene expression by stimulation of dopamine D2 receptor in transfected NG108-15 cells. J Neurochem 82:316-328.

Van Duyne GD (2001) A structural view of Cre-loxP site-specific recombination. Annu Rev Bioph Biom 30:87-104.

Vogt MA, Chourbaji S, Brandwein C, Dormann C, Sprengel R, Gass P (2008) Suitability of tamoxifen-induced mutagenesis for behavioral phenotyping. Experimental Neurology 211:25-33.

Walaas SI, Lai Y, Gorelick FS, DeCamilli P, Moretti M, Greengard P (1988) 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.

Wang H, Shimizu E, Tang YP, Cho M, Kyin M, Zuo W, Robinson DA, Alaimo PJ, Zhang C, Morimoto H, Zhuo M, Feng R, Shokat KM, Tsien JZ (2003) 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.



Chapter 3

The molecular, temporal and region-specific requirements of the beta isoform of Calcium/Calmodulin-dependent protein kinase type 2 (CAMK2B) in mouse locomotion

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ABSTRACT

Genetic approaches using temporal and brain region-specific restricted gene deletions have provided a wealth of insight in the brain regions and temporal aspects underlying spatial and associative learning. However, for locomotion such extensive studies are still scarce.

Previous studies demonstrated that *Camk2b*$^{-/-}$ mice, which lack the β isoform of Calcium/Calmodulin-dependent protein kinase 2 (CAMK2B), show very severe locomotion deficits. However, where these locomotion deficits originate is unknown. Here we made use of novel *Camk2b* mutants (*Camk2b*$^{f/f}$ and *Camk2b*^{T287A}), to explore the molecular, temporal and brain region-specific requirements of CAMK2B for locomotion. At the molecular level we found that normal locomotion requires Calcium/Calmodulin mediated activation of CAMK2B, but CAMK2B autonomous activity is largely dispensable. At a systems level, we found that global deletion of *Camk2b* in the adult mouse causes only mild locomotion deficits, suggesting that the severe locomotion deficits of *Camk2b*$^{-/-}$ mice are largely of developmental origin. However, early onset deletion of *Camk2b* in cerebellum, striatum or forebrain did not recapitulate the locomotion deficits, suggesting that these deficits cannot be attributed to a single brain area.

Taken together, these results provide the first insights into the molecular, temporal and region-specific role of CAMK2B in locomotion.

INTRODUCTION

The Calcium/Calmodulin-dependent protein kinase II (CaMKII, from hereon called CAMK2) family members are pivotal to normal synaptic plasticity and learning in the hippocampus, cortex and cerebellum. The family consists of four different isoforms, (alpha, beta, gamma and delta) of which CAMK2A is the most studied (for review, see (Lisman et al., 2002; 2012; Hell, 2014)). In the last decade, the role of CAMK2B in the brain has gained attention largely due to the generation of new mutant mice (Van Woerden et al., 2009; Borgesius et al., 2011; Bachstetter et al., 2014; Gao et al., 2014).

In sharp contrast to CAMK2A, CAMK2B has been shown to play an important role in locomotion, since two independent CAMK2B mutants both show severe locomotion deficits (Van Woerden et al., 2009; Bachstetter et al., 2014). Like CAMK2A, CAMK2B is highly expressed in the brain (Cheng et al., 2006) and it has been shown that CAMK2B plays an enzymatic as well as a structural role in both hippocampal and cerebellar plasticity (Van Woerden et al., 2009; Borgesius et al., 2011). This structural role of CAMK2B comes from an additional domain within CAMK2B, the F-actin binding domain, which enables CAMK2B to cluster CAMK2A to the actin cytoskeleton (Shen et al., 1998; Shen and Meyer, 1999; Borgesius et al., 2011). Indeed, most hippocampal phenotypes observed in *Camk2b*$^{-/-}$ mutants require CAMK2B protein, but not its enzymatic function, since the *Camk2b*^{A303R} mutants, in which CAMK2B cannot be activated, do not show an overt hippocampal phenotype (Borgesius et al., 2011). At the cerebellar parallel fiber–Purkinje cell synapse, CAMK2B appears to have both a structural role as an enzymatic role (Van Woerden et al., 2009) but it is unknown to what extend the severe locomotion deficits seen in *Camk2b*$^{-/-}$ mice, arise from loss of the enzymatic or the structural role of CAMK2B.

Expression of CAMK2B starts around E12.5, whereas expression of CAMK2A starts postnatal, around P1 (Bayer et al., 1999). According to this difference in temporal expression, one might expect that CAMK2B is involved in embryonic development, whereas CAMK2A is not. Indeed, it has been shown that deletion of CAMK2A in an adult brain gives a similar phenotype as germ-line deletion of CAMK2A with respect to learning and synaptic plasticity (Achterberg et al., 2014). Similar studies have not been performed for CAMK2B, but considering the early onset of expression, it is conceivable that germ-line deletion of CAMK2B results in a more severe phenotype compared to deletion in adult animals.

Since CAMK2B has been shown to play an important role in controlling the direction of synaptic plasticity at the parallel fiber–Purkinje cell synapse (Van Woerden et al., 2009),

the Purkinje cells are a likely candidate for being responsible for the locomotion deficits observed in the *Camk2b*$^{-/-}$ mice. However, there are several other CAMK2B-expressing brain areas involved in motor control, such as motor cortex, several nuclei in brainstem, spinal cord and basal ganglia (e.g. striatum and subthalamic nucleus). Besides the hippocampus (Borgesius et al., 2011; Okuno et al., 2012), cerebellum (Van Woerden et al., 2009; Gao et al., 2014; Nagasaki et al., 2014) and lateral habenula (Li et al., 2013), CAMK2B function in different specific motor control areas has not been studied.
For this study we generated an autophosphorylation-deficient CAMK2B mutant (*Camk2b*^{T287A}) and made use of the previously described CAMK2B mutant which can no longer bind Calcium/Calmodulin (*Camk2b*^{A303R}) (Borgesius et al., 2011) to study the structural and enzymatic role of CAMK2B in locomotion using the accelerating rotarod. Additionally, we generated a floxed *Camk2b* mutant to study the temporal and brain region-specific role of CAMK2B in motor behaviour. We show that Calcium/Calmodulin-dependent activation of CAMK2B is essential for normal locomotion, but surprisingly, CAMK2B autonomous activity is largely dispensable. Additionally, we found that normal locomotion requires CAMK2B to be present during development, and that the locomotion deficits observed in the *Camk2b* mutant cannot be assigned to a single brain area.

RESULTS

The role of Calcium/Calmodulin-dependent and autonomous activity of CAMK2B in locomotion

We have previously shown that *Camk2b*$^{-/-}$ mice (in which exon 11 was deleted) show severe locomotion deficits on the rotarod and the balance beam (Van Woerden et al., 2009). Since CAMK2 can have both structural and enzymatic functions in the brain, we first set out to understand which molecular aspects of CAMK2B are important for normal locomotion.

Mutating Alanine 303 to Arginine (A303R) in CAMK2B, interferes with Calcium/Calmodulin binding and activation of CAMK2B, and renders CAMK2B into a persistently F-actin bound state (Shen et al., 1998; Borgesius et al., 2011). To investigate whether this mutation has any effect on motor behaviour, we tested the *Camk2b*$^{A303R/A303R}$ mice on the rotarod. Even though this mutant has normal hippocampal learning and plasticity (Borgesius et al., 2011), *Camk2b*$^{A303R/A303R}$ mice showed a severe locomotion deficit, not being able to stay on the rod for more than 5-10 seconds (effect of genotype: $F_{1,18}$=39.05, p<0.001; effect of time: $F_{1,18}$=2.48, p=0.05; interaction: $F_{1,18}$=2.44, p=0.05, repeated measures ANOVA; Fig. 1a).

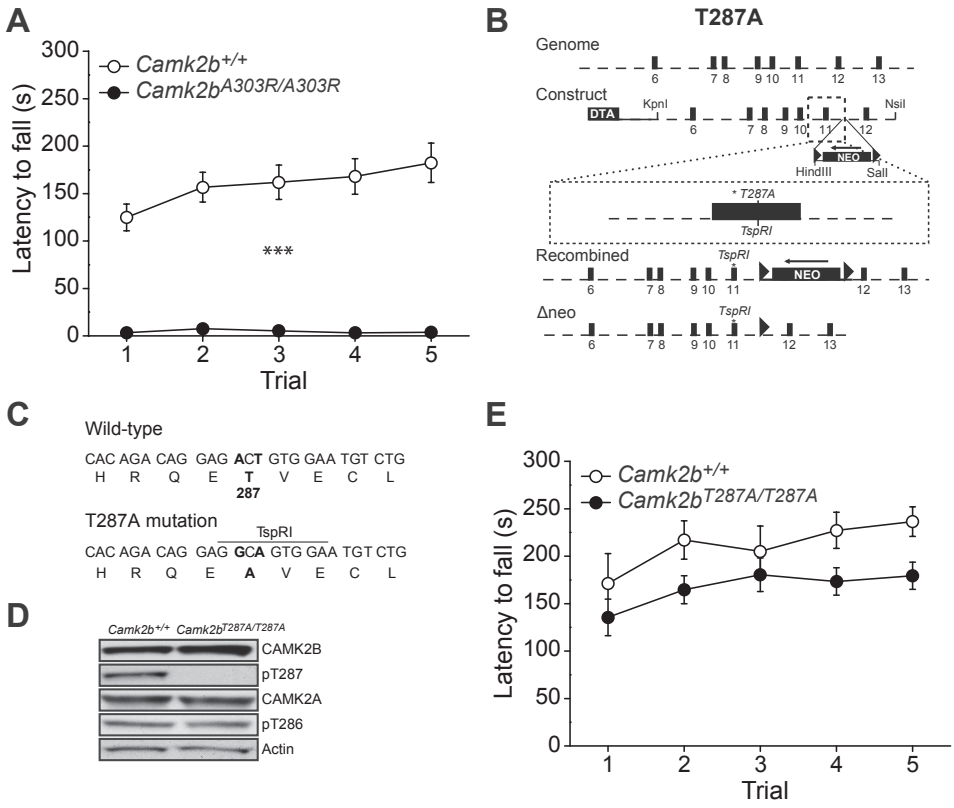


Figure 1. A crucial role for Calcium/Calmodulin-dependent activity, but not autonomous activity of CAMK2B in locomotion. Locomotion was tested using the accelerating rotarod. **(A)** *Camk2b*$^{A303R/A303R}$ mice (n=6) show a severe impairment in locomotion compared to *Camk2b*$^{+/+}$ control littermates (n=14). **(B)** Schematic picture for the generation of the *Camk2b*^{T287A} mutants. Exons are depicted as black boxes. The asterisk in exon 11 indicates the mutation at Thr287, where a new TspRI restriction site was introduced. 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 depicts the mutant *Camk2b*^{T287A} locus after homologous recombination. ΔNEO depicts the mutant *Camk2b*^{T287A} locus after *Cre* recombination. **(C)** Sequence of Thr287 in exon 11 showing the specific mutation made to induce Thr287Ala and introducing the TspRI restriction site used for genotyping. **(D)** Western blots probed with a phospho-specific antibody against ph-T287 reveal no detectable Thr287 phosphorylation in the hippocampus of *Camk2b*$^{T287A/T287A}$ mice. Note that the Thr287Ala mutation has no effect on ph-Thr286, CAMK2A, and CAMK2B protein levels. Actin levels are shown as loading control. **(E)** *Camk2b*$^{T287A/T287A}$ mice (n=14) show no impairment in locomotion compared to *Camk2b*$^{+/+}$ control littermates (n=9). Error bars indicate SEM.

The *Camk2b*^{A303R} mutation interferes with the Calcium/Calmodulin dependent activity, as well as the autonomous (Calcium/Calmodulin independent) activity of CAMK2B. To specifically investigate the role of autonomous activity in locomotion, we generated an autophosphorylation-deficient CAMK2B mouse mutant in which Threonine 287 is substituted by an Alanine, thus blocking autonomous CAMK2B activity (*Camk2b*$^{T287A/T287A}$) (Fig. 1b and c). Protein quantification using western blot indeed revealed a near complete absence of T287-phosphorylated CAMK2B in *Camk2b*$^{T287A/T287A}$ mice without changes in T286-phosphorylated CAMK2A or total levels of both CAMK2A and CAMK2B (Fig. 1d; for quantification see Table 1).

Table 1. Overview of western blot quantification performed on the Camk2b mutants in percentage of controls.

	1 Day Paradigm					
	Effect of Genotype		Effect of Time		Interaction	
Mouse Line	F-value	p-value	F-value	p-value	F-value	p-value
L7-Cre	$F_{(3,45)}$=0.292	0.8311	$F_{(3,45)}$=19.52	**0.0001**	$F_{(3,45)}$=1.554	0.1090
GABAa6-Cre	$F_{(1,14)}$=1.388	0.2585	$F_{(1,14)}$=2.706	**0.0393**	$F_{(1,14)}$=1.273	0.2916
RGS9-Cre	$F_{(1,12)}$=2.289	0.1562	$F_{(1,12)}$=0.362	0.8348	$F_{(1,12)}$=0.993	0.4208
EMX-Cre	$F_{(1,28)}$=2.235	0.1461	$F_{(1,28)}$=14.14	**0.0001**	$F_{(1,28)}$=2.724	**0.0330**

	5 Day Paradigm					
	Effect of Genotype		Effect of Time		Interaction	
Mouse Line	F-value	p-value	F-value	p-value	F-value	p-value
L7-Cre	$F_{(3,45)}$=1.734	0.1723	$F_{(3,45)}$=49.49	**0.0001**	$F_{(3,45)}$=1.833	**0.0451**
GABAa6-Cre	$F_{(1,14)}$=3.697	0.0751	$F_{(1,14)}$=7.707	**0.0001**	$F_{(1,14)}$=0.878	0.4828
RGS9-Cre	$F_{(1,12)}$=1.314	0.2739	$F_{(1,12)}$=2.615	**0.0090**	$F_{(1,12)}$=1.119	0.3555
EMX-Cre	$F_{(1,28)}$=5.824	**0.0224**	$F_{(1,28)}$=36.55	**0.0001**	$F_{(1,28)}$=2.276	0.0654

WT = wildtype; Het = heterozygous knockout; KO = knockout. Cre– = Cre-negative *Camk2b* mutant; Cre+ = Cre-positive *Camk2b* mutant. Number of samples is depicted in brackets.

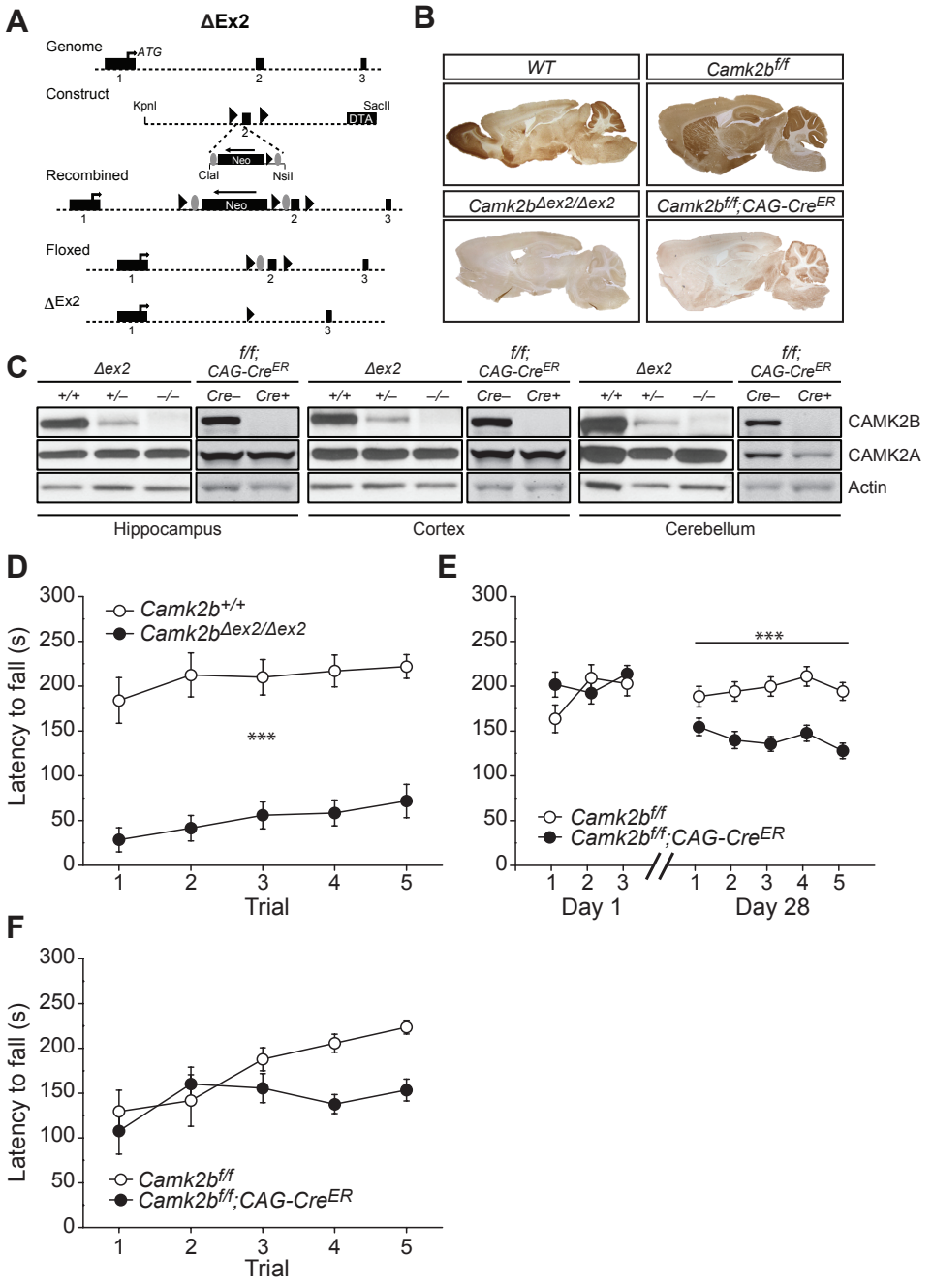
Camk2b$^{T287A/T287A}$ mice showed a trend towards reduced locomotion compared to their wildtype littermates, however this difference was not significant (effect of genotype: $F_{1,21}$=3.72, p=0.07; effect of time: $F_{1,21}$=6.47, p<0.001; interaction: $F_{1,21}$=0.71, p=0.59, repeated measures ANOVA; Fig. 1e). These results indicate, in contrast to the Calcium/Calmodulin-dependent activation, the contribution of CAMK2B autonomous activity in normal locomotion is marginal.

Temporal involvement of CAMK2B in locomotion

Expression of CAMK2B starts during early development (E12.5) (Bayer et al., 1999), hence the motor deficits seen in the *Camk2b*$^{-/-}$ mice could very well be due to a crucial role for CAMK2B during development. To assess temporal contribution of CAMK2B in the severe locomotion deficits, we generated a novel floxed allele of *Camk2b*, with *LoxP* sites around exon 2 (containing the catalytic site) of the *Camk2b* gene (*Camk2b*$^{f/f}$), which was crossed into the C57Bl/6 background (see Methods and Fig. 2a). To ensure that the locomotion deficits are also present in the C57Bl/6 background (the previous mutants were tested in an F2 129P2-C57Bl/6 hybrid background), we crossed the *Camk2b*$^{f/f}$ mice with a *Cag-cre* transgene, deleting exon 2 from germline (*Camk2b*$^{\Delta ex2/\Delta ex2}$). Using immunocytochemistry and western blots, we confirmed the global deletion of CAMK2B in this line. As expected, *Camk2b*$^{\Delta ex2/\Delta ex2}$ showed loss of CAMK2B without changes in the expression of CAMK2A (Fig. 2b and c; Table 1). Furthermore, *Camk2b*$^{\Delta ex2/\Delta ex2}$ mice showed a severe locomotion deficit compared to their wildtype littermates (effect of genotype: $F_{1,14}$=48.01, p<0.001, repeated measures ANOVA; Fig. 2d), which indicates that the motor deficits are present in both F2 129P2-C57Bl/6 hybrid mice (Van Woerden et al., 2009) as well as congenic (16 backcrosses) C57Bl/6 mice.

After having shown that our novel *Camk2b* mutant indeed recapitulates the severe locomotion deficit described before, we continued determining the developmental component of CAMK2B in the locomotion deficit. Therefore *Camk2b*$^{f/f}$ mice were crossed with *CAG-Cre*^{ER}, giving us Tamoxifen-dependent temporal control over gene deletion. As expected, 12-14 week old *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice showed loss of CAMK2B 4 weeks after 4 daily consecutive injections with Tamoxifen and showed no changes in levels of CAMK2A (Fig. 2b and c; Table 1).

We tested 8-10 week old mice prior to Tamoxifen-induced deletion on the rotarod and found no difference between genotypes (effect of genotype: $F_{1,38}$=0.53, p=0.47, repeated measure ANOVA), indicating that before Tamoxifen mediated gene deletion, the *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice do not have a locomotion deficit (Fig. 2e, *left*). When tested 28 days after Tamoxifen-induced deletion, *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice showed a clear locomotion deficit on the accelerating rotarod (effect of genotype: $F_{1,38}$=24.25, p<0.001, repeated measures ANOVA; Fig. 2e, *right*), however, this phenotype was not as severe as upon germ-line deletion. To make sure that the milder phenotype was not caused by the initial rotarod testing before gene deletion, we induced *Camk2b* gene deletion in a naïve cohort of 8-10 week old mice, and tested the mice 4 weeks after gene deletion. Although these *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice showed no improvement of locomotion over time compared to their *Camk2b*$^{f/f}$ littermates without expression of CRE


A
ΔEx2
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Floxed
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WT
Camk2b f/f
Camk2b Δex2/Δex2
Camk2b f/f;CAG-CreER
C
Δex2
f/f; CAG-CreER
+/+
+/–
–/–
Cre–
Cre+
CAMK2B
CAMK2A
Actin
Hippocampus
Cortex
Cerebellum
D
Camk2b +/+
Camk2b Δex2/Δex2
Latency to fall (s)
Trial
***
E
Camk2b f/f
Camk2b f/f;CAG-CreER
Day 1
Day 28
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F
Camk2b f/f
Camk2b f/f;CAG-CreER
Latency to fall (s)
Trial

Figure 2. Temporal requirement of CAMK2B in locomotion. (A) Schematic overview of the generation of the floxed *Camk2b* and *Camk2b*$^{\Delta Ex2/\Delta Ex2}$ mice. *Camk2b* locus and targeting construct with Exons 1, 2 and 3 depicted in black boxes. *LoxP* sites are indicated by the black triangles and the *Frt* sites are indicated by the grey ovals. The Diphtheria Toxin Cassette (DTA) was inserted for positive selection. Recombined depicts the mutant *Camk2b* locus after homologous recombination. Floxed depicts the *Camk2b*$^{f/f}$ mutant locus after transient expression of the *Flp* recombinase, resulting in a floxed locus without the neomycin cassette. Δex2 depicts the *Camk2b*$^{\Delta Ex2}$ mutant locus after Cre-mediated deletion. **(B)** Immunohistochemistry stainings of CAMK2B, showing (Top to bottom, left to right): normal expression in *WT* mice and no expression in *Camk2b*$^{\Delta ex2/\Delta ex2}$ mice; normal expression in *Camk2b*$^{f/f}$ mice and deletion throughout the brain in *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice **(C)** Western blots using an antibody specific for CAMK2A and CAMK2B in *Camk2b*$^{\Delta ex2/\Delta ex2}$ and *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice and their control littermates. Actin levels are shown as loading control. Decreased levels of CAMK2B in *Camk2b*$^{wt/\Delta ex2}$ mice and no detection of CAMK2B in *Camk2b*$^{\Delta ex2/\Delta ex2}$ and *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice in hippocampus, cortex and cerebellum with no changes in levels of CAMK2A. **(D)** *Camk2b*$^{\Delta ex2/\Delta ex2}$ mice (n=8) show a significant impairment in locomotion compared to wildtype littermates (n=8). **(E)** 8-10 week old *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice (n=13) and their *Camk2b*$^{f/f}$ control littermates (n=17) were trained on the rotarod before (Day 1) and 4 weeks after Tamoxifen injections (Day 28). Before deletion, both genotypes performed equally. After deletion, *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice showed a significant impairment of locomotion compared to *Camk2b*$^{f/f}$ control littermates as shown in the 5 trials given 28 days after the first injection. **(F)** 8-10 week old *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice (n=8) show no impairment in locomotion compared to *Camk2b*$^{f/f}$ control littermates (n=8) 4 weeks after Tamoxifen injections with no prior training. Error bars indicate SEM.

(overall effect of genotype: $F_{1,14}$=4.01, p=0.06; effect of time: $F_{1,14}$=6.48, p<0.001; interaction: $F_{1,14}$=3.02, p<0.05 Fig. 2f), performance at the first two trials was indistinguishable from control mice, which is markedly different from *Camk2b*$^{\Delta ex2/\Delta ex2}$ mice (Fig. 2d)

Taken together, deletion of CAMK2B in adulthood resulted in a much milder locomotion deficit compared to germline deletion of CAMK2B, indicating a significant developmental origin for the locomotion deficits seen in *Camk2b*$^{\Delta ex2/\Delta ex2}$ mice.

Brain areas contributing to the CAMK2B dependent rotarod deficits

Lesion studies in rodents have indicated that several brain areas are involved in locomotion, the most important being the cerebellum, striatum and motor cortex (for review see (Lalonde and Strazielle, 2007)). To our knowledge, the specific contribution of each of these brain areas to rotarod motor behaviour has not been systematically assessed through genetic lesions. CAMK2B is expressed in all these brain regions, therefore we used our conditional mutant to assess which brain area, if not all, is responsible for the severe rotarod phenotype seen in *Camk2b*$^{\Delta ex2/\Delta ex2}$ mice. Knowing that loss of CAMK2B in the cerebellum reverses plasticity at the parallel fiber–Purkinje cell

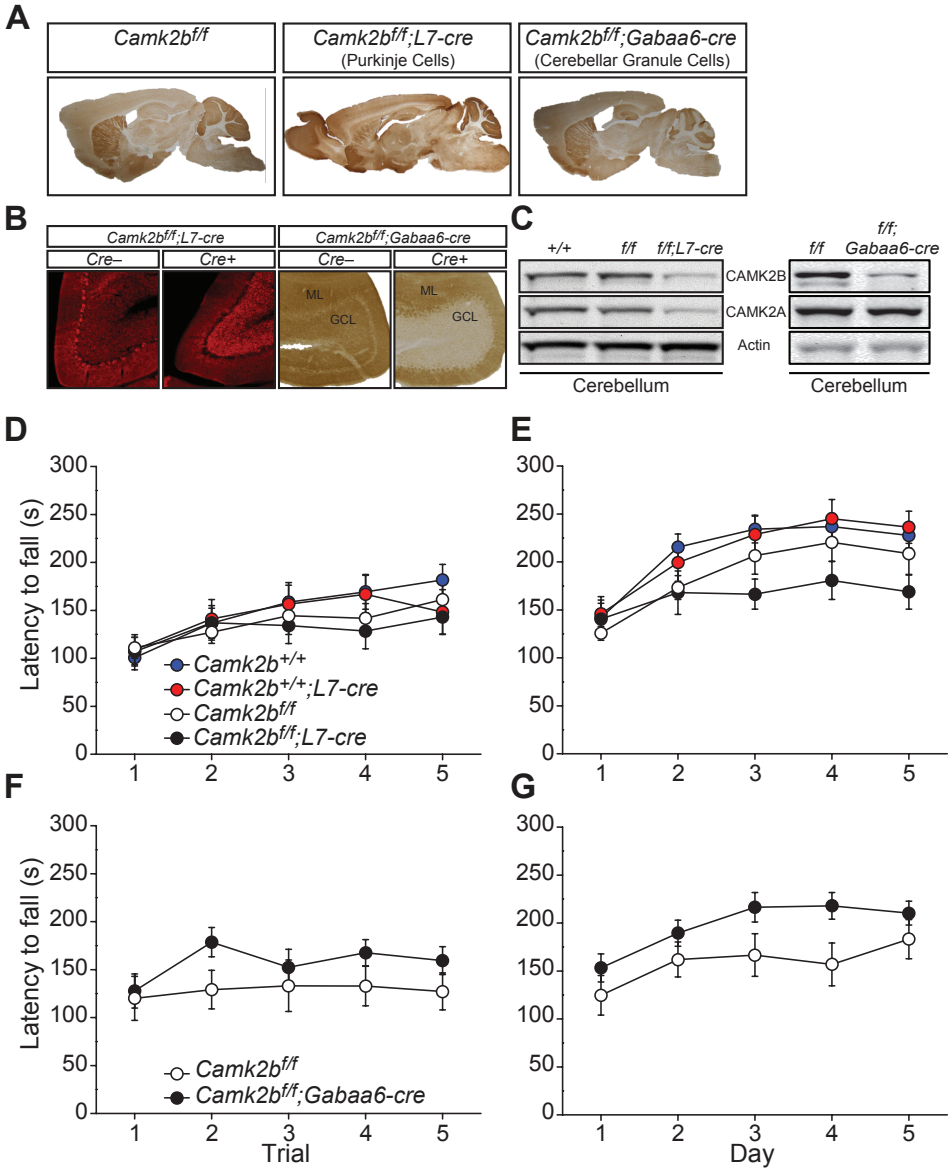

A
Camk2b f/f
Camk2b f/f;L7-cre
(Purkinje Cells)
Camk2b f/f;Gabaa6-cre
(Cerebellar Granule Cells)
B
Camk2b f/f;L7-cre
Camk2b f/f;Gabaa6-cre
Cre–
Cre+
Cre–
Cre+
ML
GCL
ML
GCL
C
+/+
f/f
f/f;L7-cre
f/f
f/f; Gabaa6-cre
CAMK2B
CAMK2A
Actin
Cerebellum
Cerebellum
D
Latency to fall (s)
300
250
200
150
100
50
0
1
2
3
4
5
Camk2b +/+
Camk2b +/+;L7-cre
Camk2b f/f
Camk2b f/f;L7-cre
E
F
Latency to fall (s)
Camk2b f/f
Camk2b f/f;Gabaa6-cre
Trial
G
Day

Figure 3. Requirement of cerebellar CAMK2B in locomotion. Motor performance (1-day paradigm; **(D)** and **(F)**) and learning (5-day paradigm; **(E)** and **(G)**) was tested using the accelerating rotarod. **(A)** Immunohistochemistry stainings of CAMK2B, showing (left to right): normal expression in *Camk2b$^{f/f}$* mice; specific deletion in cerebellar Purkinje cells in *Camk2b$^{f/f}$;L7-cre* mice and specific deletion in the cerebellar granule cells of *Camk2b$^{f/f}$;Gabaa6-cre* mice. **(B)** Immunofluorescent and immunohistochemical zoomed-in picture of the cerebellum of *Camk2b$^{f/f}$* mice, *Camk2b$^{f/f}$;L7-cre* and *Camk2b$^{f/f}$;Gabaa6-cre* mice showing specific deletion in cerebellar Purkinje Cells in the *Camk2b$^{f/f}$;L7-cre* mice and deletion in the granule cell layer (GCL) of *Camk2b$^{f/f}$;Gabaa6-cre* mice, but not in Purkinje cells of the molecular layer (ML). **(C)** Western blots using an antibody specific for CAMK2A and CAMK2B in the different *Camk2b* mutants and their control littermates. (Left) Decreased levels of CAMK2A and CAMK2B in the cerebellum of *Camk2b$^{f/f}$;L7-cre* mice. *LoxP* sites do not affect CAMK2A and CAMK2B levels. (Right) Decreased levels of CAMK2B in the cerebellum of *Camk2b$^{f/f}$;Gabaa6-cre* mice with no changes in CAMK2A levels. Actin levels are shown as loading control. **(D)** *Camk2b$^{f/f}$;L7-cre* mice (n=10) show no difference in performance compared to *Camk2b$^{f/f}$* control littermates (n=12). *Camk2b$^{+/+}$;L7-cre* (n=12) and *Camk2b$^{+/+}$* mice (n=15) do not differ in performance compared to *Camk2b$^{f/f}$;L7-cre* and *Camk2b$^{f/f}$* mice implying no effect of CRE or the flanked *LoxP* sites on rotarod performance. **(E)** *Camk2b$^{f/f}$;L7-cre* mice (n=10) show an impairment in learning compared to *Camk2b$^{f/f}$* control littermates (n=12). *Camk2b$^{+/+}$;L7-cre* (n=12) and *Camk2b$^{+/+}$* mice (n=15) do not differ in learning compared to *Camk2b$^{f/f}$;L7-cre* and *Camk2b$^{f/f}$* mice implying no effect of CRE or the flanked *LoxP* sites on rotarod learning. **(F)** *Camk2b$^{f/f}$;Gabaa6-cre* mice (n=8) show no impairment in performance compared to *Camk2b$^{f/f}$* control littermates (n=8). **(G)** *Camk2b$^{f/f}$;Gabaa6-cre* mice (n=8) and *Camk2b$^{f/f}$* control littermates (n=8) show normal learning. Error bars indicate SEM.

synapse, we started with assessing the importance of the cerebellum in the rotarod phenotype. *Camk2b$^{f/f}$* mice were crossed with two different cerebellum-specific *Cre*-lines: *L7-cre* (specific for cerebellar Purkinje cells) and *Gabaa6-cre* (specific for cerebellar granule cells). Both *Cre*-lines express the CRE protein within the first postnatal week (Barski et al., 2000; Aller et al., 2003).

Camk2b$^{f/f}$;L7-cre mice showed selective loss of CAMK2B in cerebellar Purkinje cells (Fig. 3a, b and c; Table 1) and *Camk2b$^{f/f}$;Gabaa6-cre* mice showed selective loss of CAMK2B in cerebellar granule cells. Surprisingly, *Camk2b$^{f/f}$;L7-cre* mice also showed a decrease in levels of CAMK2A in the cerebellum, whereas in *Camk2b$^{f/f}$;Gabaa6-cre* mice CAMK2A levels were unaffected (Fig. 3a and c; Table 1). Importantly, *Camk2b$^{f/f}$* mice lacking CRE recombinase did not show any detectible reduction of CAMK2B protein compared to *Camk2b$^{+/+}$* mice, indicating that the insertion of the *LoxP* sites did not interfere with *Camk2b* gene expression (Table 1).

For the rotarod experiments with the *L7-cre* mice, we used all four genotypes obtained from crossings of heterozygous *Camk2b$^{f/+}$* (with and without cre) mice: *Camk2b$^{f/f}$;L7-cre, Camk2b$^{f/f}$, Camk2b$^{+/+}$* and *Camk2b$^{+/+}$;L7-Cre*. Finding no difference in locomotion

between the *Camk2b*$^{+/+}$ and *Camk2b*$^{f/f}$ mice, we decided for subsequent experiments to cross homozygous *Camk2b*$^{f/f}$ mice (with and without cre), resulting in only *Camk2b*$^{f/f}$ and *Camk2b*$^{f/f}$*;cre* offspring. Surprisingly, neither the cerebellar Purkinje cell nor granule cell specific *Camk2b* mutants showed a rotarod deficit on the one-day training paradigm (Fig. 3d and f; for statistics see Table 2). When tested for 5 consecutive days however (to assess motor learning), *Camk2b*$^{f/f}$*;L7-cre* mice showed impaired motor learning (Fig. 3e; Table 2), whereas the granule cell specific mutants still showed no hint of any deficit compared to their littermate control mice (Fig. 3g; Table 2).

Since the loss of CAMK2B in cerebellar granule cells or Purkinje cells does not appear to contribute to the locomotion deficit as seen in *Camk2b*$^{\Delta ex2/\Delta ex2}$ mice, we then focused on striatum and motor cortex. *Camk2b*$^{f/f}$ mice were crossed with *Rgs9-cre* (deletion specifically in most medium spiny projection neurons of the striatum (Rahman et al., 1999)) and *Emx-cre* (deletion in glutamatergic pyramidal neurons in cortex and hippocampus) transgenic mice to test respectively striatal and cortical involvement in the rotarod phenotype. *Rgs9-cre* is expressed from P8 onwards (Dang et al., 2006) and *Emx-cre* is expressed from E10.5 (Iwasato et al., 2004). *Camk2b*$^{f/f}$*;Rgs9-cre* mice showed selective loss of CAMK2B in striatum (Fig. 4a and b; Table 1) and *Camk2b*$^{f/f}$*;Emx-cre* mice showed selective loss of CAMK2B in glutamatergic pyramidal neurons in hippocampus and cortex with no changes in levels of CAMK2A in both mutants (Fig. 4a and b; Table 1). Importantly, none of the specific lines showed notable off-target deletion in other brain areas (data not shown).

Notably, neither the striatal nor forebrain-specific *Camk2b* mutants showed a rotarod deficit on the one-day training paradigm (Fig. 4c and e; for statistics see Table 2). When tested for 5 consecutive days these region-specific mutants still showed no deficits compared to their controls (Fig. 4d and f; Table 2). Interestingly, the *Camk2b*$^{f/f}$*;Emx-cre* mice even showed a significantly enhanced locomotion (Fig. 4f; Table 2).

Taken together these results show that selective deletion of CAMK2B in brain areas supporting locomotion, is not sufficient to recapitulate the locomotion deficits observed in either the *Camk2b*$^{\Delta ex2/\Delta ex2}$ or the *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice. This suggests that multiple brain areas are responsible for locomotion deficits observed in *Camk2b*$^{\Delta ex2/\Delta ex2}$ mice. The only mouse line showing a small but significant effect on motor learning (5 day paradigm), but not motor performance (1 day paradigm), was the Purkinje cell specific knockout (*Camk2b*$^{f/f}$*;L7-cre*). However, this effect is still marginal compared to *Camk2b*$^{\Delta ex2/\Delta ex2}$ mice.

Table 2. Overview of statistical analysis of rotarod performances and learning of *Camk2b* mutants. A 2-way repeated measures ANOVA was used to test for performance or learning impairments (for details, see material and methods).

	T287A Hippocampus		CAG-Cre^{ER} Cortex		Hippocampus		Cerebellum	
Antibody	WT (4)	T287A (4)	Cre– (3)	Cre+ (5)	Cre–(3)	Cre+ (5)	Cre– (3)	Cre+ (5)
CAMK2B	100±27%	84±22%	100±16%	0±0%	100±15%	0.2±0.1%	100±13%	0±0%
CAMK2A	100±24%	67±11%	100±7%	126±25%	100±10%	89±3%	100±13%	57±20%
T287-phospho	100±16%	0.1±0.1%						
T286-phospho	100±11%	116±18%						

	Δex2 Cortex			Hippocampus			Cerebellum		
Antibody	WT (2)	Het (2)	KO (2)	WT (8)	Het (8)	KO (2)	WT (4)	Het (4)	KO (4)
CAMK2B	100±16%	23±8%	2±1%	100±5%	13±2%	10±4%	100±5%	17±5%	6±2%
CAMK2A	100±25%	109±35%	118±45%	100±7%	96±13%	128±34%	100±16%	64±21%	77±28%

	L7-Cre Cerebellum		GABAa6-Cre Cerebellum		RGS9-Cre Striatum		EMX-Cre Cortex		Hippocampus	
Antibody	Cre– (2)	Cre+ (2)	Cre– (5)	Cre+ (5)	Cre– (4)	Cre+ (4)	Cre– (6)	Cre+ (6)	Cre– (6)	Cre+ (6)
CAMK2B	100±11%	19±3%	100±17%	9±1%	100±12%	19±4%	100±12%	2±1%	100±8%	0±0%
CAMK2A	100±15%	30±1%	100±9%	115±7%	100±17%	104±15%	100±23%	92±7%	100±8%	87±6%

	L7-Cre Cerebellum			
Antibody	WT (4)	Cre+ (2)	WT (4)	Cre- (2)
CAMK2B	100±23%	27±5%	100±23%	139±15%
CAMK2A	100±7%	18±1%	100±7%	61±15%

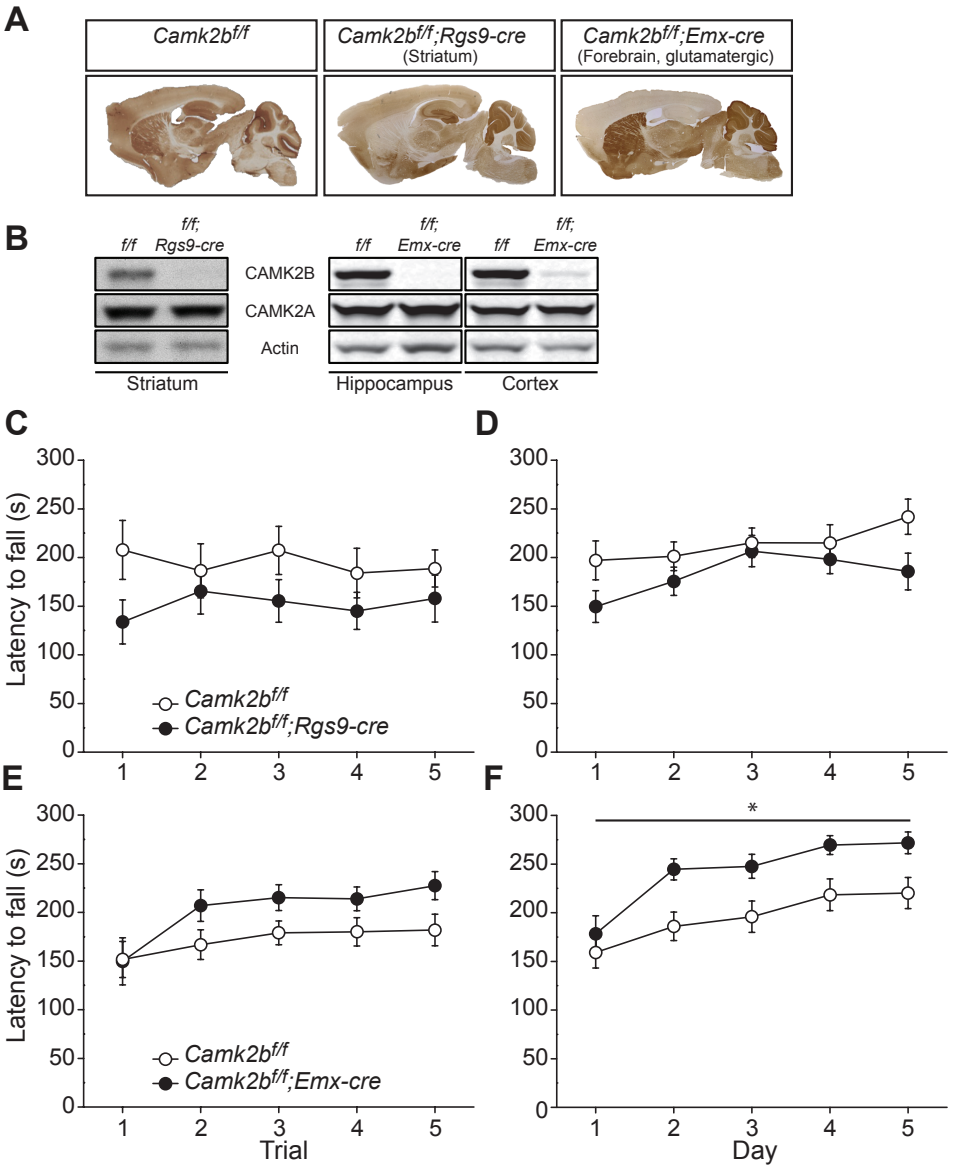

A
Camk2b f/f
Camk2b f/f;Rgs9-cre
(Striatum)
Camk2b f/f;Emx-cre
(Forebrain, glutamatergic)
B
f/f
f/f; Rgs9-cre
f/f
f/f; Emx-cre
f/f
f/f; Emx-cre
CAMK2B
CAMK2A
Actin
Striatum
Hippocampus
Cortex
C
D
Latency to fall (s)
300
250
200
150
100
50
0
1
2
3
4
5
Camk2b f/f
Camk2b f/f;Rgs9-cre
E
F
Latency to fall (s)
Camk2b f/f
Camk2b f/f;Emx-cre
*
Trial
Day

Figure 4. Requirement of striatal, hippocampal and cortical CAMK2B in locomotion. Motor performance (1-day paradigm; **(C)** and **(E)**) and learning (5-day paradigm; **(D)** and **(F)**) was tested using the accelerating rotarod. **(A)** Immunohistochemistry stainings of CAMK2B, showing (left to right): normal expression in *Camk2b*$^{f/f}$ mice; specific deletion in the striatum of *Camk2b*$^{f/f}$*;Rgs9-cre* and specific hippocampal and cortical deletion in *Camk2b*$^{f/f}$*;Emx-cre* mice. **(B)** Western blots using an antibody specific for CAMK2A and CAMK2B in the different *Camk2b* mutants and their control littermates. Actin levels are shown as loading control. Decreased levels of CAMK2B in striatal tissue of *Camk2b*$^{f/f}$*;Rgs9-cre* mice (left). Decreased hippocampal and cortical levels of CAMK2B in *Camk2b*$^{f/f}$*;Emx-cre* mice (right). No changes in levels of CAMK2A were observed in both mutants. **(C)** *Camk2b*$^{f/f}$*;Rgs9-cre* mice (n=7) show no impairment in performance compared to *Camk2b*$^{f/f}$ control littermates (n=7). **(D)** *Camk2b*$^{f/f}$*;Rgs9-cre* mice (n=7) and *Camk2b*$^{f/f}$ control littermates (n=7) show normal learning. **(E)** *Camk2b*$^{f/f}$*;Emx-cre* mice (n=13) show no impairment in performance compared to *Camk2b*$^{f/f}$ control littermates (n=17). **(F)** *Camk2b*$^{f/f}$*;Emx-cre* mice (n=13) and *Camk2b*$^{f/f}$ control littermates (n=17) show enhanced performance but no impairment in learning. Error bars indicate SEM.

DISCUSSION

Using several novel as well as previously described *Camk2b* mouse mutants, we dissected the molecular, temporal and systems requirements for CAMK2B in locomotion. At the molecular level we showed that even though the autonomous activity of CAMK2B in locomotion is dispensable, the ability of CAMK2B to bind Calcium/Calmodulin is crucial for normal locomotion. At the developmental level we found that deletion of *Camk2b* in the adult mouse causes a much milder locomotion deficit compared to germ line *Camk2b* deletion. Finally, at a systems level we showed that early onset deletion of *Camk2b* in any of the major motor areas of the brain (cerebellum, striatum or forebrain) did not recapitulate the locomotion deficit of global *Camk2b* deletion. Taken together these results suggest that the locomotion deficits are mainly of a developmental origin, as well as a result of interplay between multiple regions (systems level).

We showed here that loss of autophosphorylation of CAMK2B at the T287 site does not significantly affect rotarod behaviour, although a small trend could be seen. In contrast, mutating the Calcium/Calmodulin binding site of CAMK2B, such that it cannot bind Calcium/Calmodulin and rendering CAMK2B bound to actin (*Camk2b*$^{A303R/A303R}$), led to a severe locomotion deficit. Whether the inability to bind Calcium/Calmodulin and becoming active, or the inability to be released from actin causes the deficits observed, remains to be investigated. CAMK2B forms heteromers with CAMK2A (Brocke et al., 1999) and ratios of CAMK2A/CAMK2B can differ between cell types (Miller and Kennedy, 1985; Brocke et al., 1999). It has been shown for the hippocampus (in dissociated neuronal cultures and using *Camk2b*$^{A303R/A303R}$ mice) that CAMK2B kinase activity is not

important for synaptic plasticity or hippocampal learning, but that CAMK2B is required for regulating the subcellular location of CAMK2A (Shen et al., 1998; Borgesius et al., 2011). However, in Purkinje cells, CAMK2B not only regulates the subcellular localization of CAMK2A, but also plays an important enzymatic role in regulating the direction of plasticity at the parallel fiber–Purkinje cell synapse (Van Woerden et al., 2009). These different requirements likely reflect the differences in the CAMK2A/CAMK2B ratio in the different brain areas. Thus, when the relative expression level of CAMK2A is low, both the enzymatic and the non-enzymatic function of CAMK2B may be important, whereas in areas where CAMK2A is abundant, CAMK2B serves mainly to regulate the subcellular localization of CAMK2A. It would be interesting to investigate the CAMK2A/CAMK2B ratio in the specific motor areas of the brain to get more insight in which brain areas are involved in the severe locomotion deficits seen in the *Camk2b*$^{-/-}$ and *Camk2b*$^{A303R/A303R}$ mice. Notably, *Camk2b*$^{f/f}$*;L7-cre* mice showed a significant decrease in CAMK2A in western blot when only CAMK2B was deleted. A similar trend could be found in the cerebellum of *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} and in Camk2b$^{-/-}$ mice (Van Woerden et al., 2009). These results imply that specifically in Purkinje cells, which are the only cells in the cerebellum expressing CAMK2A, CAMK2B stabilizes CAMK2A, and that absence of CAMK2B therefore results in reduced levels of CAMK2A. However, the exact mechanism behind this regulation requires further research.

From a developmental point of view, we found that deleting most of *Camk2b* in the adult mice resulted in a significant locomotion impairment showing its necessity for normal motor behaviour. However, the phenotype was rather mild compared to the germ-line knock out mouse, indicating that acute deletion of *Camk2b* does not dramatically affect neuronal function. These results indicate that the severe locomotion deficits of *Camk2b*$^{-/-}$ mice largely arise during development of the nervous system. Unfortunately, little is known about the role of both the CAMK2 isoforms in brain development. For CAMK2A, which starts to be expressed around P1, we recently showed that deletion of *Camk2a* in adulthood is as detrimental as a germline deletion of *Camk2a* for hippocampal learning and plasticity (Achterberg et al., 2014), indicating an important post-developmental role. CAMK2B however, starts to be expressed already during early embryonic development (around E12.5) (Karls et al., 1992; Bayer et al., 1999). Therefore it is likely that CAMK2B plays a significant role during development. Previous studies looking at the developmental role of CAMK2B have shown an important role for CAMK2B in neurite extension and spine formation in acute knock-down experiments in neuronal cultures (Fink et al., 2003). However, no morphological changes were found in the *Camk2b*$^{-/-}$ mice (Van Woerden et al., 2009; Bachstetter et al., 2014), nor in neuronal cultures obtained from *Camk2b*$^{-/-}$ mice (Borgesius et al., 2011), thus it is unclear how a germ-line deletion of *Camk2b* can be so profoundly affected.

Locomotion deficits can be caused by dysfunction of several different brain areas (as reviewed by (Lalonde and Strazielle, 2007)). Using lesion studies it was shown that the cerebellum and thalamus are important in rotarod behaviour (Caston et al., 1995; Jeljeli et al., 2000; 2003), but lesioning the dorsal striatum did not cause motor impairments on the rotarod (Thullier et al., 1996). As for the motor cortex, some lesion studies have been performed, but the effect of the lesions was not assessed by the rotarod task. However, a recent pharmacological study showed that mGluR5 inhibition in primary motor cortex affected rotarod performance, which was not observed upon mGluR5 inhibition in dorsolateral striatum (Guimaraes et al., 2015). In contrast, deletion of the *Nmdar1* gene from P8 onwards using *Rgs9-cre* mice in the striatum resulted in performance and learning deficits on the rotarod (Dang et al., 2006). Taken together, these studies all indicate that, depending on the lesion or genetic change, several specific brain areas could potentially be responsible for deficits in motor performance as measured with the rotarod.

Surprisingly, restricted deletion of CAMK2B in the major motor areas of the brain did not reveal a specific brain area or cell type to be responsible for the severe rotarod phenotype observed in the *Camk2b*$^{-/-}$ mice. In these groups we not only assessed motor performance, but also motor learning by testing the mice over 5 consecutive days. Even though there are small variations in performance and learning in the control groups between the different experiments, the effects of *Camk2b* deletion on locomotion was only marginal compared to the profound phenotypes seen in *Camk2b*$^{-/-}$, *Camk2b*$^{\Delta ex2/\Delta ex2}$ and *Camk2b*$^{A303R/A303R}$ mice. There are three possible explanations for our findings. First, onset of *Camk2b* gene deletion in our mutants is likely to be too late. Three of the *cre*-lines used in this study start *cre* expression after onset of CAMK2B expression (*L7-cre* starts expression at P1 (Barski et al., 2000), *Gabaa6-cre* starts expression at P5-P7 (Aller et al., 2003), and *Rgs9-cre* expression is seen as early as P8 (Dang et al., 2006)). Thus, these lines start off with normal embryonic CAMK2B expression. Since we do not know the critical time window for CAMK2B in rotarod behaviour, it could be that in these mice CAMK2B is deleted too late in these separate brain regions or cell types to cause a significant effect on rotarod behaviour, comparable to *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice, where adult deletion resulted only in a mild phenotype when CAMK2B was deleted throughout the brain. Only the *Emx-cre* line, which expresses *cre* from E10.5 (Iwasato et al., 2004), induces deletion of *Camk2b* before onset of CAMK2B expression (E12.5). Interestingly, deletion in this *cre*-line resulted in enhanced, instead of impaired performance. Second, *Camk2b* deletion in thalamus, brainstem and the deep cerebellar nuclei is only obtained in the *Camk2b*$^{\Delta ex2/\Delta ex2}$ mice. Since it has been shown that these areas are involved in locomotion (Lalonde and Strazielle, 2007), there is a possibility that the motor deficits arise from those areas. Third, the severity of the locomotion deficits observed in the

Camk2b$^{-/-}$ mice (Van Woerden et al., 2009) could indicate a distributed role for CAMK2B in several brain areas and cell types, each one of them contributing a little to the deficit. For example, we cannot exclude that deleting *Camk2b* in Purkinje-cells and cerebellar granule cells simultaneously would have an effect on locomotion. Additionally, even though the different *cre*-lines used in this study were very effective at deleting *Camk2b* throughout multiple brain areas and cell types (as judged by immunuhistochemstry and Western blot), we cannot exclude the possibility that a small number of cells did not undergo deletion, and prevented the lack of a severe phenotype.

Taken together we conclude that the nature of severe the locomotion deficits observed in the *Camk2b*$^{-/-}$ is predominantly of developmental origin and cannot be attributed to one specific motor area or cell type in the brain. Moreover we conclude that the Calcium/Calmodulin-dependent activation of CAMK2B is essential for normal locomotion, but that the autonomous activity of CAMK2B is largely dispensable for normal locomotion. These findings are in sharp contrast to the role of CAMK2A in spatial learning, where loss of CAMK2A in adult mice recapitulates the phenotype of *Camk2a*$^{-/-}$ mice, where selective loss of CAMK2A in the hippocampus causes spatial learning deficits, and where loss of autonomous CAMK2A activity causes severe spatial learning deficits (Giese et al., 1998; Achterberg et al., 2014).

MATERIALS AND METHODS

Animals

Camk2b$^{A303R/A303R}$ *and Camk2b*$^{T287A/T287A}$ mice were tested in a 129P2-C57Bl/6OlaHsd F2 hybrid background. *Camk2b*$^{\Delta ex2/\Delta ex2}$ mice were backcrossed > 16 times into C57Bl/6JOlaHsd background. All conditional mice used in this study were backcrossed 10-12 times into the C57BL/6JOlaHsd background and crossed with cre lines maintained in the C57BL/6JOlaHsd background. Mice were genotyped when they were 7-10 days old, and re-genotyped after the mice were sacrificed. Genotyping records were obtained and kept by a technician not involved in the experimental design, performance and analysis. All mice were tested between 2-3 months of age, except the *Camk2b*$^{f/f}$*;Rgs9-cre* group, which was tested between 3-5 months of age. All mice were kept group-housed in IVC cages (Sealsafe 1145T, Tecniplast) with bedding material (Lignocel BK 8/15 from Rettenmayer) on a 12/12 h light/dark cycle in 21°C (±1°C), humidity at 40-70% and with food pellets (801727CRM(P) from Special Dietary Service) and water available *ad libitum*. For all experiments mutants were compared to WT or *cre*-negative homozygous floxed littermates. All groups were matched for age and sex and all experiments were done

during daytime. Experimenters were blind for genotype throughout experiments and data analysis. All research was performed in accordance with and approved by a Dutch Animal Ethical Committee (DEC) for animal research.

Generation of Camk2b$^{A303R/A303R}$ and Camk2b$^{T287A/T287A}$ mice

Generation of the *Camk2b*$^{A303R/A303R}$ mice has been described previously (Borgesius et al., 2011). The *Camk2b*^{T287A} targeting construct to generate *Camk2b*$^{T287A/T287A}$ *mice* 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'-GGTACCTGAGGAAGGTGCC AGCTCTGTCCC-3' and 3' primer: 5'–GTCGACCAGGGTAGTCACGGTTGTCC-3' (5.3 Kb; exon denotation according to ENSMUST00000019133) and exon 11-12 using 5' primer: 5'-GC GGCCGCCTGTTAAAGGAATGGTTCTC-3' and 3' primer: 5'-ATGCATCTAAAAGGCAGGCAGG ATGATCTGC-3' (6 Kb) 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 verified by sequencing. Site directed mutagenesis was used to introduce the Thr287Ala point mutation, which induced a TspRI restriction site (Figure 1). For counter selection, a gene encoding Diphtheria toxin chain A (DTA) was inserted at the 5' end 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 Leukaemia inhibitory factor (LIF). After selection with G418 (200 mg/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 subsequently inter-crossed to obtain F2 129P2-C57Bl/6 hybrid mice to generate homozygous mutants and wild-type littermate controls.

Generation of floxed Camk2b and Camk2b$^{\Delta ex2/\Delta ex2}$ mice

The floxed *Camk2b* 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 intron 1 using 5' primer: 5'-TTTGGTACCGCATTGTGGGCATCTATGAAG-3' and 3' primer: 5'–AAAGGATCCAGTCAGCTGGAATGAGACGTG-3' (2.6 Kb; intron and exon denotation according to ENSMUST00000019133), exon 2 using 5' primer: 5'-TTTATGCATGATAGACC TGGGTGTTACACAG-3' and 3' primer: 5'-AAAGTCGACCTTCAGGTCTGGGACAGAG-3' (583 bp) and intron 2 using 5' primer: 5'-TTTGCGGCCGCAGAAGTCCTCATATTGGGGAGG-3' and 3' primer: 5'-AAACCGCGGTGACTCCTAATGCAGAAGACACC-3' (4 kb) were amplified

using High Fidelity Taq Polymerase (Roche) on ES cell genomic DNA. The 5' fragment containing part of intron 1 was inserted before the first *LoxP* site whereas exon 2 and part of intron 2 were cloned on either site of a PGK-Neomycin selection cassette, which is flanked by *frt* and *LoxP* sites (for the schematics see Figure 2). Exon 2 and flanking intronic sequences were sequenced to verify that the absence of secondary mutations. 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 Leukaemia inhibitory factor (LIF). After selection with G418 (200 mg/ml), targeted clones were identified by PCR (long-range PCR from Neomycin resistance gene to the region flanking the targeted sequence). To delete the Neomycin resistance cassette and obtain the conditional construct, the correctly targeted clones were transiently transfected with an *flp* recombinase (Figure 2). Finally, a clone with correct karyotype was injected into blastocysts of C57Bl/6 mice. Male chimeras were crossed with female C57BL/6JOlaHsd mice (Harlan). The resulting F1 heterozygous *Camk2b*$^{f/+}$ mice, which were backcrossed 10-12 times with C57BL/6JOlaHsd mice before generating the brain region and temporally restricted mutants as described below. To obtain the *Camk2b*$^{\Delta ex2/\Delta ex2}$ mice, the *Camk2b*$^{f/+}$ mice were crossed with transgenic *Cag-cre* mice (Sakai and Miyazaki, 1997). The *Camk2b*$^{\Delta ex2/+}$ heterozygous offspring were backcrossed >16 times in C57BL/6JOlaHsd to obtain a congenic line.

Generation of Camk2b$^{f/f}$ conditional mutants

For the generation of conditional mutants, female *Camk2b*$^{f/f}$ mice (backcrossed 10-12 times with C57BL/6JOlaHsd) were crossed with male transgenic Cre lines maintained in C57BL/6JOlaHsd. To obtain temporal control, *Camk2b*$^{f/f}$ mice were crossed with *CAG-Cre*^{ER} mice (RRID:IMSR_JAX:004682; (Hayashi and McMahon, 2002)). F1 heterozygous floxed female *Camk2b*$^{f/+}$ mice were then crossed with male heterozygous floxed mice expressing *CAG-Cre*^{ER} to obtain the desired F2 genotypes: homozygous floxed *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} with transgenic expression of *CAG-Cre*^{ER} (mutants) and *Camk2b*$^{f/f}$ without *CAG-Cre*^{ER} expression (controls). A similar crossing was performed to obtain Purkinje cell specific deletion of CAMK2B, using *L7/pcp-2 cre* transgenic mice (RRID:IMSR_JAX:004146; (Oberdick et al., 1990; Barski et al., 2000), and to obtain glutamatergic and telencephalic-restricted deletion of CAMK2B, using *Emx1-cre* transgenic mice (RRID:IMSR_RBRC01345; (Iwasato et al., 2004)). Deletion of *Camk2b* in cerebellar granule cells was obtained by crossing *Camk2b*$^{f/f}$ mice with *Gabaa6-cre* transgenic mice (Aller et al., 2003). To generate striatal specific deletion of CAMK2B we crossed *Camk2b*$^{f/f}$ mice with *Rgs9-cre* mice (Dang et al., 2006).

Tamoxifen injections

Adult *Camk2b*$^{f/f}$ and *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice (8-10 weeks of age) were injected with Tamoxifen intraperitoneally (Sigma-Aldrich) (0.1mg/gr of bodyweight) for 4 consecutive days. To keep the dose of Tamoxifen constant throughout injection days we kept a tight injection scheme, injecting mice 24+/–1 hour after the previous injection. Tamoxifen was dissolved in sunflower oil (20mg/ml). Behavioural testing was assessed 4 weeks after the first injection. Even though Tamoxifen does not have an effect on emotional reactivity, neurological functioning or learning (Vogt et al., 2008) we injected both *Camk2b*$^{f/f}$ and *Camk2b*$^{f/f}$*;CAG-Cre*^{ER} mice to control for any possible effects of Tamoxifen.

Rotarod

The accelerating rotarod (Ugo Basile, Comerio Varese, Italy, 7650) contains a cylinder 3cm in diameter and can train 5 mice at the same time. Rotarod speed starts at 4 r.p.m., which increases to 40 r.p.m. at 270 seconds. The experiment stopped at 300 seconds. Latency to fall was measured in seconds after a mouse (i) fell of, (ii) clung to the rod for 3 consecutive rotations or (iii) clung to the rod for 2 rotations twice within 10 seconds. Mice were trained with an inter-trial interval of 45 minutes. We used two different paradigms on the accelerating rotarod. For motor performance (which we define as locomotion throughout the text) we used 5 consecutive trials with naïve mice (1 day paradigm). To assess motor learning we took the average of the first two trials of the first day and continued training the mice for another 4 consecutive days with 2 trials per day (5 day paradigm).

Immunohistochemistry and immunofluorescence

Mice were anaesthetized with pentobarbital and perfused transcardially with PBS followed by freshly prepared 4% paraformaldehyde solution (PFA, Sigma). Brains were taken out after perfusion and post-fixed for 1.5 hours in PFA and afterwards kept in 30% sucrose solution overnight. Immunohistochemistry was performed on free-floating 40µm thick sagittal cryostat sections. Sections were washed in PBS once and afterwards primary antibodies were added (anti-CAMK2B, 1:2000, #ab34703, Abcam) diluted in PBS containing 2% NHS, 0.5% Triton-X 100 and 150mM bovine serum albumin (BSA) and kept at 4°C overnight for 48 hours. Two days later sections were washed 3 times with PBS and then secondary antibodies were added (biotinylated goat anti-rabbit IgG antibody, Vector Laboratories, Burlinghame, CA; 1:200) diluted in PBS containing 2% NHS, 0.5% Triton-X 100 and 150mM bovine serum albumin (BSA) for 1 to 2 hours on room temperature. For diaminobenzidine (DAB) staining, sections were processed using a standard avidin-biotin-immunoperoxidase complex method (ABC, Vector Laboratories, USA) and 0.05% DAB as the chromogen. Sections were

mounted on slices using chrome(3)potassiumsulfatedodecahydrate and left to dry. The next day, slides were dehydrated in alcohol, cleared with xylene and covered using Permount (Fisher Scientific, USA). For immunofluorescence, the same CAMK2B was used as in immunohistochemistry (1:1000) and we used Cy3 rabbit (1:200) as a secondary antibody. After 1-2 hours incubation of the secondary antibody at room temperature sections were washed four times in PB (0.05M) and mounted on slices using chrome(3) potassiumsulfatedodecahydrate and left to dry. Finally, sections were covered using Mowiol (Sigma-Aldrich).

Western blot

Mice were anaesthetized using isoflurane and sacrificed by decapitation. Brain samples were taken out quickly and stored in liquid nitrogen. Upon protein determination lysates were first prepared and brain samples were homogenized in lysis buffer (10mM Tris-HCl 6.8, 2.5% SDS, 2mM EDTA). Protein concentration in the samples was determined and then lysate concentrations were adjusted to 1mg/ml. Western blots were probed with primary antibodies against either CAMK2A (6G9, 1:40.000, Abcam), CAMK2B (CB-β1, 1:10.000, Invitrogen), Actin (MAB1501R, 1:20.000, Chemicon) or Ph-T286/T287 (autophosphorylated αCaMKII and CAMK2B antibody; #06-881; 1:5000; Upstate Cell Signaling Solutions) and secondary antibodies (goat anti-mouse and/or goat anti-rabbit, both 1:3000, AffiniPure #115-007-003 and #111-007-003). Blots were stained with Enhanced ChemoLuminescence (ECL) (#32106, Pierce) or stained and quantified using LI-COR Odyssey Scanner and Odyssey 3.0 software. Quantification of western blot in ECL was done using ImageJ.

Data analysis and statistics

All experiments and analyses were performed blind to genotype. All behavioural tests were analysed using a 2-WAY repeated measures ANOVA to determine the performance and learning of the genotypes, with genotype as the between subjects factor and the repeated measures as within subject factor. α was set at 0.05. All values represent average +/- SEM. Group sizes for each genotype are depicted in the figure legends. All statistics were performed in Graphpad Prism. *$p < 0.05$, ***$p < 0.001$.

REFERENCES

Achterberg KG, Buitendijk GHS, Kool MJ, Goorden SMI, Post L, Slump DE, Silva AJ, Van Woerden GM, Kushner SA, Elgersma Y (2014) Temporal and Region-Specific Requirements of αCaMKII in Spatial and Contextual Learning. Journal of Neuroscience 34:11180–11187.

Aller MI, Jones A, Merlo D, Paterlini M, Meyer AH, Amtmann U, Brickley S, Jolin HE, McKenzie ANJ, Monyer H, Farrant M, Wisden W (2003) Cerebellar granule cell Cre recombinase expression. Genesis 36:97–103.

Bachstetter AD, Webster SJ, Tu T, Goulding DS, Haiech J, Watterson DM, Van Eldik LJ (2014) Generation and Behavior Characterization of CaMKIIβ Knockout Mice Skoulakis EMC, ed. PLoS ONE 9:e105191.

Barski JJ, Dethleffsen K, Meyer M (2000) Cre recombinase expression in cerebellar Purkinje cells. Genesis 28:93–98.

Bayer KU, Löhler J, Schulman H, Harbers K (1999) 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.

Borgesius NZ, Van Woerden GM, Buitendijk GHS, Keijzer N, Jaarsma D, Hoogenraad CC, Elgersma Y (2011) βCaMKII plays a nonenzymatic role in hippocampal synaptic plasticity and learning by targeting αCaMKII to synapses. Journal of Neuroscience 31:10141–10148.

Brocke L, Chiang LW, Wagner PD, Schulman H (1999) Functional implications of the subunit composition of neuronal CaM kinase II. J Biol Chem 274:22713–22722.

Caston J, Jones N, Stelz T (1995) Role of preoperative and postoperative sensorimotor training on restoration of the equilibrium behavior in adult mice following cerebellectomy. Neurobiology of Learning and Memory 64:195–202.

Cheng D, Hoogenraad CC, Rush J, Ramm E, Schlager MA, Duong DM, Xu P, Wijayawardana SR, Hanfelt J, Nakagawa T, Sheng M, Peng J (2006) Relative and absolute quantification of postsynaptic density proteome isolated from rat forebrain and cerebellum. Mol Cell Proteomics 5:1158–1170.

Dang MT, Yokoi F, Yin HH, Lovinger DM, Wang Y, Li Y (2006) Disrupted motor learning and long-term synaptic plasticity in mice lacking NMDAR1 in the striatum. Proceedings of the National Academy of Sciences of the United States of America 103:15254–15259.

Fink CC, Bayer K-U, Myers JW, Ferrell JE, Schulman H, Meyer T (2003) Selective regulation of neurite extension and synapse formation by the beta but not the alpha isoform of CaMKII. Neuron 39:283–297.

Gao Z, Van Woerden GM, Elgersma Y, De Zeeuw CI, Hoebeek FE (2014) Distinct roles of α- and βCaMKII in controlling long-term potentiation of GABAA-receptor mediated transmission in murine Purkinje cells. Front Cell Neurosci 8:16.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ (1998) Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. Science 279:870–873.

Guimaraes IM, Carvalho TG, Ferguson SS, Pereira GS, Ribeiro FM (2015) The metabotropic glutamate receptor 5 role on motor behavior involves specific neural substrates. Mol Brain 8:24.

Hayashi S, McMahon AP (2002) Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev Biol 244:305–318.

Hell JW (2014) CaMKII: Claiming Center Stage in Postsynaptic Function and Organization. Neuron 81:249–265.

Iwasato T, Nomura R, Ando R, Ikeda T, Tanaka M, Itohara S (2004) Dorsal telencephalon-specific expression of Cre recombinase in PAC transgenic mice. Genesis 38:130–138.

Jeljeli M, Strazielle C, Caston J, Lalonde R (2000) Effects of centrolateral or medial thalamic lesions on motor coordination and spatial orientation in rats. Neurosci Res 38:155–164.

Jeljeli M, Strazielle C, Caston J, Lalonde R (2003) Effects of ventrolateral-ventromedial thalamic lesions on motor coordination and spatial orientation in rats. Neurosci Res 47:309–316.

Karls U, Müller U, Gilbert DJ, Copeland NG, Jenkins NA, Harbers K (1992) 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.

Lalonde R, Strazielle C (2007) Brain regions and genes affecting postural control. Progress in Neurobiology 81:45–60.

Li K, Zhou T, Liao L, Yang Z, Wong C, Henn F, Malinow R, Yates JR, Hu H (2013) βCaMKII in Lateral Habenula Mediates Core Symptoms of Depression. Science 341:1016–1020.

Lisman J, Schulman H, Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. Nat Rev Neurosci 3:175–190.

Lisman J, Yasuda R, Raghavachari S (2012) Mechanisms of CaMKII action in long-term potentiation. Nat Rev Neurosci 13:169–182.

Miller SG, Kennedy MB (1985) 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.

Nagasaki N, Hirano T, Kawaguchi S-Y (2014) Opposite Regulation of Inhibitory Synaptic Plasticity by α and β Subunits of Ca2+/calmodulin-dependent Protein Kinase II. The Journal of Physiology.

Oberdick J, Smeyne RJ, Mann JR, Zackson S, Morgan JI (1990) A promoter that drives transgene expression in cerebellar Purkinje and retinal bipolar neurons. Science 248:223–226.

Okuno H, Akashi K, Ishii Y, Yagishita-Kyo N, Suzuki K, Nonaka M, Kawashima T, Fujii H, Takemoto-Kimura S, Abe M, Natsume R, Chowdhury S, Sakimura K, Worley PF, Bito H (2012) Inverse Synaptic Tagging of Inactive Synapses via Dynamic Interaction of Arc/Arg3.1 with CaMKII&beta. Cell 149:886–898.

Rahman Z, Gold SJ, Potenza MN, Cowan CW, Ni YG, He W, Wensel TG, Nestler EJ (1999) Cloning and characterization of RGS9-2: a striatal-enriched alternatively spliced product of the RGS9 gene. J Neurosci 19:2016–2026.

Sakai K, Miyazaki JI (1997) A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the cre transgene transmission. Biochem Biophys Res Commun 237:318–324.

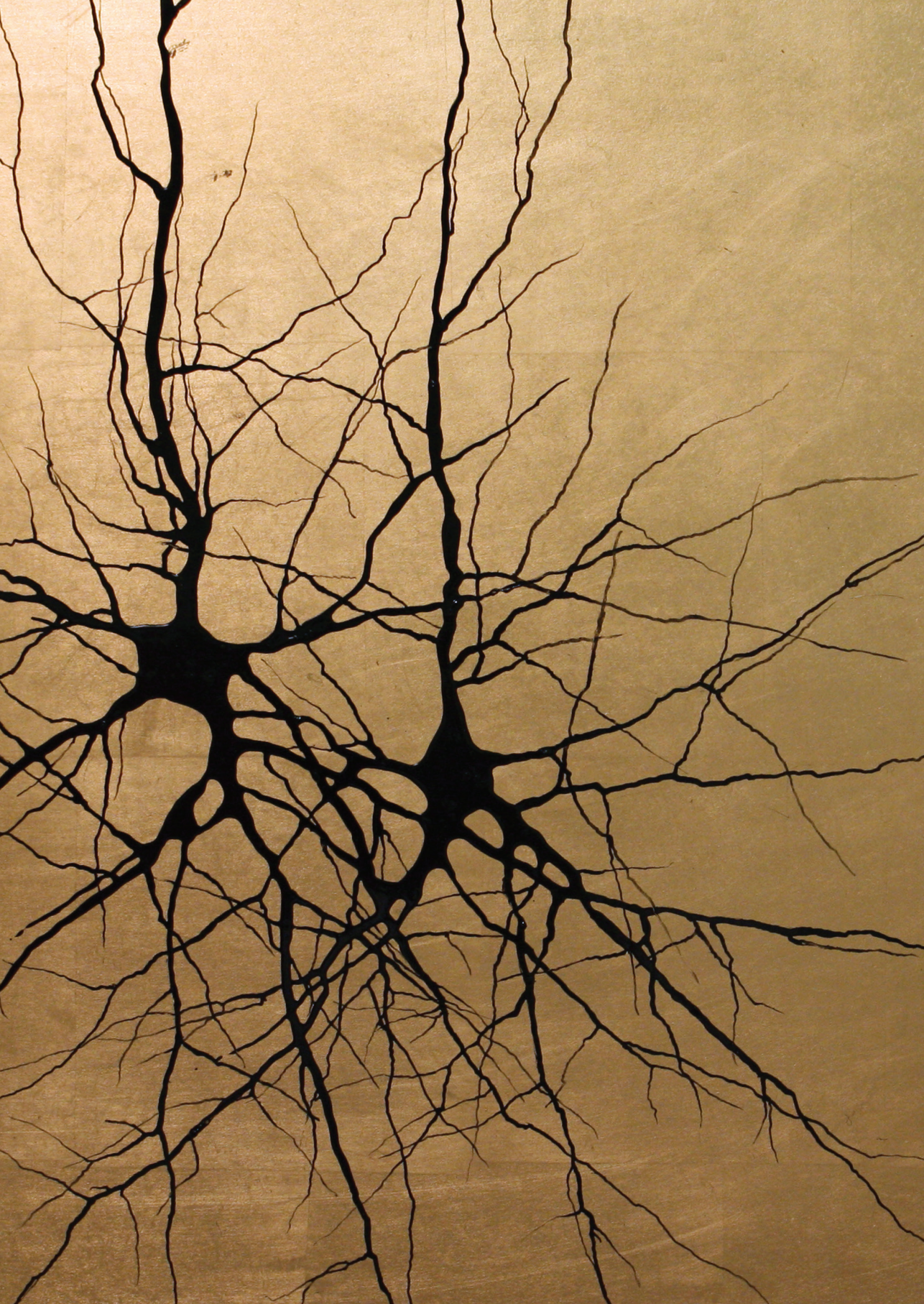
Shen K, Meyer T (1999) Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. Science 284:162–166.

Shen K, Teruel MN, Subramanian K, Meyer T (1998) CaMKIIbeta functions as an F-actin targeting module that localizes CaMKIIalpha/beta heterooligomers to dendritic spines. Neuron 21:593–606.

Thullier F, Lalonde R, Mahler P, Joyal CC, Lestienne F (1996) Dorsal striatal lesions in rats. 1: Effects on exploration and motor coordination. Arch Physiol Biochem 104:300–306.

Van Woerden GM, Hoebeek FE, Gao Z, Nagaraja RY, Hoogenraad CC, Kushner SA, Hansel C, De Zeeuw CI, Elgersma Y (2009) betaCaMKII controls the direction of plasticity at parallel fiber-Purkinje cell synapses. Nature Publishing Group 12:823–825.

Vogt MA, Chourbaji S, Brandwein C, Dormann C, Sprengel R, Gass P (2008) Suitability of tamoxifen-induced mutagenesis for behavioral phenotyping. Exp Neurol 211:25–33.



Chapter 4

CAMK2-dependent signaling in neurons is essential for survival

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ABSTRACT

Ca^{2+}/Calmodulin-dependent protein kinase II (CAMK2) is a key player in synaptic plasticity and memory formation. Mutations in either *Camk2a* or *Camk2b*, cause intellectual disability in humans, and severe plasticity and learning deficits in mice, indicating the presence of unique functions for each isoform. However, considering the high homology between CAMK2A and CAMK2B, it is conceivable that for critical functions, one isoform compensates for the absence of the other isoform, and that the full functional spectrum of neuronal CAMK2 remains to be revealed.

Here we show that deletion of both CAMK2 isoforms at germline or adult age is lethal. Moreover, Ca^{2+}-dependent activity as well as autonomous activity of CAMK2 is crucial for survival. Together, these results reveal an essential role for CAMK2 signalling in early postnatal development as well as in adulthood, and indicate that the full spectrum of CAMK2 requirements is masked due to partial overlapping functions of CAMK2A and CAMK2B.

Significance statement:

CAMK2A and CAMK2B have been studied for over 30 years for their role in neuronal functioning. However, most studies were performed using single knockout mice. Since the two isoforms show high homology with respect to structure and function, it is likely that some redundancy exists between the two isoforms, meaning that for critical functions CAMK2B compensates for the absence of CAMK2A and *vice versa*, leaving these functions to uncover. In this study, we generated CAMK2A/CAMK2B double-mutant mice, and observed that loss of CAMK2, as well as the loss of Ca^{2+}-dependent and Ca^{2+}-independent activity of CAMK2 is lethal. These results indicate that despite 30 years of research the full spectrum of CAMK2 functioning in neurons remains to be unraveled.

INTRODUCTION

Since the discovery of the CAMK2 protein family in the 1970s, more than 2000 papers have been published in which the function of CAMK2A or CAMK2B, the most abundant CAMK2 isoforms in the brain, has been studied. The generation of different *Camk2a* mutants (of which the knockout was already published 25 years ago (Silva et al., 1992a; 1992b)) and *Camk2b* mutants, greatly contributed to the understanding of the role of these 2 isoforms in neuronal functioning, learning and plasticity in mice (Mayford et al., 1995; Giese et al., 1998a; Elgersma et al., 2002; Borgesius et al., 2011; Achterberg et al., 2014; Kool et al., 2016). Very recently, the important role of CAMK2A and CAMK2B for normal human neurodevelopment was shown (Küry et al., 2017; Stephenson et al., 2017; Akita et al., 2018).

CAMK2 forms a holoenzyme of approximately 12 subunits, which can consist of both CAMK2A and CAMK2B subunits. This CAMK2 holoenzyme is able to convert a short high frequency signal into a long-term change in synaptic strength (for review see (Lisman et al., 2002; Hell, 2014)). Upon calcium influx Ca^{2+}/Calmodulin binds CAMK2 in the regulatory domain (Vallano, 1989). This binding allows the release of a pseudosubstrate region of the protein from the catalytic domain. When two adjacent subunits within the holoenzyme are activated by Ca^{2+}/Calmodulin, one subunit can phosphorylate the neighboring subunit on Thr286 (CAMK2A) or Thr287 (CAMK2B) leaving this subunit autonomously active (Ca^{2+}-independent activity) when calcium levels drop to baseline (Miller and Kennedy, 1986; Hanson et al., 1994). However, upon detachment of Ca^{2+}/Calmodulin from CAMK2, Thr305/Thr306 (CAMK2A) or Thr306/Thr307 (CAMK2B) within the calmodulin binding region can be phosphorylated thereby preventing future binding of Ca^{2+}/Calmodulin (thus Ca^{2+}-dependent activity).

The importance of these autophosphorylation events for CAMK2 function, was shown by generating CAMK2 point mutants, in which the Thr286 or Thr305/Thr306 were mutated to either phosphomimic residues (e.g. Thr305Asp), or phosphodead residues (e.g. Thr286Ala). All of these mutations resulted in learning and plasticity phenotypes (Mayford et al., 1995; Giese et al., 1998a; Elgersma et al., 2002). Besides an important enzymatic function, there are also studies showing that CAMK2A and CAMK2B play important structural roles. For example, CAMK2A has been shown to play an important structural role in the pre-synapse in short-term plasticity (Hojjati et al., 2007) and CAMK2B plays an important structural role in determining the localization of CAMK2A during hippocampal plasticity, through its F-actin binding domain (Borgesius et al., 2011). Thus, taken together many studies have shown unique functions for CAMK2A and CAMK2B in neuronal functioning. However, CAMK2A and CAMK2B are highly

homologous, thus it is conceivable that there is substantial redundancy in function, and that these functions of CAMK2 are missed when studying the *Camk2a* or *Camk2b* single mutants.

In this study we tried to reveal novel CAMK2 functions by studying different *Camk2a* /*Camk2b* double mutants. We found that *Camk2a*/*Camk2b* double knockout mice (*Camk2a*$^{-/-}$;*Camk2b*$^{-/-}$) die immediately after birth. Surprisingly, blocking Ca^{2+}-dependent (*Camk2a*$^{T305D/T305D}$;*Camk2b*$^{A303R/A303R}$) as well as Ca^{2+}-independent (*Camk2a*$^{286A/286A}$;*Camk2b*$^{287A/287A}$) activity in both CAMK2A and CAMK2B is also lethal, since these mice also die immediately after birth. Additionally, not only germline deletion of *Camk2*, but also loss of both CAMK2A and CAMK2B in adulthood is lethal, with a median survival of 37 days after gene deletion initiation. When looking at neuronal function in the absence of CAMK2 in the inducible *Camk2a*/*Camk2b* double knockout mice (*Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR}), we found that loss of both isoforms in adult animals leads to a complete abolishment of CA3-CA1 LTP but that the absence of both CAMK2 isoforms does not affect basal synaptic strength. To investigate whether pre- or postsynaptic CAMK2 is essential for this loss of LTP, we used a mouse model with a genetic ablation of *Camk2a* and *Camk2b* specifically in the excitatory neurons of the CA3 area. This revealed that presynaptic CAMK2A and CAMK2B are not required for LTP. Taken together we show that CAMK2 is crucial for survival during early postnatal development as well as adulthood. This indicates that despite the enormous wealth of literature on CAMK2 functions, its full spectrum is still not uncovered and that the role of CAMK2 signaling in neurons is much more important than was previously thought.

MATERIAL AND METHODS

Animals

In this study the following mice were used: *Camk2a*$^{-/-}$ (Camk2a^{tm3Sva}, MGI:2389262) and *Camk2b*$^{-/-}$ mice to generate *Camk2a*$^{+/+}$;*Camk2b*$^{+/+}$ (WT mice); *Camk2a*$^{+/-}$;*Camk2b*$^{-/-}$ (mice heterozygous for *Camk2a* and knockout for *Camk2b*); *Camk2a*$^{-/-}$;*Camk2b*$^{+/-}$ (mice knockout for *Camk2a* and heterozygous for *Camk2b*); *Camk2a*$^{-/-}$;*Camk2b*$^{-/-}$ (*Camk2a* and *Camk2b* double knockout mice); *Camk2a*$^{T286A/T286A}$ (Camk2a^{tm2Sva}, MGI:2158733) and *Camk2b*$^{T287A/T287A}$ mice to generate *Camk2a*$^{+/T286A}$;*Camk2b*$^{T287A/T287A}$ (mice heterozygous for a T286A knock-in mutation in *Camk2a* and homozygous for a T287A knock-in mutation in *Camk2b*); *Camk2a*$^{T286A/T286A}$;*Camk2b*$^{+/T287A}$ (mice homozygous for T286A in *Camk2a* and heterozygous for T287A in *Camk2b*); *Camk2a*$^{T286A/T286A}$;*Camk2b*$^{T287A/T287A}$ (homozygous T286A and T287A knock-in mutations in *Camk2a* and *Camk2b* respectively); *Camk2a*$^{T305D/T305D}$ (Camk2a^{tm5Sva}, MGI: 2389272) and *Camk2b*$^{A303R/A303R}$ (Camk2b$^{tm2.1Yelg}$, MGI:5285573)

mice to generate *Camk2a*$^{+/T305D}$;*Camk2b*$^{A303R/A303R}$ (mice heterozygous for a T305D knock-in mutation in *Camk2a* and homozygous for a A303R knock-in mutation in *Camk2b*); *Camk2a*$^{T305D/T305D}$;*Camk2b*$^{+/A303R}$ (mice homozygous for T305D in *Camk2a* and heterozygous for A303R in *Camk2b*); *Camk2a*$^{T305D/T305D}$;*Camk2b*$^{A303R/A303R}$ (homozygous T305D and A303R knock-in mutations in *Camk2a* and *Camk2b* respectively); *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ (homozygous floxed *Camk2a* (Camk2a$^{tm1.1Yelg}$, MGI:5662417) and *Camk2b* mice with no *Cre* expression; controls); *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} (homozygous floxed *Camk2a* and *Camk2b* mice with transgenic *Cre* expression throughout the body after injection with tamoxifen (Tg(CAG-cre/Esr1*)5Amc; MGI:2182767) and *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CA3-Cre* (knockout mutants for *Camk2a* and *Camk2b* specifically in the CA3 region of the hippocampus (Grik4$^{tm1.1(cre)Slab}$; MGI: 4398684).

All mice were backcrossed >16 times in a C57BL/6J background and were group-housed in IVC cages (Sealsafe 1145 T, Tecniplast) with bedding material (Lignocel BK 8/15 from Rettenmayer) on a 12/12 h light/dark cycle in 21 °C (±1 °C), humidity at 40–70% and with chow (No 1 maintenance autoclave pellets, Special Diets Services) and water available *ad libitum*. Experimenters were blind to all genotypes throughout experiments and data analysis. Mice were genotyped when they were 7 days old, and re-genotyped after the mice were sacrificed. Genotyping records were obtained and kept by a technician not involved in the experimental design, performance and analysis. All experiments were done during the light phase, with animals between 2 and 4 months of age. All experiments were done with approval of the local Dutch Animal Ethical Committee (DEC) for animal research and were in accordance with the European Communities Council Directive (86/609/EEC).

Generation of mouse mutants

The generation of both the floxed and knockout *Camk2a* (Elgersma et al., 2002; Achterberg et al., 2014) and *Camk2b* (Borgesius et al., 2011; Kool et al., 2016) mouse mutants has been described previously. All knock-in mutants used in this study have been published before as well: *Camk2a*^{T286A} (Giese et al., 1998a); *Camk2a*^{T305D} (Elgersma et al., 2002); *Camk2b*^{T287A} (Kool et al., 2016); *Camk2a*^{A303R} (Borgesius et al., 2011). To generate a CA3 specific deletion of the *Camk2a* and *Camk2b* genes we crossed *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice with *Grik4-Cre-Neo* (Grik4$^{tm1.1(cre)Slab}$; MGI: 4398684, kindly provided by Ralf Schoepfer and York Rudhard) (Filosa et al., 2009) (in this study referred to as *CA3-Cre* mice). *CA3-Cre* expression starts as early as P5 and is predominantly restricted to the CA3 area of the hippocampus. To make sure that full deletion of the gene of interest had taken place, experiments were started at a minimum age of 8 weeks.

Tamoxifen injections

Adult *Camk2a$^{f/f}$;Camk2b$^{f/f}$*, *and Camk2a$^{f/f}$;Camk2b$^{f/f}$;CAG-Cre^{ESR}* mice (8-10 weeks of age) were intraperitoneally injected with Tamoxifen (Sigma-Aldrich) (0.1mg/g of bodyweight) for 8 consecutive days. To keep the dose of Tamoxifen constant throughout injection days we kept a tight injection scheme, injecting mice 24+/–1 hour after the previous injection. Tamoxifen was dissolved in sunflower oil (20mg/ml). For electrophysiological experiments we sacrificed adult mice (12-16 weeks old) 25 days after the 1st Tamoxifen injection. Though Tamoxifen is not known to have an effect on emotional reactivity, neurological functioning or learning (Vogt et al., 2008) we injected both *Camk2a$^{f/f}$;Camk2b$^{f/f}$* and *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CAG-Cre^{ESR}* mice to control for any possible effects of Tamoxifen.

EEG recordings

The surgical procedure for EEG recordings have been described previously (Abs et al., 2013). At the onset of measurements, mice were connected to a wireless EEG recorder (NewBehavior AG, Zurich, Switzerland) and recorded for approximately 16 hours. Recordings were analysed by an observer blind to genotype and were only checked for the onset of seizures, a pattern of repetitive spike discharge followed by a progressive evolution in spike amplitude, which were not observed in any of our recordings.

Electrophysiology

Mice were sacrificed after being anesthetized with isoflurane (Nicholas Piramal (I) Limited, London) and the brain was taken out quickly and submerged in ice-cold oxygenated (95%) and carbonated (5%) artificial cerebrospinal fluid (ACSF) (<4.0°) containing the following substances (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. Using a vibratome 400-μm-thick sagittal slices were made for CA3-CA1 experiments and 400-μm-thick coronal slices for CA3-CA3 experiments. Hippocampal sections were dissected out afterwards and maintained at room temperature for at least 1.5 hours in an oxygenated and carbonated bath to recover before experiments were initiated. At the onset of experiments hippocampal slices 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 30°C. Extracellular recording of field EPSP (fEPSPs) and stimulation were done using bipolar platinum (Pt)/iridium (Ir) electrodes (Frederick Haer Companu, Bowdoinham, ME). Stimulus duration of 100µs for all experiments was used. In CA3-CA1 measurements, the stimulating electrode and recording electrode were placed on the CA3-CA1 Schaffer collateral afferents and apical dendrites of CA1 pyramidal cells (both 150-200µm from s. pyramidale) respectively. In CA3-CA3 measurements both the stimulating electrode and recording electrode were

placed on the stratum radiatum of the CA3 area. The stratum lucidum was carefully avoided. Upon placement of the electrodes slices were given 20-30 minutes to rest before continuing measurements. All paired-pulse facilitation (PPF) experiments were stimulated at one-third of slice maximum. Varying intervals were used in PPF: 10, 25, 50, 100, 200 and 400ms. CAMK2-dependent LTP was evoked using four different tetani: (i) 100 Hz (1 train of 1 second at 100 Hz, stimulated at one-third of slice maximum), (ii) 200 Hz (4 trains of 0.5 seconds at 200 Hz, spaced by 5s), stimulated at one-third of slice maximum), (iii) Theta burst (2 trains of 4 stimuli at 100 Hz, 200 ms apart, stimulated at two-thirds of slice maximum) and (iv) CA3-CA3 LTP (2 trains of 1 second at 100 Hz 10 seconds apart, stimulated at one-third of slice maximum). A possible caveat in fEPSP measurements in the CA3 area is distinguishing between the mossy fiber pathway and the commissural (CA3-CA3) pathway. Therefore, we took several measures to make sure we recorded from the commissural pathway. First, we used antidromic stimulation in the CA3 area. Second, we made use of the electrophysiological parameters PPF and 1Hz frequency facilitation that differ between these two pathways. Mossy fiber transmission shows very strong facilitation (± 215% for PPF and ± 250% for 1 Hz) (Scanziani et al., 1997), hence, we chose an upper limit of 180% for PPF and 130% for 1Hz, and excluded all slices exceeding those limits. Finally, at the end of all experiments we used a pharmacological approach using DCG-IV (3mM) to distinguish between both pathways. DCG-IV (3mM) is known to reduce mossy fiber transmission by 80% (Kirschstein et al., 2004). This way, we felt confident that we only included data from experiments where we specifically stimulated CA3-CA3 synapses. Chemical LTD was induced using a 5-minute wash-in of DHPG (100 µM; TOCRIS Biosciences) 20 mins after establishing a stable baseline. For PKA-dependent LTP a similar baseline was established before chemical induction. We added picrotoxin (50uM) to the ACSF throughout the experiment and LTP was induced chemically (cLTP) by bath application of picrotoxin (50uM), Forskolin (50uM) and Rolipram (0.1uM) for 15 minutes, after which bath circulation was returned to ACSF with only picrotoxin (50uM). During LTP slices were stimulated once per minute. Potentiation was measured as the normalized increase of the mean fEPSP slope for the duration of the baseline. During induction of chemical LTP slices were stimulated at half of slice maximum. Only stable recordings were included and this judgment was made blind to genotype. Average LTP was defined as the mean last 10 minutes of the normalized fEPSP slope.

Western Blot

Mice were anaesthetized using isoflurane and sacrificed by decapitation. Brain samples (or acute hippocampal slices in the case of the western blots after the electrophysiology experiments) were taken out quickly and stored in liquid nitrogen. Lysates were then

first prepared and brain samples were homogenized in lysis buffer (10mM Tris-HCl 6.8, 2.5% SDS, 2mM EDTA). Protein concentration in the samples was determined and lysate concentrations were adjusted to 1mg/ml. Western blots were probed with primary antibodies against either CAMK2A (6G9, 1:40.000, Abcam), CAMK2B (CB-β1, 1:10.000, Invitrogen) or Actin (MAB1501R, 1:20.000, Chemicon) and secondary antibodies (goat anti-mouse and/or goat anti-rabbit, both 1:3000, AffiniPure #115-007-003 and #111-007-003). Blots were stained either with Enhanced ChemoLuminescence (ECL) (#32106, Pierce) or stained and quantified using LI-COR Odyssey Scanner and Odyssey 3.0 software. Quantification of western blot in ECL was done using ImageJ.

Immunohistochemistry and immunofluorescence

First mice were anaesthetized with pentobarbital and perfused transcardially with PBS followed by freshly prepared 4% paraformaldehyde solution (PFA, Sigma). Brains were taken out after perfusion and post-fixed for 1.5 hours in PFA and afterwards kept in 30% sucrose solution overnight. Free floating 40 µm thick frozen sections were made and for immunohistochemistry, a standard avidin-biotin-immunoperoxidase complex method (ABC, Vector Laboratories, USA) with CAMK2A (MAB3119, 1:10,000; Chemicon) as the primary antibody and diaminobenzidine (0.05%) as the chromogen was used. For immunofluorescence, free floating 40 µm thick sections were washed in PBS once and afterwards primary antibodies were added (anti-CAMK2B, 1:1000, #ab34703, Abcam for immunofluorescence; anti-CAMK2A, 1:10,000, MAB3119, Chemicon for immunohistochemistry) diluted in PBS containing 2% NHS, 0.5% Triton-X 100 and 150mM bovine serum albumin (BSA) and kept at 4°C overnight for 48 hours. Two days later sections were washed 3 times with PBS and secondary antibodies were added (Cy3 rabbit, 1:200 for immunofluorescence) diluted in PBS containing 2% NHS, 0.5% Triton-X 100 and 150mM bovine serum albumin (BSA). After 1-2 hours incubation of the secondary antibody at room temperature sections were washed four times in PB (0.05M) and mounted on slides using chrome(3)potassiumsulfatedodecahydrate and left to dry. Finally, for immunofluorescence, sections were covered using Mowiol (Sigma-Aldrich). For immunohistochemistry, the slices were, after drying, dehydrated in alcohol, cleared with xylene and covered using Permount (Fisher Scientific, USA).

Half-life calculations

For the protein degradation curves, mice received Tamoxifen injections and were killed 4, 8, 10, 12, 15, 18, 21 and 24 days after the start of the experiment (n=2 for each time point). Protein levels were measured using Western Blot and data was plotted using Prism data analysis software (Prism Inc.).

Data analysis and statistics

All statistical tests were performed using a 2-WAY repeated measures ANOVA to determine the effect of genotype in the experiments. In LTP experiments, the last 10 data points were used for comparison. α was set at 0.05. All values represent average +/- SEM. Group sizes can be found in the figure legends. All values are based on number of slices measured. Each experimental group contained at minimum 4 different mice. All statistical tests were performed either using Graphpad Prism or SPSS Statistics v22.0.

RESULTS

Loss of both CAMK2A and CAMK2B results in neonatal death

To unravel the full spectrum of CAMK2 functions, the CAMK2A/CAMK2B double heterozygous mice (Camk2a$^{-/+}$;Camk2b$^{-/+}$) were intercrossed to obtain F2 CAMK2A/CAMK2B double knockout mice (*Camk2a*$^{-/-}$;*Camk2b*$^{-/-}$)(see Methods). Genotyping performed at day 7 on 222 pups, revealed 0 *Camk2a*$^{-/-}$;*Camk2b*$^{-/-}$ double mutants, whereas 14 pups were expected based on a Mendelian distribution (Figure 1a), indicating that the double mutant might be lethal. Moreover, all of the *Camk2a*$^{+/-}$;*Camk2b*$^{-/-}$ mice died within 42 days after birth, whereas only 28% of the *Camk2a*$^{-/-}$;*Camk2b*$^{+/-}$ died within the same period, indicating that complete loss of CAMK2B is less well tolerated than complete loss of CAMK2A. Importantly, other genotypes obtained by this breeding (such as *Camk2a*$^{+/-}$;*Camk2b*$^{+/-}$ mice, data not shown) appeared just as vital as wild-type mice. To understand whether the lethality of *Camk2a*$^{-/-}$;*Camk2b*$^{-/-}$ mice was pre- or postnatal, mice were monitored immediately from birth on. We observed that a small number of the born pups died within the first day after birth, which all appeared to be *Camk2a*$^{-/-}$;*Camk2b*$^{-/-}$ upon genotyping. Taken these pups into account, we found that 6 out of 100 pups were *Camk2a*$^{-/-}$;*Camk2b*$^{-/-}$ double mutants, which is the expected number of double mutants, indicating that the *Camk2a*$^{-/-}$;*Camk2b*$^{-/-}$ mutants are born at normal frequency (c^2: 9.4, p=0.31), but die within one day after birth. When observing the pups directly after birth it was not possible to predict which pup would die, since the *Camk2a*$^{-/-}$;*Camk2b*$^{-/-}$ pups did not show notable growth retardation or morphological changes, and a milk spot was visible in the abdomen, indicating that it was not lack of food intake that killed the pups. Additionally, immunohistochemistry showed no gross morphological changes in brains of pups on P0 (data not shown). Taken together, this shows that simultaneous loss of both CAMK2A and CAMK2B results in neonatal death, indicating a critical role of CAMK2 during this period, which cannot be revealed by studying the CAMK2 isoforms in isolation.

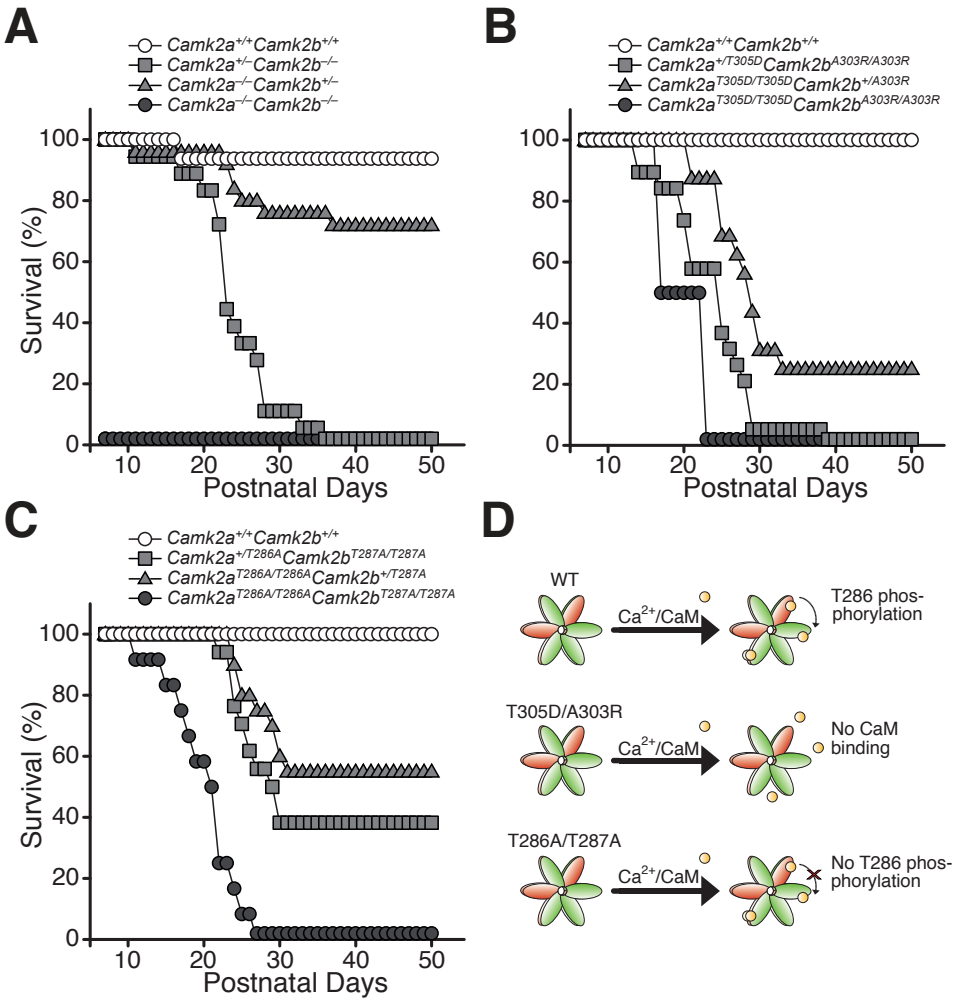


Figure 1: Multiple *Camk2a* mutants crossed with *Camk2b* mutants and their survival in percentage of their total group size. ***a,*** Double knockout mice for both *Camk2a* and *Camk2b* (*Camk2a*$^{-/-}$*Camk2b*$^{-/-}$) die on P0. Homozygosity for *Camk2b* (with one functioning allele of *Camk2a*; *Camk2a*$^{+/-}$*Camk2b*$^{-/-}$, n=18) has a more severe impact on survival than homozygosity for *Camk2a* (and one functioning allele of *Camk2b*; *Camk2a*$^{-/-}$*Camk2b*$^{+/-}$, n=25). *Camk2a*$^{+/+}$*Camk2b*$^{+/+}$ were used as controls (n=16). ***b,*** Homozygous loss of Ca^{2+}-dependent activity of both CAMK2A and CAMK2B (*Camk2a*$^{T305D/T305D}$*Camk2b*$^{A303R/A303R}$, n=2) results in early death. Homozygosity for a A303R knock-in mutation in *Camk2b* and a heterozygous T305D knock-in mutation for *Camk2a* (*Camk2a*$^{+/T305D}$*Camk2b*$^{A303R/A303R}$, n=18) has a more severe impact on survival than a homozygous knock-in mutation for *Camk2a* and a heterozygous A303R knock-in mutation for *Camk2b* (*Camk2a*$^{T305D/T305D}$*Camk2b*$^{+/A303R}$, n=15). *Camk2a*$^{+/+}$*Camk2b*$^{+/+}$ were used as controls (n=4). ***c,*** Homozygous loss of autonomous activity of both CAMK2A and CAMK2B (*Camk2a*$^{T286A/T286A}$*Camk2b*$^{T287A/T287A}$, n=12) results in early death. Again, homozygosity for a T287A knock-in mutation in *Camk2b* (and a heterozygous T286A knock-in mutation for *Camk2a*; *Camk2a*$^{+/}$

^{T286A}*Camk2b*$^{T287A/T287A}$, n=34) has a more severe impact on survival than a homozygous knock-in mutation for *Camk2a* (and a heterozygous T287A knock-in mutation for *Camk2b*; *Camk2a*$^{T286A/T286A}$*Camk2b*$^{+/T287A}$, n=20). *Camk2a*$^{+/+}$*Camk2b*$^{+/+}$ were used as controls (n=14). With the exception of 1 mouse in the first experiment (***a***), all *Camk2a*$^{+/+}$*Camk2b*$^{+/+}$ mice survived a minimum of up to 50 days postnatally. ***d,*** Model showing the effect of the different mutations used on the activity of the holoenzyme for the survival experiments. Green = CAMK2A, red = CAMK2B and yellow = Calcium/Calmodulin.

Loss of Ca^{2+}-independent and Ca^{2+}-dependent activity of CAMK2 results in neonatal death

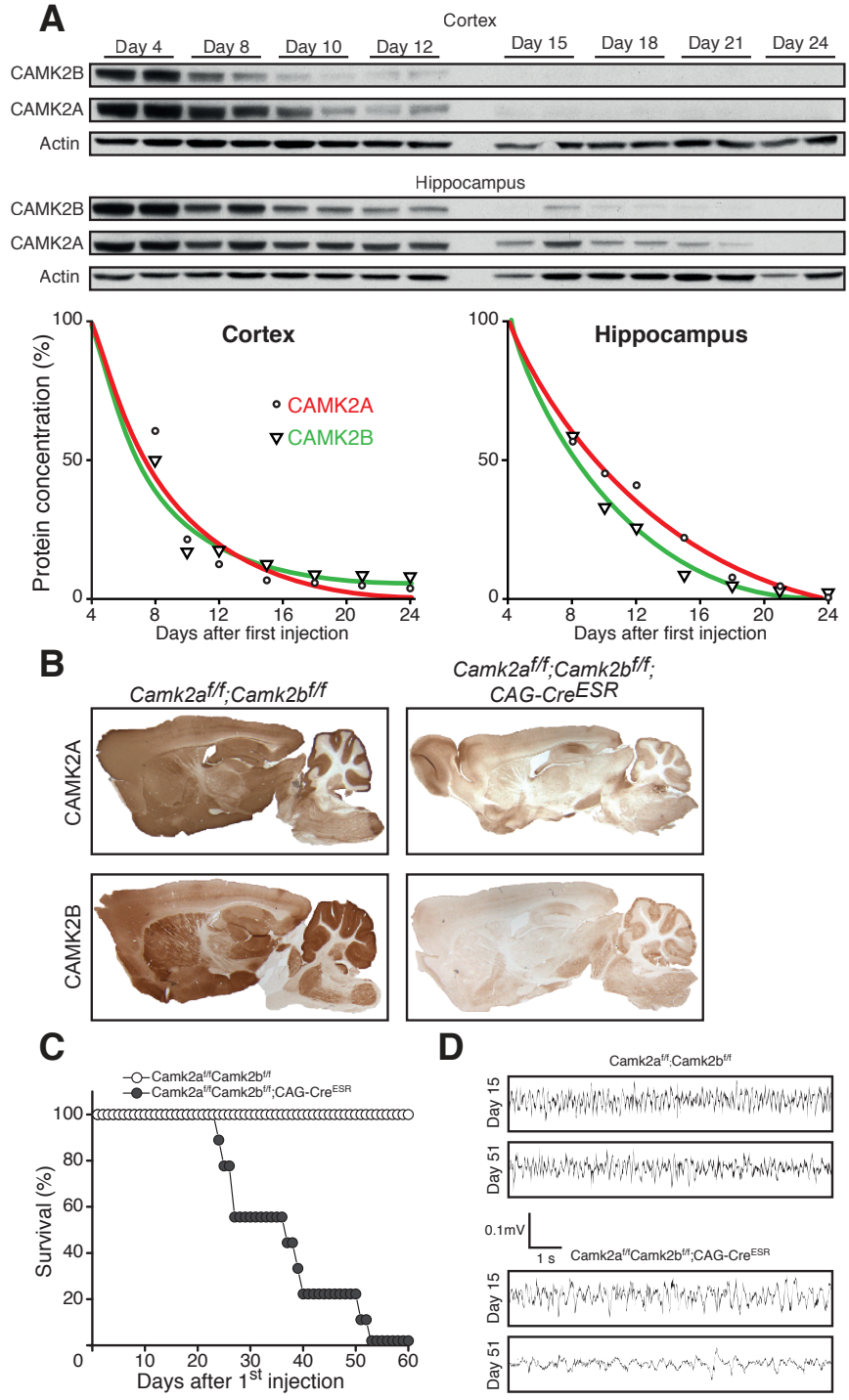
Activity of CAMK2 is governed by multiple phosphorylation sites, of which the Thr286 (Thr287 in CAMK2B) is important for Ca^{2+}-independent activity (Figure 1d, bottom panel) and Thr305/Thr306 (Thr306/Thr307 in CAMK2B) for the Ca^{2+}-dependent activity (Figure 1d, middle panel), since they are located within the Ca^{2+}/Calmodulin binding site on CAMK2. Considering the phenotypes of the *Camk2a* and *Camk2b* single mutants, we know that the CAMK2A phosphomimic mutation at Thr305 (CAMK2A-T305D), which blocks Ca^{2+}/Calmodulin binding and keeps CAMK2 in its inactivated state, is more detrimental than not having CAMK2A at all (Elgersma et al., 2002). This is also the case for a similar mutation in CAMK2B (CAMK2B-A303R) with respect to locomotion (Kool et al., 2016), although this is not the case for hippocampal learning (Borgesius et al., 2011). Hence, we expected that a double mutant of CAMK2A-T305D and CAMK2B-A303R might be lethal as well. Indeed, when intercrossing *Camk2a*$^{+/T305D}$;*Camk2b*$^{+/A303R}$ double mutant mice we found that upon P7, only 2 *Camk2a*$^{T305D/T305D}$;*Camk2b*$^{A303R/A303R}$ mutants were found in a total of 126 pups (number expected was 8). The 2 pups that survived until P7 still died a premature death within 16-23 days after birth (Figure 1b). Similar to the CAMK2A;CAMK2B double mutants, homozygous mutations in the Ca^{2+}/Calmodulin binding of CAMK2B were less well tolerated than comparable mutations in CAMK2A (Figure 1b).

We then tested whether Ca^{2+}-independent activity (also known as autonomous activity) was essential for life. To that end, we intercrossed *Camk2a*$^{+/T286A}$;*Camk2b*$^{+/T287A}$ double mutant mice, such that autophosphorylation of both CAMK2A and CAMK2B at the Thr286/287 site is prevented (Giese et al., 1998a; Kool et al., 2016). Surprisingly, despite the fact that these mice still have Ca^{2+}-dependent activity, we found that the *Camk2a*$^{T286A/T286A}$;*Camk2b*$^{T287A/T287A}$ mice started dying from P11 onwards and had all died by P33 (Figure 1c). Again homozygous mutations in CAMK2B were less well tolerated than comparable mutations in CAMK2A. This indicates that CAMK2 autonomous activity is essential for survival.

Adult deletion of CAMK2A and CAMK2B is lethal

The premature death observed in the various CAMK2A/CAMK2B double mutants described above, indicate a crucial role for CAMK2-dependent signaling during development. Using inducible *Camk2a* and *Camk2b* knockout mice, we have recently shown that CAMK2-dependent signaling is also important after brain development (Achterberg et al., 2014; Kool et al., 2016). Notably, the phenotypes observed when deleting the *Camk2a* gene in adult mice are as severe as when deleting the gene at germline (Achterberg et al., 2014). Therefore we postulated that deletion of both CAMK2A and CAMK2B could potentially also be lethal in adult mice. We generated inducible *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice which were injected with Tamoxifen at 8 weeks of age to induce deletion of both *Camk2a* and *Camk2b*. Up till 4 days after the first injection, the protein levels remained the same, after that time point the levels reduced exponentially. Both CAMK2A and CAMK2B showed similar half-lives and decay constants in the cortex (CAMK2A: half-life: 3.5 days; CAMK2B: half-life: 2.8 days) as well as in the hippocampus (CAMK2A: half-life: 5.3 days; CAMK2B: half-life: 4.4, Figure 2a). Immunohistochemical stainings at 21 days after gene deletion showed that despite a few CAMK2A or CAMK2B positive cells, most brain areas were devoid of CAMK2 staining (Figure 2b). Around 15-19 days (depending on the brain region) after the first injection, the levels of CAMK2A and CAMK2B dropped below 10%, after which the *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice started to die. All injected *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice died within 24-53 days, with a median survival of 37 days (Figure 2c). When observing the mice we found that the mice did not show any obvious alterations in behavior until their last 24 hours, during which they would stop moving, eating and drinking.

Figure 2: Adult loss of CAMK2A and CAMK2B is lethal but does not lead to epileptic activity. (A) Western blot of cortical (top) and hippocampal (bottom) lysates using antibodies targeted against CAMK2A and CAMK2B. Actin was used as loading control. Days after first injection are indicated above the blots. Lower left graph: non-linear regression curve showing protein degradation in cortex, showing faster protein degradation of both CAMK2A and CAMK2B than in the hippocampus (n=2 for each time point). Lower right graph: non-linear regression curve showing protein degradation in hippocampus, where CAMK2B degradation is faster than CAMK2A degradation. **(B)** Immunohistological stainings showing effective loss after Tamoxifen injections of CAMK2A (*Top*) and CAMK2B (*Bottom*) in *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice 21 days after gene deletion. **(C)** Loss of both CAMK2A and CAMK2B (*Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR}) in adulthood is lethal. Both groups of mice (*Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} (n=9) versus *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ (n=8)) received Tamoxifen injections (see Methods). **(D)** Example traces of EEG recordings of *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} and *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ control mice. Despite the changing activity pattern in *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice, no seizure activity was detected in any recordings. The bottom trace is of a *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mouse that died less than 24 hours later.

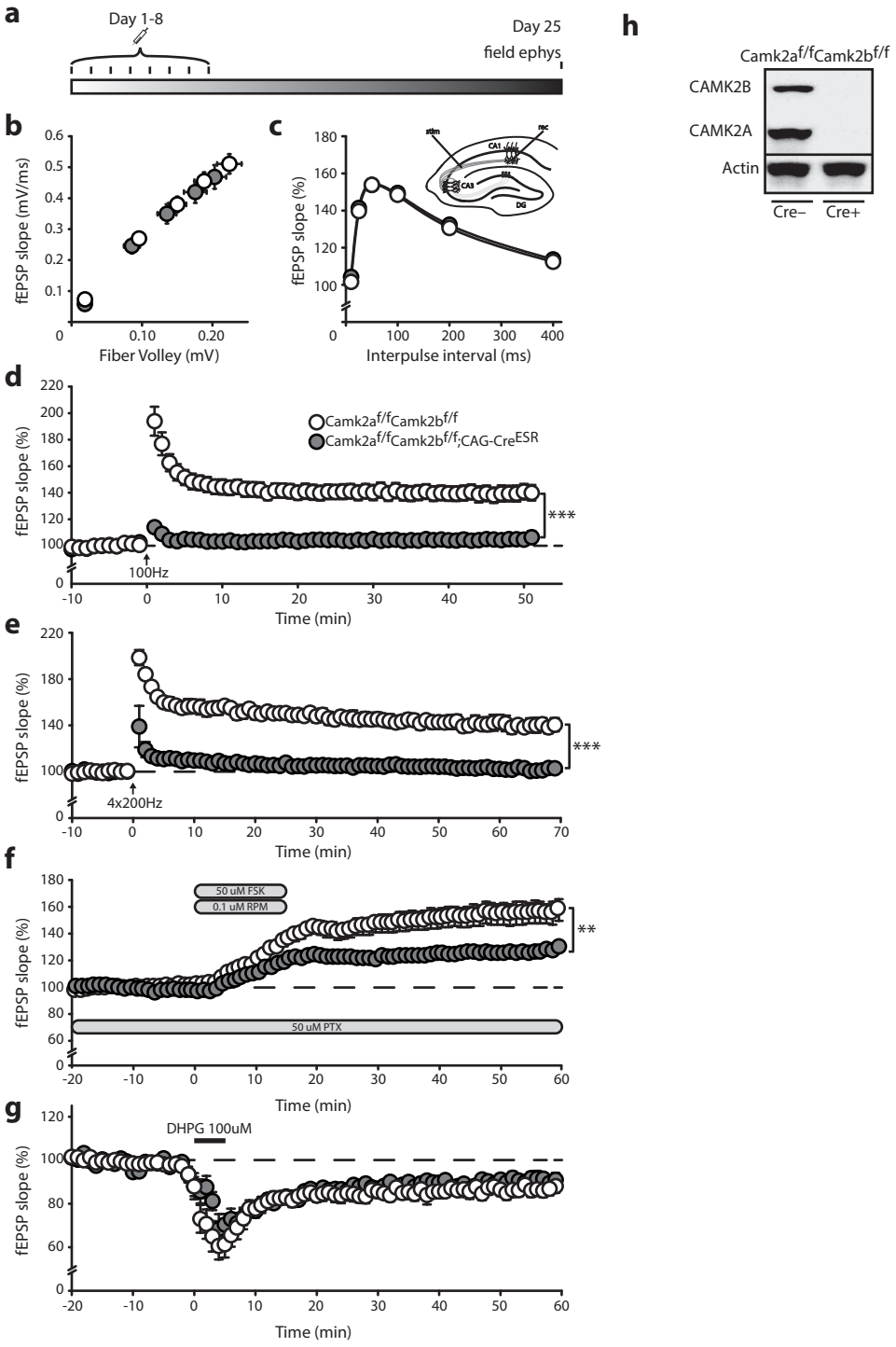

A
Cortex
Day 4
Day 8
Day 10
Day 12
Day 15
Day 18
Day 21
Day 24
CAMK2B
CAMK2A
Actin
Hippocampus
CAMK2B
CAMK2A
Actin
Cortex
Hippocampus
Protein concentration (%)
CAMK2A
CAMK2B
Days after first injection
B
Camk2a f/f;Camk2b f/f
Camk2a f/f;Camk2b f/f;
CAG-Cre ESR
CAMK2A
CAMK2B
C
Camk2a f/fCamk2b f/f
Camk2a f/fCamk2b f/f;CAG-Cre ESR
Survival (%)
Days after 1st injection
D
Camk2a f/f;Camk2b f/f
Day 15
Day 51
0.1mV
1 s
Camk2a f/fCamk2b f/f;CAG-Cre ESR
Day 15
Day 51

Importantly, *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice without *CAG-Cre*^{ESR} (control group) all survived. Even though we did not observe any seizures in the *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice, we performed continuous EEG recordings on a subset of the mice, to monitor epileptic activity more carefully. None of the tested mice showed epileptic activity in their EEG recordings (Figure 2d), also not just before death (Figure 2d, lower panel). Analysis of these brains obtained just after death did not reveal any gross morphology changes (Figure 2b). These data indicate that also in adult mice, CAMK2-dependent signaling is crucial for survival.

Loss of CAMK2A and CAMK2B completely abolishes LTP

We tested the effect of the combined loss of CAMK2A and CAMK2B on basal synaptic transmission and LTP. Thus far, previous reports on conventional and inducible *Camk2a* and *Camk2b* single knockout mice showed an impairment of LTP upon *Camk2* gene deletion with approximately 50% of residual LTP left compared to wild-type levels (Hinds et al., 1998; Elgersma et al., 2002; Borgesius et al., 2011; Achterberg et al., 2014). It is likely that the remaining fraction of LTP present in these mutant mice is provided by the remaining isoform present (CAMK2B in the case of *Camk2a* mutant mice and *vice versa*). To test this, we injected 8 week-old mice and chose 25 days after the first injection as the moment of sacrifice and electrophysiological testing, corresponding to

Figure 3: CAMK2A and CAMK2B are essential for CA3-CA1 LTP. (A) Timeline showing the loss of CAMK2A and CAMK2B upon induction of genomic deletion with Tamoxifen injections (see Methods). Mice were sacrificed 25 days after the first injection to conduct electrophysiological experiments. **(B)** *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice (fiber volley: (n=30, from 11 mice), fEPSP slope: (n=42, from 11 mice)) show normal basal synaptic transmission compared to *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice (fiber volley: (n=29, from 15 mice), fEPSP slope: (n=50, from 15 mice)). **(C)** *Inset*: Schematic overview of LTP induction in the CA3-CA1 pathway (also, see Methods). stim = stimulating electrode; rec = recording electrode; DG = dentate gyrus. *Figure*: *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice (n=40, from 11 mice) show normal PPF compared to *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice (n=51, from 15 mice). **(D)** *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice (n=16, from 6 mice) show a complete loss of 100Hz LTP compared to *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice (n=21, from 9 mice). **(E)** *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice (n=11, from 4 mice) show a complete loss of 200Hz LTP compared to *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice (n=9, from 5 mice). **(F)** *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice (n=28, from 7 mice) show impaired Forskolin/Rolipram-induced (50uM/0.1uM) LTP compared to *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice (n=29, from 7 mice). FSK = Forskolin; RPM = Rolipram; PTX = Picrotoxin **(G)** *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice (n=9, from 5 mice) show normal DHPG-induced (100uM) LTD compared to *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice (n=13, from 6 mice). **(H)** Western blot showing efficient loss of both CAMK2A and CAMK2B in the acute hippocampal slices of *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice with normal CAMK2A and CAMK2B expression in *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice. Actin levels are shown as loading control. Numbers depicted in brackets represent number of slices, followed by number of mice. Error bars indicate SEM.


a
Day 1-8
Day 25
field ephys
b
fEPSP slope (mV/ms)
Fiber Volley (mV)
c
fEPSP slope (%)
Interpulse interval (ms)
stim
rec
CA1
CA3
DG
d
fEPSP slope (%)
Camk2a$^{f/f}$Camk2b$^{f/f}$
Camk2a$^{f/f}$Camk2b$^{f/f}$;CAG-Cre^{ESR}
***
100Hz
Time (min)
e
fEPSP slope (%)
***
4x200Hz
Time (min)
f
fEPSP slope (%)
50 uM FSK
0.1 uM RPM
**
50 uM PTX
Time (min)
g
fEPSP slope (%)
DHPG 100uM
Time (min)
h
Camk2a$^{f/f}$Camk2b$^{f/f}$
CAMK2B
CAMK2A
Actin
Cre–
Cre+

the moment when CAMK2 levels have dropped to a minimum but well before most of these mice start dying, to keep confounding effects of dying on the LTP measurements to a minimum (Figure 3a). We measured basal synaptic transmission, paired-pulse facilitation (PPF), LTP and DHPG-induced LTD in the well-studied CA3-CA1 Schaffer collateral pathway in acute hippocampal slices. In agreement with the lack of gross brain morphology changes, *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice still showed normal basal synaptic transmission as fiber volley amplitude, fEPSP slope and their ratio did not differ significantly between both *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} and *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice (effect of genotype: fiber volley: $F_{1,57}$=0,53, p=0.47; fEPSP slope: $F_{1,90}$=0.74, p=0.39; repeated measures ANOVA; Figure 3b). Subsequently PPF was not impaired in *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice (effect of genotype: PPF: $F_{1,89}$=0.34, p=0.56; repeated measures ANOVA; Figure 3c). We then tested LTP by giving a 100Hz tetanus and found a complete abolishment of LTP 50 minutes after induction in *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice. (effect of genotype: 100Hz LTP: $F_{1,35}$=19.86, p<0.001; repeated measures ANOVA; Figure 3d). We then tested a much stronger LTP induction protocol (4 trains of 200Hz for 0.5 seconds, spaced 5 seconds apart) known to activate different pools of CAMK2 in the spines (Lee et al., 2009). This LTP induction protocol yields normal LTP in *Camk2b*$^{-/-}$ mice and only partially reduces LTP in *Camk2a*$^{-/-}$ mice (Borgesius et al., 2011). However, like in the 100 Hz LTP protocol, *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mutants showed complete absence of LTP in the in the 4 x 200Hz protocol (effect of genotype: 200Hz LTP: $F_{1,18}$=27.19, p<0.001; repeated measures ANOVA; Figure 3e). To investigate if other LTP inducing pathways were similarly affected, we tested PKA-dependent plasticity, using a 15-minute wash-in of Forskolin (50uM) and Rolipram (0.1uM) in the presence of Picrotoxin (50uM) to induce LTP chemically (cLTP). We found that also this LTP pathway was affected in the *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice, although considerable potentiation was still observed (effect of genotype: PKA LTP: $F_{1,55}$=9.75, p<0.01; repeated measures ANOVA; Figure 3f). Finally, we found no involvement of CAMK2A and CAMK2B in DHPG-induced LTD (effect of genotype: DHPG LTD: $F_{1,20}$=1.05, p=0.32; repeated measures ANOVA; Figure 3g). As a control for the efficiency of gene deletion, we performed western blot analysis on the acute hippocampal slices used in these experiments. As expected, the slices of *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice showed a clear absence of CAMK2A and CAMK2B (Figure 3h).

Presynaptic CAMK2 is dispensable for CA3-CA1 LTP

CAMK2 was originally found as a presynaptic protein, involved in the phosphorylation of Synapsin I (DeLorenzo et al., 1979; Kennedy and Greengard, 1981; Kennedy et al., 1983). Additionally, more recent literature shows involvement of CAMK2A in vesicle release and short-term presynaptic plasticity as well as a role for presynaptic CAMK2 in

LTP in culture conditions (Llinás et al., 1985; Lin et al., 1990; Nichols et al., 1990; Chapman et al., 1995; Hinds et al., 2003; Ninan and Arancio, 2004; Lu and Hawkins, 2006; Hojjati et al., 2007; Jiang et al., 2008; Pang et al., 2010; Achterberg et al., 2014). Therefore, it is likely that loss of both presynaptic as well as postsynaptic CAMK2 contributes to the LTP deficits described above. To investigate the requirement of presynaptic CAMK2 for LTP induction, we deleted *Camk2* in the CA3 region of the hippocampus, without affecting CAMK2 expression in the other hippocampal regions, by crossing *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice with a *Cre*-line in which Cre is under the control of the GRIK4 promotor (Glutamate Ionotropic Receptor Kainate Type Subunit 4). This gene is highly expressed in CA3 neurons, but absent in CA1 neurons (Filosa et al., 2009). We confirmed the specificity of this cre-line in *Camk2a*$^{f/f}$;*CA3-Cre* and *Camk2b*$^{f/f}$;*CA3-Cre* mice. These mice showed specific deletion of CAMK2A and CAMK2B in the pyramidal cells of the CA3 region of the hippocampus at the age of 8 weeks, with no deletion in other parts of the hippocampus (Figure 4a). Note that the mossy fibers coming from the dentate gyrus still express CAMK2A and can now be readily observed crossing through the CA3 area. We used immunofluorescence to confirm the deletion of CAMK2B in these mice. Although confirmation of the deletion of CAMK2B is complicated by the residual expression of CAMK2B in oligodendrocytes (Cahoy et al., 2008; Waggener et al., 2013) and interneurons (Lamsa et al., 2007), we could still observe a decrease in fluorescence in the stratum pyramidale of the CA3 region of the hippocampus (Figure 4a).

As we observed in the *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice, synaptic transmission and PPF were not affected in the absence of presynaptic CAMK2A and CAMK2B (effect of genotype: fiber volley: $F_{1,39}$=0.15, p=0.70; fEPSP: $F_{1,39}$=0.89, p=0.35; PPF: $F_{1,83}$=0.89, p=0.35; repeated measures ANOVA; Figure 4b and c). As different LTP induction protocols rely on different molecular pathways (Grover and Teyler, 1990; Cavuş and Teyler, 1996; Raymond and Redman, 2002) and are reported to differ in their dependence on presynaptic CAMK2A (Lu and Hawkins, 2006), we tested 100Hz as well as theta burst (2 Theta, see methods) LTP-induction protocols. Surprisingly, both the 100Hz and theta burst LTP-inducing protocols resulted in normal LTP in *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CA3-Cre* mice (effect of genotype: 100Hz LTP: $F_{1,32}$=0.002, p=0.96; Theta burst LTP: $F_{1,17}$=0.001, p=0.98; repeated measures ANOVA; Figure 4d and e), indicating that presynaptic CAMK2A and CAMK2B are not required for LTP at the CA3-CA1 synapse of the hippocampus. Thus the complete loss of high-frequency stimulation (HFS) induced LTP in *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice is completely caused by loss of postsynaptic CAMK2.

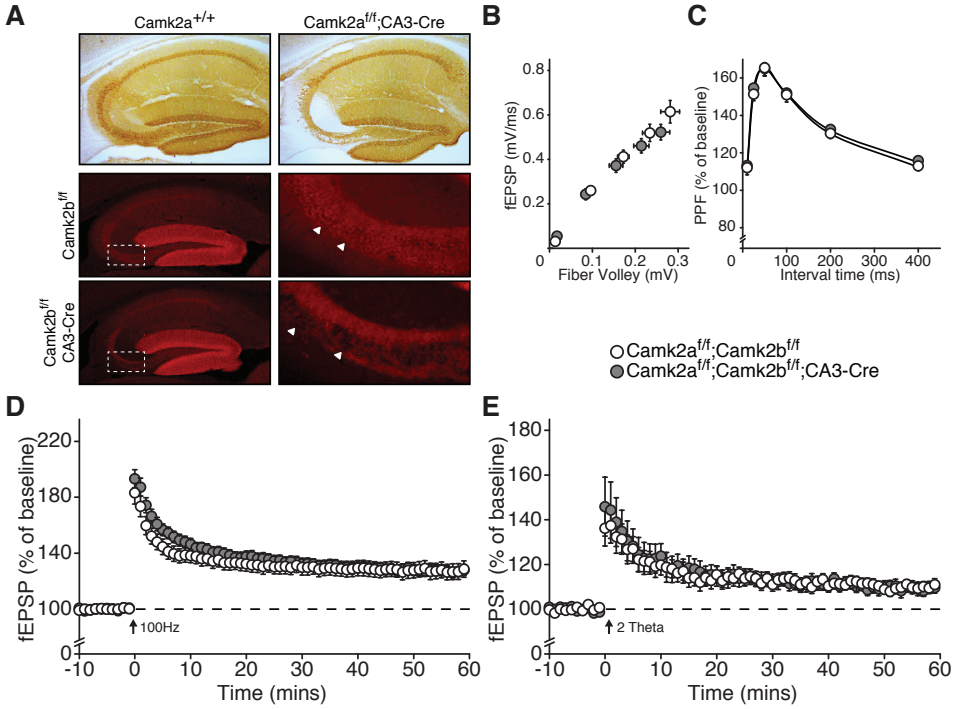


Figure 4: Presynaptic CAMK2A and CAMK2B are not necessary for CA3-CA1 LTP. (A) Specific deletion of CAMK2A (*Top*) and CAMK2B (*Middle and Bottom*) in the CA3 area of the hippocampus. Immunohistochemical stainings showing deletion of CAMK2A in the CA3 area in *Camk2a$^{f/f}$;CA3-Cre* mice (*Upper Right*). Note the absence of CAMK2A staining in all layers of the CA3 area except for mossy fibers coming from the dentate gyrus that still express CAMK2A. Immunofluorescent images showing deletion of CAMK2B in the CA3 area in *Camk2b$^{f/f}$;CA3-Cre* mice (*Bottom right*). Note that interneurons, oligodendrocytes and mossy fibers coming from the dentate gyrus still express CAMK2B. The middle and bottom right pictures are enlarged images of boxed areas. Arrowheads show CA3 pyramidal cell somas in *Camk2b$^{f/f}$* mice (*middle right*) and absence of fluorescence in cell somas in *Camk2b$^{f/f}$;CA3-Cre* mice (*bottom right*). **(B)** *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* mice (fiber volley: (n=19, from 6 mice), fEPSP slope: (n=19, from 6 mice)) show normal basal synaptic transmission compared to *Camk2a$^{f/f}$;Camk2b$^{f/f}$* mice (fiber volley: (n=22, from 6 mice), fEPSP slope: (n=22, from 6 mice)). **(C)** *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* mice (n=41, from 6 mice) show normal PPF compared to *Camk2a$^{f/f}$;Camk2b$^{f/f}$* mice (n=44, from 6 mice). **(D)** *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* mice (n=20, from 6 mice) show normal 100Hz LTP compared to *Camk2a$^{f/f}$;Camk2b$^{f/f}$* mice (n=14, from 6 mice). **(E)** *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* mice (n=9, from 4 mice) show normal theta burst LTP compared to *Camk2a$^{f/f}$;Camk2b$^{f/f}$* mice (n=10, from 4 mice). Numbers in brackets represent number of slices, followed by number of mice. Error bars indicate SEM.

Role of CAMK2A and CAMK2B in plasticity at the CA3-CA3 synapse

Control over gene deletion in the CA3 area of the hippocampus does not only provide a great tool for investigating a presynaptic role in the well-studied CA3-CA1 synapse, it can also be used to investigate the role of CAMK2A and CAMK2B both pre- and postsynaptically in the associational/commissural pathway (CA3-CA3 synapse). The CA3-CA3 synapse in the hippocampus has been widely proposed to play a pivotal role in spatial processing and previous studies have shown that LTP at this synapse is NMDA-dependent (Debanne et al., 1998). Therefore, we examined the role of CAMK2A and CAMK2B in the properties of the CA3-CA3 synapse in our *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* mutant.

Although CA3-CA3 basal synaptic transmission and PPF were not affected in the *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* mice (effect of genotype: fiber volley: $F_{1,45}$=0.18, p=0.68; fEPSP slope: $F_{1,49}$=0.03, p=0.87; PPF: $F_{1,36}$=0.92, p=0.34, repeated measures ANOVA; Figure 5a, b and c), deletion of both CAMK2A and CAMK2B in the CA3 region of the hippocampus resulted in a significant impairment of LTP at the CA3-CA3 synapse (effect of genotype: $F_{1,32}$=6.89, p=0.01; repeated measures ANOVA; Figure 5d). Pyramidal neurons in the CA3 region of the hippocampus receive inputs from different pathways. The major source of inputs originates from the CA3 itself through the commissural pathway (CA3-CA3 synapse), but also the mossy fibers coming from the dentate gyrus (DG-CA3 synapse) form an important source. To distinguish between these different pathways, we used three different approaches: (i) antidromic stimulation (Figure 5a), (ii) differences in physiological characteristics of 1Hz facilitation and PPF and (iii) differences in sensitivity to DCG-IV (see Methods). Importantly, 1Hz stimulation did not show any facilitation (1Hz facilitation (10th stimulus): *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* 106.8 ± 2.1 vs *Camk2a$^{f/f}$;Camk2b$^{f/f}$* 103.8 ± 2.3; 1Hz Facilitation (10 stimuli, not shown): effect of genotype: $F_{1,40}$=1.56, p=0.22, repeated measures ANOVA). Furthermore, 3mM DCG-IV reduced transmission by less than 20% in our experiments (81.9 ± 2.7% and 78.1 ± 3.3% transmission in the last 10 minutes for *Camk2a$^{f/f}$;Camk2b$^{f/f}$* and *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* respectively; DCG-IV: t=1.12, p=0.27; unpaired two tailed t-test; Figure 5e). Therefore, we are confident that we selectively stimulated CA3-CA3 connections.

Notably, the phenotype observed in the *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* mice appeared to be the result of the combined deletion of CAMK2A and CAMK2B and not due to absence of CAMK2A or CAMK2B alone, as the same parameters were not affected in the *Camk2a$^{f/f}$;CA3-Cre* or *Camk2b$^{f/f}$;CA3-Cre* mice, in which only CAMK2A or CAMK2B is deleted from the CA3 region of the hippocampus (effect of genotype: *Camk2a$^{f/f}$;CA3-Cre* LTP: $F_{1,43}$=0.43, p=0.52; *Camk2b$^{f/f}$;CA3-Cre* LTP: $F_{1,50}$=0.004, p=0.95, repeated measures ANOVA; Figure 5f and g). Since we did not observe an effect in the expression level of

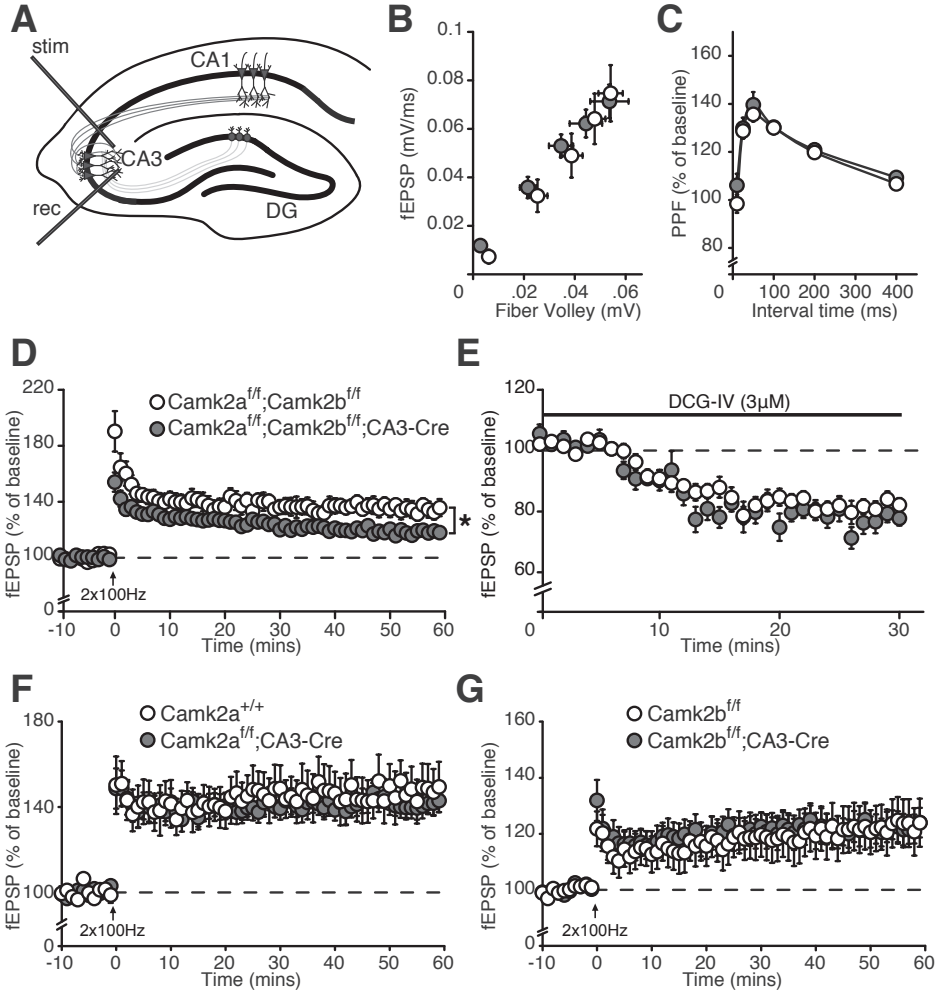


Figure 5: Redundancy of CAMK2A and CAMK2B in CA3-CA3 LTP. (A) Schematic overview of LTP induction in the CA3-CA3 pathway (also, see Methods). stim = stimulating electrode; rec = recording electrode; DG = dentate gyrus. **(B)** *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* mice (fiber volley: (n=27, from 4 mice), fEPSP slope: (n=23, from 4 mice)) mice show normal basal synaptic transmission compared to *Camk2a$^{f/f}$;Camk2b$^{f/f}$* mice (fiber volley: (n=24, from 4 mice) fEPSP slope: (n=24, from 4 mice)). **(C)** *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* (n=17, from 4 mice) mice show normal PPF compared to *Camk2a$^{f/f}$;Camk2b$^{f/f}$* (n=21, from 4 mice) mice. **(D)** *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* (n=16, from 4 mice) show reduced 100Hz LTP compared to *Camk2a$^{f/f}$;Camk2b$^{f/f}$* (n=18, from 4 mice) mice. **(E)** DCG-IV only minimally blocks the fEPSP signal in both *Camk2a$^{f/f}$;Camk2b$^{f/f}$;CA3-Cre* (n=21, from 4 mice) and *Camk2a$^{f/f}$;Camk2b$^{f/f}$* (n=21, from 4 mice) mice. **(F)** *Camk2a$^{f/f}$;CA3-Cre* (n=29, from 8 mice) show normal 100Hz LTP compared to *Camk2a$^{+/+}$* (n=16, from 5 mice) mice. **(G)** *Camk2b$^{f/f}$;CA3-Cre* (n=28, from 8 mice) show normal 100Hz LTP compared to *Camk2b$^{f/f}$* (n=24, from 8 mice) mice. Numbers in brackets represent number of slices, followed by number of mice. Error bars indicate SEM.

CAMK2A in *Camk2a*$^{f/f}$ animals without Cre in comparison with wild-type mice (data not shown), we used the wild-type littermate *Camk2a*$^{+/+}$ mice as the control group for one of these experiments (Figure 5f). Importantly, basal synaptic transmission and PPF were both not significantly different and the control experiments for mossy fiber stimulation 1Hz facilitation and DCG-IV wash-in were all within normal range and not significantly different for both *Camk2a*$^{f/f}$*;CA3-Cre* and *Camk2b*$^{f/f}$*;CA3-Cre* mice (data not shown). Taken together, these data show that either CAMK2A or CAMK2B needs to be expressed at the CA3-CA3 synapse for normal CA3-CA3 LTP. Hence, this confirms the notion that some functions of CAMK2 cannot be uncovered using *Camk2a* or *Camk2b* single mutants.

DISCUSSION

CAMK2A and CAMK2B are the most abundant proteins in the brain and are shown to be crucial for learning, memory and plasticity in mice and for normal neurodevelopment in humans (Silva et al., 1992a; 1992b; Van Woerden et al., 2009; Borgesius et al., 2011; Li et al., 2013; Küry et al., 2017; Stephenson et al., 2017; Akita et al., 2018). Due to the high homology in their structure, we contemplated that the full spectrum of CAMK2 functions has yet to be revealed, since crucial functions can potentially be masked, due to compensation by the non-deleted isoform. In this study we confirm this hypothesis with the following findings. 1) Loss of CAMK2A and CAMK2B simultaneously is lethal both during development as well as in adult mice. 2) Loss of the combined CAMK2A and CAMK2B Ca^{2+}-dependent or Ca^{2+}-independent activity is lethal. 3) Simultaneous loss of CAMK2A and CAMK2B results in complete absence of LTP at the CA3-CA1 synapse in the hippocampus. 4) Simultaneous loss of CAMK2A and CAMK2B but not of CAMK2A or CAMK2B alone results in LTP deficits at the CA3-CA3 synapse. Taken together these results show that there is quite some redundancy in the CAMK2A and CAMK2B isoform function, and that crucial functions of CAMK2 remain to be uncovered.

It remains to be investigated why absence or dysregulation of CAMK2 function results in lethality. CAMK2 is critical for NMDA receptor-dependent signaling, and it is known that absence of the NR1 subunit of the NMDA receptor also results in neonatal death due to respiratory failure in these mice (Forrest et al., 1994). CAMK2 activity mediates the emergence and maintenance of synchronous activity in the preBötzinger complex, a center in the brainstem important for respiratory motor output (Mironov, 2013). Thus respiratory distress could underlie the lethality seen in the *Camk2a*$^{-/-}$*;Camk2b*$^{-/-}$ mutants. Also NR2B null mutants are not viable (Kutsuwada et al., 1996), which is due to a defect in the suckling response. Indeed lethality was rescued when the pups were hand-fed (Kutsuwada et al., 1996). We do not think that a similar mechanism underlies the lethality seen in our mice, since all pups had milk in their stomach just before they

died. Moreover, when looking at the catalytically inactive *Camk2a*$^{T305D/T305D}$;*Camk2b*$^{A303R/A303R}$ mice, it seems unlikely that either feeding or breathing deficits are the cause of death, since some of these mice survive until P17 or later. These findings further indicate that enzymatic activity of CAMK2 is dispensable in the first 2 weeks after birth and that the survival of these mice depends solely on the presence of an enzyme that cannot be activated by Ca^{2+}/Calmodulin. This may point out an essential structural role of CAMK2. It is also interesting that *Camk2a*$^{T286A/T286A}$;*Camk2b*$^{T287A/T287A}$ mice die, indicating that the autonomous (Ca^{2+}-independent) activity of the CAMK2 holoenzyme is required for life. However, it is notable that no gross brain morphology differences were found in our double mutants nor in the NR1 or the NR2B null mutants (Forrest et al., 1994; Kutsuwada et al., 1996), which is consistent with the finding that the brain does not need CAMK2-NMDA receptor dependent plasticity or functional synapses to develop anatomically (Verhage et al., 2000; Sando et al., 2017; Sigler et al., 2017).

The complete loss of HFS-induced LTP upon deletion of both CAMK2A and CAMK2B is perhaps not surprising considering the impairments both *Camk2a*$^{-/-}$ (Hinds et al., 1998; Elgersma et al., 2002) and *Camk2b*$^{-/-}$ (Borgesius et al., 2011) mice show in this kind of LTP. The complete loss of LTP in *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} mice strongly suggests that the residual LTP left in the single knockout mice is mediated by the remaining isoform. This is further strengthened by the observation that single amino acid mutations in *Camk2a* mutant mice such as *Camk2a*^{T286A} (Giese et al., 1998b) and *Camk2a*^{T305D} (Elgersma et al., 2002) have more detrimental effects on LTP than the loss of the *Camk2a* isoform as a whole. This could well be explained by the dominant-negative effects of these *Camk2a* point mutations on the proper functioning of the heteromeric CAMK2A-CAMK2B holoenzyme.

In contrast to impaired LTP, basal synaptic transmission and PPF in the CA3-CA1 area were unaffected in absence of both CAMK2A and CAMK2B. Given the clear abundance of CAMK2 in synapses both pre- and postsynaptically and its major role in AMPA phosphorylation, GluN2B binding and AMPA insertion (reviewed in (Lisman et al., 2012)), it is surprising that in the absence of these major isoforms synaptic strength stays intact. This suggests that synaptic strength in the CA3-CA1 synapse can be maintained independently of CAMK2A and CAMK2B.

Forskolin-induced LTP has been shown to be primarily PKA-dependent (Sokolova et al., 2006), but also NMDA-dependent components have been described (Otmakhov et al., 2004). Thus the impairment seen in cLTP in our *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ESR} could be caused by the selective loss of the NMDA (CAMK2)-dependent pathway while leaving the PKA-dependent pathway intact.

The finding that presynaptic CAMK2 is dispensable for LTP at the CA3-CA1 synapse is surprising, considering that CAMK2 was first discovered as a presynaptic protein (DeLorenzo et al., 1979; Kennedy and Greengard, 1981; Kennedy et al., 1983). Indeed, CAMK2A has long been shown to play a role in presynaptic plasticity and regulation of neurotransmitter release at the CA3-CA1 synapse in brain slices (Llinás et al., 1985; Lin et al., 1990; Nichols et al., 1990; Chapman et al., 1995; Hinds et al., 2003; Hojjati et al., 2007; Jiang et al., 2008; Pang et al., 2010; Achterberg et al., 2014). Additionally, a previous study reported a role for presynaptic CAMK2 in LTP in dissociated hippocampal neurons (Ninan and Arancio, 2004). In this study, the role of presynaptic CAMK2 was assessed using different CAMK2 inhibitors, which when applied resulted in reduced LTP. Thus, in light of these studies, we expected to find a requirement for presynaptic CAMK2A and CAMK2B in synaptic transmission and LTP at the CA3-CA1 synapse. However, we found that presynaptic CAMK2 is completely indispensable for LTP at the Schaffer collateral pathway. There are a few reasons to explain the discordance between literature and our findings. First, in our model CAMK2 is already absent from as early as postnatal day 5. We cannot exclude that the lack of effect in our experiments is due to presynaptic compensatory mechanisms during this phase of development. Second, to study the role of presynaptic CAMK2 in LTP in hippocampal cultures, CAMK2 inhibitors were used to block presynaptic CAMK2 activity. These are general CAMK2 blockers, blocking not only CAMK2A or CAMK2B, but also CAMK2D and CAMK2G. It could be that in our experiments that CAMK2D and CAMK2G are compensating for the absence of CAMK2A and CAMK2B. Taken together, it is clear that complete understanding of the precise role of presynaptic CAMK2 at the CA3-CA1 Schaffer collateral synapse is still lacking.

CA3-CA3 LTP shares many characteristics with Schaffer collateral (CA3-CA1) LTP. Most notably, both CA3-CA3 and CA3-CA1 LTP depend on NMDA receptor activation and a postsynaptic rise in Ca^{2+} (Bradler and Barrionuevo, 1990; Zalutsky and Nicoll, 1990; Debanne et al., 1998; Bains et al., 1999; Debanne et al., 1999; Smith and Swann, 1999; Pavlidis et al., 2000; Kakegawa et al., 2004). We show here that CA3-CA3 LTP is impaired when deleting both CAMK2A and CAMK2B, but not upon deletion of either CAMK2A or CAMK2B, indicating that one of the 2 CAMK2 isoforms needs to be present to support normal LTP. These results are in agreement with a previous study using a pharmacological approach, which described a pre- and postsynaptic role for CAMK2 in LTP at the CA3-CA3 synapse (Lu and Hawkins, 2006). Using a CAMK2 synthetic peptide (281-309) CAMK2 inhibitor, on organotypic slice cultures the authors saw a decrease of 50% in theta burst LTP when CAMK2 was blocked pre- or postsynaptically. This inhibitor does not distinguish between the 4 different CAMK2 isoforms, leaving the question which CAMK2 isoform is responsible for the LTP deficit open. Here we showed that the loss of either CAMK2A or CAMK2B isoform does not affect LTP, but that only the simultaneous

absence of both CAMK2A and CAMK2B causes a CA3-CA3 LTP deficit. Interestingly, whereas CA3-CA1 LTP is completely abolished in absence of CAMK2, CA3-CA3 LTP is only reduced indicating that there are some fundamental differences underlying CA3-CA1 LTP compared to CA3-CA3 LTP.

Taken together, our results show that despite our vast knowledge about CAMK2, there are many aspects of CAMK2 function that remain to be uncovered.

REFERENCES

Abs E, Goorden SMI, Schreiber J, Overwater IE, Hoogeveen-Westerveld M, Bruinsma CF, Aganović E, Borgesius NZ, Nellist M, Elgersma Y (2013) TORC1-dependent epilepsy caused by acute biallelic Tsc1 deletion in adult mice. Ann Neurol.

Achterberg KG, Buitendijk GHS, Kool MJ, Goorden SMI, Post L, Slump DE, Silva AJ, Van Woerden GM, Kushner SA, Elgersma Y (2014) Temporal and Region-Specific Requirements of αCaMKII in Spatial and Contextual Learning. Journal of Neuroscience 34:11180–11187.

Akita T et al. (2018) De novo variants in CAMK2A and CAMK2B cause neurodevelopmental disorders. Ann Clin Transl Neurol 5:280–296.

Bains JS, Longacher JM, Staley KJ (1999) Reciprocal interactions between CA3 network activity and strength of recurrent collateral synapses. Nat Neurosci 2:720–726.

Borgesius NZ, Van Woerden GM, Buitendijk GHS, Keijzer N, Jaarsma D, Hoogenraad CC, Elgersma Y (2011) βCaMKII plays a nonenzymatic role in hippocampal synaptic plasticity and learning by targeting αCaMKII to synapses. Journal of Neuroscience 31:10141–10148.

Bradler JE, Barrionuevo G (1990) Heterosynaptic correlates of long-term potentiation induction in hippocampal CA3 neurons. Neuroscience 35:265–271.

Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, Lubischer JL, Krieg PA, Krupenko SA, Thompson WJ, Barres BA (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. Journal of Neuroscience 28:264–278.

Cavuş I, Teyler T (1996) Two forms of long-term potentiation in area CA1 activate different signal transduction cascades. J Neurophysiol 76:3038–3047.

Chapman PF, Frenguelli BG, Smith A, Chen CM, Silva AJ (1995) The alpha-Ca2+/calmodulin kinase II: a bidirectional modulator of presynaptic plasticity. Neuron 14:591–597.

Debanne D, Gähwiler BH, Thompson SM (1998) Long-term synaptic plasticity between pairs of individual CA3 pyramidal cells in rat hippocampal slice cultures. The Journal of Physiology 507 (Pt 1):237–247.

Debanne D, Gähwiler BH, Thompson SM (1999) Heterogeneity of synaptic plasticity at unitary CA3-CA1 and CA3-CA3 connections in rat hippocampal slice cultures. Journal of Neuroscience 19:10664–10671.

DeLorenzo RJ, Freedman SD, Yohe WB, Maurer SC (1979) Stimulation of Ca2+-dependent neurotransmitter release and presynaptic nerve terminal protein phosphorylation by calmodulin and a calmodulin-like protein isolated from synaptic vesicles. Proceedings of the National Academy of Sciences of the United States of America 76:1838–1842.

Elgersma Y, Fedorov NB, Ikonen S, Choi ES, Elgersma M, Carvalho OM, Giese KP, Silva AJ (2002) Inhibitory autophosphorylation of CaMKII controls PSD association, plasticity, and learning. Neuron 36:493–505.

Filosa A, Paixão S, Honsek SD, Carmona MA, Becker L, Feddersen B, Gaitanos L, Rudhard Y, Schoepfer R, Klopstock T, Kullander K, Rose CR, Pasquale EB, Klein R (2009) Neuron-glia communication via EphA4/ephrin-A3 modulates LTP through glial glutamate transport. Nature Publishing Group 12:1285–1292.

Forrest D, Yuzaki M, Soares HD, Ng L, Luk DC, Sheng M, Stewart CL, Morgan JI, Connor JA, Curran T (1994) Targeted disruption of NMDA receptor 1 gene abolishes NMDA response and results in neonatal death. Neuron 13:325–338.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ (1998a) Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. Science 279:870–873.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ (1998b) Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. Science 279:870–873.

Grover LM, Teyler TJ (1990) Two components of long-term potentiation induced by different patterns of afferent activation. Nature 347:477–479.

Hanson PI, Meyer T, Stryer L, Schulman H (1994) Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. Neuron 12:943–956.

Hell JW (2014) CaMKII: Claiming Center Stage in Postsynaptic Function and Organization. Neuron 81:249–265.

Hinds HL, Goussakov I, Nakazawa K, Tonegawa S, Bolshakov VY (2003) Essential function of alpha-calcium/calmodulin-dependent protein kinase II in neurotransmitter release at a glutamatergic central synapse. Proceedings of the National Academy of Sciences of the United States of America 100:4275–4280.

Hinds HL, Tonegawa S, Malinow R (1998) CA1 long-term potentiation is diminished but present in hippocampal slices from alpha-CaMKII mutant mice. Learn Mem 5:344–354.

Hojjati MR, Van Woerden GM, Tyler WJ, Giese KP, Silva AJ, Pozzo-Miller L, Elgersma Y (2007) Kinase activity is not required for alphaCaMKII-dependent presynaptic plasticity at CA3-CA1 synapses. Nat Neurosci 10:1125–1127.

Jiang X, Lautermilch NJ, Watari H, Westenbroek RE, Scheuer T, Catterall WA (2008) Modulation of CaV2.1 channels by Ca2+/calmodulin-dependent protein kinase II bound to the C-terminal domain. Proceedings of the National Academy of Sciences of the United States of America 105:341–346.

Kakegawa W, Tsuzuki K, Yoshida Y, Kameyama K, Ozawa S (2004) Input- and subunit-specific AMPA receptor trafficking underlying long-term potentiation at hippocampal CA3 synapses. Eur J Neurosci 20:101–110.

Kennedy MB, Bennett MK, Erondu NE (1983) Biochemical and immunochemical evidence that the "major postsynaptic density protein" is a subunit of a calmodulin-dependent protein kinase. Proceedings of the National Academy of Sciences of the United States of America 80:7357–7361.

Kennedy MB, Greengard P (1981) Two calcium/calmodulin-dependent protein kinases, which are highly concentrated in brain, phosphorylate protein I at distinct sites. Proceedings of the National Academy of Sciences of the United States of America 78:1293–1297.

Kirschstein T, Brelie von der C, Steinhäuser M, Vinçon A, Beck H, Dietrich D (2004) L-CCG-I activates group III metabotropic glutamate receptors in the hippocampal CA3 region. Neuropharmacology 47:157–162.

Kool MJ, van de Bree JE, Bodde HE, Elgersma Y, Van Woerden GM (2016) The molecular, temporal and region-specific requirements of the beta isoform of Calcium/Calmodulin-dependent protein kinase type 2 (CAMK2B) in mouse locomotion. Sci Rep 6:26989.

Kutsuwada T, Sakimura K, Manabe T, Takayama C, Katakura N, Kushiya E, Natsume R, Watanabe M, Inoue Y, Yagi T, Aizawa S, Arakawa M, Takahashi T, Nakamura Y, Mori H, Mishina M (1996) Impairment of suckling response, trigeminal neuronal pattern formation, and hippocampal LTD in NMDA receptor epsilon 2 subunit mutant mice. Neuron 16:333–344.

Küry S et al. (2017) De Novo Mutations in Protein Kinase Genes CAMK2A and CAMK2B Cause Intellectual Disability. Am J Hum Genet 101:768–788.

Lamsa KP, Heeroma JH, Somogyi P, Rusakov DA, Kullmann DM (2007) Anti-Hebbian long-term potentiation in the hippocampal feedback inhibitory circuit. Science 315:1262–1266.

Lee S-JR, Escobedo-Lozoya Y, Szatmari EM, Yasuda R (2009) Activation of CaMKII in single dendritic spines during long-term potentiation. Nature 458:299–304.

Li K, Zhou T, Liao L, Yang Z, Wong C, Henn F, Malinow R, Yates JR, Hu H (2013) βCaMKII in Lateral Habenula Mediates Core Symptoms of Depression. Science 341:1016–1020.

Lin JW, Sugimori M, Llinás RR, McGuinness TL, Greengard P (1990) Effects of synapsin I and calcium/calmodulin-dependent protein kinase II on spontaneous neurotransmitter release in the squid giant synapse. Proceedings of the National Academy of Sciences of the United States of America 87:8257–8261.

Lisman J, Schulman H, Cline H (2002) The molecular basis of CaMKII function in synaptic and behavioural memory. Nat Rev Neurosci 3:175–190.

Lisman J, Yasuda R, Raghavachari S (2012) Mechanisms of CaMKII action in long-term potentiation. Nat Rev Neurosci 13:169–182.

Llinás R, McGuinness TL, Leonard CS, Sugimori M, Greengard P (1985) Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. Proceedings of the National Academy of Sciences of the United States of America 82:3035–3039.

Lu F-M, Hawkins RD (2006) Presynaptic and postsynaptic Ca(2+) and CamKII contribute to long-term potentiation at synapses between individual CA3 neurons. Proceedings of the National Academy of Sciences of the United States of America 103:4264–4269.

Mayford M, Wang J, Kandel ER, O'Dell TJ (1995) CaMKII regulates the frequency-response function of hippocampal synapses for the production of both LTD and LTP. Cell 81:891–904.

Miller SG, Kennedy MB (1986) Regulation of brain type II Ca2+/calmodulin-dependent protein kinase by autophosphorylation: a Ca2+-triggered molecular switch. Cell 44:861–870.

Mironov SL (2013) Calmodulin and calmodulin kinase II mediate emergent bursting activity in the brainstem respiratory network (preBotzinger complex). The Journal of Physiology 591:1613–1630.

Nichols RA, Sihra TS, Czernik AJ, Nairn AC, Greengard P (1990) Calcium/calmodulin-dependent protein kinase II increases glutamate and noradrenaline release from synaptosomes. Nature 343:647–651.

Ninan I, Arancio O (2004) Presynaptic CaMKII is necessary for synaptic plasticity in cultured hippocampal neurons. Neuron 42:129–141.

Otmakhov N, Khibnik L, Otmakhova N, Carpenter S, Riahi S, Asrican B, Lisman J (2004) Forskolin-Induced LTP in the CA1 Hippocampal Region Is NMDA Receptor Dependent. J Neurophysiol 91:1955–1962.

Pang ZP, Cao P, Xu W, Sudhof TC (2010) Calmodulin Controls Synaptic Strength via Presynaptic Activation of Calmodulin Kinase II. Journal of Neuroscience 30:4132–4142.

Pavlidis P, Montgomery J, Madison DV (2000) Presynaptic protein kinase activity supports long-term potentiation at synapses between individual hippocampal neurons. J Neurosci 20:4497–4505.

Raymond CR, Redman SJ (2002) Different calcium sources are narrowly tuned to the induction of different forms of LTP. J Neurophysiol 88:249–255.

Sando R, Bushong E, Zhu Y, Huang M, Considine C, Phan S, Ju S, Uytiepo M, Ellisman M, Maximov A (2017) Assembly of Excitatory Synapses in the Absence of Glutamatergic Neurotransmission. Neuron 94:312–321.e313.

Scanziani M, Salin PA, Vogt KE, Malenka RC, Nicoll RA (1997) Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. Nature 385:630–634.

Sigler A, Oh WC, Imig C, Altas B, Kawabe H, Cooper BH, Kwon H-B, Rhee J-S, Brose N (2017) Formation and Maintenance of Functional Spines in the Absence of Presynaptic Glutamate Release. Neuron 94:304–311.e304.

Silva AJ, Paylor R, Wehner JM, Tonegawa S (1992a) Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science 257:206–211.

Silva AJ, Stevens CF, Tonegawa S, Wang Y (1992b) Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science 257:201–206.

Smith KL, Swann JW (1999) Long-term depression of perforant path excitatory postsynaptic potentials following synchronous network bursting in area CA3 of immature hippocampus. Neuroscience 89:625–630.

Sokolova IV, Lester HA, Davidson N (2006) Postsynaptic Mechanisms Are Essential for Forskolin-Induced Potentiation of Synaptic Transmission. J Neurophysiol 95:2570–2579.

Stephenson JR, Wang X, Perfitt TL, Parrish WP, Shonesy BC, Marks CR, Mortlock DP, Nakagawa T, Sutcliffe JS, Colbran RJ (2017) A Novel Human CAMK2A Mutation Disrupts Dendritic Morphology and Synaptic Transmission, and Causes ASD-Related Behaviors. Journal of Neuroscience 37:2216–2233.

Vallano ML (1989) Separation of isozymic forms of type II calcium/calmodulin-dependent protein kinase using cation-exchange chromatography. J Neurosci Methods 30:1–9.

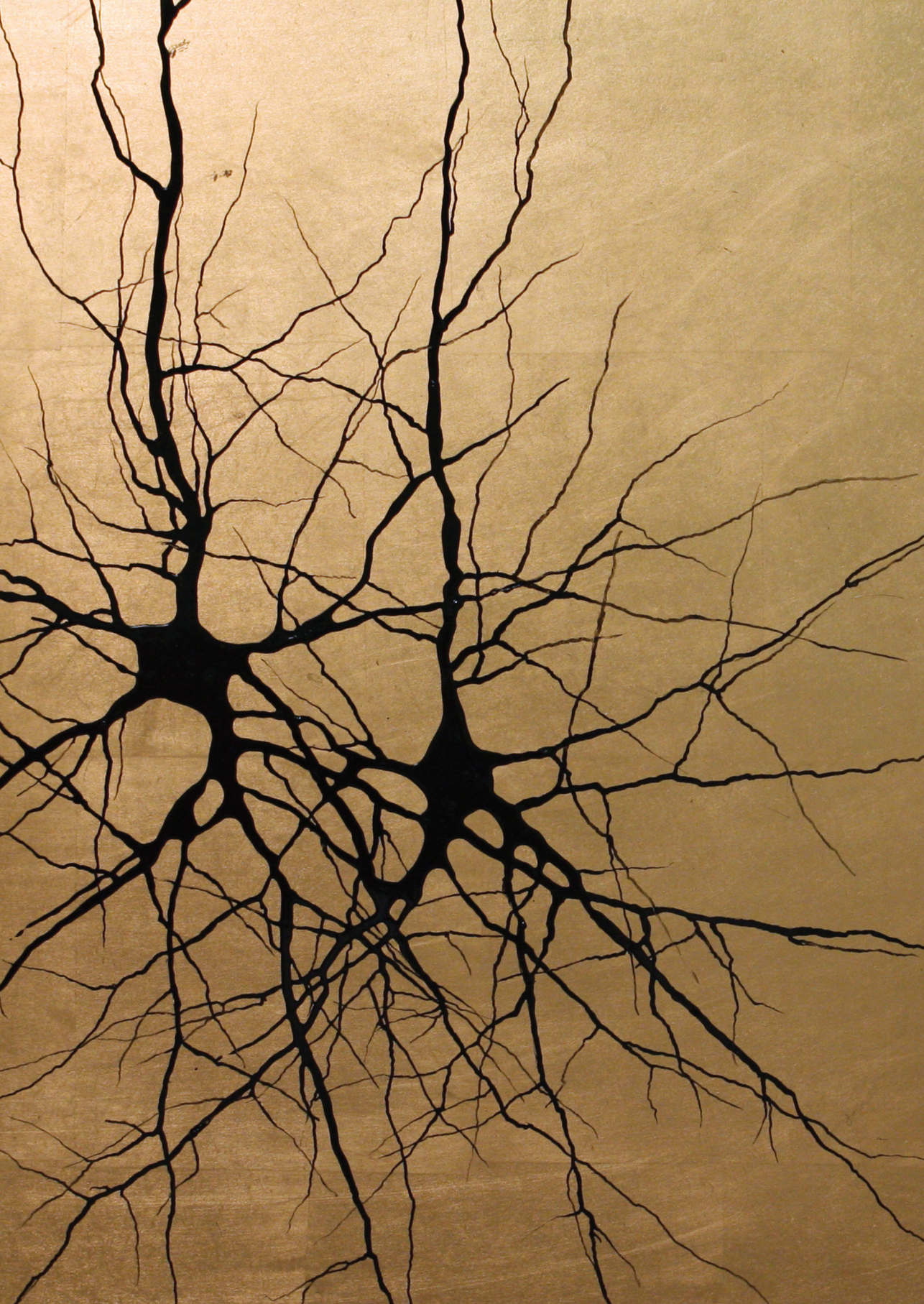
Van Woerden GM, Hoebeek FE, Gao Z, Nagaraja RY, Hoogenraad CC, Kushner SA, Hansel C, De Zeeuw CI, Elgersma Y (2009) betaCaMKII controls the direction of plasticity at parallel fiber-Purkinje cell synapses. Nature Publishing Group 12:823–825.

Verhage M, Maia AS, Plomp JJ, Brussaard AB, Heeroma JH, Vermeer H, Toonen RF, Hammer RE, van den Berg TK, Missler M, Geuze HJ, Sudhof TC (2000) Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science 287:864–869.

Vogt MA, Chourbaji S, Brandwein C, Dormann C, Sprengel R, Gass P (2008) Suitability of tamoxifen-induced mutagenesis for behavioral phenotyping. Exp Neurol 211:25–33.

Waggener CT, Dupree JL, Elgersma Y, Fuss B (2013) CaMKIIβ Regulates Oligodendrocyte Maturation and CNS Myelination. Journal of Neuroscience 33:10453–10458.

Zalutsky RA, Nicoll RA (1990) Comparison of two forms of long-term potentiation in single hippocampal neurons. Science 248:1619–1624.



Chapter 5

Bidirectional changes in excitability upon loss of both CAMK2A and CAMK2B

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ABSTRACT

The mammalian Ca2+/calmodulin-dependent protein kinase II (CAMK2) family consists of 4 different *CAMK2* genes, encoding CAMK2A, CAMK2B, CAMK2D and CAMK2G, which have high structural homology. CAMK2A and CAMK2B are abundantly expressed in the brain; they play a unique role in proper neuronal functioning, since both CAMK2A and CAMK2B knockout mice show several behavioural and cellular phenotypes. However, our recent finding that deletion of both CAMK2A and CAMK2B is lethal indicates that they also have overlapping functions and that the full spectrum of CAMK2 function in neurons, for example excitability and synaptic transmission, remains to be uncovered.

In order to get more insight into the full spectrum of CAMK2 functions in neurons, we performed whole-cell patch clamp experiments in CA1 pyramidal neurons of inducible *Camk2a/Camk2b* double knockout mice, as well as CAMK2A and CAMK2B knockout mice. We found that whereas deletion of CAMK2A or CAMK2B alone did not change evoked firing rate, deletion of both CAMK2A and CAMK2B resulted in a decrease in evoked firing rate 10 days after deletion, which reversed to an increased evoked firing rate 21 days after deletion. Additionally, loss of both CAMK2A and CAMK2B resulted in a decreased frequency of both miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs) 21 days after deletion, but not 10 days after deletion, an effect not seen in the single mutants. Our results indicate that CAMK2 is critically important to maintain normal excitability of hippocampal CA1 pyramidal cells, as well as normal inhibitory and excitatory synaptic transmission. Together, these results provide new insights in the regulation of neuronal function by CAMK2 and highlight the importance of having both CAMK2A and CAMK2B expressed in high levels in the brain.

INTRODUCTION

The mammalian Ca2+/calmodulin-dependent protein kinase II (CAMK2) family consists of 4 different *CAMK2* genes, encoding CAMK2A, CAMK2B, CAMK2D and CAMK2G, which all share high homology in their structure (Tobimatsu and Fujisawa, 1989). CAMK2A and CAMK2B are both expressed in high abundance in neurons, accounting for up to 2% of total brain protein (Erondu and Kennedy, 1985). Studies using *Camk2a* or *Camk2b* mutant mice indicate that these proteins fulfil unique functions in the brain (Silva et al., 1992a; 1992b; Hojjati et al., 2007; Van Woerden et al., 2009; Borgesius et al., 2011; Kool et al., 2016). However, these isoforms may also have overlapping functions. In support for this hypothesis we recently found that simultaneous deletion of both CAMK2A and CAMK2B is lethal, indicating that critical functions of CAMK2 are masked in the single mutants due to isoform compensation and or redundancy (Kool et al., submitted). Here we set out to identify these functions at the single cell and network level.

The role of CAMK2A and CAMK2B in neuronal function and synaptic plasticity has been extensively studied using *Camk2a*$^{-/-}$ and/or *Camk2b*$^{-/-}$ mice (Silva et al., 1992b; Van Woerden et al., 2009; Borgesius et al., 2011; Achterberg et al., 2014), as well as by studying mutant mice with knock-in missense mutations (Mayford et al., 1996; Giese et al., 1998; Elgersma et al., 2002). In addition, pharmacological approaches have been used that interfere with kinase function of both isoforms. Inhibiting CAMK2 activity in vestibular nucleus neurons (Nelson et al., 2005) and medium spiny neurons in the striatum (Klug et al., 2012) resulted in increased intrinsic excitability. Consistent with this finding, expression of constitutive active CAMK2 in CA1 pyramidal neurons decreases neuronal excitability (Varga et al., 2004), whereas the CAMK2A-T286A mouse mutant, in which the autonomous activity of CAMK2A is blocked, shows increased excitability (Sametsky et al., 2009), suggesting that CAMK2 activity is needed to regulate neuronal excitability. Indeed, blocking CAMK2 activity in cultured cortical neurons for >4 hours increases excitability and eventually leads to cell death (Ashpole et al., 2012).

At the synaptic level most studies have focussed either on CAMK2A alone or used a broad pharmacological approach to investigate the role of CAMK2 in basal synaptic transmission. For example, in hippocampal CA1 pyramidal cells and piriform cortex pyramidal cells, CAMK2 has been shown to increase the amplitude of spontaneous or miniature inhibitory postsynaptic currents (sIPSC or mIPSC), most likely through phosphorylation of the $GABA_A$ receptor (Wei et al., 2004; Ghosh et al., 2015). CAMK2 mediates a glutamate-induced increase in miniature excitatory postsynaptic currents (mEPSC) frequency in cultured hippocampal neurons (Ninan and Arancio, 2004). Infusion of activated CAMK2 in hippocampal CA1 pyramidal cells also increases the amplitude

of evoked EPSCs (Lledo et al., 1995), whereas knockdown of CAMK2A in dissociated hippocampal cultures decreased mEPSC amplitude, but not frequency (Barcomb et al., 2014). Only one study investigated the differential effect of CAMK2A and CAMK2B on mEPSC frequency; upon overexpression in neurons opposing effects on basal synaptic strength were found, indicating that the ratio of CAMK2A and CAMK2B is important for activity-dependent synaptic homeostasis (Thiagarajan et al., 2002). Recently another study found that CAMK2A activity is essential for maintaining basal synaptic transmission and that loss of CAMK2A, but not CAMK2B, diminishes AMPA-receptor and NMDA-receptor currents (Incontro et al., 2018).

Since the majority of the above-mentioned studies used CAMK2 inhibitors, it is difficult to disentangle whether CAMK2A, CAMK2B or both are responsible for the observed effects. In this study we set out to investigate the contribution of CAMK2A and CAMK2B, alone or in combination, on neuronal excitability and basal synaptic transmission at the single cell level. To that end, we made use of single *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mutants to study the role of each of these isoforms separately, as well as inducible *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ knockout mice to understand how simultaneous loss of both isoforms affects neuronal function. Using whole-cell electrophysiology we found that only deletion of both CAMK2A and CAMK2B affects intrinsic excitability and basal synaptic transmission. We further show that induced deletion of both isoforms results in remarkable temporary changes. Whereas deletion of both CAMK2 isoforms initially results in decreased neuronal intrinsic excitability, this shifts to a marked increase in intrinsic excitability in just 10 days. The increase in intrinsic excitability coincides with a decrease of both miniature excitatory as well as inhibitory postsynaptic currents in the inducible *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ knockout mice, suggesting that the increase in excitability could be a homeostatic response of the cell to maintain its basal functions.

MATERIAL AND METHODS

Animals

Camk2a$^{-/-}$, *Camk2b*$^{-/-}$ and *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;Cag-Cre^{ER} mice were tested in a C57Bl/6JOlaHsd background and backcrossed >16 times. Mice were genotyped between 7-10 days of age and re-genotyped after the mice were sacrificed. Genotyping records were obtained and kept by a technician not involved in the design, execution or analysis of the experiments.

Electrophysiological recordings in *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mice were performed between 17 and 30 days of age. *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice were older when

tested. Tamoxifen injections began at the age of 21 days until a maximum age of 30 days. Three groups were used: D10 (10 days after the first tamoxifen injection), D15 (15 days after the 1st Tamoxifen injection) and D21 (21 days after the 1st Tamoxifen injection). In absolute age, these time points corresponded with 31-40 days of age for mice of the D10 group, 36-45 days of age for mice of the D15 group and 42-50 days of age for mice of the D21 group.

All mice were kept group-housed in IVC cages (Sealsafe 1145T, Tecniplast) with bedding material (Lignocel BK 8/15 from Rettenmayer) on a 12/12 h light/dark cycle in 21°C (±1°C), humidity at 40-70% and with food pellets (801727CRM(P), Special Dietary Service) and water available *ad libitum*. For all experiments, mutants were compared to either WT or *Cre*–negative homozygous floxed littermates. Groups were matched for age and sex. All experiments were done during daytime and experimenters were blind for genotype throughout experiments and data analysis. All research has been performed in accordance with and approved by a Dutch Animal Ethical Committee (DEC) for animal research.

Generation of Camk2a$^{-/-}$, Camk2b$^{-/-}$ and Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER} mice

The generation of *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mice has been described previously (Elgersma et al., 2002; Kool et al., 2016) *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;Cag-Cre^{ER} mice were generated by crossing *Camk2a*$^{f/f}$ (Achterberg et al., 2014) and *Camk2b*$^{f/f}$ (Kool et al., 2016) mice.

Tamoxifen injections

Camk2a$^{f/f}$;*Camk2b*$^{f/f}$ and *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice (as early as P21) were injected with 0.1mg/gr of bodyweight Tamoxifen (Sigma-Aldrich) intraperitoneally for 4 consecutive days. To keep Tamoxifen dose constant throughout injection days we kept a tight injection scheme, injecting mice 24±1 hour after the previous injection. Injections were given each day around noon. Tamoxifen was dissolved in sunflower oil (20mg/ml). Even though Tamoxifen is not known to have an effect on physiology, we injected both *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ and *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice with Tamoxifen to control for any possible effects.

Electrophysiology

Brain slices were prepared from mice using standard techniques. Briefly, mice were decapitated under deep isoflurane anaesthesia, brains were quickly removed and 320 µm thick transverse slices were cut using a vibratome (HM650V; Microm) in ice-cold

cutting solution, containing (in mM): 126 Choline Chloride, 2.5 KCl, 1.25 NaH_2PO_4, 10 $MgSO_4$, 0.5 $CaCl_2$, 16.7 glucose, 26 $NaHCO_3$ bubbled with 95% O_2 and 5% CO_2. After cutting, slices were stored in the same cutting solution for 30 seconds at 35°C and then transferred to 35°C artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 2 $MgSO_4$, 2 $CaCl_2$ bubbled with 95% O_2 and 5% CO_2 (osmolarity between 300-310 mOsm/kg H_2O). Afterwards, slices were kept in ACSF at room temperature for a minimum of 1 hour before onset of experiments.

Whole-cell patch-clamp recordings were made from pyramidal neurons from area CA1 in the hippocampus. Neurons were visualized using differential interference contrast (DIC) and infrared video microscopy optics on an Olympus BX51W1 microscope. Pipettes were pulled from borosilicate glass capillaries and had a resistance of 3-5 MΩ when filled with intracellular solutions containing (in mM): 120 K-gluconate, 10 KCl, 10 HEPES, 4 Mg-ATP, 10Na_2-Phosphocreatine, 0.3 GTP (pH adjusted to 7.3 with KOH) and 0.5% biocytin (osmolarity between 275-280 mOsm/kg H_2O). Pyramidal neurons were identified based on their firing pattern, morphology under the microscope as well as their morphology after biocytin staining. Recordings were made with a patch-clamp amplifier (Multiclamp 700B; Axon Instruments, Foster City, CA, USA). Signals were low-pass filtered at 2 kHz and digitized at 10 kHz. Only cells with series resistance under 30 MOhm were included. Series resistance was monitored during the experiments and not compensated for. Cells were rejected when series resistance changed by more than 20%. Resting membrane potential was measured in bridge mode (I=0). Other passive membrane properties (membrane resistance, access resistance, membrane capacitance and tau values) were measured immediately after obtaining whole-cell access using the membrane test in Clampex (Axon Instruments). Firing pattern, action potential characteristics and threshold were analysed using Clampfit 10.3 (Axon Instruments). Physiological responses were evoked using a series of depolarizing constant-current pulses of 750 ms duration at 30 pA intervals from a holding potential of -68 mV or by using 1 ms, 1 or 1.5 nA constant-current pulses given at 10 Hz for 750 ms. Firing rate was measured as the number of action potentials elicited during the 750ms long constant-current pulse for each current step, calculated in Hertz and action potentials were identified and counted by the researcher in Clampfit. Action potential characteristics were measured on the 1st and 3rd action potential at the first step that induced action potentials. The action potential amplitude was calculated as the difference between threshold and peak. For rise and decay kinetics of the action potentials, maximum rise and decay slope were calculated. Action potential half width was calculated as the width of the action potential at half of the maximum amplitude. Action potential threshold was calculated taking the first derivative of the action potential, and measuring the corresponding voltage when the derivative exceeded 2 mV/ms. Membrane potentials were corrected

for a (calculated) liquid junction potential of -8 mV. KN62 (Tocris Bioscience) was used in a concentration of 20 μM and autocamtide-2 related inhibitory peptide II (AIP-II; Merck Millipore) at 4 μM.

Miniature synaptic currents (mEPSCs and mIPSCs) were analysed using Mini Analysis (Synaptosoft Inc, Decatur, GA, USA). Events were detected with a threshold criterion of 3 times root-mean-square (RMS) of baseline noise. After detection, recordings were manually checked for false positive or missed events while the experimenter was still blind for genotype. mEPSCs were pharmacologically isolated using tetrodotoxin (TTX) (1 μM) and picrotoxin (50 μM). mIPSCs were isolated using TTX (1 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM) and D,L-2-amino-5-phosphopentanoic acid (AP5; 50 μM). Cells were held at -70 mV when measuring miniature events. For mIPSCs a high chloride internal solution was used, containing (in mM): 2 NaCl, 141 KCl, 1 $CaCl_2$, 10 EGTA, 2 Mg-ATP, 0.3 Na-GTP, 10 HEPES, 10 Na-phosphocreatine (pH 7.25, 295 mOsm/kg H_2O).

Western blot

Western blot analysis was done as described previously (Kool et al., 2016). In short, western blots were probed with primary antibodies against CAMK2A (6G9, 1:40.000, Abcam), CAMK2B (CB-β1, 1:10.000, Invitrogen) and actin (MAB1501R, 1:20.000, Chemicon) and secondary antibodies (goat anti-mouse and/or goat anti-rabbit, both 1:3000, Affinipure #115-007-003 and #111-007-003). Blots were stained and quantified using LI-COR Odyssey Scanner and Odyssey 3.0 software.

Immunofluorescence

Immunofluorescence has been done as described previously (Kool et al., 2016).

Data analysis and statistics

All excitability tests were analysed using a 2-way repeated measures ANOVA to determine a main effect of genotype. For all miniatures, averages were compared using a Student's t-test. α was set at 0.05. All values represent average ± SEM. Number of mice and number of slices are depicted in the figure legends. All statistics were performed in Graphpad Prism. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

RESULTS

Absence of either CAMK2A or CAMK2B does not change the intrinsic excitability of CA1 pyramidal neurons

In order to decipher the role of CAMK2A and/or CAMK2B in neuronal excitability, we measured the physiological responses upon depolarizing current stimulations in CA1 pyramidal neurons in the hippocampus of both *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mice. For both mutants we found no difference in passive membrane properties such as input resistance, capacitance and resting membrane potential compared to their littermate controls (see Table 1). Additionally, we found no changes in excitability in both mutants (*Camk2a*$^{-/-}$: effect of genotype: $F_{(1,42)}$=0.06, p=0.81; *Camk2b*$^{-/-}$: effect of genotype: $F_{(1,60)}$=0.22, p=0.64; 2way repeated measures ANOVA; Figure 1).

Table 1: An overview of all passive membrane properties of all mice used in Figure 1 and 2. Cre+ = *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER}. Cre- = *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$.

	Camk2a$^{-/-}$			*Camk2b*$^{-/-}$		
Parameter	WT (12)	KO (10)	p-value	WT (11)	KO (20)	p-value
Rm (MΩ)	229.3±23.6	238.3±46.9	ns	220.4±30.4	244.4±15.6	ns
Ra (MΩ)	21.1±1.4	22.1±2.8	ns	14.4±1.4	17.1±0.9	ns
Cm (pF)	115.2±13.6	108±17.3	ns	110.6±10.2	99.2±8.8	ns
Vm (mV)	-72.3±0.9	-72.4±1.4	ns	-71.5±1.2	-70.4±0.9	ns
tau (ms)	2.7±0.3	3.3±0.7	ns	1.8±0.3	1.8±0.2	ns

	Camk2a$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ER} Day 10			Day 15			Day 21		
Parameter	Cre– (13)	Cre+ (10)	p-value	Cre– (8)	Cre+ (10)	p-value	Cre– (15)	Cre+ (18)	p-value
Rm (MΩ)	240.7±9.8	220.6±19.3	ns	238.3±20.6	210.4±17.1	ns	227.6±13.2	252.7±12.6	ns
Ra (MΩ)	23.8±1.9	22.8±1.8	ns	24±1.6	25.7±1.1	ns	24.6±1.3	23.8±1.1	ns
Cm (pF)	90.8±7.4	91.9±13.2	ns	72.6±8.2	85.5±3.9	ns	87.2±5.3	82.6±5.1	ns
Vm (mV)	-72.7±1.8	-71.0±1.7	ns	-74±2.1	-73.9±2.3	ns	-74.1±0.7	-76.3±1.4	ns
tau (ms)	2.4±0.3	2.7±0.3	ns	2.5±0.4	2.7±0.3	ns	2.5±0.2	2.2±0.2	ns

	Camk2a$^{f/f}$;*Camk2b*$^{f/f}$;*CAG-Cre*^{ER} OIL D10			OIL D21		
Parameter	Cre– (13)	Cre+ (14)	p-value	Cre– (12)	Cre+ (13)	p-value
Rm (MΩ)	241.5±15.4	227.9±15.2	ns	239.1±15.3	243.3±12.3	ns
Ra (MΩ)	24.3±1.4	25.4±1.2	ns	24.9±1.3	21.8±1.8	ns
Cm (pF)	84.7±5.4	80.5±4.3	ns	94.1±8.1	75.8±4.3	ns
Vm (mV)	-71.2±1.3	-71.5±1.3	ns	-70.5±1.4	-72.6±1.1	ns
tau (ms)	2.4±0.2	2.6±0.2	ns	3.1±0.3	2.1±0.3	0.0164

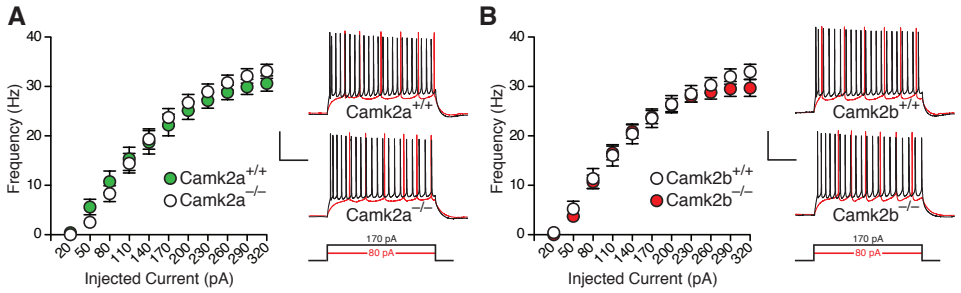


Figure 1: Loss of only CAMK2A and CAMK2B does not change excitability in hippocampal pyramidal CA1 neurons. (A) Excitability is unchanged in pyramidal neurons in *Camk2a*$^{-/-}$ (n=23) compared to *Camk2a*$^{+/+}$ (n=21) mice. **(B)** Excitability is unchanged in pyramidal neurons in *Camk2b*$^{-/-}$ (n=34) compared to *Camk2b*$^{+/+}$ (n=28) mice. N indicates the number of neurons measured. Scale bars indicate time (x) = 200ms and voltage (y) = 40mV.

Absence of both CAMK2A and CAMK2B causes a bidirectional change in excitability over time

Since we recently showed clear redundancy in some functions for CAMK2A and CAMK2B (Kool et al., submitted), we considered the possibility that also at the level of neuronal excitability, loss of CAMK2A is compensated for by CAMK2B and *vice versa*. Therefore, we decided to measure excitability in mice lacking both CAMK2A and CAMK2B. Because *Camk2a*$^{-/-}$;*Camk2b*$^{-/-}$ mice die at P1, we made use of our inducible homozygous floxed *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice (Kool et al., submitted). Simultaneous deletion of *Camk2a* and *Camk2b* in adult mice results in lethality 24-53 days after onset of deletion. Hence we decided to measure the neuronal changes over time in the three weeks following gene deletion but before the mice become clearly affected. We injected *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice with Tamoxifen from postnatal day 21 (P21) onwards for 4 consecutive days and measured neuronal excitability on three different time points: 10 days (D10), 15 days (D15) and 21 days (D21) after the 1st injection. Western blot analysis of prefrontal cortex tissue of the brains used for the whole cell experiments showed a 60% decrease of CAMK2A and an 85% decrease of CAMK2B protein on D10, which was further decreased on D15. Maximal reduction of protein level was obtained on D21 with <14% of CAMK2A and <5% of CAMK2B left in lysates (Figure 2A). First, we compared passive membrane properties, including input resistance, capacitance and resting membrane potential, of hippocampal CA1 pyramidal neurons at the different time points. Overall, we detected no differences in passive membrane properties on D10, D15 and D21 (see Table 1). Next, we measured voltage responses in CA1 pyramidal neurons upon depolarizing current injections. On D10, *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice showed a *decrease* in excitability (effect of genotype: $F_{(1,21)}$=7.64, p=0.012; Figure 2B). To our surprise, when measuring

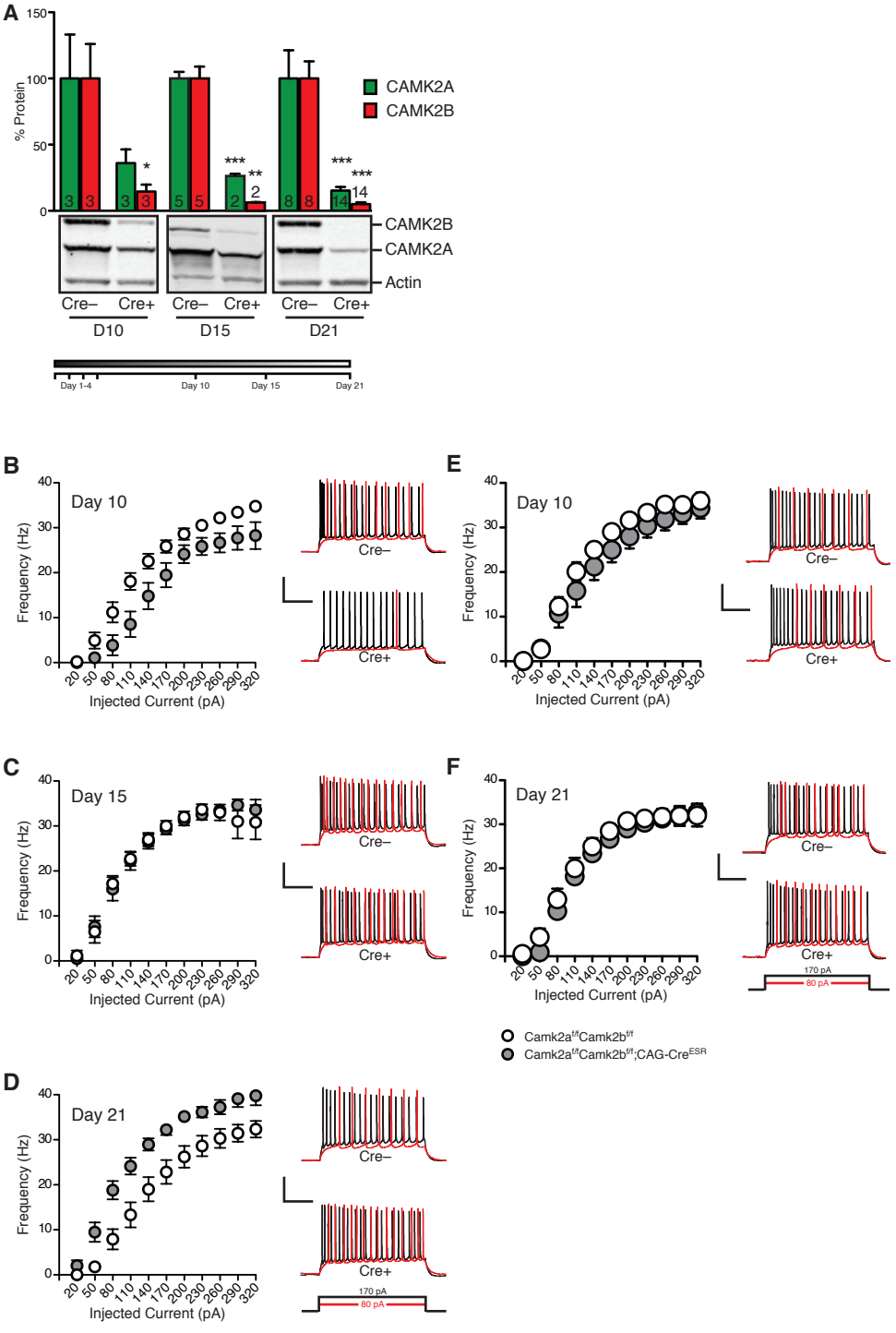

A
% Protein
CAMK2A
CAMK2B
Cre–
Cre+
D10
D15
D21
Actin
Day 1-4
Day 10
Day 15
Day 21
B
Day 10
Frequency (Hz)
Injected Current (pA)
C
Day 15
D
Day 21
E
Day 10
F
Day 21
170 pA
80 pA
Camk2a^f/f Camk2b^f/f
Camk2a^f/f Camk2b^f/f;CAG-Cre^ESR

Figure 2: Loss of both CAMK2A and CAMK2B causes a bidirectional change in excitability in time (A) CAMK2A and CAMK2B protein quantification using Western Blot of prefrontal cortex of *Camk2a$^{f/f}$;Camk2b$^{f/f}$-CAG-Cre^{ER}* mice (Cre+) versus *Camk2a$^{f/f}$;Camk2b$^{f/f}$* mice (Cre–) 10, 15 and 21 days after the first Tamoxifen injection. Quantification of all Cre+ samples was compared to normalized Cre– samples from the same age (set at 100%). Statistics: Cre- vs Cre+: CAMK2A; D10: t=1.8, p=0.14; D15: t=9.2, p<0.001; D21: t=5.2, p<0.0001; CAMK2B; D10: t=3.2], p=0.032; D15: t=6.3, p<0.01; D21: t=9.8, p<0.0001; unpaired two tailed t-test. Actin was used as a loading control and number of samples is depicted in the bars. **(B)** Cre+ mice (n=10) show decreased excitability 10 days after onset of gene deletion compared to Cre– mice (n=13). **(C)** Cre+ mice (n=10) show no change in excitability 15 days after onset of gene deletion compared to Cre– mice (n=8). **(D)** Cre+ mice (n=18) show increased excitability 21 days after onset of gene deletion compared to Cre– mice (n=15). **(E)** Cre+ mice (n=14) show no change in excitability 10 days after injection of vehicle compared to Cre– mice (n=13). **(F)** Cre+ mice (n=13) show no change in excitability 21 days after injection of vehicle compared to Cre– mice (n=12). N indicates the number of neurons measured, except for **A**. Scale bars in **B-F** indicate time (x) = 200ms and voltage (y) = 40mV.

pyramidal neurons on D15, excitability was no longer significantly different between the *Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER}* mutants and controls (effect of genotype: $F_{(1,16)}$=0.02, p=0.89; 2way repeated measures ANOVA; Figure 2C), whereas on D21 *Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER}* pyramidal neurons showed an *increase* in excitability compared to controls (effect of genotype: $F_{(1,31)}$=15.25, p<0.001; 2-way repeated measures ANOVA; Figure 2D). Importantly, *Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER}* mice injected with vehicle (sunflower oil without Tamoxifen) showed no changes in excitability both 10 and 21 days after the first vehicle injection (D10: effect of genotype: $F_{(1,25)}$=0.94, p=0.34; D21: effect of genotype: $F_{(1,23)}$=0.95, p=0.34; 2-way repeated measures ANOVA; Figure 2E and F). Taken together, whereas excitability was not changed in *Camk2a$^{-/-}$* and *Camk2b$^{-/-}$* single knockout mice, simultaneous deletion of both CAMK2A and CAMK2B initially induced a *decrease* in excitability followed by an *increase* in excitability.

CAMK2 is known to have both a catalytic as well as a structural role in neurons (Giese et al., 1998; Elgersma et al., 2002; Hojjati et al., 2007; Borgesius et al., 2011). To distinguish between the structural and catalytic role of CAMK2 in the observed phenotypes, excitability was measured again in CA1 pyramidal neurons of *Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER}* mice; this time not after injection with Tamoxifen, but in the presence or absence of two types of CAMK2 inhibitors: the calmodulin-binding competitor KN62 and substrate-binding competitor AIP-II (autocamtide-2 related inhibitory peptide II). Neither wash-in of 20uM KN62, nor 2.5h incubation of AIP-II affected excitability in CA1

pyramidal cells (KN62: effect of genotype: $F_{(1,26)}$=0.24, p=0.63; AIP-II: effect of genotype: $F_{(1,20)}$=0.08, p=0.79; 2-way repeated measures ANOVA; Figure 3A and B), suggesting that the changes in excitability are not due to acute loss of CAMK2 activity.

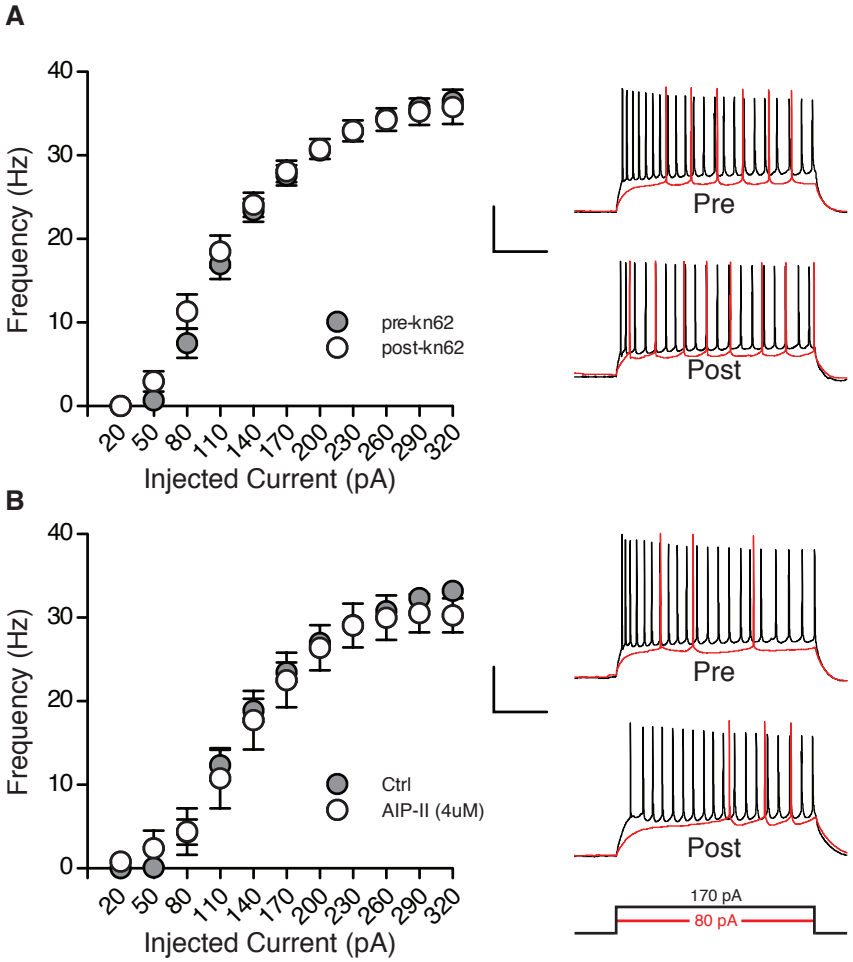


Figure 3: Blocking CAMK2 activity for several hours does not change excitability. (A) KN62 wash-in for 10 minutes does not change excitability in CA1 pyramidal (n=14) compared to control conditions with no KN62 (n=14). **(B)** Incubation of Autocamtide-2 related inhibitory peptide II (AIP-II) for 2.5 hours does not change excitability in CA1 pyramidal neurons (n=10) compared to control conditions with no AIP-II (n=12). In these experiments *Camk2a*$^{f/f}$*;Camk2b*$^{f/f}$*;Cag-Cre*^{ER} mice who received no Tamoxifen injections were used and were therefore used as controls. N indicates the number of neurons measured. Scale bars indicate time (x) = 200ms and voltage (y) = 40mV.

Absence of CAMK2 changes action potential threshold

Changes in excitability could result from changes in the active properties of CA1 neurons. Thus we analysed the action potential properties including the threshold, maximum amplitude, rise and decay slopes, and half-width. We analysed the properties of the first and the third action potential at the first step that induced action potentials from mice on D10 and D21. We found no changes in amplitude, maximum rise slope, maximum decay slope or action potential half-width on D10 (Figure 4). However, the threshold at which the action potential was generated on D10 for the first and third action potential was significantly more positive (Figure 4D). However, 21 days after onset of deletion, we found that mice lacking CAMK2 had a significantly more negative threshold, with no changes in amplitude, maximum rise slope, maximum decay slope or action potential half-width (Figure 5). Also, using a more physiological approach to elicit action potentials using high current squared pulses for 1 ms at 10Hz, we still found a more negative threshold in *Camk2a*$^{f/f}$*;Camk2b*$^{f/f}$*;Cag-Cre*^{ER} mice on D21 (Cre- vs Cre+: t=2.3, p=0.29; unpaired two tailed t-test; Figure 5G). Taken together, loss of CAMK2 significantly changed the action potential threshold first to a more positive level, which then shifted to a more negative level.

Reduction of both inhibitory and excitatory inputs upon loss of CAMK2

Hypo- and hyperexcitability of neurons is often caused by changes in the inhibition to excitation ratio (Isaacson and Scanziani, 2011; Zhou and Yu, 2018). CAMK2A and CAMK2B are highly expressed at the synapse, regulating functional as well as structural plasticity (Lee et al., 2009; Kim et al., 2015) and changes in the ratio of CAMK2A and CAMK2B are shown to influence unitary synaptic strength as well as mEPSC frequency (Thiagarajan et al., 2002). Thus, it is plausible that loss of one or both CAMK2 isoforms affects the inhibitory and/or excitatory input a neuron receives, resulting in changes in excitability. Therefore, we measured both miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs) in hippocampal pyramidal cells of both *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mice. Importantly, mEPSC and mIPSC frequency and amplitude were unaffected in both *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mice (*Camk2a*$^{-/-}$: mEPSC frequency: t=0.01, p=0.99; mEPSC amplitude: t=0.27, p=0.79; mIPSC frequency: t=0.05, p=0.96; mIPSC amplitude: t=0.51, p=0.62; *Camk2b*$^{-/-}$: mEPSC frequency: t=0.37, p=0.71; mEPSC amplitude: t=0.78, p=0.45; mIPSC frequency: t=1.98, p=0.06; mIPSC amplitude: t=0.66, p=0.51; unpaired two tailed t-test; Figure 6). We then hypothesized that redundancy could play a role on the level of mEPSC and mIPSC frequency and amplitude similar as in excitability. We measured *Camk2a*$^{f/f}$*;Camk2b*$^{f/f}$*;Cag-Cre*^{ER} mice on the two time points at which excitability was changed: 10 days (D10) and 21 days (D21) after the 1st injection. Surprisingly, on D10, mEPSCs and mIPSCs showed no difference in frequency and amplitude between

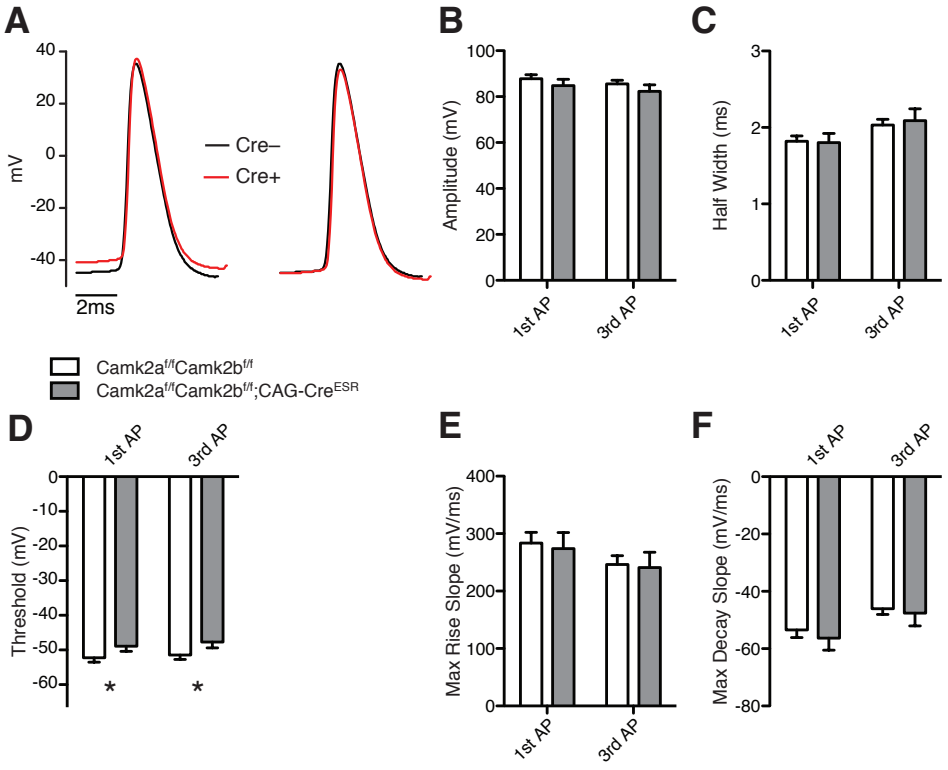


Figure 4: Action potential characteristics 10 days after onset of gene deletion of Camk2a and Camk2b in Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER} mice. (A) (*Left*) Average action potential for *Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER}* mice (Cre+; red) and *Camk2a$^{f/f}$;Camk2b$^{f/f}$* mice (Cre–; black). Averages were taken from the first action potential at the first depolarizing step that induced action potentials. Scale bar indicates time (x) = 2ms and absolute voltage is shown on the y-axis. (*Right*) Same averages as left, but now overlapped to allow easier comparison for overall shape. **(B)** No difference in amplitude of action potentials in the first and third action potential between Cre+ and Cre– mice. **(C)** No difference in the half width of action potentials in the first and third action potential between Cre+ and Cre– mice. **(D)** Cre+ mice show a significantly more depolarized action potential threshold in the first and third action potential compared to Cre– mice. **(E)** No difference in maximum rise slope of action potentials in the first and third action potential between Cre+ and Cre– mice. **(F)** No difference in maximum decay slope of action potentials in the first and third action potential between Cre+ and Cre– mice. For all graphs: Cre+ (n=10), Cre– (n=13). N indicates number of neurons measured.

Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER} mice and *Camk2a$^{f/f}$;Camk2b$^{f/f}$* control littermates (Cre- vs Cre+: mEPSC frequency: t=0.65, p=0.52; mEPSC amplitude: t=1.66, p=0.11; mIPSC frequency: t=1.0, p=0.31; mIPSC amplitude: t=0.32, p=0.75; unpaired two tailed t-test; Figure 7A and B). On D21 however, we found a significant decrease in mEPSC and mIPSC

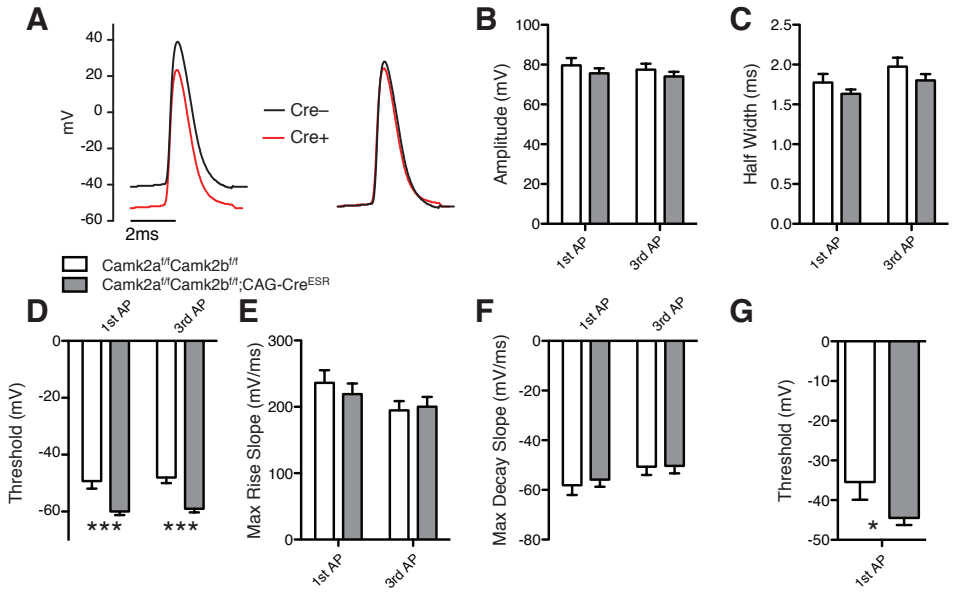


Figure 5: Action potential characteristics 21 days after onset of gene deletion of Camk2a and Camk2b. (A) (*Left*) Average action potential for *Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER}* mice (Cre+; red) and *Camk2a$^{f/f}$;Camk2b$^{f/f}$* mice (Cre–; black). Averages were taken from the first action potential from the first depolarizing step that induced action potentials. Scale bar indicates time (x) = 2ms and absolute voltage is shown on the y-axis. (*Right*) Same averages as left, but now overlapped to allow easier comparison for overall shape. **(B)** No difference in amplitude of action potentials in the first and third action potential between Cre+ and Cre– mice. **(C)** No difference in the half width of action potentials in the first and third action potential between Cre+ and Cre– mice. **(D)** Cre+ mice show a significantly more hyperpolarized action potential threshold in the first and third action potential compared to Cre– mice. **(E)** No difference in maximum rate of rise of the first or the third action potential between Cre+ and Cre– mice. **(F)** No difference in maximum decay slope of action potentials in the first and third action potential between Cre+ and Cre– mice. For all graphs **(A-F)**: Cre+ (n=18), Cre– (n=11). **(G)** a more physiological protocol using 1ms squared pulses at 10Hz instead of continuous depolarization still show a significantly more hyperpolarized action potential threshold in Cre+ (n=22) compared to Cre– (n=9). N indicates number of neurons measured.

frequency but not in amplitude (Cre- vs Cre+: mEPSC frequency: t=2.14, p=0.0398; mEPSC amplitude: t=0.71, p=0.48; mIPSC frequency: t=3.49, p<0.01; mIPSC amplitude: t=1.2, p=0.22; unpaired two tailed t-test; Figure 7C and D). To understand whether the reduced frequency was due to loss of synapses, neurons were filled with biocytin and the number of spines as well as their shape was analysed. Whereas there was no change in the density of spines per 10 micron length of dendrite, primary dendrites, branches and total neurite length (number of spines: t=0.06, p=0.95; unpaired two tailed t-test;

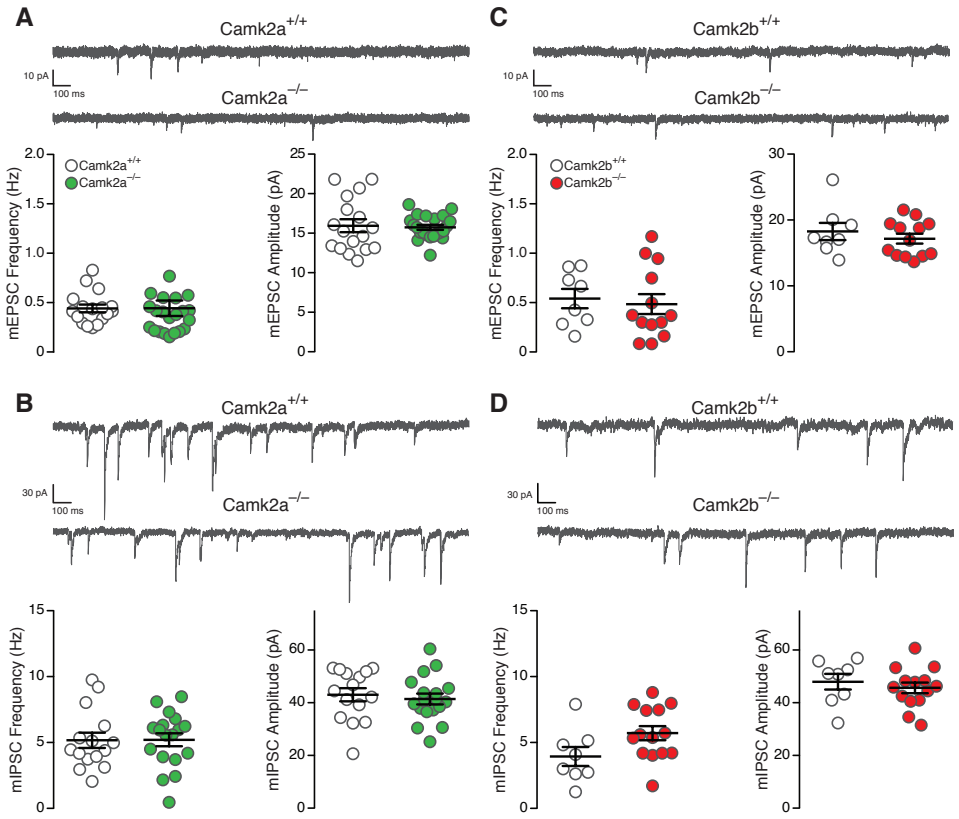


Figure 6: No changes in mEPSC and mIPSC frequency and amplitude of Camk2a$^{-/-}$ and Camk2b$^{-/-}$. (A) Representative traces (top) and frequency and amplitude (bottom) of miniature excitatory postsynaptic currents (mEPSC) measured from CA1 pyramidal cells. *Camk2a*$^{-/-}$ mice (n=21) show no change in frequency (*Camk2a*$^{+/+}$ vs *Camk2a*$^{-/-}$: 0.44 vs 0.44 Hz) and amplitude (*Camk2a*$^{+/+}$ vs *Camk2a*$^{-/-}$: 15.9 vs 15.7 pA) compared to *Camk2a*$^{+/+}$ mice (n=17). **(B)** Representative traces (top) and frequency and amplitude (bottom) of miniature inhibitory postsynaptic currents (mIPSC) measured from CA1 pyramidal cells. *Camk2a*$^{-/-}$ mice (n=18) show no change in frequency (*Camk2a*$^{+/+}$ vs *Camk2a*$^{-/-}$: 5.17 vs 5.21 Hz) and amplitude (*Camk2a*$^{+/+}$ vs *Camk2a*$^{-/-}$: 43.03 vs 41.42 pA) compared to *Camk2a*$^{+/+}$ mice (n=15). **(C)** Representative traces (top) and frequency and amplitude (bottom) of miniature excitatory postsynaptic currents (mEPSC) measured from CA1 pyramidal cells. *Camk2b*$^{-/-}$ mice (n=13) show no change in frequency (*Camk2b*$^{+/+}$ vs *Camk2b*$^{-/-}$: 0.54 vs 0.50 Hz) and amplitude (*Camk2b*$^{+/+}$ vs *Camk2b*$^{-/-}$: 18.26 vs 17.03 pA) compared to *Camk2b*$^{+/+}$ mice (n=8). **(D)** Representative traces (top) and frequency and amplitude (bottom) of miniature inhibitory postsynaptic currents (mIPSC) measured from CA1 pyramidal cells. *Camk2b*$^{-/-}$ mice (n=14) show no change in frequency (*Camk2b*$^{+/+}$ vs *Camk2b*$^{-/-}$: 3.95 vs 5.71 Hz) and amplitude (*Camk2b*$^{+/+}$ vs *Camk2b*$^{-/-}$: 47.97 vs 45.65 pA) compared to *Camk2b*$^{+/+}$ mice (n=8). Scale for all mEPSCs indicates time (x) = 100ms and current (y) = 10pA: Scale for all mIPSCs indicates time (x) = 100ms and current (y) = 30pA. N indicates number of neurons measured.

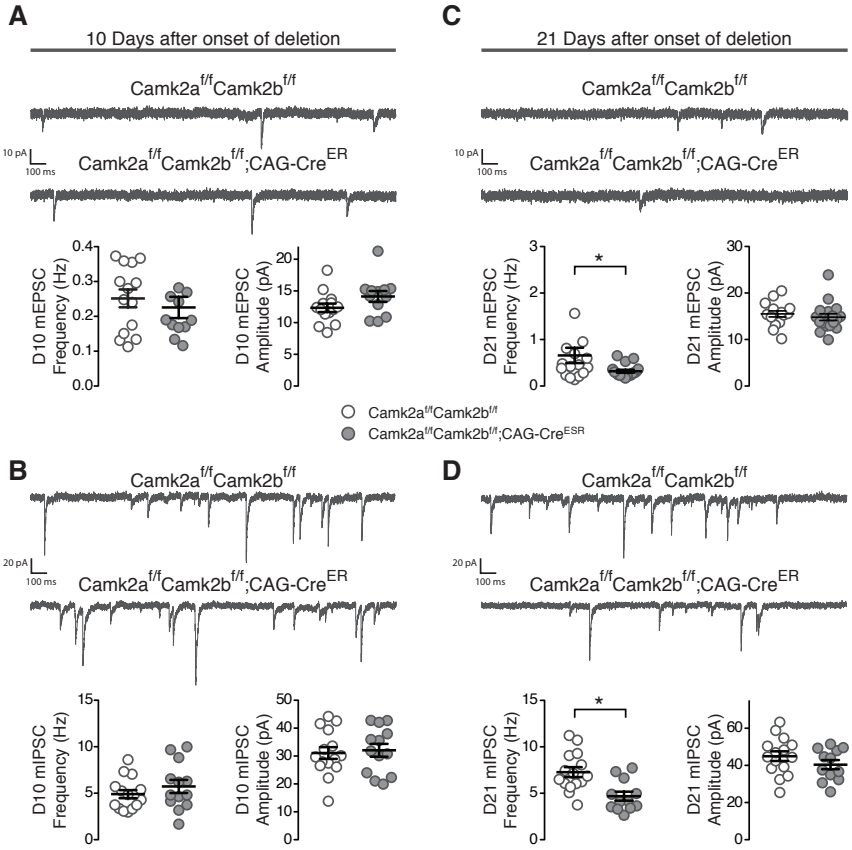


Figure 7: A decrease in mEPSC and mIPSC frequency 21 days after onset of gene deletion of *Camk2a* and *Camk2b*. (A) Representative traces (top) and frequency and amplitude (bottom) of miniature excitatory postsynaptic currents (mEPSC) measured from CA1 pyramidal cells 10 days after onset of gene deletion. *Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER}* mice (Cre+; n=11) show no change in frequency (Cre+ vs Cre–: 0.23 vs 0.25 Hz) and amplitude (Cre+ vs Cre–: 14.13 vs 12.35 pA) compared to *Camk2a$^{f/f}$;Camk2b$^{f/f}$* mice (Cre–; n=14). **(B)** Representative traces (top) and frequency and amplitude (bottom) of miniature inhibitory postsynaptic currents (mIPSC) measured from CA1 pyramidal cells 10 days after onset of gene deletion. *Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER}* mice (Cre+; n=13) show no change in frequency (Cre+ vs Cre–: 5.73 vs 4.90 Hz) and amplitude (Cre+ vs Cre–: 32.1 vs 31.1 pA) compared to *Camk2a$^{f/f}$;Camk2b$^{f/f}$* mice (Cre–; n=15). **(C)** Representative traces (top) and frequency and amplitude (bottom) of mEPSCs measured from CA1 pyramidal cells 21 days after onset of gene deletion. *Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER}* mice (Cre+; n=18) show a decrease in frequency (Cre+ vs Cre–: 0.32 vs 0.66 Hz) but not in amplitude (Cre+ vs Cre–: 14.82 vs 15.51 pA) of mEPSCs compared to *Camk2a$^{f/f}$;Camk2b$^{f/f}$* mice (Cre–; n=16). **(D)** Representative traces (top) and frequency and amplitude (bottom) of mIPSCs measured from CA1 pyramidal cells 21 days after onset of gene deletion. *Camk2a$^{f/f}$;Camk2b$^{f/f}$;Cag-Cre^{ER}* mice (Cre+; n=12) show a decrease in frequency (Cre+ vs Cre–: 4.70 vs 7.28 Hz) but not in amplitude (Cre+ vs Cre–: 40.40 vs 44.97 pA) of mIPSCs compared to *Camk2a$^{f/f}$;Camk2b$^{f/f}$* mice (Cre–; n=15). Scale for all mEPSCs indicates time (x) = 100ms and current (y) = 10pA: Scale for all mIPSCs indicates time (x) = 100ms and current (y) = 20pA. N indicates number of neurons measured.

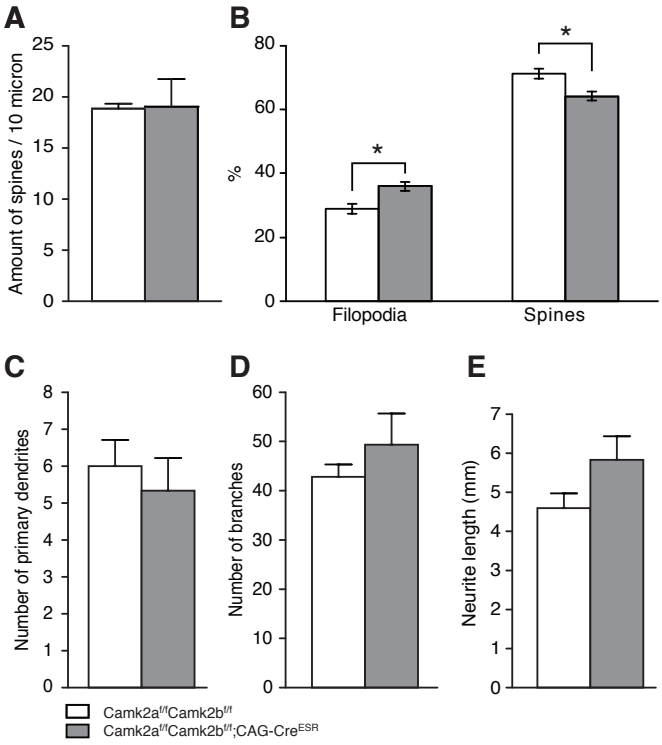


Figure 8: Loss of CAMK2A and CAMK2B causes an increase of immature spines (filopodia). **(A)** No change in the density of spines per 10 micron length of dendrite in *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice compared to *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice. **(B)** *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice show significantly more immature spines than *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice. For **(A)** and **(B)** 544 spines/filopodia from 4 neurons from 3 different *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice were used versus 540 spines/filopodia from 3 neurons from 3 different *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice. **(C)** No change in the number of primary dendrites in *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice compared to *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice. **(D)** No change in the number of neuronal branches in *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice compared to *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice. **(E)** No change in the total neurite length in *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice compared to *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice. For graphs **(C-E)** 3 neurons were analysed from 3 different *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice and 5 neurons from 4 different *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$ mice.

Figure 8A, C-E), we did find a trend towards more immature spines (filopodia) in the *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice (filopodia vs. spines: t=3.29, p=0.046; unpaired two tailed t-test; Figure 8B). Taken together, these results imply that the hypo-excitability seen in the *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice at day 10 after induction of gene deletion, is not caused by initial changes in the inhibition to excitation ratio.

DISCUSSION

Here we made use of the *Camk2a*$^{-/-}$, the *Camk2b*$^{-/-}$ as well as the *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice to study the effect of loss of either CAMK2A or CAMK2B or both simultaneously on neuronal excitability in hippocampal CA1 pyramidal neurons. Whereas loss of only CAMK2A or CAMK2B did not change the excitability, simultaneous loss of CAMK2A and CAMK2B resulted in a bidirectional change in excitability over time. Upon acute loss of both CAMK2 isoforms, excitability of hippocampal CA1 pyramidal cells *decreased,* but over time this was converted to an *increase* in excitability. These changes are accompanied by an initial increase of action potential threshold followed by a decrease in the threshold for which the underlying mechanisms are unknown. The changes in excitability seem not to be caused by changes in inhibition and excitation, since initially these inputs are not affected in our mini analysis; only at the latest time point a decrease was found in the frequency of both inhibitory and excitatory synaptic inputs.

It is interesting that an effect on excitability was observed only when CAMK2A and CAMK2B are both absent. This indicates that either one of them can fulfill the function needed to maintain normal intrinsic neuronal excitability. Several studies showed involvement of the kinase activity of CAMK2 in neuronal excitability, most of which indicate that CAMK2 functions to inhibit neuronal excitability. In vestibular nucleus neurons, loss of CAMK2 activity results in an increase of intrinsic excitability mediated through BK-type calcium-activated potassium channels (Nelson et al., 2005). In Drosophila, CAMK2 was found to phosphorylate the *ether à go-go* (EAG) potassium channel, and inhibition of CAMK2 results in hyper-excitability (Wang et al., 2002). The $K_v4.2$ channel is also a substrate for CAMK2, and increased CAMK2 activity results in a decrease in excitability (Varga et al., 2004). In cardiac tissue and the axon initial segment of Purkinje cells, b_{IV}-spectrin-dependent targeting of CAMK2 to $Na_v1.5$ allows for phosphorylation of $Na_v1.5$ on S571, altering excitability (Hund et al., 2010).

Surprisingly, we find a bidirectional change in excitability over time, starting with hypo-excitability. There are several explanations for this switch: i) dose-dependence; we find at 10 days after gene deletion still 40% of CAMK2A and 15% of CAMK2B left in the prefrontal cortex. Since we have recently shown that the half-life in the hippocampus is slightly longer than in the cortex (Kool et al., submitted), the expression levels of CAMK2 in the hippocampus at this time point are likely somewhat higher than what we measured in the prefrontal cortex. This would mean that in total a fair amount of the kinase is still present when we see the decrease in excitability. However, the *Camk2a*$^{-/-}$ or *Camk2b*$^{-/-}$ mice, in which there is still 50% of CAMK2 present, did not show any change in excitability. It is

possible that adult deletion in our mouse model prohibits the neuron to compensate for loss of 50% of the most abundant protein, possibly showing the direct involvement of CAMK2 in neuronal excitability. Upon further decrease of CAMK2 levels, perhaps the neuron tries to compensate for the total loss of CAMK2 and the accompanying loss of input on the synapses by homeostatically increasing the excitability at the soma. ii) subcellular localization of CAMK2 loss; CAMK2 is present in the soma, in dendrites as well as in spines. Additionally, there is a pool of CAMK2A mRNA present in the dendrites (Burgin et al., 1990; Benson et al., 1992). Depending on which pool of CAMK2 is depleted first upon acute gene deletion, different channels and pathways will be affected, initially resulting in a more positive threshold for action potential firing, hence hypo-excitability. Upon loss of CAMK2 in more subcellular locations, other pathways get involved, resulting eventually in a decrease in action potential firing, hence hyper-excitability. Interestingly, when applying 2 different CAMK2 inhibitors we did not find any change in excitability, indicating that acute inhibition in slices does not directly affect neuronal excitability. Thus, whether the shift from hypo- to hyper-excitability seen in our *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice is caused by a structural or kinetic function of CAMK2 or maybe a combination of both is subject for future research.

We observed a decrease in the frequency but not in the amplitude of miniature excitatory and inhibitory postsynaptic currents only 21 days after deletion of CAMK2A and CAMK2B, which is not seen in *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mice. However, at 10 days after gene deletion, no changes in mPSCs are observed. This indicates that the changes in excitability are not caused by changes in inhibitory and excitatory input.

At 21 days, it is possible that the neuron tries to compensate for the synaptic loss of CAMK2A and CAMK2B and the accompanying decrease in mPSC input by decreasing the action potential threshold. Loss of CAMK2B has already been associated with a decreased volume of spines and the conversion of mature spines into immature spines (Okamoto et al., 2007). Moreover, CAMK2 activity has been shown to be involved in unsilencing silent synapses (Liao et al., 2001). Our observation of more immature, filopodia-shaped synapses in the *Camk2a*$^{f/f}$;*Camk2b*$^{f/f}$;*Cag-Cre*^{ER} mice indeed supports the idea that CAMK2 is important for synapse formation and/or maintenance and indicates that the neuron tries to compensate for the loss of CAMK2 at the synapse by increasing somatic excitability. However, we cannot exclude the opposite possibility, in which the neuron compensates for the hyperexcitability by decreasing mPSC frequency. Furthermore, it is still possible that the changes in mPSCs and in excitability are 2 independent mechanisms, caused by deletion of CAMK2A and CAMK2B simultaneously.

The mouse models used in our study have a different onset of gene deletion. The *Camk2a*$^{f/f}$*;Camk2b*$^{f/f}$*-CAG-Cre*^{ER} mice develop normal CAMK2 expression up to the moment of Tamoxifen injections, usually starting at P21. However, *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mice lack CAMK2A or CAMK2B from germline. This could mean that differences observed can also be explained by differences during (embryological) development. However, for CAMK2A it was recently shown that adult deletion results in similar LTP and hippocampal-dependent learning deficits as germline deletion (Achterberg et al., 2014). Furthermore taking into account the late onset of CAMK2A expression (P1) (Bayer et al., 1999), it is unlikely that CAMK2A plays a role during the embryological development. Its role in early postnatal development remains to be uncovered. CAMK2B starts to be expressed at E12.5 (Karls et al., 1992; Bayer et al., 1999), and is therefore more likely to play an important role early in development. Indeed, for locomotion, germline deletion of *Camk2b* is more disruptive than deletion of *Camk2b* in adulthood (Kool et al., 2016), indicating an important role for CAMK2B in development for locomotion If this is also true for other phenotypes remains to be investigated. However, it is still possible that the lack of effect we see in the *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ single mutants is not only due to compensation of the other CAMK2 isoform still being present, but through other compensatory mechanisms kicking in during early development, and that adult deletion of CAMK2A or CAMK2B in inducible single mutants would cause similar changes in excitability and unitary synaptic transmission as seen in the inducible double Camk2a/Camk2b mutants studied here.

Taken together our data show critical involvement in neuronal excitability as well as synapse formation and shed new light on the full spectrum of CAMK2 function in neurons. Additionally, our data indicates that acute deletion of a gene can uncover novel functions, which could remain unseen using conventional knockout methods.

REFERENCES

Achterberg KG, Buitendijk GHS, Kool MJ, Goorden SMI, Post L, Slump DE, Silva AJ, Van Woerden GM, Kushner SA, Elgersma Y (2014) Temporal and Region-Specific Requirements of αCaMKII in Spatial and Contextual Learning. Journal of Neuroscience 34:11180–11187.

Ashpole NM, Song W, Brustovetsky T, Engleman EA, Brustovetsky N, Cummins TR, Hudmon A (2012) Calcium/calmodulin-dependent protein kinase II (CaMKII) inhibition induces neurotoxicity via dysregulation of glutamate/calcium signaling and hyperexcitability. J Biol Chem 287:8495–8506.

Barcomb K, Buard I, Coultrap SJ, Kulbe JR, O'Leary H, Benke TA, Bayer KU (2014) Autonomous CaMKII requires further stimulation by Ca2+/calmodulin for enhancing synaptic strength. The FASEB Journal.

Bayer KU, Löhler J, Schulman H, Harbers K (1999) 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.

Benson DL, Gall CM, Isackson PJ (1992) Dendritic localization of type II calcium calmodulin-dependent protein kinase mRNA in normal and reinnervated rat hippocampus. Neuroscience 46:851–857.

Borgesius NZ, Van Woerden GM, Buitendijk GHS, Keijzer N, Jaarsma D, Hoogenraad CC, Elgersma Y (2011) βCaMKII plays a nonenzymatic role in hippocampal synaptic plasticity and learning by targeting αCaMKII to synapses. Journal of Neuroscience 31:10141–10148.

Burgin KE, Waxham MN, Rickling S, Westgate SA, Mobley WC, Kelly PT (1990) In situ hybridization histochemistry of Ca2+/calmodulin-dependent protein kinase in developing rat brain. J Neurosci 10:1788–1798.

Elgersma Y, Fedorov NB, Ikonen S, Choi ES, Elgersma M, Carvalho OM, Giese KP, Silva AJ (2002) Inhibitory autophosphorylation of CaMKII controls PSD association, plasticity, and learning. Neuron 36:493–505.

Erondu NE, Kennedy MB (1985) Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain. J Neurosci 5:3270–3277.

Ghosh S, Reuveni I, Lamprecht R, Barkai E (2015) Persistent CaMKII activation mediates learning-induced long-lasting enhancement of synaptic inhibition. Journal of Neuroscience 35:128–139.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ (1998) Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. Science 279:870–873.

Hojjati MR, Van Woerden GM, Tyler WJ, Giese KP, Silva AJ, Pozzo-Miller L, Elgersma Y (2007) Kinase activity is not required for alphaCaMKII-dependent presynaptic plasticity at CA3-CA1 synapses. Nat Neurosci 10:1125–1127.

Hund TJ, Koval OM, Li J, Wright PJ, Qian L, Snyder JS, Gudmundsson H, Kline CF, Davidson NP, Cardona N, Rasband MN, Anderson ME, Mohler PJ (2010) A βIV-spectrin/CaMKII signaling complex is essential for membrane excitability in mice. J Clin Invest 120:3508–3519.

Incontro S, Díaz-Alonso J, Iafrati J, Vieira M, Asensio CS, Sohal VS, Roche KW, Bender KJ, Nicoll RA (2018) The CaMKII/NMDA receptor complex controls hippocampal synaptic transmission by kinase-dependent and independent mechanisms. Nature Communications 9:2069.

Isaacson JS, Scanziani M (2011) How Inhibition Shapes Cortical Activity. Neuron 72:231–243.

Karls U, Müller U, Gilbert DJ, Copeland NG, Jenkins NA, Harbers K (1992) 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.

Kim K, Lakhanpal G, Lu HE, Khan M, Suzuki A, Kato-Hayashi M, Narayanan R, Luyben TT, Matsuda T, Nagai T, Blanpied TA, Hayashi Y, Okamoto K (2015) A Temporary Gating of Actin Remodeling during Synaptic Plasticity Consists of the Interplay between the Kinase and Structural Functions of CaMKII. Neuron 87:813–826.

Klug JR, Mathur BN, Kash TL, Wang H-D, Matthews RT, Robison AJ, Anderson ME, Deutch AY, Lovinger DM, Colbran RJ, Winder DG (2012) Genetic inhibition of CaMKII in dorsal striatal medium spiny neurons reduces functional excitatory synapses and enhances intrinsic excitability. PLoS ONE 7:e45323.

Kool MJ, van de Bree JE, Bodde HE, Elgersma Y, Van Woerden GM (2016) The molecular, temporal and region-specific requirements of the beta isoform of Calcium/Calmodulin-dependent protein kinase type 2 (CAMK2B) in mouse locomotion. Sci Rep 6:26989.

Lee S-JR, Escobedo-Lozoya Y, Szatmari EM, Yasuda R (2009) Activation of CaMKII in single dendritic spines during long-term potentiation. Nature 458:299–304.

Liao D, Scannevin RH, Huganir R (2001) Activation of silent synapses by rapid activity-dependent synaptic recruitment of AMPA receptors. Journal of Neuroscience 21:6008–6017.

Lledo PM, Hjelmstad GO, Mukherji S, Soderling TR, Malenka RC, Nicoll RA (1995) Calcium/calmodulin-dependent kinase II and long-term potentiation enhance synaptic transmission by the same mechanism. Proceedings of the National Academy of Sciences of the United States of America 92:11175–11179.

Mayford M, Bach ME, Huang YY, Wang L, Hawkins RD, Kandel ER (1996) Control of memory formation through regulated expression of a CaMKII transgene. Science 274:1678–1683.

Nelson AB, Gittis AH, Lac du S (2005) Decreases in CaMKII Activity Trigger Persistent Potentiation of Intrinsic Excitability in Spontaneously Firing Vestibular Nucleus Neurons. Neuron 46:623–631.

Ninan I, Arancio O (2004) Presynaptic CaMKII is necessary for synaptic plasticity in cultured hippocampal neurons. Neuron 42:129–141.

Okamoto K-I, Narayanan R, Lee SH, Murata K, Hayashi Y (2007) The role of CaMKII as an F-actin-bundling protein crucial for maintenance of dendritic spine structure. Proceedings of the National Academy of Sciences of the United States of America 104:6418–6423.

Sametsky EA, Disterhoft JF, Ohno M (2009) Autophosphorylation of αCaMKII downregulates excitability of CA1 pyramidal neurons following synaptic stimulation. Neurobiology of Learning and Memory 92:120–123.

Silva AJ, Paylor R, Wehner JM, Tonegawa S (1992a) Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science 257:206–211.

Silva AJ, Stevens CF, Tonegawa S, Wang Y (1992b) Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science 257:201–206.

Thiagarajan TC, Piedras-Renteria ES, Tsien RW (2002) alpha- and betaCaMKII. Inverse regulation by neuronal activity and opposing effects on synaptic strength. Neuron 36:1103–1114.

Tobimatsu T, Fujisawa H (1989) Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. J Biol Chem 264:17907–17912.

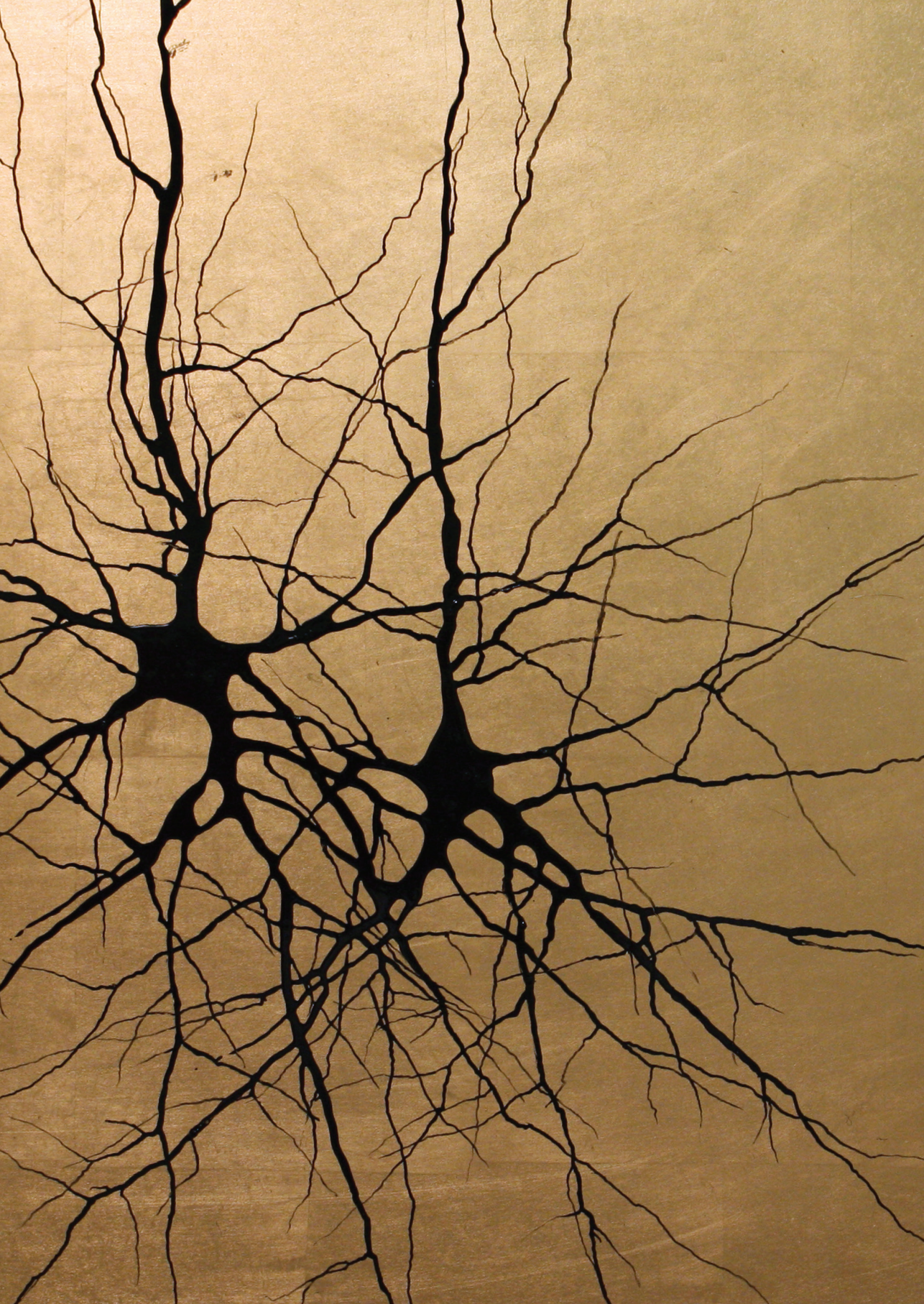
Van Woerden GM, Hoebeek FE, Gao Z, Nagaraja RY, Hoogenraad CC, Kushner SA, Hansel C, De Zeeuw CI, Elgersma Y (2009) betaCaMKII controls the direction of plasticity at parallel fiber-Purkinje cell synapses. Nature Publishing Group 12:823–825.

Varga AW, Yuan L-L, Anderson AE, Schrader LA, Wu G-Y, Gatchel JR, Johnston D, Sweatt JD (2004) Calcium-calmodulin-dependent kinase II modulates Kv4.2 channel expression and upregulates neuronal A-type potassium currents. Journal of Neuroscience 24:3643–3654.

Wang Z, Wilson GF, Griffith LC (2002) Calcium/calmodulin-dependent protein kinase II phosphorylates and regulates the Drosophila eag potassium channel. J Biol Chem 277:24022–24029.

Wei J, Zhang M, Zhu Y, Wang JH (2004) Ca2+–calmodulin signalling pathway up-regulates GABA synaptic transmission through cytoskeleton-mediated mechanisms. Neuroscience 127:637–647.

Zhou S, Yu Y (2018) Synaptic E-I Balance Underlies Efficient Neural Coding. Front Neurosci 12:243.



Chapter 6

General Discussion

DISCUSSION

The aim of this thesis was to further elucidate the roles of CAMK2A and CAMK2B, both their individual as well as their collective role, in neuronal and cognitive functioning.

Throughout the chapters in this thesis the Cre-LoxP system has played a central role to investigate protein functioning in the different mouse mutants. The Cre-LoxP system has the advantage to explore functions of the gene of interest in both a temporal as well as a spatial manner. We first used this technique to target *Camk2a* and *Camk2b* separately in **Chapter 2** and **3**. We explored both the spatial and temporal roles of these proteins and compared them to phenotypes that have been observed in the global *Camk2a* and *Camk2b* knockout models.

In **Chapter 2** we focussed specifically on CAMK2A. We generated a floxed mouse model and investigated the spatiotemporal role of CAMK2A in spatial and contextual learning and LTP. We found that forebrain specific deletion of CAMK2A impairs learning but cerebellar specific deletion does not. Furthermore, adult deletion of CAMK2A results in impaired LTP at the Schaffer-collateral pathway, similar as in conventional *Camk2a* knockout mice. These results highlight the necessity of CAMK2A in the forebrain but not in cerebellar Purkinje cells in spatial and contextual learning. Furthermore, these results showed that the presence of CAMK2A at the time of learning is an absolute requirement.

In **Chapter 3** we used a similar approach. We used several *Camk2b* mouse mutants, two of which were new, and tested them on the accelerating Rotarod. Already CAMK2B was known for its important role in locomotion, as conventional *Camk2b* knockout mice show profound locomotion deficits on the Rotarod (Van Woerden et al., 2009; Bachstetter et al., 2014). We further tested the molecular, temporal and region-specific requirements for this deficit. First, we found that autonomous activity of CAMK2B (using CAMK2B-T287A mice) is dispensable for locomotion. In sharp contrast, loss of Ca^{2+}-dependent activity in CAMK2B-A303R mice has a severe impact on locomotion performance. Second, onset of deletion in adulthood resulted in a relatively mild impairment in locomotion, suggesting that the locomotion deficit in *Camk2b*$^{-/-}$ mice, is mainly of developmental origin. Third, with respect to spatial requirements, early onset of gene deletion in cerebellum, striatum or forebrain did not recapitulate this deficit, arguing that this deficit cannot be attributed to a single brain area.

The findings of these two chapters can be discussed both on a temporal and spatial level. On a temporal level, we gained more information on the role of the separate isoforms in development. The phenotypes we found in **Chapter 2** in the *Camk2a*$^{f/f}$ mice

are remarkably consistent with previous research performed in *Camk2a*$^{-/-}$ mice (Silva et al., 1992a; 1992b; Elgersma et al., 2002). These findings make it unlikely that CAMK2A plays an important role in (postnatal) brain development necessary to sustain normal LTP and hippocampal-dependent learning. Additionally, the late onset expression of CAMK2A at P1 (Bayer et al., 1999) and the dependency of neonatal LTP on PKA but not on CAMK2 (Yasuda et al., 2002) further suggest that CAMK2A does not have an important developmental role. However, to definitively exclude a developmental role for CAMK2A, another approach using the Cre-LoxP system could be used. Instead of flanking exon 2 of *Camk2a* with LoxP sites, as has been done in the *Camk2a*$^{f/f}$ mice, the *Camk2a* gene could be preceded by a Stop-cassette flanked by LoxP sites. Hence, expression of the gene is withheld until the moment of Tamoxifen injection. This way, mice could reach adulthood mimicking the knockout condition and upon deletion of the Stop-cassette expression of CAMK2A is restored. The interesting question then becomes whether LTP is fully reinstated and if the phenotypes in hippocampal-dependent learning are rescued. If this is indeed the case, then a developmental role for CAMK2A could be excluded in LTP and hippocampal-dependent learning. A similar approach using this technique has recently been used for Angelman syndrome, which showed that rescue of behavioural phenotypes (but not LTP) required gene reinstatement early in life (Silva-Santos et al., 2015). These experiments would require a new set of mutant *Camk2* mice, but these rescue experiments answer a fundamentally important question for the treatment of intellectual disability in humans, in particular for individuals where malfunctioning of CAMK2 is directly causing intellectual disability (Küry et al., 2017 and Akita et al., 2018).

In contrast to CAMK2A, we did find a developmental role for CAMK2B in locomotion. In **Chapter 3** we showed that the adult onset of *Camk2b* deletion resulted in a locomotion phenotype which was rather mild compared to *Camk2b*$^{-/-}$ mice. This implies that CAMK2B has a more substantial role during development than in adulthood. We have chosen to delete CAMK2B 8-10 weeks postnatally. Since expression starts already at embryonic day 12.5 (Bayer et al., 1999) this means that the brain in these mice has been allowed to mature in the presence of CAMK2B for a substantial amount of time before it was deleted. It could be expected that earlier onset of deletion will result in a stronger locomotion phenotype. Conversely, the same could hold if time between *Camk2b* deletion and testing of locomotion increases. This would mean that the longer CAMK2B is absent in our mouse model, the more detrimental the locomotion phenotype will be. Whether this hypothesis is true, would be worth investigating.

Earlier I discussed the possibility of reinstating CAMK2A expression in adulthood using Stop-cassettes flanked by LoxP sites. A similar approach could be used for the locomotion deficits present in *Camk2b*$^{-/-}$ mice. Will reinstating CAMK2B rescue these locomotion

deficits? And would early reinstatement have a more profound rescue effect than late reinstatement? Some of the *Camk2b* patients that were recently identified (Küry et al., 2017) showed delayed gross motor development. Again, these rescue experiments could provide us with crucial information on whether reinstating normal CAMK2B expression in patients would be feasible to treat these impairments in the future.

On a spatial level we gained more information which brain regions are involved in the phenotypes present in the global knockout mice. For example, in **Chapter 2** we found that CAMK2A in the forebrain but not in the cerebellum is important for LTP and spatial learning. However, considering the size of the forebrain we still targeted a relatively broad area in the brain. Future research could further dissect the role of CAMK2A in different smaller brain areas or within different subfields of these brain areas. For example, different areas in the hippocampus are known for different functions. The CA3 area has been postulated in pattern recognition and therefore essential in spatial learning, partially due to the recurrent connections between different CA3 pyramidal cells (Marr, 1971). Would loss of CAMK2A in this area underlie the spatial learning deficits? A previous study using knockout mice for NMDA-receptor subunit NR1 specifically in CA3 have already shown an important role for NMDA-receptor dependent LTP in pattern recognition and completion (Nakazawa et al., 2002).

When we deleted CAMK2B in different brain regions in **Chapter 3**, we failed to locate a single brain area responsible for the locomotion deficit observed in *Camk2b*$^{-/-}$ mice. It might be possible that we missed this either because (i) the promoters driving the Cre in our mouse models start expressing Cre too late allowing normal embryonic development, (ii) we failed to target the brain area involved in the locomotion deficit or (iii) we missed it because we only targeted CAMK2B in one brain region at a time whereas the locomotion deficit might arise from the summation of relative small deficits in multiple brain regions. In order to distinguish between these options, we could either switch using new Cre-driver lines or cross already existing Cre-lines to expand the deletion over multiple brain regions. An example of the former could be to use Cre-lines targeting other brain regions not used in our study important for locomotion, such as the thalamus or deep cerebellar nuclei. An example of expanding the deletion over multiple brain regions would be using the GABAa6-Cre and L7-Cre simultaneously, thereby targeting a bigger part of the cerebellum. However, considering the inter-experimental variation present when using the Rotarod, one could argue whether exploring these options using the Rotarod will be feasible.

Next to dissecting temporal and spatial roles of the different isoforms, in **Chapter 3** we also focussed on the molecular requirements of CAMK2B in the locomotion phenotype.

We found that binding of Ca^{2+}/Calmodulin to CAMK2B is crucial for locomotion (as seen in *Camk2b*$^{A303R/A303R}$ mice), whereas subsequent autophosphorylation is not (as seen in *Camk2b*$^{T287A/T287A}$ mice). However, upon binding of Ca^{2+}/Calmodulin two events occur: CAMK2B becomes active and the protein detaches from actin. Even though this occurs subsequently, they can still be seen as two separate events. Using *Camk2b*$^{A303R/A303R}$ mice we could not distinguish whether it is the impairment in activating CAMK2B or whether it is the inability to detach from actin that underlies the locomotion deficit.

In **Chapters 4** and **5** we continued using the Cre-LoxP system now targeting *Camk2a* and *Camk2b* simultaneously. Considering the remarkable similarity on genetic and protein level, we hypothesized that certain functions could be carried out by both CAMK2A and CAMK2B. Targeting both genes allowed us to explore redundancy between the two isoforms. Below, our findings will be further discussed in the light of this redundancy.

In **Chapter 4** we explored potential redundancy and uncovered new functions of CAMK2 by deleting or mutating *Camk2a* and *Camk2b* simultaneously. Complete loss of CAMK2A and CAMK2B is lethal both during development as well as in adulthood. Moreover, loss of Ca^{2+}-dependent and Ca^{2+}-independent activity is lethal. On a physiological level, loss of CAMK2A and CAMK2B in adulthood causes a complete loss of LTP at the CA3-CA1 synapse in the hippocampus. This could not be attributed to a presynaptic role of CAMK2A and CAMK2B in this synapse, as presynaptic deletion of these two proteins did not impair LTP expression. Finally, we found that simultaneous deletion of CAMK2A and CAMK2B at the CA3-CA3 synapse impairs LTP, whereas deletion of only CAMK2A or CAMK2B does not. Taken together, these results show yet unknown roles for CAMK2A and CAMK2B that were masked in previous studies by redundancy of the isoform still present in the single knockout models.

In **Chapter 5** we continued exploring the redundancy of CAMK2A and CAMK2B in our inducible *Camk2a*$^{f/f}$*;Camk2b*$^{f/f}$ mouse model. We investigated the role of CAMK2A and CAMK2B in neuronal excitability and unitary synaptic transmission on a single cell level in CA1 pyramidal cells using whole-cell electrophysiology. We found a bidirectional change in excitability in time in CA1 pyramidal cells upon loss of CAMK2A and CAMK2B. Loss of CAMK2A and CAMK2B decreased neuronal excitability 10 days after onset of deletion, which was absent 15 days after onset of deletion and subsequently followed by an increase in excitability 21 days after onset of deletion. At the synaptic level, loss of CAMK2A and CAMK2B resulted in a decrease in mIPSC and mEPSC frequency and amplitude 21 days after onset of deletion, but not after 10 days. Importantly, these

changes in excitability and mEPSC and mIPSC frequency and amplitude were not found in conventional *Camk2a*$^{-/-}$ and *Camk2b*$^{-/-}$ mice. Finally, the decrease in mEPSC and mIPSC frequency was accompanied by higher percentage of immature spines (filopodia).

These results further emphasize the redundancy of CAMK2A and CAMK2B. Only upon simultaneous deletion of CAMK2A and CAMK2B could these phenotypes be uncovered. This redundancy could be explained by the evolutionary origins of the *Camk2a* and *Camk2b* genes. As mentioned in the introduction of this thesis, it is very likely that *Camk2a* and *Camk2b* share a common ancestral gene. As assessed in rats, different CAMK2 isoforms have a 89-93% sequence homology in the catalytic and regulatory domain (Tobimatsu and Fujisawa, 1989). Therefore, it is not surprising that they can have an overlap in substrates. This is exemplified by CAMK2's role in survival, CA3-CA3 LTP, neuronal excitability and unitary synaptic transmission, where single *Camk2a* or *Camk2b* knockout mice show no phenotypes. These findings argue for more studies on CAMK2 double knockout or knock-in mouse models for CAMK2 to explore novel functions that were previously hidden in single knockout or knock-in mice.

Unfortunately, we were not able to identify the cause of death in our mouse mutants. We can however speculate, based on studies performed investigating proteins closely related to CAMK2. For example, knockout animals for NMDA-receptor subunits such as NR1 and NR2B, die shortly after birth (Forrest et al., 1994; Kutsuwada et al., 1996), similar as our *Camk2a*$^{-/-}$*;Camk2b*$^{-/-}$ mice. These mouse models have been found to die from respiratory failure and impaired suckling response, respectively. An impaired suckling response is unlikely, given that we found milk in the stomachs of P0 pups. However, it is possible that respiratory failure could be the cause of death for *Camk2a*$^{-/-}$*;Camk2b*$^{-/-}$ mice. Indeed, a role for CAMK2 in the emergence and maintenance of synchronous activity in the preBötzinger complex (preBotC), a respiratory center in the brainstem important for the motor output of respiration, was found (Mironov, 2013). In this study, blocking CAMK2 activity using KN93 attenuated bursting activity of the preBotC. Another reason why respiratory failure is a likely cause of death in *Camk2a*$^{-/-}$*;Camk2b*$^{-/-}$ mice is based on the importance of a well-functioning respiratory system after birth. *In utero* the organism is not depending on respiration. After birth however, respiration is critical for sustaining life. It is not unlikely that in the absence of CAMK2 in the preBotC in these mice the induction of a respiratory rhythm fails. For adult mice however, this argument is harder to defend, mainly since some mice lived more than 50 days after the induction of deletion, well after levels of CAMK2 in the brain have dropped to a minimum. Still, loss of CAMK2 in the preBotC could dysregulate the synchronous activity in the preBotC in such a way that the mice can sustain for multiple days, but eventually will die because of this dysregulation.

In addition to a new-found role for CAMK2 in survival, our electrophysiological findings in the hippocampus shed some important light on the role of CAMK2 in plasticity throughout the brain. The finding that loss of CAMK2A and CAMK2B completely eliminated LTP elicited by high frequency stimulation at the CA3-CA1 synapse, while it was only partially impaired in the CA3-CA3 synapse underscores the differences that are present in the brain in mechanisms underlying LTP on a spatial level. The CA3-CA1 synapse may be an example of a synapse where CAMK2 has a greater role for expression of LTP, whereas in the CA3-CA3 synapse other protein kinases such as PKA, PKG or PKC (as suggested by Lu and Hawkins, 2006) may act in concert with CAMK2 to express LTP. However, we have shown that PKA-dependent LTP is also present at the CA3-CA1 synapse and is only partially impaired there when CAMK2A and CAMK2B are absent. Also on a temporal level plasticity is known to be regulated differently. For example in the CA3-CA1 synapse, early expression of LTP (<9 postnatal days) is not regulated by CAMK2 but by PKA (Yasuda et al., 2002). Thus, whereas the CA3-CA1 synapse could well be the most-studied synapse in the mammalian brain, scientists should be cautious in extrapolating observations from the CA3-CA1 synapse taken from one developmental stage to other synapses in the brain or other developmental stages.

When we performed whole-cell electrophysiology on a single cell level, we found a bidirectional change in excitability over time. What is the reason for this change over time? One explanation could be that the effect of excitability is dose-dependent. For example, on day 10 when there is a decrease in excitability, levels of CAMK2A and CAMK2B are at 60% and 15%, respectively. Upon day 21 when there is an increase in excitability, levels have dropped to 14% for CAMK2A and less than 5% for CAMK2B. Another explanation could be that the loss of CAMK2 directly triggers this decrease in excitability, which is later homeostatically (over)compensated by an increase in excitability. The difference between these two scenarios is that in the former the hyperexcitability of D21 is directly related to the loss of CAMK2, whereas in the latter the hyperexcitability is an indirect (compensatory) effect.

Loss of CAMK2A and CAMK2B also leads to a decrease in unitary synaptic transmission 21 days after onset of gene deletion. Interestingly, this loss of unitary synaptic transmission in **Chapter 5** (decrease in frequency of mEPSCs and mIPSCs) precedes the basal synaptic transmission tested in field electrophysiology on day 25 in **Chapter 4**, where we observed normal synaptic transmission. These two observations seem hard to reconcile. In whole-cell electrophysiology we selectively measured input on a single hippocampal pyramidal cell in the presence of tetrodotoxin (TTX), thereby blocking action potential-induced vesicle release. In field electrophysiology we measure a field of pyramidal neurons simultaneously with no blockers present and the evoked

release of presynaptic vesicles. There are multiple explanations for this discrepancy in outcome between these two experimental approaches: (i) the effects we measure using whole-cell electrophysiology are relatively small and dissipate when measured using field electrophysiology or (ii) the effects are only noticeable when action potentials are blocked (i.e. during unitary synaptic transmission), but are compensated by action potential-dependent release. Future research could try to distinguish between these options and find an explanation for this seeming inconsistency.

The change in excitability in time in **Chapter 5** highlights the importance of working with inducible knockout mice. By using inducible knockout mice, we kept track of the changes occurring in time, demonstrating that the increase in excitability on day 21 is preceded by a decrease in excitability on day 10. It is conceivable that similar changes occur in conventional knockout mice as well, as a response of the neuron to the absence of one of its crucial proteins. However, this could easily be missed by researchers since these compensatory mechanisms are likely to kick in already during development and most experiments are done after that time window. Therefore, certain phenotypes in conventional knockout mice could be directly attributed to the loss of the gene whereas the notion that the phenotype could also be a compensatory mechanism (i.e. an indirect effect) will be missed. For example, in our double knockout mice the hyperexcitability at day 21 could be a compensatory mechanism for the initial hypo-excitability on day 10. The point that needs to be stressed here is that any phenotype can thus be a direct consequence of the loss of a protein or it can be caused as a compensatory reaction towards the absence of the protein. Therefore, when researchers are exploring phenotypes they should be cautious for indirect effects when investigating conventional knockout models and be aware of compensatory mechanisms. One way to better track changes occurring upon loss of a protein is by studying inducible knockout mouse models and follow the changes over time.

During the years when our insight in CAMK2A and CAMK2B function accumulated using animal models, the question has always been: are there human patients with *CAMK2* mutations? Now, nearly 25 years after the generation of the first knockout mouse for *Camk2a* in 1992 (Silva et al., 1992a; 1992b) we are starting to find point mutations or haploinsufficiency in humans (Iossifov et al., 2014; Vincent et al., 2014; Küry et al., 2017; Akita et al., 2018). Compared to other genes causing intellectual disability however, it took a significant amount of time before the first human patients with mutations in one of the *CAMK2* genes were found. On the contrary, in other diseases causing intellectual disability (e.g. *FMRP* in Fragile X or *UBE3A* in Angelman Syndrome) the underlying genetic cause is already known for quite some time. One reason could be that these genetic diseases present a typical cluster of symptoms making them easier to recognize

clinically. Another potential reason is that genetic screening is very expensive, which could make clinicians reluctant to perform these tests when the symptoms at hand cannot be treated with drugs or surgically, symptoms such as intellectual disability or delay in motor development. At present however, prices of sequencing a human genome have plummeted ever since 2007 (Hayden, 2014), now reaching prices under $1000 per genome and prices are expected to drop even further for the coming years. This creates great opportunities to genetically screen vast numbers of patients with intellectual disability. Most certainly, some of these patients will have mutations in one of the *CAMK2* genes. When more patients are found, it will be easier to cluster these patients. This could be done according to which *CAMK2* gene is affected or according to the effect of the mutation (for example, whether it is a loss or gain of function mutation) or even the localization of the mutation (which domain is affected). Beyond doubt, this will further increase our understanding of CAMK2 in cognitive functioning in humans.

Conclusively, the research in this thesis increases our understanding of Ca^{2+}/calmodulin-dependent protein kinase II in the brain both on a spatial as well as a temporal level. CAMK2A and CAMK2B are two majorly abundant proteins in the mammalian brain, controlling a great deal of different processes. For example, on the basis of this thesis CAMK2 can be found to control processes ranging from locomotion and spatial navigation on a behavioural level to excitability at a somatic level and LTP at a synaptic level. As noted in the previous paragraph, with the recent discovery of humans with mutations in *CAMK2*, future research is expected to start investigating human patients alongside animal models. The next decades will be very promising in terms of describing the full spectrum of clinical features in which *CAMK2* mutations can present themselves. We might still be a long way from curing patients with a *CAMK2* mutation, but one small step at a time, we will get closer to obtaining a therapy that can benefit patients with a mutation in one of the *CAMK2* genes.

REFERENCES

Akita T et al. (2018) De novo variants in CAMK2A and CAMK2B cause neurodevelopmental disorders. Ann Clin Transl Neurol 5:280–296.

Bayer KU, Löhler J, Schulman H, Harbers K (1999) 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.

Elgersma Y, Fedorov NB, Ikonen S, Choi ES, Elgersma M, Carvalho OM, Giese KP, Silva AJ (2002) Inhibitory autophosphorylation of CaMKII controls PSD association, plasticity, and learning. Neuron 36:493–505.

Forrest D, Yuzaki M, Soares HD, Ng L, Luk DC, Sheng M, Stewart CL, Morgan JI, Connor JA, Curran T (1994) Targeted disruption of NMDA receptor 1 gene abolishes NMDA response and results in neonatal death. Neuron 13:325–338.

Hayden EC (2014) Technology: The $1,000 genome. Nature 507:294–295.

Iossifov I et al. (2014) The contribution of de novo coding mutations to autism spectrum disorder. Nature:1–17.

Kutsuwada T, Sakimura K, Manabe T, Takayama C, Katakura N, Kushiya E, Natsume R, Watanabe M, Inoue Y, Yagi T, Aizawa S, Arakawa M, Takahashi T, Nakamura Y, Mori H, Mishina M (1996) Impairment of suckling response, trigeminal neuronal pattern formation, and hippocampal LTD in NMDA receptor epsilon 2 subunit mutant mice. Neuron 16:333–344.

Küry S et al. (2017) De Novo Mutations in Protein Kinase Genes CAMK2A and CAMK2B Cause Intellectual Disability. The American Journal of Human Genetics 101:768–788.

Lu F-M, Hawkins RD (2006) Presynaptic and postsynaptic Ca(2+) and CamKII contribute to long-term potentiation at synapses between individual CA3 neurons. Proceedings of the National Academy of Sciences of the United States of America 103:4264–4269.

Marr D (1971) Simple memory: a theory for archicortex. Philos Trans R Soc Lond, B, Biol Sci 262:23–81.

Mironov SL (2013) Calmodulin and calmodulin kinase II mediate emergent bursting activity in the brainstem respiratory network (preBotzinger complex). The Journal of Physiology 591:1613–1630.

Nakazawa K, Quirk MC, Chitwood RA, Watanabe M, Yeckel MF, Sun LD, Kato A, Carr CA, Johnston D, Wilson MA, Tonegawa S (2002) Requirement for hippocampal CA3 NMDA receptors in associative memory recall. Science 297:211–218.

Silva AJ, Paylor R, Wehner JM, Tonegawa S (1992a) Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science 257:206–211.

Silva AJ, Stevens CF, Tonegawa S, Wang Y (1992b) Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science 257:201–206.

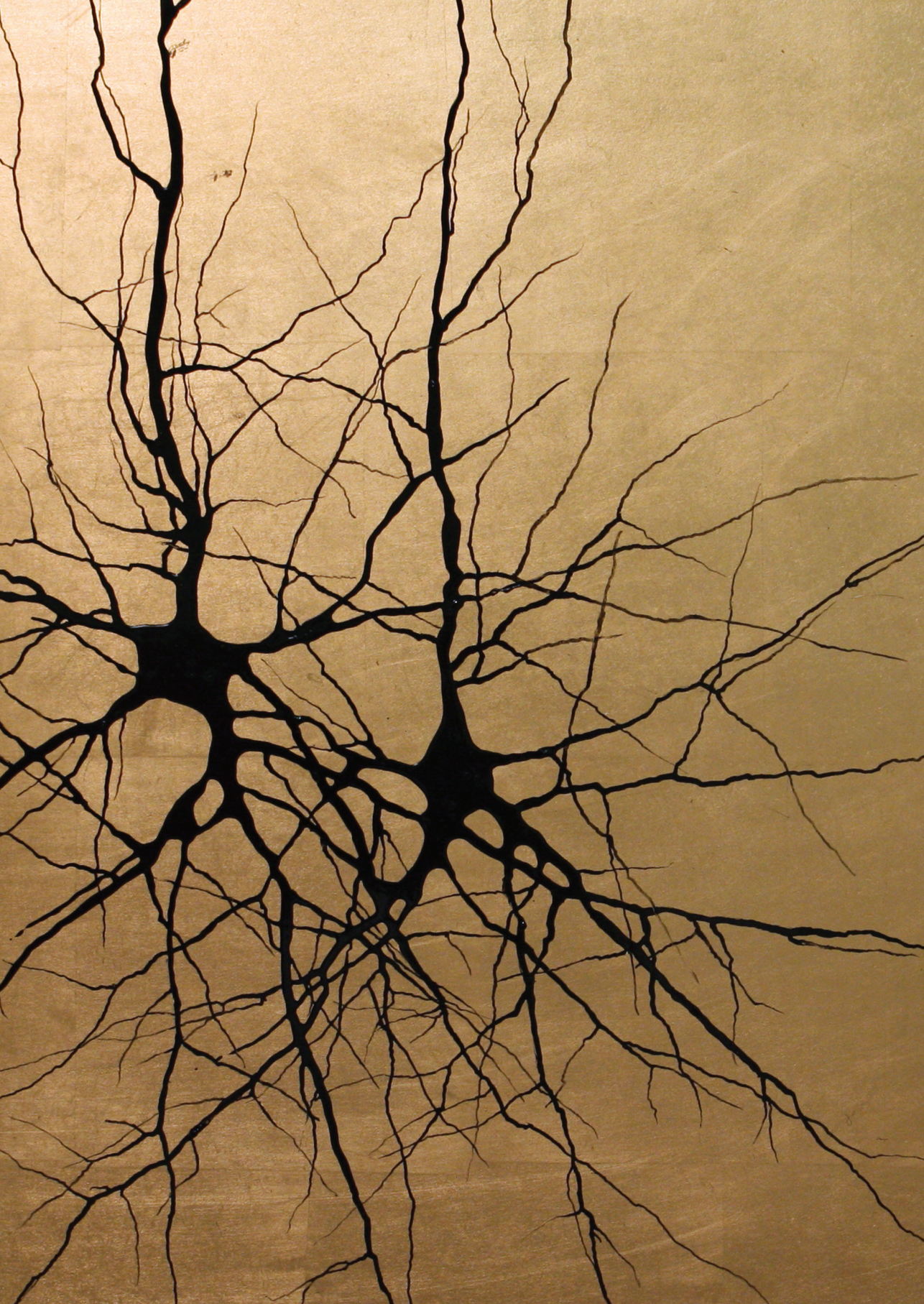
Silva-Santos S, Van Woerden GM, Bruinsma CF, Mientjes E, Jolfaei MA, Distel B, Kushner SA, Elgersma Y (2015) Ube3a reinstatement identifies distinct developmental windows in a murine Angelman syndrome model. J Clin Invest.

Tobimatsu T, Fujisawa H (1989) Tissue-specific expression of four types of rat calmodulin-dependent protein kinase II mRNAs. J Biol Chem 264:17907–17912.

Van Woerden GM, Hoebeek FE, Gao Z, Nagaraja RY, Hoogenraad CC, Kushner SA, Hansel C, De Zeeuw CI, Elgersma Y (2009) betaCaMKII controls the direction of plasticity at parallel fiber-Purkinje cell synapses. Nature Publishing Group 12:823–825.

Vincent M, Collet C, Verloes A, Lambert L, Herlin C, Blanchet C, Sanchez E, Drunat S, Vigneron J, Laplanche J-L, Puechberty J, Sarda P, Geneviève D (2014) Large deletions encompassing the TCOF1 and CAMK2A genes are responsible for Treacher Collins syndrome with intellectual disability. Eur J Hum Genet 22:52–56.

Yasuda H, Barth AL, Stellwagen D, Malenka RC (2002) A developmental switch in the signaling cascades for LTP induction. Nat Neurosci 6:15–16.



Appendix

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Curriculum Vitae
PhD Portfolio
Dankwoord

ENGLISH SUMMARY

In the field of learning and memory, CAMK2 is one of the most studied proteins. In addition, CAMK2 is also one of the most abundant proteins in the mammalian brain. Over 30 years of research has established a clear role for CAMK2 in learning, memory and long-term potentiation (LTP). This thesis focuses on CAMK2A and CAMK2B, the two major brain isoforms that can form a CAMK2 dodecamer. Specifically, the spatial and temporal roles of CAMK2A and CAMK2B individually were investigated, as well as the redundancy of CAMK2A and CAMK2B.

In **Chapter 2** we investigated the spatial and temporal requirements of CAMK2A in spatial and contextual learning. We generated a new mouse model in which genetic deletion of CAMK2A could either be induced in specific parts of the brain or globally in adulthood. We found that CAMK2A expressed in forebrain, but not in the cerebellar Purkinje cells, is essential for spatial learning. Moreover, adult deletion of CAMK2A impaired spatial learning and LTP at a similar level as in conventional *Camk2a* knockout mice. These results highlight the importance of CAMK2A at the moment of learning and LTP expression.

A similar approach was used for CAMK2B in **Chapter 3**. Here we investigated the spatiotemporal requirements of CAMK2B in locomotion, since the conventional *Camk2b*$^{-/-}$ mutant has a severe locomotion deficit. Additionally, we used two point mutants to study the molecular requirements for locomotion. Starting with the molecular requirements, we found that autonomous activity of CAMK2B (using CAMK2B-T287A mice) is dispensable, whereas loss of Ca^{2+}-dependent activity of CAMK2B (using CAMK2B-A303R mice) is detrimental for normal locomotion. On a temporal level, loss of CAMK2B in adulthood results in a relatively mild impairment in locomotion, suggesting a developmental role for CAMK2B in locomotion. On a spatial level we could not recapitulate the locomotion deficit by deleting CAMK2B in single brain regions such as cerebellum, striatum or forebrain arguing that this deficit does not arise from a single brain region.

In **Chapter 4** we targeted both CAMK2A and CAMK2B simultaneously. This allowed us to uncover new functions of CAMK2 which were masked by redundancy of CAMK2A in *Camk2b*$^{-/-}$ mutant mice and CAMK2B in *Camk2a*$^{-/-}$ mutant mice. One of these new functions we unmasked was a role for CAMK2 in survival. We found that deletion of CAMK2A and CAMK2B simultaneously either from germline or at the adult age is lethal. Moreover, loss of autonomous and Ca^{2+}-dependent activity of CAMK2 is lethal. On a physiological level, we found that LTP at the CA3-CA1 synapse was completely absent

upon genetic deletion of *Camk2a* and *Camk2b* in adulthood. This was not due to the loss of CAMK2A and CAMK2B in the presynaptic neuron, as genetic deletion presynaptically did not alter LTP. Finally, we found that at the CA3-CA3 synapse plasticity rules are governed differently as compared to the CA3-CA1 synapse with respect to CAMK2. Only upon loss of CAMK2A and CAMK2B simultaneously is LTP impaired at the CA3-CA3 synapse. This is in contrast with the CA3-CA1 synapse, where loss of only CAMK2A or CAMK2B already partially impairs LTP. Together these results show that CAMK2A and CAMK2B can compensate for each other for some critical functions and that investigating single knockout mutants is not sufficient to apprehend the full spectrum of CAMK2A and CAMK2B.

We went one step further in **Chapter 5** by investigating new functions of CAMK2 on a single cell level. There we explored the loss of CAMK2A and CAMK2B and the subsequent effect on neuronal excitability and unitary synaptic transmission in hippocampal CA1 pyramidal cells. We found a bidirectional change in excitability following CAMK2A and CAMK2B deletion. 10 days after deletion we found a decrease in excitability when CAMK2A and CAMK2B were deleted. This effect was gone at 15 days after onset of deletion and subsequently followed by an increase in excitability 21 days after onset of deletion. At the synaptic level we found that loss of CAMK2A and CAMK2B resulted in a decrease of mIPSC and mEPSC frequency and amplitude 21 days after deletion, but not yet after 10 days of deletion. All these effects in neuronal excitability and unitary synaptic transmission were only present in mice where CAMK2A and CAMK2B were deleted simultaneously. When only one of these isoforms was deleted, these effects were no longer present.

Together these results further elucidate the mechanisms of CAMK2 in behavior, on a network level and on a single cell level. Understanding the mechanisms of CAMK2 on these different levels will one day provide new insights for treating patients with intellectual disability based on mutations in the human *CAMK2* gene.

NEDERLANDSE SAMENVATTING

CAMK2 is een van de meest bestudeerde enzymen binnen het veld van leren en geheugen en sterk oververtegenwoordigd in het brein van zoogdieren. Meer dan 30 jaar fundamenteel onderzoek laat een essentiële rol zien voor CAMK2 in leren, geheugen en lange-termijn potentiatie (LTP). Deze fundamentele kennis blijkt nu van onkenbaar belang, sinds er dankzij verbeterde en goedkopere DNA-technieken nu CAMK2 mutaties gevonden worden in individuen met een (tot nog toe) onverklaarde verstandelijke beperking. Echter in dit proefschrift wordt nog met name de fundamentele rol van CAMK2A en CAMK2B bestudeerd, twee zeer veel voorkomende isoformen, die in het brein samen een CAMK2 enzymcomplex kunnen vormen. Zowel de individuele rol van CAMK2A en CAMK2B in verschillende hersengebieden en in verschillende ontwikkelingsstadia (de spatiele en temporele rol) als de gezamenlijke rol van CAMK2A en CAMK2B komen aan bod.

In **hoofdstuk 2** onderzochten we de rol van CAMK2A in verschillende hersengebieden en in verschillende ontwikkelingsstadia in ruimtelijk en contextueel leren. We maakten een nieuw muis model waarin we CAMK2A genetisch konden verwijderen ofwel in specifieke gebieden in het brein of in het gehele brein op volwassen leeftijd. We laten zien dat CAMK2A in de voorhersenen essentieel is voor ruimtelijk leren, maar dat CAMK2A in de Purkinje cellen van de kleine hersenen (cerebellum) geen rol speelt in dit gedrag. Deletie van het gen *Camk2a*, en daarbij deletie van het enzym CAMK2A, op volwassen leeftijd verhinderde het ruimtelijk leren en de expressie van LTP. Dit is vergelijkbaar met de conventionele *Camk2a* knockout muis waarin CAMK2A nooit aanwezig is geweest. Deze resultaten tonen aan dat aanwezigheid van CAMK2A belangrijk is op het moment van leren en voor het creëren van LTP.

Eenzelfde methode werd gebruikt voor CAMK2B in **hoofdstuk 3**. Hier onderzochten we de spatiele en temporele rol van CAMK2B in motoriek. De conventionele *Camk2b* knockout muismutant heeft hierin een sterk fenotype. Aanvullend maakten we gebruik van twee knock-in mutanten om de moleculaire rol van CAMK2B voor motoriek te onderzoeken. We vonden dat calcium-onafhankelijk (autonome) activiteit van CAMK2B verwaarloosbaar is voor de motorische fenotypes. Daarentegen was de calcium-afhankelijke activiteit van CAMK2B cruciaal voor normale motoriek. Wat betreft het temporele aspect vonden we dat verlies van CAMK2B op latere leeftijd een relatief milder fenotype in de motoriek gaf in vergelijking met de conventionele CAMK2B knockout muis. Dit betekent dat er een rol voor CAMK2B in motoriek is weggelegd in de ontwikkeling. Wanneer we CAMK2B alleen in het cerebellum, striatum of in de

voorhersenen weghaalden zagen we geen fenotype in de motoriek. Het lijkt er dus op dat de motoriek van de knockout muis niet uit een van deze hersengebieden alleen komt, maar waarschijnlijk uit meerdere tegelijkertijd.

In **hoofdstuk 4** onderzochten we zowel CAMK2A als CAMK2B door muizen te onderzoeken die beide isoformen niet hadden vanaf de conceptie of op volwassen leeftijd verloren. Hierdoor werden we in staat gesteld nieuwe functies van CAMK2 te ontdekken. Deze waren eerder mogelijk niet zichtbaar door compensatie van CAMK2A in de *Camk2b* knockout muis of CAMK2B in de *Camk2a* knockout muis. Een van deze nieuwe functies was een cruciale rol voor CAMK2 in de overleving. We vonden dat genetische deletie van *Camk2a* en *Camk2b* zowel vanaf de conceptie als geïnduceerde deletie op volwassen leeftijd resulteerde in vroegtijdige sterfte. Ook het verlies van autonome en calcium-afhankelijke activiteit van CAMK2 resulteerde in vroegtijdige sterfte. Op elektrofysiologisch niveau vonden we dat LTP compleet afwezig was in de CA3-CA1 synaps van de hippocampus in muizen waar *Camk2a* en *Camk2b* genetisch werden verwijderd op volwassen leeftijd. In een ander muismodel vonden we dat de afwezigheid van LTP niet kwam niet door verlies van presynaptisch CAMK2A en CAMK2B. Tot slot vonden we dat de regels voor plasticiteit in de CA3-CA3 synaps verschillen van die in de CA3-CA1 synaps. Alleen bij verlies van zowel CAMK2A als CAMK2B was er een fenotype in LTP-expressie in de CA3-CA3 synaps, niet bij verlies van alleen CAMK2A of CAMK2B. Dit is in tegenstelling tot de CA3-CA1 synaps waar verlies van alleen CAMK2A of CAMK2B een fenotype in LTP-expressie geeft. Deze resultaten laten zien dat CAMK2A en CAMK2B voor elkaar kunnen compenseren voor een aantal cruciale functies zoals overleving en LTP. Tevens laten deze resultaten zien dat het onderzoek van alleen *Camk2a* of *Camk2b* knockout muizen niet voldoende is om het volledige functionele spectrum van CAMK2A of CAMK2B te begrijpen.

In **hoofdstuk 5** gingen we een stap verder door op zoek te gaan naar nieuwe functies van CAMK2 op het niveau van één neuron. Op dat niveau verkenden we het effect van verlies van CAMK2A en CAMK2B tegelijk, op de prikkelbaarheid en de synaptische transmissie in pyramidale cellen in het gebied CA1 van de hippocampus. We vonden een tweezijdig effect op de prikkelbaarheid na het verwijderen van CAMK2A en CAMK2B. 10 dagen na de genetische deletie vonden we een afname van deze prikkelbaarheid, wat niet meer zichtbaar was 15 dagen na de start van de genetische deletie en verschoof naar een toename van prikkelbaarheid 21 dagen na genetische deletie. Op synaptisch niveau vonden we dat verlies van CAMK2A en CAMK2B resulteerde in een afname in de frequentie en amplitude van kleine signalen tussen neuronen (mIPSCs en mEPSCs) 21 dagen na de start van genetische deletie, maar niet eerder. Al deze effecten in prikkelbaarheid en synaptische transmissie waren alleen zichtbaar in muizen waarbij

tegelijk CAMK2A en CAMK2B werden verwijderd. Deze effecten waren afwezig als alleen CAMK2A of CAMK2B werd verwijderd. Deze resultaten laten ook hier zien dat CAMK2A en CAMK2B elkaar kunnen compenseren.

Tezamen dragen deze bevindingen bij aan het begrip van hoe CAMK2 werkt op het niveau van gedrag, op netwerkniveau en op het niveau van één enkel neuron. Het begrijpen van de mechanismes waarmee CAMK2 werkt op deze verschillende niveaus zal in de toekomst nieuwe inzichten leveren in de behandeling van patiënten met een verstandelijke beperking veroorzaakt door mutaties in een van de *CAMK2* genen.

LIST OF PUBLICATIONS:

This thesis:

Achterberg KG, Buitendijk GHS, **Kool MJ**, Goorden SMI, Post L, Slump DE, Silva AJ, Van Woerden GM, Kushner SA, Elgersma Y (2014) Temporal and Region-Specific Requirements of αCaMKII in Spatial and Contextual Learning. *Journal of Neuroscience* 34:11180–11187.

Kool MJ, van de Bree JE, Bodde HE, Elgersma Y, Van Woerden GM (2016) The molecular, temporal and region-specific requirements of the beta isoform of Calcium/Calmodulin-dependent protein kinase type 2 (CAMK2B) in mouse locomotion. *Scientific Reports* 6:26989.

Kool MJ, Borgesius NZ, van de Bree JE, Elgersma-Hooisma M, Buitendijk GHS, Aghadavoud Jolfaei M, Elgersma Y, van Woerden GM. CAMK2-dependent signaling in neurons is essential for survival. *Under Review in the Journal of Neuroscience*

Kool MJ, Rotaru DC, Bodde HE, Elgersma Y, van Woerden GM. Bidirectional changes in excitability upon loss of both CAMK2A and CAMK2B. *Manuscript in preparation*

Other Publications:

Bruinsma CF, Savelberg SMC, **Kool MJ**, Jolfaei MA, Van Woerden GM, Baarends WM, Elgersma Y (2016) An essential role for UBE2A/HR6A in learning and memory and mGLUR-dependent long-term depression. *Human Molecular Genetics* 25:1–8.

CURRICULUM VITAE

Martijn Jacob Kool was born on December 31, 1986 and raised in Rotterdam. After graduating from high school at the Erasmiaans Gymnasium in Rotterdam he was admitted to medical school in 2006 at the Erasmus University Rotterdam. Parallel to medical school, he followed the Erasmus MC research master program in Neuroscience under the supervision of prof. dr. Y. Elgersma investigating the molecular mechanisms underlying learning and memory. After graduating from the neuroscience master program in 2012 he decided to postpone his medical school and started as a PhD-candidate in the same field as his master thesis under the supervision of dr. G.M van Woerden and prof. dr. Y. Elgersma. During his work as a PhD student Martijn was member and subsequently chairman of the Science Café committee, an initiative from the Erasmus University Rotterdam to bring science to the general public in a series of monthly lectures by high-profile scientists. Additionally, Martijn participated in ScienceBattle, a cultural initiative to bring science to theatres where PhD-students in theatres across the Netherlands compete in trying to sell the relevance of their scientific work to a broad audience. Since September 2016 he started with his final two years of medical school while finishing his PhD. In September 2018 Martijn graduated from medical school and he's currently working at the E.R. of the Franciscus Vlietland Hospital. Martijn is married to Marieke, together they live in Rotterdam.

PHD PORTFOLIO

Name: Martijn Jacob Kool
PhD Period: 2012-2016
Department: Neuroscience
Promotor: Prof.dr. Y. Elgersma
Co-promotor: Dr. G.M. van Woerden
Research School: Graduate School Neurosciences Amsterdam Rotterdam (ONWAR)

1. PhD Training	Year	Workload (ECTS)
Article 9 Course required for animal research	2012	1.5
Biostatistical methods I: basic principles (NIHES)	2013	5
Interneuron Summer School Amsterdam	2014	1.5
Biomedical English Writing and Communication	2016	4
Conferences		
E-Phys Brain Conference, department of Neuroscience	2013-2015	0.5
ONWAR annual PhD meeting, Driebergen (poster presentation)	2012-2013	1
Interneuron Summer School Amsterdam (Conference part)	2014	0.5
Society for Neuroscience annual meeting Chicago (poster presentation)	2015	1.5
Seminars and workshops		
Neuroscience department seminar series	2010-2016	2
Seminar at Essen University (presentation)	2017	1
2. Teaching		
Medical curriculum "Functional anatomy of the musculoskeletal system"	2013-2016	10
"Anatomy of the eye" classes for medical students	2013	0.5
"General anatomy of the body" classes for medical students	2015	0.5
Workshop Hippocampal Field Recording for Master of Neuroscience students	2012-2016	3
Supervising Masters theses		
H.E. Bodde (Master of Neuroscience student)	2015-2016	1.5
Other		
Chairman Wetenschapscafe Rotterdam	2014-2016	2.5

DANKWOORD

Als je denkt dat je het lastigste gehad hebt, komt er nog het dankwoord. Er zijn een hoop mensen die ik wil bedanken.

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Andere commissieleden, prof. dr. Borst, prof. dr. Kushner en prof. dr. Hoebeek. Beste Gerard, bedankt voor het kritisch beoordelen van mijn thesis en voor de vele hulp rondom de elektrofysiologie de afgelopen jaren. Beste Steven, bedankt voor alle intrigerende discussies en de mooie colleges. Beste Freek, bedankt voor de discussies en de leuke tijd in Chicago! Prof. dr. Kessels en dr. Nadif Kasri, bedankt voor het plaatsnemen in de commissie.

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